Title: **The RING domain of PIASy is involved in the suppression of Bone morphogenetic protein-signaling pathway**

Running title: Regulation of BMP signaling by PIASy

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ABSTRACT

Bone morphogenetic proteins (BMPs) play central roles in differentiation, development, and physiologic tissue remodeling. Recently, we have demonstrated that a protein inhibitor of activated STAT, PIASy suppresses TGF-β signaling by interacting with Sma and MAD-related protein 3 (Smad3). In this study, we examined a PIASy dependent inhibitory effect on BMP signaling. PIASy expression was induced by BMP-2 stimulation and suppressed BMP-2-dependent Smad activity in hepatoma cells. Furthermore, BMP-2-regulated Smads directly bound to PIASy. We also demonstrated that the RING domain of PIASy played an important role in PIASy-mediated suppression of Smad activity. We here provide evidence that the inhibitory action of PIASy on BMP-regulated Smad activity was due to direct physical interactions between Smads and PIASy through its RING domain.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF-) superfamily that have been implicated in tissue growth and remodeling (1, 2, 3). BMPs were initially identified by the ability of bone extracts to induce bone formation at extraskeletal sites (2). BMPs bind to two types of transmembrane receptors, denoted type I and type II BMP receptors, which have serine/threonine kinase activity (3). Upon ligand binding, type II receptors phosphorylate the type I receptors. The activated type I receptors then phosphorylate downstream Smads, Smad1, Smad5 or Smad8, which are transcription factors that regulate gene expression in response to BMPs (4, 5, 6).

BMP signaling has been also implicated in the metastatic potential of several human cancers (7, 8, 9, 10, 11). BMP stimulates cell adhesion to types I and IV collagens, fibronectin, and vitronectin as well as down-regulated expression of integrins (1, 2, 12), following by an increased cell spreading. BMP signaling also induces the expression of several distinct target genes, including members of the Id gene family through Smad proteins (13, 14, 15).

In attempt to identify novel Smad partners, we screened a mouse embryo cDNA library with yeast two-hybrid system using the MH2 domain of Smad7 as bait. We recently identified PIASy, which is one of the protein inhibitor of activated STAT family protein, as a protein that interacts specifically with Smad7 (16). They have been originally identified as a cofactor that inhibits the transcriptional activation potential of STAT (signal transducers and activators of transcription) and in mammals five PIAS proteins (PIAS1, 3, x, x and y) have been reported (17). PIAS1 and PIAS3 were initially cloned as transcriptional repressors of the Jak-STAT signaling pathway (18, 19). PIAS3 was originally identified as a specific co-repressor of signal transducer and activator of transcription 3 (STAT3)(20). PIAS3 binds to STAT3 and inhibits its DNA-binding activity and thereby interferes with STAT3-mediated gene activation. PIAS1, another member of PIAS family, was originally identified as a co-

repressor of STAT1 (18). PIAS family proteins also function as a transcriptional cofactor for nuclear receptors (20, 21, 22). Recently, PIAS family proteins have been proposed to function as a small ubiquitin-related modifier (SUMO)-E3 ligase (23). PIAS1 and PIASy were shown to catalyze sumoylation of p53, LEF-1 and Tcf-4, respectively (24, 25, 26, 27). PIAS family proteins have the RING domain, and their SUMO-E3 ligase activities are dependent on this domain (23). PIASy was demonstrated to interact with Smad3 and antagonizes Smad3-dependent transcriptional activation (16, 28). We also showed that expression of PIASy is induced by TGF-□ (16).

In this study, we demonstrate a novel molecular mechanism for the inhibitory actions of PIASy on BMP-2 function: there are direct physical and functional interactions between BMP-regulated Smads and PIASy. We also demonstrate that the RING domain of PIASy plays an important role in PIASy-mediated suppression of Smad activity and the sequestration of Smad in nuclear bodies. These findings provide new insights into the regulation of BMP-2 -signaling pathway that may have implications in reproductive physiology and the process of oncogenesis.

MATERIALS AND METHODS

Reagents and antibodies

Human recombinant BMP-2 was purchased from Strathmann Biotech GmbH (Germany). Expression vectors, FLAG-tagged Smad1, Smad8, Smad6, BMPR-IA (QD), 12xGCCG-LUC (29, 30), T□R-I (T204D) (31) and p3TP-LUC (31), were kindly provided by Dr. K. Miyazono (Tokyo Univ., Tokyo, Japan) and Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). Myc-tagged PIASy RING mutant ($PIASy^{CA}$) (27) were kindly provided by Dr. A Kikuchi (Hiroshima Univ., Hiroshima, Japan). PIASy mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG antibody was purchased from Sigma (St Louis, MO).

