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**Regulation of TGF- β Signaling by Protein Inhibitor Activated of STAT, PIASy
through Smad3**

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SUMMARY

Smads proteins play a key role in the intracellular signaling of the TGF- β family of growth factors, which exhibits a diverse set of cellular responses, including cell proliferation and differentiation. In particular, Smad7 acts as an antagonist of TGF- β signaling, which could determine the intensity or duration of its signaling cascade. In this study, we identified protein inhibitor of STAT, PIASy as a novel interaction partner of Smad7 by yeast two-hybrid screening using the MH2 domain of Smad7 as bait. The association of Smad7 and PIASy was confirmed using co-expressed tagged proteins in 293T cells. Moreover, we found that other Smads including Smad3 also associated with PIASy through its MH2 domain and PIASy suppressed TGF- β -mediated activation of Smad3. PIASy also stimulated the sumoylation of Smad3 in vivo. Furthermore, endogenous PIASy expression was induced by TGF- β in Hep3B cells. These findings provide the first evidence that a PIAS family protein, PIASy associates with Smads and involves in the regulation of TGF- β signaling using the negative feedback loop.

INTRODUCTION

Transforming growth factor- β (TGF- β) is a member of the TGF- β superfamily, which also includes the activins/inhibins, bone morphogenetic proteins (BMPs), and other members, such as Müllerian inhibiting substance and Lefty (1). These factors regulate growth, differentiation, apoptosis, migration, and secretion of important molecules, such as components of the extracellular matrix, adhesion molecules, hormones, and cytokines in a variety of cell types, affecting morphogenesis, tissue repair, tumor suppression, and immunoregulation (1). TGF- β family also plays important roles in various pathological conditions, ranging from abnormal tissue repair state to cancer development (1, 2).

TGF- β signaling is mediated through transmembrane receptors located at the cell surface (T β Rs) that are serine/threonine kinases, which in turn use the highly conserved members of the Smad (Sma and MAD-related protein) family of transcription factors to transduce their signals to the nucleus (3). Two of the receptor-regulated Smads (R-Smads), Smad2 and Smad3, transduce signals for TGF- β . On the other hand, Smad4 is a common mediator (Co-Smad) and acts as a heterodimeric partner for Smad2 and Smad3 for efficient DNA binding and transcriptional activation (4, 5). When T β Rs are activated by the binding of their cognate ligands, Smad 2 and Smad3 are phosphorylated by the type I receptor (T β R-I) serine-threonine kinase. Phosphorylated Smad2 and Smad3 then form stable hetero-complexes with Smad4 that translocate into the nucleus and activate transcription.

Inhibitory Smads, such as Smad6 and Smad7, bind activated T β RI, thereby preventing phosphorylation of R-Smads (6, 7, 8). In addition, Smad7 interacts with the E3-ubiquitin ligases Smurf1 or Smurf2 in the nucleus and induces ubiquitination and degradation of the

TGF- β receptors (9, 10). Transcriptional activation by Smads partly requires the aid of nuclear coactivators such as CBP and p300 (11, 12). Conversely, transcriptional repression is effected by interaction with nuclear corepressors such as SnoN and c-Ski (13, 14). These findings indicate that Smad7 regulate intensity and/or duration of TGF- β signaling by interaction with the several molecules, and it could be possible other signaling regulatory molecule(s) may co-operate with Smad7.

In attempt to identify novel Smad7 partners, we screened a mouse embryo cDNA library with yeast two-hybrid system using the MH2 domain of Smad7 as bait. Here, we identify PIASy, which is one of the protein inhibitor of activated STAT family protein, as a protein that interacts specifically with Smad7. 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 β , x γ and y) have been reported (15). PIAS1 and PIAS3 were initially cloned as transcriptional repressors of the Jak-STAT signaling pathway (16, 17). (PIAS3) was originally identified as a specific corepressor of signal transducer and activator of transcription 3 (STAT3)(17). 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 (16). PIAS family proteins also function as a transcriptional cofactor for nuclear receptors (18, 19, 20). Recently, PIAS family proteins have been proposed to function as a small ubiquitin-related modifier (SUMO)-E3 ligase (21). PIAS1 and PIASy were shown to catalyze sumoylation of p53 and LEF-1, respectively (22, 23, 24). PIAS family proteins have RING finger-like domain, and their SUMO-E3 ligase activities are dependent on this domain (21).

