The tumor suppressor, Smad4/DPC4, is a common signal transducer in transforming growth factor-β (TGF-β) signaling. In this study, we demonstrated that the protein inhibitor of activated STAT1 (PIAS1) regulates the signaling potential of Smad4 through a sumoylation-dependent mechanism. PIAS1 was shown to be an E3 ligase for Smad4 sumoylation in vitro and in vivo. PIAS1 physically interacted with Smad4 in a TGF-β-inducible manner. A minimal SUMO E3 ligase domain and Smad4-binding domain were defined on PIAS1 protein. The RING finger domain of PIAS1 was essential for its E3 ligase function. Although PIAS1 enhanced the Smad4-dependent transcriptional activation of TGF-β signaling, a mutant lacking the RING domain inhibited the sumoylation of Smad4 in a dominant negative manner and, as a result, abolished the transcriptional response of TGF-β. These data demonstrate that PIAS1 protein positively modulates TGF-β responses as a SUMO E3 ligase for Smad4.

Substrates of SUMO conjugation often contain a tetrapeptide motif, i.e., ψKX(D/E) (where ψ is hydrophobic and X is any amino acid). Structural analysis of the complex between Ubc9 and its substrate, RanGAP1, demonstrates clearly how the motif is specifically recognized by Ubc9 and elucidates nicely why RanGAP1 sumoylation may not depend on an E3 ligase (20). However, more recent reports support the presence of E3 enzymes for sumoylation (21–24). In particular, a family of SUMO E3 ligases, termed protein inhibitors of activated STAT (PIAS), has recently been discovered (21, 22). The PIAS protein family consists of five members: PIAS1, PIAS3, PIASα, PIASβ, and PIASγ. PIAS1 and PIAS3 were originally shown to interact with STAT1 and STAT3, respectively, and inhibit their functions (25, 26). Recently other PIAS proteins have been shown to interact as coregulators for nuclear receptors (27–32).

The first demonstration of a mammalian PIAS protein as a SUMO E3 ligase was shown in the involvement of PIAS1 in p53 sumoylation (33). PIAS proteins resemble the RING-class ubiquitin E3 ligases and bind to Ubc9 through the RING finger domain (34). The RING finger domain is highly conserved among PIAS proteins and is supported by the observation that, similar to PIAS1, PIASα also promotes the sumoylation of CtBP (12), p53, and e-Jun (35). Although PIASα binds to the androgen receptor through the RING domain (29, 31), PIAS1 binds to p53 independently of the RING (35). Amid the increasing numbers of transcription factors that interact with PIAS proteins, it is believed that the coregulator function of PIAS proteins could operate, at least partly, through PIAS-mediated sumoylation of the transcription factors (18, 19).

Recently our laboratory and others revealed that Smad4 is a sumoylated protein (8, 36, 37). It appears that Smad4 sumoylation may involve the function of a PIAS protein (37, 38). Furthermore, PIASy also regulates TGF-β signaling through its interaction with Smad3 (39, 40). Yet, whether PIAS proteins act as SUMO E3 ligases for Smads has not been resolved in a well defined system. To understand the role of PIAS proteins in TGF-β signaling, we determined the in vivo and in vitro function of PIAS1 and other family members in Smad4 sumoylation, the physical association of PIAS1 and Smad4, and the physiological consequence in TGF-β-induced transcription. We delineated the minimal requirement for the SUMO E3 ligase activity and Smad4-binding activity of PIAS1 protein. Interest-

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The ubiquitin/proteasome pathway controls the turnover of products of many oncogenes and tumor suppressor genes and thus plays a major role in cancer development (1–5). Recently, a number of ubiquitin-related proteins have also been found in eukaryotic cells. These proteins, including the small ubiquitin-like modifier 1 (SUMO1), utilize a conjugation system that is similar to ubiquitination (6, 7). In particular, SUMO is activated in an ATP-dependent manner by an E1 enzyme consisting of the Aos1/Uba2 heterodimer and then transferred to the SUMO-conjugating E2 enzyme Ubc9 and subsequently ligated to the substrate. In contrast to ubiquitination, SUMO1 modifications of target proteins do not promote their degradation. Furthermore, SUMO1 modification reduces ubiquitination of tumor suppressor Smad4 (8) and may also preclude ubiquitination of IκB (9). The functional consequences of SUMO1 modification vary depending on the target. For example, SUMO1 conjugation is necessary for RanGAP1 localization to the nuclear pore complex (10, 11), for nuclear import of the transcription corepressor CtBP (12), and for localization of promyelocytic leukemia to the nuclear bodies (13). For many other proteins, their covalent modifications by SUMO1 modulate their biological activities (7, 14–19).