Cell culture, transfection, and luciferase assays

Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol (33). Human hepatoma cell line Hep3B was maintained in DMEM containing 10% FCS (34). Before stimulation, the cells were cultured for 12 h in DMEM containing 1% FCS followed by treatment with BMP-2 (31, 32). Hep3B cells (2-2.5x10^5 in a 6-cm dish) were transfected by using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. Luciferase assay was performed as described (33). The cells were harvested 48 h after transfection and lysed in 100 □l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and □-galactosidase activities according

to the manufacturer's instructions. Luciferase activities were normalized to the β-galactosidase activities. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously (33). Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on 5-20% SDS-PAGE and transferred to Immobilon filter (Millipore; Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Northern blot and RT-PCR analysis

After 12 h of incubation in 1% FCS, Hep3B cells were treated with BMP-2 (50 ng/ml) for the indicated time. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in Northern blot analysis according to the established procedures (34). A nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) and radiolabelled cDNA probes were used, where indicated. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Human Id1 and G3PDH primers were used as described previously (35, 36).

RESULTS AND DISCUSSION

PIASy expression is induced by BMP-2 and suppresses BMP-2-mediated transcription in Hep3B cells.

In our previous report, we demonstrated that TGF-β-induced PIASy suppressed TGF-β-stimulated gene expression in a human hepatoma cell line, Hep3B cells (16). We then examined whether PIASy expression is also regulated by BMP-2 in Hep3B cells. Hep3B cells were either left untreated or treated with BMP-2, and PIASy expression was monitored by Northern blot analysis. As shown in Fig. 1A, about 3-fold induction of PIASy expression was observed by treatment with BMP-2 in Hep3B cells. The level of PIASy mRNA expression increased about 3-fold at 6 h, and did not alter until 24 h. We also examined BMP-2-induced Id1 expression through Smads (13, 14, 15). Id proteins interact with a variety of basic helix-loop-helix (bHLH) and non-bHLH transcription factors that contribute to normal growth regulation. Therefore, dysregulated Id expression may contribute to oncogenesis (37, 38, 39), and their expression is increased in a variety of human cancers (40). When we monitored the same RNA samples by RT-PCR, BMP-2 treatment strongly induced transient expression of Id1 at 3-6h after stimulation in Hep3B cells.

To examine the effect of PIASy expression on BMP-2 signaling in Hep3B, we next performed the transient transfection assay. The BMP-2-mediated transcriptional responses were measured by using 12xGCCG-LUC, which is a reporter construct that directly detects Smad phosphorylation through BMP receptors (30). Hep3B cells were transfected with 12xGCCG-LUC together with empty vector or PIASy, and treated with BMP-2 and LUC activities were determined. As shown in Fig. 1B, PIASy expression showed a significant decrease of BMP-2-stimulated 12xGCCG-LUC activation in Hep3B cells. These results

demonstrated that expression of PIASy is induced by BMP-2 and inhibits BMP-2/Smad-mediated transcription activity in Hep3B cells.

PIASy suppresses BMP-signaling in 293T cells.

To delineate the interactions between PIASy and BMP-signaling pathways further, we carried out transient transfection experiments in 293T cells using the respective receptors and the downstream activators for the BMP signaling, Smad1. In addition, in most of these experiments, a constitutively active form of BMP type IA receptor, BMPR-IA(QD), was used (30, 41). Expression of BMPR-IA(QD) in 293T cells resulted in an induction of 12xGCCG-LUC as previously described (34). When 293T cells were transfected with 12xGCCG-LUC together with BMPR-IA (QD), LUC expression was increased by 2-2.5-fold (Fig. 2A). Additional expression of Smad1 augmented 12xGCCG-LUC expression by 5-6-fold (Fig. 2A). We then examined the effect of expression of PIASy on BMP signaling in 293T cells. 293T cells were transfected with an expression vector for BMPR-IA(QD), Smad1 and 12xGCCG-LUC together with or without an inhibitory Smad, Smad6 or PIASy. As shown in Fig. 2A, PIASy expression but not PIAS1, PIAS3 and PIASX suppressed BMPR-IA (QD)/Smad1-mediated 12xGCCG-LUC activation in a dose-dependent (Fig. 2B). These results suggest that BMP/Smad-mediated signal is suppressed by PIASy in a specific manner.

Physical interactions between PIASy and BMP-regulated Smads in vivo.

One of the possible mechanisms that would be consistent with the data described above is that there are direct physical interactions between PIASy and BMP-regulated Smads. To investigate the association of PIASy with BMP-regulated Smads in vivo, 293T cells were

transfected with either FLAG-tagged Smad1, Smad8 or Smad6 together with Myc-tagged PIASy. As shown in Fig. 3A, comparable amounts of Smads were expressed in each cell. Similarly, PIASy was expressed in samples containing Smads. Western blot analysis of associated proteins with an anti-FLAG antibody revealed that PIASy interacts with Smad1, Smad8 and Smad6 in 293T cells.