We find that PIASy interacts with other Smads including Smad3 and antagonizes Smad3-dependent transcriptional activation by T β R-I. Coexpression of PIASy with Smad3 results in the modification of Smad3 with SUMO, suggesting that PIASy functions as a SUMO-E3 ligase for Smad3. We also show that expression of PIASy is induced by TGF- β . Our findings provide additional mechanisms of the negative regulation of TGF- β signaling by PIASy, which may be due to the sumoylation of Smad3 by PIASy using the negative feedback loop.

EXPERIMENTAL PROCEDURES

Reagents and antibodies

Human recombinant TGF- β 1 was purchased from Strathmann Biotech GmbH (Hamburg, Germany). MG132 was purchased from Peptide Institute (Osaka, Japan). Expression vectors, FLAG-tagged Smad2, Smad3, Smad4, Smad6, Smad7, and a series of Smad3 mutants (25) were kindly provided by Dr. K. Miyazono (Tokyo Univ., Tokyo, Japan). T β R-I (T204D), p3TP-LUC (26), Myc-tagged human PIASy, HA-tagged PIASy, human PIASX β (27) in pcDNA3 were kindly provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY), Dr. H. Ariga and Dr. T. Taira (Hokkaido Univ., Sapporo, Japan) respectively. Anti-HA and anti-Myc, antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Sigma (St Louis, MO). Anti-SUMO-1 antibody was purchased from Medical & Biological Laboratories (Nagoya, Japan).

Construction of fusion proteins with Smad7 or Smad6 MH2 domain and the Gal 4 DNA binding domain

Full-length mouse Smad7 and Smad6 cDNAs were obtained from Dr. K. Miyazono (Tokyo Univ., Tokyo, Japan). To generate a bait construct, with the MH2 domain of Smad7 and Smad6, polymerase chain reaction (PCR) was used to amplify the portion of the cDNA encoding amino acid residues 260-427 for Smad7 and residues 331-496 for Smad6 (primer sequences are available upon request). The PCR product was digested with EcoRI and XhoI and inserted into pGBKT7 digested with EcoRI and Sall (downstream of the Gal4

activation domain). All constructs were sequenced to verify integrity of the constructs.

Yeast two-hybrid screen

Gal4-Smad7 MH2 was constructed by fusing the Smad7 (residues 260-427) coding sequence in-frame to the Gal4 DNA-binding domain in the pGBKT7 vector as described the above. *Saccharomyces cerevisiae*, strain AH109 cells transformed with pGal4-Smad7 MH2, followed by mating with a pretransformed mouse 11-day embryo MATCHMAKER cDNA library in Y187 cells (Clontech, Palo Alto, CA), were plated onto media that lacked tryptophan, leucine and histidine and had been supplemented with 5 mM 3-amino-1, 2, 4-triazole (Sigma, MO). Approximately 3×10^7 colonies were screened for growth in the absence of histidine. Plasmid DNAs derived from positive clones were extracted from yeasts, and sequenced. Clones were re-introduced into yeast strain AH109 along with either empty pGBKT7, pGBKT7-Smad7 MH2, pGBKT7-Smad6 MH2 (residues 331-496) to verify the Smad /clone interaction.

Cell culture, transfection, and luciferase assays

Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol (28). Human hepatoma cell line Hep3B was maintained in DMEM containing 10% FCS (29). Before stimulation, the cells were cultured for 12 h in DMEM containing 1% FCS followed by treatment with TGF- β (30, 31). Hep3B cells ($2-2.5 \times 10^5$ 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 (28). The cells were harvested 48 h after transfection and lysed in 100 μ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β -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 (28). Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M sodium orthovanadate, 1 μ M phenylmethylsulfonyl fluoride and 10 μ g/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).