Substrates of SUMO conjugation often contain a tetrapeptide motif, i.e., ψKX(D/E) (where ψ is hydrophobic and X is any amino acid). Structural analysis of the complex between Ubc9 and its substrate, RanGAP1, demonstrates clearly how the motif is specifically recognized by Ubc9 and elucidates nicely why RanGAP1 sumoylation may not depend on an E3 ligase (20). However, more recent reports support the presence of E3 enzymes for sumoylation (21–24). In particular, a family of SUMO E3 ligases, termed protein inhibitors of activated STAT (PIAS), has recently been discovered (21, 22). The PIAS protein family consists of five members: PIAS1, PIAS3, PIASα, PIASβ, and PIASγ. PIAS1 and PIAS3 were originally shown to interact with STAT1 and STAT3, respectively, and inhibit their functions (25, 26). Recently other PIAS proteins have been shown to interact as coregulators for nuclear receptors (27–32).

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PIAS1 Is a Smad4-SUMO E3 Ligase

PIAS1 formed a TGF-β-inducible complex with Smad4. PIAS1 promoted sumoylation of Smad4 both in vitro and in vivo. Accordingly, PIAS1 profoundly stimulated TGF-β-dependent transcription. Deletion or point mutations in the RING domain of PIAS1 inhibited TGF-β-induced reporter gene expression in a dominant negative manner. Therefore, PIAS-mediated sumoylation plays an important role in SMAD-dependent TGF-β signaling.

EXPERIMENTAL PROCEDURES

Molecular Cloning—Mammalian expression plasmids for epitope (HA, FLAG-, or His)-tagged Smad4 and SUMO1 have been described previously (8, 36). PIAS1 was obtained by PCR and cloned into the EcoRI (at the 5’ end) and SalI (at the 3’ end) in pXPF2F or pXPF3H, which are derived from pRK5 for the generation of N-terminally FLAG-tagged or HA-tagged proteins, respectively. Deletion mutants of PIAS1 containing aa 2–480, 121–480, 200–480, and 300–480, as well as point mutations at Cys-345, Cys-350, and Cys-355 into Ser residues were also obtained by PCR and similarly cloned into the pXPF3H vector. Details of the primer sequences and cloning strategies can be obtained upon request.

The bacterial expression plasmid for glutathione S-transferase (GST)-tagged Smad4 has been described previously (45). Plasmids for GST-PIAS proteins were constructed by transferring the inserts containing the PIAS coding region from mammalian plasmids into pGEX2TK (Ammersham Biosciences). Recombinant GST fusion proteins were produced in the Escherichia coli BL21 strain and purified by glutathione-Sepharose beads (Ammersham Biosciences). For in vitro sumoylation, recombinant Smad4 was separated from the GST tag by thrombin (0.2 units/μl) cleavage in phosphate-buffered saline. We observed that GST-Smad4 fusion protein is a poor substrate for in vitro sumoylation.

Ni-NTA Precipitation, Immunoprecipitation, and Western Blot—For Ni-NTA precipitation, HeLa cells were transfected with expression plasmids for His-tagged Smad4, FLAG-tagged SUMO1, and HA-PIAS as specified in the text and/or figures. Forty-eight h after transfection, the cells were harvested and sonicated in guanidine lysis buffer (6 M guanidinium HCl, 0.1 M NaPO₄, 0.01 M Tris-Cl, pH 8). His-tagged Smad4 was then immobilized on Ni-NTA beads (Qiagen) and, after extensive washing, eluted in elution buffer (200 mM imidazole, 5% SDS, 0.15 M Tris, pH 6.7, 30% glycerol, 0.72 μM β-mercaptoethanol). Eluted proteins were separated by SDS-PAGE, transferred onto nitrocellulose (Schleicher & Schuell), and detected with anti-His or anti-Smad4 antibody (Santa Cruz Biotechnology) for the Smad4 protein and anti-FLAG (Sigma) or anti-SUMO1 antibody (Zymed Laboratories Inc.) for the SUMO-conjugated Smad4 protein. Antibody-bound proteins were visualized by horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence (Pierce).