We next determined which domains of PIASy mediate interactions with Smad1, using a series of deletion mutants of PIASy as shown in Fig 3B. Expression vectors encoding FLAG-tagged Smad1 and/or a series of Myc-tagged PIASy mutants were transiently transfected into 293T cells. Cells were lysed, and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an anti-Myc antibody. As shown in Figure 3C, PIASy(1-100) lacking almost C-terminal domain was unable to bind Smad1. However, PIASy(1-202) and PIASy (100-510) showed a little lower binding affinity. These results indicate that PIASy-Smad1 interaction requires the N-terminal domain of PIASy. The data also suggest that the N-terminal domain of PIASy may contain a secondary binding site for Smad1.

The RING domain of PIASy is involved in the suppression of Smads-mediated transcription.

PIASy has the RING domain, which has been identified as an important functional determinant of ubiquitin and SUMO E3 ligases (24, 25, 26). Sumoylation proteins have been shown to localize to specific subnuclear structures, known as PML-containing nuclear bodies (42). The recruitment of target proteins to subnuclear structures might be the critical determinant for the regulation of these proteins, and the sumoylation may be a consequence of the sequestration. To examine the involvement of the RING domain on PIASy-mediated

suppression of Smad activity, we used a PIASy RING mutant ($PIASy^{CA}$), in which Cys342 and Cys347 were mutated to alanine. $PIASy^{CA}$ was shown to have no sumoylation activity on Tcf-4 (27). We first examine a complex formation between Smad1 and $PIASy^{CA}$ in 293T cell. FLAG-tagged Smad1 together with Myc-tagged PIASy or $PIASy^{CA}$ were transfected into 293T cells. Cells were lysed and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western Blot analysis with an anti-Myc antibody. As shown in Fig. 4A, both of PIASy and $PIASy^{CA}$ bound to Smad1. These results suggest that the RING mutation has no effect on the interaction between PIASy and Smad1. We next examined the effect of $PIASy^{CA}$ expression on BMPR-IA(QD)/Smad1-induced Smad activation. As shown in Fig. 4B, PIASy but not $PIASy^{CA}$ showed an effective suppression of Smad activation by BMPR-IA(QD)/Smad1. $PIASy^{CA}$ failed to suppress Smad activation effectively. These results suggest that sumolyation of Smads by PIASy through the RING domain may be important PIASy-mediated suppression of BMP-induced Smad activation. We finally examined co-localization of Smad1 with PIASy in COS7 cells (Fig. 4C). When Smad1 together with BMPR-IA(QD) was expressed in COS7 cells, it showed a diffuse nuclear distribution, whereas PIASy but not $PIASy^{CA}$ was localized predominantly to punctate structures in the nucleus. Co-expression of Smad1 with PIASy but not with $PIASy^{CA}$ resulted in a remarkable redistribution of Smad1 into punctate structures, which co-localized with PIASy. These results suggest that PIASy targets Smad1 to punctate structures in the nucleus such as a subset of PML nuclear bodies. Taken together, these observations show that the RING domain of PIASy is important not for the suppression of Smad activity but also for the sequestration of Smad1 in nuclear bodies.

Concluding remarks

Recent studies have documented the interaction of a large number of intracellular proteins with the effector molecules Smads to influence BMP signaling (43, 44). Whereas some of these proteins have been found to functionally cooperate together and activate Smads, others were found to repress Smad activity. Several molecular mechanisms have been proposed for the inactivation of BMP/Smads signaling pathway. In this study, we showed that BMP-2 induced PIASy expression suppressed BMP-2-mediated Smad activation in hepatoma cells. We also demonstrated that a PIAS family protein, PIASy interacted with BMP-regulated Smads and antagonized Smads-dependent transcriptional activation by BMPR. Furthermore, this inhibitory effect required the RING domain of PIASy, which mediates the sequestration of Smad1 in nuclear bodies.

Smad-dependent proteasome-mediated degradation of Smads themselves but also of cytoplasmic and nuclear Smad partners is an important aspect of TGF-β/BMP signaling as well. Smurf1 and Smurf2, which are HECT E3 ubiquitin ligases, selectively interact with BMP-regulated Smads to trigger their ubiquitination and subsequent degradation, and hence their inactivation (45, 46). Smurf1 and Smurf2 also bind to Smad7 and are recruited to the activated TGF-β receptor I by Smad7, leading to proteasomal degradation of the receptor (47, 48). Recently, four members of the mammalian PIAS (protein inhibitor of activated STAT) family, PIAS1, PIASx, PIASx and PIASy, and a yeast PIAS homologue, Siz1, have been reported to have SUMO-E3 ligase activities toward various target proteins, including p53 and LEF1, and affect their transcriptional activity (24, 25, 26). We and others demonstrated that PIASy stimulated the sumoylation of Smad3 in vivo, followed by suppression of TGF-β-mediated transactivation (16, 28).