Sumoylation assay

Human 293T cells were transiently transfected with the indicated vectors and at 36 h after transfection, the cells were treated with 10 μ M MG132 (Peptide Institute, Osaka, Japan) for 4 h to inhibit proteasomal degradation of Smad3. Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M sodium orthovanadate, 1 μ M phenylmethylsulfonyl fluoride, 10 μ M MG132 and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). Western blotting was performed as described the above.

Indirect Immunofluorescence

Monkey COS7 cells were maintained in DMEM containing 10% FCS transfected with FLAG-Smad3 and Myc-tagged PIASy together with T β R-I (T204D) by the calcium phosphate precipitation protocol. 48 h after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with a mouse anti-anti-FLAG antibody (M2, Sigma) or with a rabbit anti-HA antibody (MBL). The cells were then reacted with an FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (CHEMICON, Temecula, CA) and observed under a confocal laser fluorescent microscope.

Northern blot and RT-PCR analysis

After 12 h of incubation in 1% FCS, Hep3B cells were treated with TGF- β (100U/ml) for the indicated time. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in Northern analysis according to the established procedures (29). 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 PIAS3 and G3PDH primers were used as described previously (30, 31).

RESULTS

Identification of PIASy as an interaction partner of Smads

To identify proteins that could be involved in the negative regulation of TGF- β signaling, we screened a mouse 11-day embryo cDNA library using the MH2 domain of mouse Smad7 as bait. Several Smad7 MH2-interacting proteins were identified from a screening of approximately 3×10^7 yeast transformants. DNA sequencing analysis revealed that one of the positive clones that interacted specifically with Gal4 DNA binding domain-fused Smad7 MH2 was identical with a member of the protein inhibitor of activated STAT family PIASy that contains a 144 amino acid insertion (residue 1-144) in the N-terminal region. To demonstrate specificity of binding, the plasmid was isolated from the positive two-hybrid clone and introduced back into *S. cerevisiae* along with either Smad7 MH2 domain or Smad6 MH2 domain fused to the DNA binding domain of Gal4 or empty vector (Gal4 DNA binding domain alone). Neither Smad7 MH2 domain nor Smad6 MH2 domain resulted in activation of the reporter genes (data not shown). After mating the indicated yeast, growth occurs only in the presence of either Smad7 MH2 or Smad6 MH2 (Fig. 1A), demonstrating that PIASy interacted with the MH2 domain of both Smad7 and Smad6 in this assay.

To investigate the association of PIASy with other Smads including Smad7 in vivo, 293T cells were transfected with either FLAG -tagged Smad2, Smad3, Smad4, Smad6 or Smad7 together with Myc-tagged PIASy. As shown in Fig. 1B, comparable amounts of Smads were expressed in each cell lysate. Similarly, PIASy was expressed well in samples containing Smads. Western blot analysis of associated proteins with an anti-FLAG antibody revealed that PIASy interacts with all Smads in 293T cells, although the PIASy-Smad4

interaction was weak. As shown in Fig 1A, the MH2 domain of the inhibitory Smad, Smad6 and Smad7 interacted with PIASy. We then examined whether PIASy interacts with Smad3 via the similar domain, using either N- or C-terminal deletion mutants of Smad3 (25) (Fig. 4A). Expression vectors encoding Myc-tagged PIASy and/or FLAG-tagged full-length Smad3 or one of its four deletion mutants were transiently transfected into 293T cells. Cells were lysed, and subjected to immunoprecipitation with an anti-Myc antibody. Immunoprecipitates were then used in Western blot analysis with an anti-FLAG antibody. As shown in Figure 1B, whereas the full-length Smad3 interacted with PIASy, the C-terminal deletion mutants lacking the MH2 or L+MH2 domains were unable to bind PIASy. In contrast, the N-terminal mutants in which MH1 or MH1+L domains are deleted retained interactions with PIASy. These results indicate that efficient PIASy-Smad3 interactions require the MH2 domain of Smad3.