For Smad4-PIAS interactions using immunoprecipitations, HeLa cells were transfected with expression plasmids for FLAG-Smad4 and HA-PIAS. Forty-eight h after transfection, the cells were harvested in FLAG lysis buffer (46). To detect PIAS-bound Smad4 and to retrieve PIAS proteins, the cell lysates were subjected to immunoprecipitation using anti-HA antibody (12CA5, Roche Applied Science), and then to detect PIAS1-bound Smad4 (as similarly described (12)), the cell lysates were subjected to Western blotting using anti-FLAG antibody (M2, Sigma).

In Vitro Sumoylation—In vitro sumoylation assay was performed as described by Lin et al. (12). Recombinant SUMO1, Uba2/Aos1, and Ubc9 were produced from E. coli as described as GST proteins in Pichler ly et al. (23) in a typical sumoylation reaction, 20 ng of SUMO1, 20 ng of Uba2/Aos1, 10 ng of Ubc9, 100 ng of substrate (Smad4 or RanGAP), and PIAS1 (100 ng or as specified in the text/figures) were incubated for 30 min at 30 °C in the presence of ATP. SUMO conjugates were separated on SDS-PAGE and analyzed by Western blotting using anti-SUMO or anti-Smad4 antibodies.

GST in Vitro Binding and Pull-down Assays—For the in vitro binding assay, 35S-labeled PIAS proteins, produced in vitro by using a T7 kit (Promega), were incubated with 1 μg of GST or GST-Smad4 as indicated in the text. GST-Smad4-bound PIAS was retrieved by using glutathione-Sepharose beads at 4 °C for 1 h. After extensive washing with GST fusions and binding proteins in 25 mM Tris-Cl, pH 8, 300 mM NaCl, and 1% Triton X-100, Smad4-bound PIAS was separated by SDS-PAGE and visualized by autoradiography. PIAS1-mediated pull down of full-length protein or domains was similarly done.

For PIAS1 pull down of endogenous Smad4, exponentially growing HaCaT cells were treated with 400 pm TGF-β (2 h) and harvested in cell lysis buffer containing 25 mM Tris-Cl, pH 8, 300 mM NaCl, and 0.5% Triton X-100. The lysates were precleared with 10 μg of GST protein (on beads) for 1 h at 4 °C, incubated with 2 μg of glutathione-Sepharose-bound GST-PIAS1 for 2 h at 4 °C, washed four times with the lysis buffer, and then analyzed by anti-Smad4 Western blotting.

Transcription Reporter Assays—Plasmid SMAD-binding elements (SBE)-luc, which contains the luciferase gene under control of the SBE (41), was used to measure TGF-β-induced transcription. Transfections, TGF-β treatment, and reporter assays were carried out as described (8, 36). TGF-β-responsive HaCaT and Mv1Lu cells were transfected by using LipofectAMINE (Invitrogen). Generally, exponentially grown cells at 30% confluency were transfected with expression plasmids for PIASs and/or reporter plasmids. The amount of transfected DNA was always the same and the binding vector DNA whenever needed. 40–45 h after transfection, cells were treated with 200 pm TGF-β for 12 h. Cells were then harvested for measurement of luciferase and β-galactosidase activities. All assays were done in duplicate and repeated more than three times, and all values were normalized for transfection efficiency against β-galactosidase activity.
RESULTS

PIAS1 Acts as a SUMO E3 Ligase in Smad4 Sumoylation—We have recently discovered that Smad4 is sumoylated in vivo at two conserved lysine residues, Lys-113 and Lys-159 (36). We then decided to determine whether Smad4 is a direct substrate for the SUMO1 conjugation in a well-defined in vitro reconstitution experiment using purified recombinant proteins. The assay contained recombinant Smad4, SUMO1, Ubc9, and E1 enzyme Aos2/Uba2 heterodimer, and ATP. When Smad4 protein was incubated with different components of SUMO1, E1 or E2 enzyme alone, we observed that Smad4 was not SUMO1-modified (data not shown). Even when all components were added to the reaction mixture, no additional bands of Smad4 with slow mobility were observed (Fig. 1, lane 3). Conversely, the positive control RanGAP1 was efficiently sumoylated under the same conditions (lane 1). These data suggest that an additional cellular factor, such as an E3 ligase, may be critical for Smad4 sumoylation.