Our findings provide a novel mechanism of the negative regulation of BMP signaling by PIASy, which may due to the sequestration of Smads in nuclear bodies by the RING domain of PIASy using the negative feedback loop. More detailed understanding of the

interactions between activated Smads and PIASy will provide not only critical information on embryogenesis, organogenesis, and tissue homeostasis, but may also be instrumental in the development of new treatment strategies in related diseases such as cancer.

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FIGURE LEGENDS

Fig. 1. **PIASy expression is induced by BMP-2 and suppresses BMP-2-induced transcription in Hep3B cells.**

(A)BMP-2-induced PIASy expression in Hep3B cells. 20 μg of total RNA isolated from cells treated or untreated with BMP-2 (50 ng/ml) for the indicated times were used in Northern blot analysis of PIASy expression. The same blot was probed with Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as control (lower panel). Relative intensities (Rel. Int.) of the bands shown below the autoradiograms were determined by densitometric analysis. Same RNA samples were subjected to RT-PCR analysis using Id1 (upper panel) and G3PDH (lower panel) primers. RT-PCR products were separated on a 1% agarose gel. This figure is representative of three separate experiments.

(B) Hep3B cells (6-well plate) were transfected with 12xGCCG-LUC (1 μg) together with empty vector or PIASy (3μg). 48 h after transfection, cells were stimulated with BMP-2(50 ng/ml) for an additional 12h. Cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 2. **PIASy inhibits BMP-mediated transcription.**

(A) 293T cells (12-well plate) were transfected with 12xGCCG-LUC (0.3μg) and/or BMPR-IA(QD) and Smad1 or Smad6 or PIASy as indicated. 48 h after transfection, cells were harvested and relative luciferase activities were measured.

(B) 293T cells (12-well plate) were transfected with 12xGCCG-LUC (0.3μg) and/or BMPR-IA(QD) and Smad1 (100ng) and/or the indicated amounts of PIAS1, PIAS3 or PIASxβ. 48 h after transfection, LUC activities were determined.

Fig. 3. **Physical interactions between PIASy and BMP-regulated Smads.**

(A) 293T cells ($1x10^7$) were transfected with Myc-tagged PIASy (10 g) and/or FLAG-tagged Smad3, Smad1, Smad8, and Smad6 (10 g). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-Myc antibody, and immunoblotted with anti-FLAG antibody (upper panel) or anti-Myc antibody (middle panel). Total cell lysates (1%) were blotted with anti-FLAG antibody as indicated (lower panel). IgH, heavy chain of immunoglobulin.

(B) Schematic line diagrams of PIASy. PIASy contains a putative chromatin-binding SAP domain, a C2HC3 RING domain (RING), and a C-terminal serine-rich and acidic domain (Ser/Acidic).

(C) Mapping the Smad1 interaction domain of PIASy. 293T cells ($1x10^7$) were transfected with FLAG-tagged Smad1 (10 g) and/or full-length PIASy or PIASy mutants. 48 h after transfection, cells were lysed and immunoprecipitated with an anti-ER antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration position of full-length PIASy or PIASy mutants. IgH, heavy chain of immunoglobulin

Fig. 4. **Involvement of the RING domain in PIASy-mediated suppression of Smad activation.**

(A) 293T cells ($1x10^7$) were transfected with FLAG-tagged Smad1(10 g) together with or without Myc-tagged PIASy (10 g) or Myc-tagged $PIASy^{CA}$ (10 g). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody as indicated (lower panel).

(B) 293T cells (12-well plate) were transfected with 12xGCCG-LUC (0.3□g) and/or BMPR-IA(QD) (100ng) and Smad1 (100ng), together with or without an increasing amounts of PIASy or PIASy^{CA} as indicated. 48 h after transfection, cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

(C) COS7 cells were cotransfected with FLAG-tagged Smad1 and Myc-tagged PIASy or Myc-tagged PIASy^{CA} by the calcium phosphate precipitation protocol. 48 h after transfection, cells were fixed, reacted with an anti-FLAG polyclonal antibody and an anti-Myc monoclonal antibody, and visualized with a FITC-conjugated anti-rabbit antibody (Smad1) and a rhodamine-conjugated anti-mouse antibody (PIASy or PIASy ^{CA}). These figures were merged. The same slide was also stained with DAPI.