Colocalization of PIASy and Smad3

To determine the subcellular localization of PIASy and activated Smad3, expression vectors for Myc-tagged PIASy and FLAG-tagged Smad3 together with a constitutively active form of TGF- β type I receptor, T β R-I(T204D), which activates Smad3, were transfected into COS7 cells. Co-expression of T β R-I(T204D) together with Smad3 in COS7 cells resulted in a maximal Smad3-mediated transcription (data not shown). 48 h after transfection, the cells were stained with anti-Myc and anti-FLAG antibodies, and they were visualized with rhodamine and FITC-conjugated secondary antibodies, respectively, under a confocal laser microscope (Fig. 2A-C). Smad3 is previously shown to tend to translocate spontaneously into nucleus and observed as a diffuse pattern in cells (32, 33).

Active Smad3 was localized mainly in the nucleus with a slightly diffuse pattern (Fig. 2A). In the previous study, PIASy was shown to localize predominantly to punctate structures in the nucleus (21). As shown in Fig. 2B, PIASy was tightly localized to punctate structures in the nucleus. Our results also showed that both PIASy and a portion of activated Smad3 were located in the nucleus, and they were found to be co-localized in a characteristic nuclear dot structure after demonstration of the merged figure, in which the red and green colors turned yellow (Fig. 2C). These results suggest that both activated Smad3 and PIASy co-localize in the nucleus within dotted structures.

Repression of TGF- β signaling by PIASy

To examine the functional relevance of the Smads/PIASy interaction in the context of TGF- β signaling pathway, we performed the transient transfection assay using a human embryonic kidney carcinoma cell line, 293T. The TGF- β -mediated transcriptional responses were measured by p3TP-LUC, which is one of the standard reporters for assessing TGF- β activity (26). In these experiments, a constitutively active form of TGF- β type I receptor, T β R-I(T204D) (26), was used which stimulated p3TP-LUC more effectively than TGF- β plus wild-type T β R-I in 293T cells (31). When 293T cells were transfected with p3TP-LUC together with an expression vector for T β R-I(T204D), LUC expression was increased by 5-6-fold (Fig. 3A). The additional expression of Smad3 enhanced p3TP-LUC activity, whereas additional expression of Smad7 inhibited p3TP-LUC in a dose-dependent manner. We then examined the effect of PIASy expression on TGF- β signaling in this model system. When 293T cells were transfected with an expression vector for PIASy, T β R-I(T204D)/Smad3 and p3TP-LUC, PIASy dramatically suppressed T β R-I(T204D)/Smad3-induced p3TP-LUC activity in a dose dependent manner

(Fig. 3A, B). The Smad7-mediated suppression of T β R-I(T204D)-induced p3TP-LUC activity was further stimulated by PIASy expression, which was reversed by the additional Smad3 expression (Fig. 3C). These results indicate that the inhibitory effects of PIASy on T β R-I(T204D)-induced transcriptional activity is mediated by the direct interaction between PIASy and Smad3. Furthermore, expression of PIASX β , another member of PIAS family, had no effect on T β R-I(T204D)/Smad3-induced p3TP-LUC activity (Fig. 3D).

We then tested the domain(s) of Smad3 that is involved in the inhibition of TGF- β signaling by PIASy. As shown in Fig. 3E, expression of PIASy significantly reduced T β R-I(T204D)/Smad3-induced p3TP-LUC activation. Expression of N-terminal deletion mutants of Smad3, but not the C-terminal deletion mutants of Smad3 lacking the MH2 domain, largely reversed the PIASy-mediated inhibition of p3TP-LUC expression. These results also suggest that the MH2 domain of Smad3 mediates the inhibition of TGF- β signaling by PIASy.