We then tested whether the recently identified SUMO E3 ligase PIAS1 could mediate SUMO1 modification on Smad4. PIAS1 has been shown to be an E3 ligase for CtBP (12), p53 (33) and c-Jun sumoylation (35). We found that in the presence of PIAS1, two bands of the higher molecular mass product (80–100 kDa) were observed by Western blotting using an anti-SUMO antibody. The higher molecular mass bands appearing in these in vitro modification reactions were further identified as Smad4-SUMO conjugates, because the same bands were recognized by an anti-Smad4 antibody. As shown in Fig. 1A, lower blot, the slow migrating Smad4 species represented SUMO1-conjugates (lane 4). In contrast, the K113R/K159R mutant was not sumoylated (Fig. 1A, lane 5; Fig. 1B, lane 8), confirming the in vivo data that Lys-113 and Lys-159 represent the sumoylation sites on the Smad4 molecule (36). Further analysis indicated the involvement of other PIAS proteins. PIASxβ, and to a lesser extent PIASγ, could promote sumoylation of Smad4 (data not shown). However, this study only focuses on the role of PIAS1.

Additionally, we conducted a dosage experiment for PIAS1 in Smad4 sumoylation. As shown in Fig. 1B, a gradual increase in PIAS1 added to the in vitro reaction mixture increased the amount of sumoylated Smad4. Consistent with our previous observations in vivo (8, 36), the two Smad4-SUMO conjugates represent a single modification on Lys-113 (the band of lower molecular mass) and Lys-159 (the band of higher molecular mass), respectively (Fig. 1B, lanes 6 and 7). Furthermore, an even more slowly migrating band could be occasionally detected, likely because of the double modification at both Lys-113 and Lys-159 (Fig. 1A, upper and lower blots). Together, data in Fig. 1 demonstrate that PIAS1 directly acts as an E3 ligase to allow SUMO1 modification of both Lys-113 and Lys-159 residues on Smad4.

PIAS1 Sumoylates Smad4 Dependent on Its RING Finger Domain—To further characterize the function of PIAS1 in Smad4 sumoylation, we determined whether the RING finger of PIAS1 is important for its E3 role toward Smad4 sumoylation. To this end, simultaneous point mutations at C345S, C350S, and C355S were introduced into PIAS1 protein (Fig. 2C, construct CS), and the effect of the mutations was tested in SUMO1 modification of Smad4 in vitro. As shown in Fig. 2A, the RING mutant failed to induce sumoylation of Smad4 (lanes 4 and 6), whereas Smad4-SUMO conjugate was observed in the presence of recombinant wild type PIAS1 protein (lanes 3 and 5). These data suggest that the functional RING domain of PIAS1 is required for its E3 ligase activity in Smad4 sumoylation.

FIG. 2. Mapping of the minimal E3 ligase domain of PIAS1. A and B, in vitro sumoylation of Smad4 by deletion mutants of PIAS1. In vitro sumoylation assay using recombinant proteins was carried out as in Fig. 1. 100 ng of recombinant Smad4 was added in lanes 2–6 in A and all lanes in B. C, schematic diagram of PIAS1 and deletion mutants. Minimal E3 ligase domain is indicated by a horizontal bracket. WT, wild type; IB, immunoblotting.
PIAS1 is essential for its E3 ligase function in Smad4 sumoylation.

Minimal E3 Domain of PIAS1 for Smad4 Sumoylation—PIAS1 is a polypeptide consisting of 650 amino acids. Besides its RING finger domain (aa 300–420), PIAS1 has a SAP domain at the N terminus that may mediate interaction with the nuclear matrix and a C-terminal domain with unknown functions. We were then interested in determining the minimal requirement of the PIAS1 sequences/domains for Smad4 sumoylation. Based on the requirement of RING domain in Smad4 sumoylation, we hypothesized that the minimal E3 ligase should at least contain the RING domain. For this purpose, a series of deletions were generated from the N and C termini of PIAS1 (Fig. 2C). Recombinant PIAS1 proteins containing these deletion mutations were produced in E. coli and purified using glutathione-Sepharose beads. The recombinant proteins were then tested for their ability to sumoylate Smad4 in vitro. As shown in Fig. 2A, deletion of the C-terminal domain (aa 481–650) did not affect its ligase function toward Smad4 sumoylation (Fig. 2A, lane 5). The mutant without the first 120 amino acids retained its ability to sumoylate Smad4, albeit less efficiently (Fig. 2B, lane 3 in comparison to lane 1). However, the RING domain alone was not sufficient to promote Smad4 sumoylation (Fig. 2B, lane 5). We also determined the essentiality of RING finger domain in the full-length and the shorter functional E3 ligase domain (aa 2–480). As expected, mutation in the RING finger (C345S/C350S/C355S) of PIAS1 caused the loss of its SUMO E3 ligase activity in Smad4 sumoylation (Fig. 2A, lanes 4 and 6). Take together, the minimal E3 ligase activity of PIAS1 is located in the region of aa 121–480, including the RING domain (summarized in Figs. 2C and 4C).