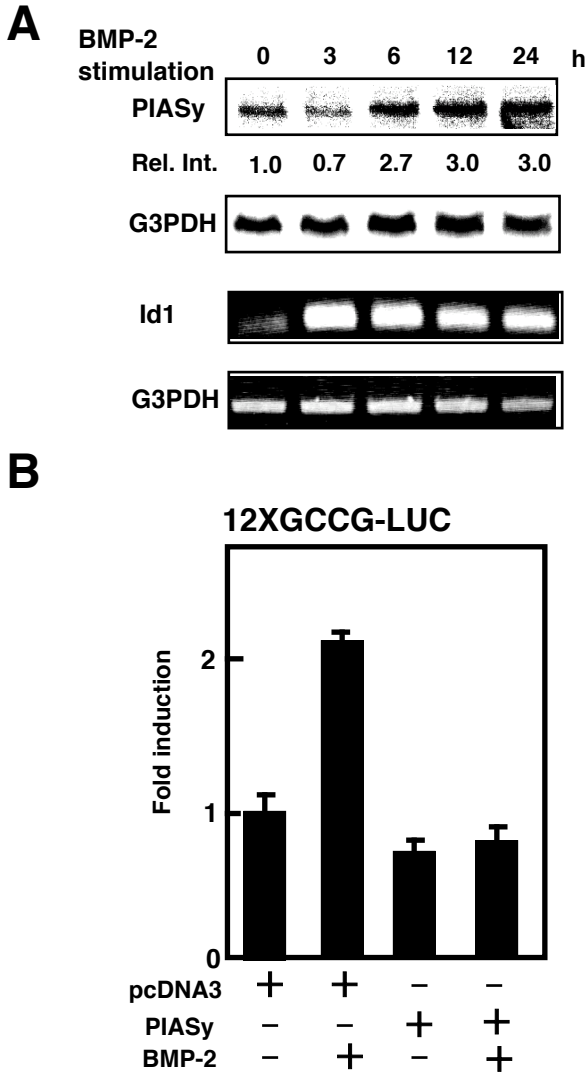
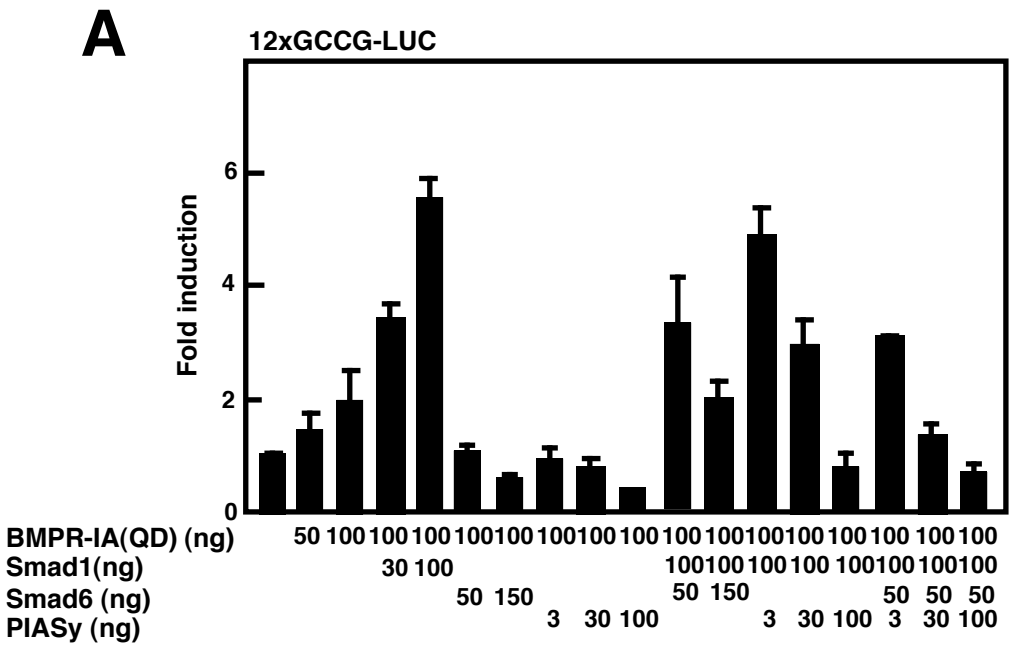

A
BMP-2 stimulation
0
3
6
12
24
h
PIASy
Rel. Int.
1.0
0.7
2.7
3.0
3.0
G3PDH
Id1
G3PDH
B
12XGCCG-LUC
Fold induction
2
1
0
pcDNA3
PIASy
BMP-2

Fig. 1.