PIASy mediates SUMO-1 modification of Smad3

One of the possible mechanisms that is consistent with the data described above is that there is post-translation modification or degradation of Smad3. To test the possibility whether PIASy-mediated inhibition of T β R-I(T204D)/Smad3-induced p3TP-LUC activation was regulated through a specific proteolytic pathway, the proteasome-specific inhibitor MG132 was added to transfected cells at concentrations of 5 and 20 μ M for 16 h. Interestingly, the results show that the proteasome-specific inhibitor MG132 could block PIASy-mediated inhibition of T β R-I(T204D)/Smad3-induced p3TP-LUC activation

(Fig. 4A). In previous studies, PIASy were shown to catalyze sumoylation of p53 and LEF-1 as SUMO E3 ligase and modify their transcriptional activation (22, 23, 24). To examine whether PIASy stimulates SUMO modification of Smad3, we transiently expressed FLAG-tagged Smad3, SUMO-1 and Myc-tagged PIASy in 293T cells. The cell extract was subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates produced were probed by Western blotting with anti-SUMO-1 or anti-FLAG antibody (Fig. 4B). A strong band with slower mobility than that of intact Smad3 was detected by anti-SUMO-1 antibody in the case of co-transfection with Smad3, SUMO-1 and PIASy expression vectors. These results suggest that Smad3 is conjugated with SUMO-1, the conjugation being mediated by PIASy. However, we could not detect any significant decrease in Smad3 content when we expressed both Smad3 and PIASy in 293T cells (Fig. 4B). This may suggest that other factors in a MG132-sensitive proteasome pathway like Smad7-associated Smurfs (9, 10) are also involved in PIASy-mediated inhibition of T β R-I(T204D)/Smad3-dependent transcription.

PIASy inhibits TGF- β -induced Smad3 activation in Hep3B cells

To examine the effect of PIASy on TGF- β signaling pathway under more physiological conditions through endogenous proteins, we first utilized a TGF- β -responsive, human hepatoma cell line, Hep3B (30), and the transient transfection assay. Hep3B cells were transfected with p3TP-LUC together with empty vector or PIASy, and treated with TGF- β and LUC activities were determined. As shown in Fig. 5A, PIASy expression showed a significant decrease of TGF- β -stimulated p3TP-LUC activation in Hep3B cells.

We next examined whether PIASy expression is regulated by TGF- β in Hep3B cells. Cells were either left untreated or treated with TGF- β , and PIASy expression was monitored by Northern analysis. Interestingly, PIASy expression was remarkably induced by treatment of TGF- β in Hep3B cells (Fig. 5B). The level of PIASy mRNA expression increased 6-fold at 6 h, 8-fold at 12 h, and did not alter until 24 h. When we monitored the same RNA samples by RT-PCR, TGF- β treatment induced Smad3-mediated PAI-1 expression, although expression level of PIAS3 was not changed by TGF- β stimulation. These results demonstrated that expression of PIASy but not PIAS3 is induced by TGF- β and affects its signaling in Hep3B cells. However, the almost maximal PAI-1 expression by TGF- β was detected at 3 h, although a strong PIASy expression by TGF- β was detected at 6 h, suggesting that PIASy may be involved in the late phase regulation or longer term refractoriness in response to TGF- β . Furthermore, we tested the effect of PIASy on endogenous PAI-1 expression by TGF- β . Hep3B cells were transfected with empty vector or PIASy, and treated with TGF- β and PAI-1 expression was monitored by RT-PCR. As shown in Fig. 5C, when cells were transfected with PIASy, endogenous PAI-1 expression was decreased by 50 % compared with that of empty vector. These results suggest that PIASy is induced by TGF- β and inhibits TGF- β /Smad3-mediated gene expression in Hep3B cells.