PIAS1 Promotes Smad4 Sumoylation in Vivo—After establishing the role of PIAS1 in Smad4 sumoylation in vitro, it was necessary and important to demonstrate its function in vivo. To this end, we transfected HeLa cells with an expression plasmid for PIAS1 and determined whether its overexpression could promote Smad4 sumoylation. As shown in Fig. 3A, although Smad4 was weakly modified with SUMO1 (lane 3), the sumoylation could be strongly stimulated with PIAS1 expression (lane 4). The increases in Smad4 sumoylation depended on the integrity of the E3 ligase function of PIAS1, as the RING mutant did not produce any effects on Smad4 sumoylation (Fig. 3B, compare lane 4 with 3).

Deletion mutants of PIAS1 were also tested for their potential stimulatory effects in Smad4 sumoylation in vivo. Similar to its ability to sumoylate Smad4 in vitro, the PIAS1 (Fig. 3B, 2–480) with the deletion of the entire C-terminal domain could efficiently promote sumoylation of Smad4 (Fig. 3B, lane 5), whereas PIAS1 (2–480CS) was unable to modify Smad4 (Fig. 3B, lane 6). The minimal E3 ligase aa 121–480 (shown in vitro, Fig. 2) had the ability to sumoylate Smad4, but its activity was significantly reduced (Fig. 3B, lane 7). Further deletion from the N-terminal end, but not into the RING domain, had a minimal effect (compare lanes 8 and 9 with 7). The deletion of the entire region after aa 316 (deletion of aa 317–650) completely removed the E3 ligase activity of PIAS1 toward Smad4 sumoylation (data not shown).

PIAS1 Contains Multiple Smad4-binding Sites in the Middle Region—The ability of PIAS1 to promote SUMO1 modification of Smad4 both in vitro and in vivo strongly supports the notion that PIAS1 is a SUMO E3 ligase for Smad4. Because an E3 ligase should have direct contact with its substrate, we anticipated a direct interaction between PIAS1 and Smad4. To determine whether the Smad4-PIAS1 interaction occurs in vitro, recombinant GST-Smad4 fusion protein were incubated with 35S-labeled PIAS1 protein generated by in vitro transcription and translation. We found that GST-Smad4 could pull down PIAS1 from the in vitro translation mixture (Fig. 4A, lane 1). In contrast, PIAS1 did not bind to GST protein (data not shown). The findings indicate a direct and specific interaction of PIAS1 with Smad4.

We next mapped the domains of PIAS1 (as in vitro translated product) for their ability to interact with recombinant GST-Smad4. Deletions from both N or C terminus of PIAS1 suggest that both N-terminal (aa 2–120) and C-terminal regions (aa 421–650) are dispensable for Smad4 binding (Fig. 4A, lanes 5 and 6). Interestingly, further deletion analysis of the middle region revealed the presence of multiple Smad4-binding sites. Both aa 121–200 and aa 200–300 independently interacted with Smad4 (Fig. 4A, lanes 8 and 9). Notably, the RING domain (aa 300–480) also bound to Smad4 (Fig. 4A, lane 10).

We also examined the Smad4 interaction with PIAS1 domains expressed in cultured 293T cells. PIAS1 domains were expressed as HA-tagged and could be detected by anti-HA Western blotting. As shown in Fig. 4B, although aa 2–121 and aa 421–650 regions had no affinity to bind to Smad4, the aa 121–480 region contained multiple Smad4-binding elements. These results were identical to the interaction pattern as demonstrated by in vitro binding shown in Fig. 4A. Thus, these data suggest that the Smad4-interacting domain consists of multiple contacts located in the region of aa 121–480 of PIAS1 (Fig. 4C).