A
12xGCCG-LUC
Fold induction
0
2
4
6
BMPR-IA(QD) (ng)
Smad1(ng)
Smad6 (ng)
PIASy (ng)
50 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100
30 100
100 100 100 100 100 100 100 100
50 150
50 150
50 50 50
3 30 100
3 30 100 3 30 100

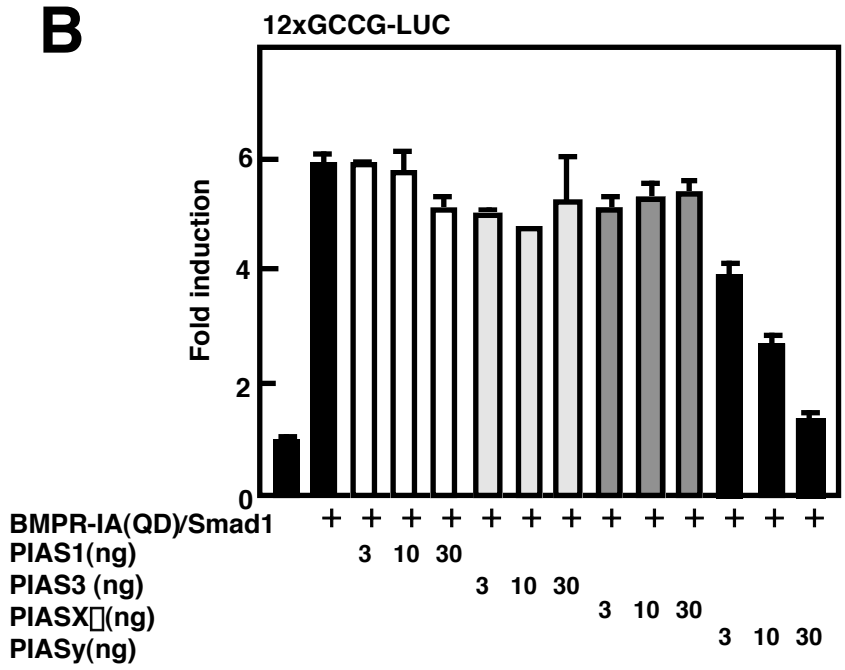

B
12xGCCG-LUC
Fold induction
0
2
4
6
BMPR-IA(QD)/Smad1
PIAS1(ng)
PIAS3 (ng)
PIASX□(ng)
PIASy(ng)
+ + + + + + + + + + + +
3 10 30
3 10 30
3 10 30
3 10 30

Fig. 2.