DISCUSSION

Dysregulation of the TGF- β signaling have been associated with a variety of clinical disorders including some cancers, renal disease, and vascular disease (2, 34). Although mutations in various components of the TGF- β pathway may account for some of these abnormalities, it has been shown that TGF- β signaling can be strongly affected by interactions with other molecules in the cell. Recent studies have documented the interaction of a large number of intracellular proteins with the effector molecules Smads to influence TGF- β signaling (35). Whereas some of these proteins have been found to functionally cooperate with and activate Smads, others were found to repress Smad activity. Several molecular mechanisms have been proposed for the inactivation of Smad/TGF- β signaling pathway. For example, TGF- β signaling is inhibited by IFN- γ (36) and tumor necrosis factor (TNF)- α (37), which induce the expression of an inhibitory Smad, Smad7. In addition, the zinc finger protein Evi-1 interacts with Smad3 and represses its DNA binding activity (38), whereas the nuclear Ski and SnoN oncoproteins have been reported to inhibit TGF- β signaling by recruitment of the transcriptional repressor N-CoR to TGF- β -responsive promoters through interaction with Smad proteins (39, 40, 41). Smad2/3 interacts with Ski through its C-terminal MH2 domain in a TGF- β -dependent manner. We also demonstrated that estrogen receptors suppress TGF- β signaling by associating with, and acting as a transcriptional co-repressor for Smad3 (42). Ubiquitin-ligases, Smurf1 and Smurf2 bind to Smad7 and are recruited to the activated T β R-I by Smad7, leading to proteasomal degradation of the receptor (9, 10). Another protein called STRAP, originally identified as a T β RI-interacting protein, also associates with Smad7 (43). STRAP recruits

Smad7 to activated T β R-I, and stabilizes the Smad7-T β R-I association, preventing R-Smad phosphorylation by T β R-I and subsequent intracellular signaling. A recently identified Smad7-interacting protein Yes-associated protein, YAP65 functions as an inhibitor of TGF- β signaling in a manner similar to that of STRAP (44).

SUMO modification proceeds by a three-step enzyme shuttle analogous to ubiquitin addition (45). For ubiquitin, an ATP-dependent activation step couples ubiquitin by a thiolester bond to the E1, ubiquitin-activating enzyme. In turn, ubiquitin is transferred to the reactive cysteine of one of several E2 ubiquitin-conjugating enzymes. Typically, an E3 ubiquitin ligase combines with the charged E2 to facilitate formation of an isopeptide bond between ubiquitin and the target protein (21). E3s typically use protein-protein interaction domains to bind to and select specific targets and either a zinc-binding RING finger domain or a HECT domain to stimulate polyubiquitin chain formation. Recently, four members of the mammalian PIAS (protein inhibitor of activated STAT) family, PIAS1, PIASx, PIASy and PIASz, 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 (21-24).

In this study, we demonstrated that one of PIAS family protein, PIASy interacts with other Smads including Smad3 and antagonizes Smad3-dependent transcriptional activation by T β R-I. Coexpression of PIASy with Smad3 results in the modification of Smad3 with SUMO-1, suggesting that PIASy functions as a SUMO-E3 ligase for Smad3. Recent studies showed that Smad4 but not Smad1 and Smad3 associates with Ubc9 and is modified by SUMO-1(46, 47, 48). Interestingly, sumoylation of Smad4 resulted in the activation of TGF- β signaling, suggesting sumoylation might modulate TGF- β signaling positively or

negatively in cells (46, 47, 48). We also show that expression of endogenous PIASy is induced by TGF- β . Our findings provide a novel mechanism of the negative regulation of TGF- β signaling by PIASy, which may be due to the sumoylation of Smad3 by PIASy using the negative feedback loop. More detailed understanding of the cross-talk between Smads and PIASy is therefore important as this new information may provide new therapeutic approaches for the TGF- β -mediated pathological conditions.

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FOOTNOTES

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2) The abbreviations used are: PIAS, protein inhibitor of activated STAT; STAT, signal transducers and activators of transcription; TGF, transforming growth factor; LUC, luciferase; T β R-I, TGF- β type I receptor; Smad, Sma and MAD-related protein; SUMO, small ubiquitin-related modifier

FIGURE LEGENDS

Fig. 1. **Physical interactions between PIASy and Smads.**

(A) Interaction between Smads and PIASy in a yeast two-hybrid assay. Growth of transformed *S. cerevisiae* demonstrating an interaction between either Smad7 MH2 or Smad6 MH2 and PIASy. pGBKT7-Smad7 MH2, pGBKT7-Smad6 MH2, pGBKT7-p53, or empty pGBKT7 in AH109 were mated with pACT2-PIASy, pACT2-SV40 T-antigen (T) or empty pACT2 in Y187 as indicated. Colonies were then re-streaked onto high-stringency plates as described under EXPERIMENTAL PROCEDURES.