The MH1 Domain of Smad4 Mediates Its Interaction with PIAS1—Smad4 has two conserved Mad homology domains (MH) (i.e. MH1 at the N terminus and MH2 at the C terminus).
Here we also sought to determine which domains of Smad4 were involved in the interaction with PIAS1. GST-PIAS1 fusion protein was used to pull down 35S-labeled in vitro translated Smad4 domains. These experiments confirmed that PIAS1 was able to bind to full-length Smad4 in vitro (Fig. 5A, lane 5). In addition, we found that Smad4N (i.e., the MH1 domain, lane 1) and Smad4NL (the MH1 + linker, lane 2) bound to PIAS1. In contrast, neither the linker region nor the MH2 domain bound to PIAS1 (Fig. 5A, lanes 3 and 4). The findings suggest that the Smad4 interacts with PIAS1 in cells. GST-Smad4 was used to pull down PIAS1 from 293T cell lysates that had been transfected with the indicated HA-tagged PIAS1 proteins. Smad4-bound PIAS1 was detected by anti-HA antibodies (upper blots). The expression levels of different PIAS1 peptides in whole cell lysates were shown in the lower blots. Open arrows indicate the absence of corresponding bands. The filled arrows indicate the interaction. B, schematic diagram of Smad4 and deletion mutants. Deletion mutants of Smad4 are shown with shaded domains. Their interaction with PIAS1 is indicated by − (lack of detectable interaction) and + (presence of an interaction).
PIAS1 Is a Smad4-SUMO E3 Ligase

MH1 domain of Smad4 was essential and sufficient for PIAS1 binding (Fig. 5B).

Smad4-PIAS1 Interaction Is TGF-β-inducible—To study the Smad4-PIAS1 interaction in cells, 293T cells were first transfected with HA-tagged PIAS1 and FLAG-tagged Smad4, treated with and without TGF-β, and finally, the cell extracts were subjected to anti-HA immunoprecipitation followed by anti-FLAG Western blotting. In the presence of TGF-β, immunoprecipitation of PIAS1 protein using anti-HA antibody could bring down FLAG-tagged Smad4 (Fig. 6A, lane 4). This PIAS1-Smad4 interaction did not occur without TGF-β stimulation (lane 3).

We next determined whether PIAS1 interacts with endogenous Smad4 by performing GST pull-down experiments using lysates of human keratinocyte HaCaT cells. The results showed that GST-PIAS1 protein immobilized to glutathione-Sepharose 4B beads was able to interact with endogenous Smad4 (Fig. 6B, lanes 7 and 8). Interestingly, GST-PIAS1 (i.e. aa 2–316) exhibited a stronger ability to retrieve endogenous Smad4 than wild type GST-PIAS1 (compare lanes 3 and 4 with 7 and 8). TGF-β had little effect on the interaction of endogenous Smad4 with the full-length or N-terminal domain of PIAS1 (Fig. 5B, lanes 4 and 8). In contrast, GST-PIAS1 (aa 421–650) did not bind to Smad4 (lanes 5 and 6). Therefore, the data suggest that the PIAS1 protein directly interacts with Smad4 at endogenous levels.

PIAS1 Potentiates TGF-β Transcriptional Responses—To investigate the functions of PIAS1-mediated Smad4 sumoylation in TGF-β signaling, we tested whether overexpression of PIAS1 exerted stimulatory or inhibitory effects on TGF-β-induced transcription. To avoid any interference of other DNA-binding cofactors (some of which could also be targeted by sumoylation), we first examined the TGF-β-dependent induction by utilizing a Smad-responsive synthetic reporter gene SBE-luc that solely depends on Smad activation. SBE-luc contains four copies of SBE linked to the luciferase reporter gene (41). In both mink lung epithelial Mv1Lu and human keratinocyte HaCaT cells, TGF-β induced a 3–5-fold increase in the SBE-luc reporter gene activity. Notably, coexpression of PIAS1 increased the reporter gene activity in response to TGF-β (Fig. 7, A and B). The stimulatory effect of PIAS1 is comparable with that of SUMO-Ubc9, which also potently stimulated SBE reporter activity (36). In addition, the potentiation of SBE-luc activity by PIAS1 was in a dose-dependent manner (Fig. 7C). We next tested whether PIAS1 expression enhanced the activity of a natural TGF-β-inducible promoter. As shown in Fig. 7D, PIAS1 expression also stimulated the TGF-β-induced activity of the plasminogen activator inhibitor type 1 (PAI-1) promoter.