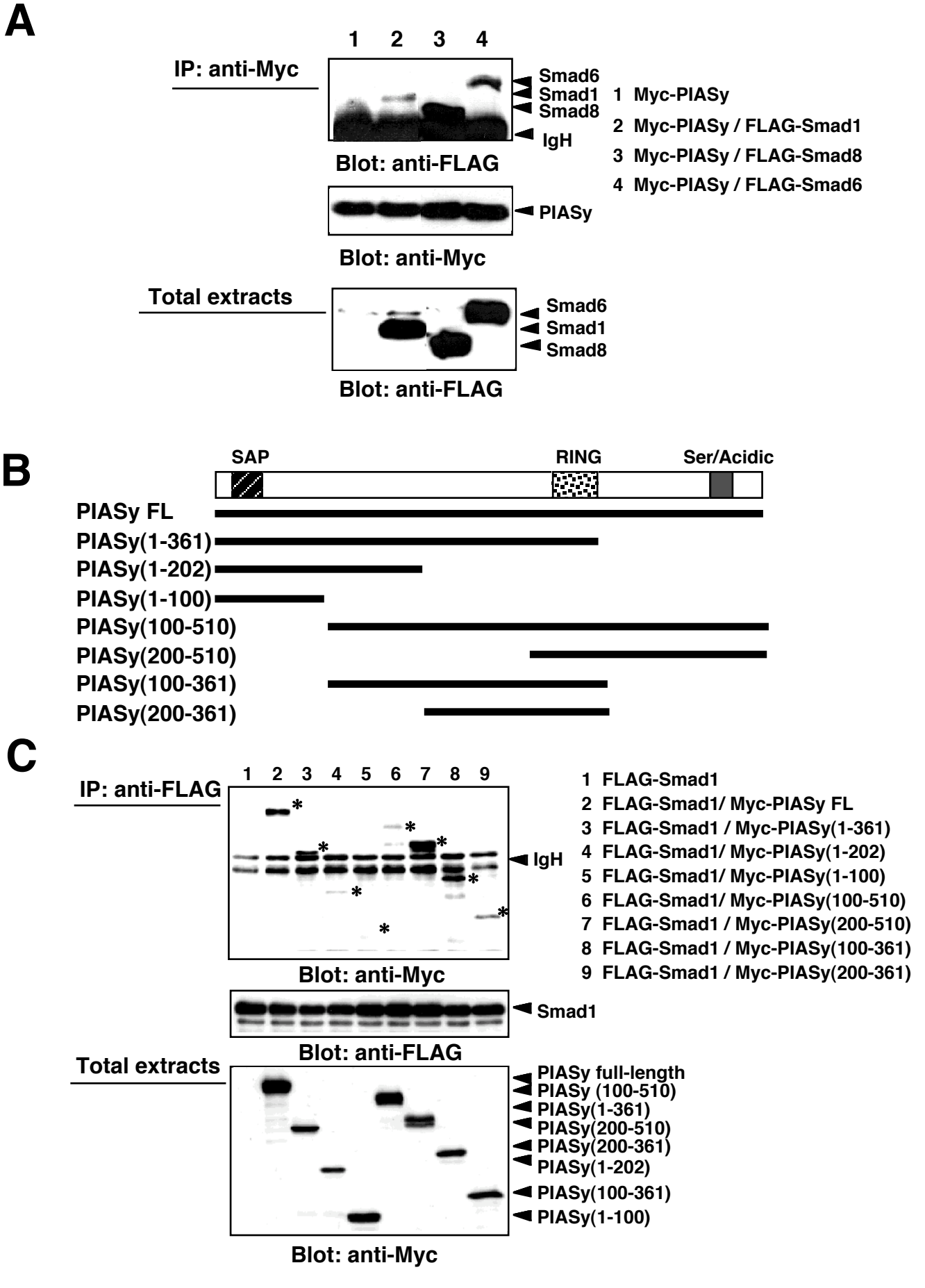
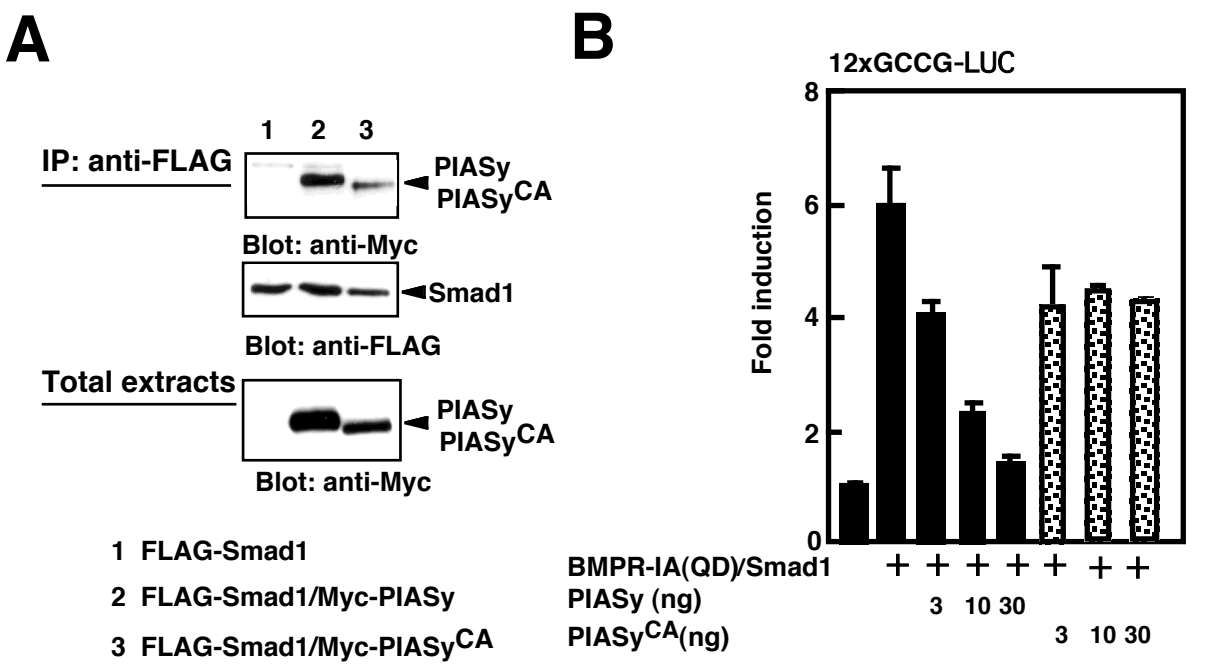