(B) 293T cells (1×10^7) were transfected with Myc-tagged PIASy (15 μ g) and/or FLAG-tagged Smad2, Smad3, Smad4 Smad6, Smad7 (15 μ g). Cell lysates were then immunoprecipitated with 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).

(C) Domain structure of Smad3 and mutant fragments are schematically shown. MH and L indicate Mad homology domain and linker domain, respectively.

(D) 293T cells (1×10^7) were transfected with a series of FLAG-tagged Smad3 mutants (15 μ g) and Myc-tagged PIASy (15 μ 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 (lower panel). The asterisks indicate the migration position of the full-length Smad3 or deletion mutants.

Fig. 2. Colocalization of activated Smad3 with PIASy in the nucleus.

COS7 cells were cotransfected with FLAG-tagged Smad3 and Myc-tagged PIASy together with T β R-I (T204D) by the calcium phosphate precipitation protocol. 48 h after transfection, cells were fixed, reacted with an anti-Myc polyclonal antibody and an anti-FLAG monoclonal antibody, and visualized with a rhodamine-conjugated anti-rabbit antibody (A) and an FITC-conjugated anti-mouse antibody (B). These figures were merged (C).

Fig. 3. PIASy inhibits TGF- β -mediated transcription.

(A) 293T cells (12-well plate) were transfected with p3TP-LUC (0.3 μ g) and/or T β R-I (T204D) and Smad3 or Smad7 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 p3TP-LUC (0.3 μ g) and/or T β R-I(T204D)(100ng) and Smad3 (30ng) and/or the indicated amounts of PIASy. 48 h after transfection, LUC activities were determined.

(C) 293T cells (12-well plate) were transfected with p3TP-LUC (0.3 μ g) and/or T β R-I(T204D)(100ng) and Smad7(30ng) and/or the indicated amounts of PIASy in the absence or the presence of Smad3(100 or 300ng). 48 h after transfection, LUC activities were determined.

(D) 293T cells (12-well plate) were transfected with p3TP-LUC (0.3 μ g) and/or T β R-I(T204D) (100ng) and Smad3 (30ng) and/or the indicated amounts of PIASX \square . 48 h after transfection, LUC activities were determined.

(E) 293T cells (12-well plate) were transfected with p3TP-LUC (0.3 μ g) and T β R-I(T204D) (100ng) and Smad3 (30ng) and/or PIASy (100ng), together with various doses (30 to 100ng) of a series of Smad3 mutants 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.

Fig. 4. PIASy mediates SUMO-1 modification of Smad3.

(A) 293T cells (12-well plate) were transfected with p3TP-LUC (0.3 μ g) and T β R-I(T204D) (100ng) and Smad3 (30ng) and/or PIASy (100ng), in the absence or presence of the increasing amounts of MG132. 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.

(B) 293T cells (1×10^7 cells) were transiently transfected with expression vectors containing FLAG-tagged Smad3(15 μ g), SUMO-1(10 μ g) and HA-tagged PIASy (15 μ g) as indicated. 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and immunoblotted with anti-SUMO-1 antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-HA antibody (lower panel). IgH, heavy chain of immunoglobulin.

Fig. 5. PIASy inhibits Smad3-mediated transcription and its expression is induced by TGF- β in Hep3B cells.

(A) Hep3B cells (6-well plate) were transfected with p3TP-LUC (1 μ g) together with empty vector or PIASy (3 μ g). 48 h after transfection, cells were stimulated with TGF- β (100 U/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.

(B) TGF- β -induced PIASy expression in Hep3B cells. 20 μ g of total RNA isolated from these cells 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 PAI-1 (upper panel), PIAS3 (middle panel) and G3PDH (lower panel) primers. RT-PCR products were separated on a 1% agarose gel.

(C) Hep3B cells (6-well plate) were transfected with empty vector or PIASy (3 μ g). 48 h after transfection, cells were stimulated with TGF- β (100 U/ml) for an additional 12h. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using PAI-1 (upper panel) and G3PDH (lower panel) primers. RT-PCR products were separated on a 1% agarose gel.