Results in Fig. 4C summarize the E3 ligase activity of RING-containing fragments. Thus, we sought to determine whether these deletion mutants retained their ability to potentiate TGF-β-induced SBE-luc activity. We found that all RING-containing PIAS1 variants were able to enhance the TGF-β response. Although the wild type and aa 2–480 had the highest stimulatory effect, smaller fragments such as aa 121–480 or aa 300–480 exhibited a moderate effect (Fig. 7E). Notably, the inactive mutant of E3 ligase (i.e. the RING mutant of PIAS1) failed to stimulate the TGF-β-dependent SBE reporter gene response (Fig. 8D). These results clearly demonstrated that forced expression of PIAS1 significantly stimulated TGF-β transcriptional responses, and this effect required the RING-dependent E3 ligase activity.

E3 Ligase-dead or Absent Mutants of PIAS1 Blocks Smad4 Sumoylation in a Dominant Negative Manner and Consequently Inhibits TGF-β Signaling—We observed that the E3 ligase-dead mutant (Fig. 3B, PIAS1(CS)) appears to inhibit in vivo SUMO conjugation of Smad4 in a dominant negative fashion. We hypothesized that a dominant negative PIAS1 mutant should lack the functional ligase activity but retain the ability to bind to Smad4. For this purpose, PIAS1(CS) (i.e. aa 2–316) might be the most favorable candidate, considering its strong binding to Smad4 (Fig. 4C). To further explore this, we compared PIAS1(CS) with another mutant of PIAS1 called PIAS1C, corresponding to PIAS1 (aa 421–650). Both PIAS1 and PIAS1C have no RING and thus exhibit no E3 ligase activity (Fig. 4C), but PIAS1(CS) retains the ability to interact with Smad4 (Fig. 4C). As shown in Fig. 8A, increases in exogenous PIAS1(CS) expression abolished the appearance of Smad4-SUMO conjugates (lanes 3 and 4), whereas PIAS1C had no effect on Smad4 modification (lanes 5 and 6). This suggests that PIAS1(CS) may compete with endogenous PIAS for the binding of Smad4 and subsequently block Smad4 sumoylation.

Such competition was further demonstrated in vitro (Fig. 8B). In a cell-free system, PIAS1 stimulated Smad4 sumoylation. Although the GST protein could not alter the pattern of PIAS1-mediated Smad4 sumoylation (lanes 2 and 3), the GST-PIAS1 fusion protein profoundly reduced the level of sumoylated Smad4 (lanes 4 and 5). In sharp contrast, GST-PIAS1C could not alter the sumoylation of Smad4 because of its inability to bind to Smad4 (lanes 6 and 7). Furthermore, the ligase-dead CS mutant of the minimal E3 ligase (aa 121–480) also blocked PIAS1-mediated Smad4-SUMO conjugation (Fig. 8B, lanes 8 and 9). The ligase-dead CS mutant of full-length PIAS1 also competes with wild type PIAS1 to inhibit the SUMO conjugation on Smad4 (Fig. 8C).

We next determined whether exogenous expression of PIAS1(CS) affects the TGF-β transcriptional response. TGF-β-
induced activity of the SBE-luc reporter gene was assayed in
HaCaT cells in the presence of PIAS1 variants. Although
TGF-β induced a 4-fold increase in the activity of the SBE-luc
reporter, the expression of PIAS1 or PIAS1 (aa 2–480) further
stimulated TGF-β-induced activity (Fig. 8D). On the
contrary, expression of PIAS1N inhibited TGF-β-induced SBE-
luc activity in a dominant negative manner. Similarly, ligase-
dead mutants of full-length PIAS1 or PIAS1 (aa 2–480), i.e.
the CS mutants, also blocked the TGF-β-induced SBE-luc
activation.