A
1 2 3 4
IP: anti-Myc
Smad6
Smad1
Smad8
IgH
1 Myc-PIASy
2 Myc-PIASy / FLAG-Smad1
3 Myc-PIASy / FLAG-Smad8
4 Myc-PIASy / FLAG-Smad6
Blot: anti-FLAG
PIASy
Blot: anti-Myc
Total extracts
Smad6
Smad1
Smad8
Blot: anti-FLAG
B
SAP
RING
Ser/Acidic
PIASy FL
PIASy(1-361)
PIASy(1-202)
PIASy(1-100)
PIASy(100-510)
PIASy(200-510)
PIASy(100-361)
PIASy(200-361)
C
1 2 3 4 5 6 7 8 9
IP: anti-FLAG
IgH
1 FLAG-Smad1
2 FLAG-Smad1/ Myc-PIASy FL
3 FLAG-Smad1 / Myc-PIASy(1-361)
4 FLAG-Smad1/ Myc-PIASy(1-202)
5 FLAG-Smad1/ Myc-PIASy(1-100)
6 FLAG-Smad1/ Myc-PIASy(100-510)
7 FLAG-Smad1 / Myc-PIASy(200-510)
8 FLAG-Smad1 / Myc-PIASy(100-361)
9 FLAG-Smad1 / Myc-PIASy(200-361)
Blot: anti-Myc
Smad1
Blot: anti-FLAG
Total extracts
PIASy full-length
PIASy (100-510)
PIASy(1-361)
PIASy(200-510)
PIASy(200-361)
PIASy(1-202)
PIASy(100-361)
PIASy(1-100)
Blot: anti-Myc

Fig. 3.


A
1 2 3
IP: anti-FLAG
PIASy
PIASyCA
Blot: anti-Myc
Smad1
Blot: anti-FLAG
Total extracts
PIASy
PIASyCA
Blot: anti-Myc
1 FLAG-Smad1
2 FLAG-Smad1/Myc-PIASy
3 FLAG-Smad1/Myc-PIASyCA
B
12xGCCG-LUC
Fold induction
0
2
4
6
8
BMPR-IA(QD)/Smad1 + + + + + + +
PIASy (ng) 3 10 30
PIASyCA(ng) 3 10 30

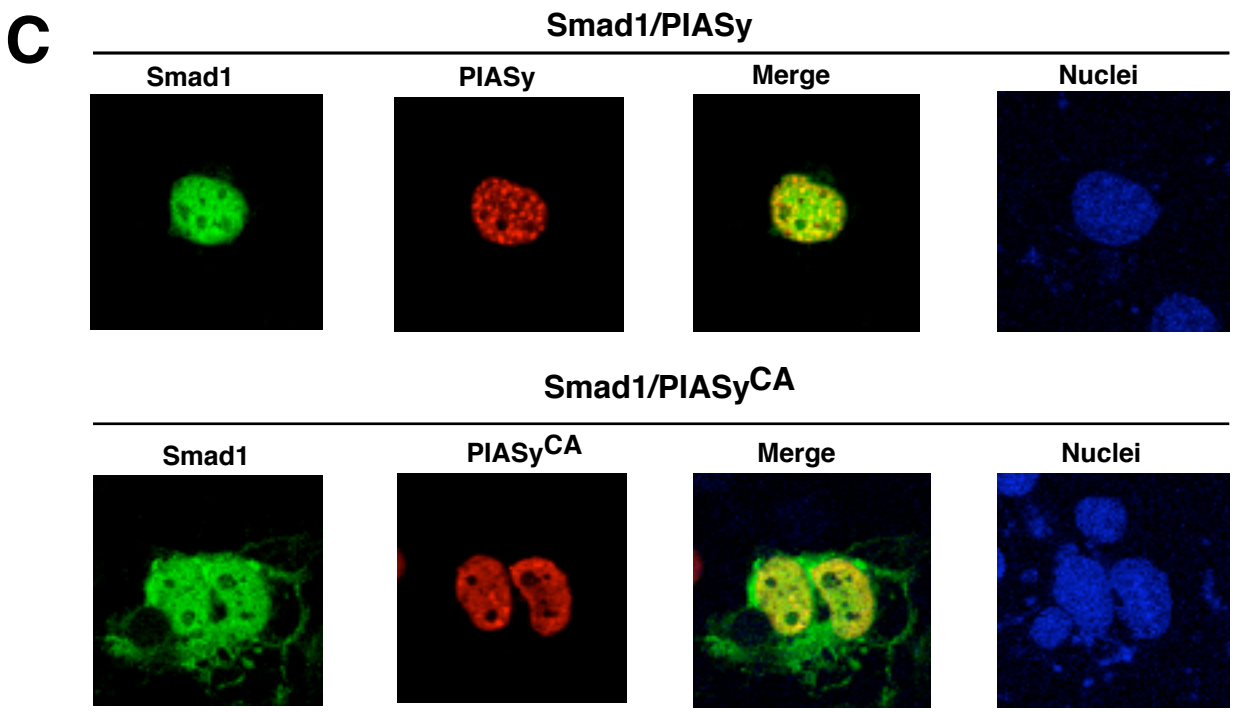

C
Smad1/PIASy
Smad1
PIASy
Merge
Nuclei
Smad1/PIASyCA
Smad1
PIASyCA
Merge
Nuclei

Fig. 4.