DISCUSSION

Post-translational modifications by ubiquitin and SUMO
pathways have emerged as common mechanisms to regulate
the steady-state levels and activity of target proteins. Improper
modifications of regulatory proteins, such as cell cycle regula-
tors and signal transducers, may play an important role in the
arising and/or progression of human cancers. The key factor
that modulates protein ubiquitination or sumoylation is the E3
ligase, which binds to the substrate protein and provides spec-
cificity to protein modifications. Three different classes of pro-
teins have been identified as SUMO E3 ligases: RanBP2, PIAS,
and hPc2. Although RanBP2 promotes sumoylation of Sp100
and HDAC4 proteins, members of the PIAS family control the
sumoylation of transcription factors of diverse groups, such as
CtBP (12), p53 (33, 35), c-Jun (35), androgen receptor (29, 30),
and LEF1 (42). Notably, PIAS1 and PIAS3 proteins were ini-
tially identified as inhibitors of STAT signaling (25, 26), and
they appear to regulate STAT activity by sumoylating STAT1
(43, 44). In this study, we provided strong evidence to demon-
strate that PIAS1 protein acts as a SUMO E3 ligase for tumor
suppressor Smad4 and positively regulates TGF-β signaling.

PIAS1 Is a Positive Regulator of TGF-β Signaling

PIAS1 is a Smad4-SUMO E3 Ligase

Fig. 7. PIAS1 stimulates TGF-β signaling. A and B, PIAS1 potentiates TGF-
β-induced SBE-luc reporter activity. Ha-
CaT (A) or Mv1Lu (B) cells were
transfected with the indicated expression
plasmids for PIAS1 or SUMO/Ubc9 to-
gether with the SBE-luc plasmid DNA.
TGF-β treatment and the luciferase assay
is described under “Experimental Proce-
dures.” C, stimulatory effect of PIAS1 on
the SBE-luc reporter activity is dosage-
dependent. HaCaT cells were used. D,
PIAS1 potentiates TGF-β-induced activ-
ity of the human PAI-1 promoter. The
reporter assay was done as in A and B.
HaCaT cells were used. E, effect of the
RING-containing domain of PIAS1. Ha-
CaT cells were used.
sumoylation plays a positive role in TGF-β/H9252 signaling (36). In fact, loss of Smad4 sumoylation profoundly abrogates TGF-β/H9252 antiproliferative and transcriptional responses (36). There are three lines of evidence supporting the E3 ligase activity of the PIAS proteins in Smad4 sumoylation. 1) PIAS1 mediates the Smad4 sumoylation in vivo and in vitro. In a well defined in vitro reconstitution reaction containing E1 and E2 enzymes, PIAS1 is essential in driving the SUMO conjugation of Smad4. The E3 ligase activity toward Smad4 sumoylation requires the functional RING finger domain, as the simultaneous Cys → Ser mutations at Cys-345, Cys-350, and Cys-355 rendered the PIAS1 protein defective in inducing Smad4 sumoylation. 2) PIAS1 protein directly interacted with Smad4 in vitro in GST-binding assays and in co-immunoprecipitation experiments. Importantly, the PIAS proteins interacted with Smad4 in a TGF-β-inducible manner. This is probably attributed to the TGF-β-induced Smad4 import into the nucleus where PIAS proteins are localized. 3) SUMO E3-defective mutants of PIAS1 inhibited TGF-β-induced transcription in a dominant negative manner, whereas PIAS proteins stimulated TGF-β signaling.

Other PIAS proteins may also participate in the regulation of TGF-β signaling. For example, PIASy has been shown previously to interact with Smad proteins (39, 40). Although PIASy interacts with Smad4 and may promote SUMO in vivo modification of Smad4 (37), we found PIASy is rather a weak E3 ligase (when compared with PIAS1 and PIASx proteins) (data not shown). Interestingly, PIASy also binds to Smad3 and acts as a transcriptional corepressor for Smad3 (39). This corepressor activity may be independent of its SUMO E3 ligase activity, as Smad3 is not SUMO-modified in vivo or in vitro (data not shown). Therefore, PIAS proteins control Smad signaling in a context-dependent manner.

Function of RING Finger and Mini-E3 Domain in Smad4 Sumoylation—PIAS proteins have a conserved RING finger.
PIAS1 Is a Smad4-SUMO E3 Ligase

PIAS1 is a Smad4-SUMO E3 ligase that mediates Smad4 sumoylation in vitro and in vivo. The mini-E3 ligase domain has the ability to sumoylate other substrates such as p53 protein. The mini-E3 ligase domain may be useful for further characterization of how it interacts with Ubc9 and identification of the mini-E3 ligase domain may be important for understanding the function of PIAS1 in Smad signaling.

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Regulation of Smad4 Sumoylation and Transforming Growth Factor-β Signaling by Protein Inhibitor of Activated STAT1

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