Sumoylation of Smad4, the Common Smad Mediator of Transforming Growth Factor-β Family Signaling*

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Transforming growth factor-β (TGF-β) and TGF-β-related factors regulate cell growth, differentiation, and apoptosis, and play key roles in normal development and tumorigenesis. TGF-β family-induced changes in gene expression are mediated by serine/threonine kinase receptors at the cell surface and Smads as intracellular effectors. Receptor-activated Smads combine with a common Smad4 to translocate into the nucleus where they cooperate with other transcription factors to activate or repress transcription. The activities of the receptor-activated Smads are controlled by post-translational modifications such as phosphorylation and ubiquitylation. Here we show that Smad4 is modified by sumoylation. Sumoylation of Smad4 was enhanced by the conjugating enzyme Ubc9 and members of the PIAS family of SUMO ligases. A major sumoylation site in Smad4 was localized to Lys-159 in its linker segment with an additional site at Lys-113 in the MH-1 domain. Increased sumoylation in the presence of the PIASy E3 ligase correlated with targeting of Smad4 to subnuclear speckles that contain SUMO-1 and PIASy. Replacement of lysines 159 and 113 by arginines or increased sumoylation enhanced the stability of Smad4, and transcription in mammalian cells and Xenopus embryos. These observations suggest a role for Smad4 sumoylation in the regulation of TGF-β signaling through Smads.

Cellular processes, like cell proliferation, differentiation, and apoptosis, are elaborated by intracellular proteins that are regulated by signaling pathways (1). Individual mediators often respond to multiple pathways and participate in multiprotein complexes of varying composition, thus requiring an exquisitely sensitive, versatile and often rapid regulation of protein function, beyond the control at the level of gene expression (2). Intracellular signaling mediators, including those that participate in transcription, are often subject to post-translational modifications that alter the activity and specificity, subcellular localization, stability, and/or interactions with other macromolecules, and are major determinants of a function of the protein (3, 4). One class of modifications involves addition of a small chemical group to amino acids, e.g., through phosphorylation, acetylation, ADP-ribosylation, or methylation, whereas the second class involves attachment of larger macromolecules, such as polypeptides in the case of ubiquitylation and sumoylation, or polysaccharides. A variety of enzymes mediate polypeptide modifications and thereby often display substrate specificity, whereas complementary enzyme sets remove these modifications, thus illustrating the reversible nature of post-translational modification processes (5). This dynamic nature and the observations that a single signaling mediator is often subject to multiple types of modification illustrate the plasticity and the combinatorial control that regulates the functions of the signaling effectors.

Ubiquitylation results in attachment of one or several 76-amino acid long ubiquitin polypeptides to Lys residues of target proteins (6). This process requires the sequential actions of a ubiquitin-activating E1 enzyme, conjugating E2 enzyme, and E3 ligase. Ubiquitin is activated by the E1 enzyme in an ATP-dependent manner and transferred as a thiol ester intermediate to one of several E2-conjugating enzymes. Covalent attachment of ubiquitin to a target protein is then mediated by one of the many E3 ligases that define substrate specificity. Polyubiquitylated proteins are usually targeted for proteasomal degradation (6).

Sumoylation involves the covalent attachment of SUMO, a ubiquitin-related polypeptide, to a Lys residue (7). However, unlike ubiquitylation, sumoylation has not been reported to target a substrate for degradation. As with ubiquitylation, sequential activities of E1, E2, and E3 enzymes are involved in sumoylation, but the identities of the enzymes are different. The SUMO activating enzyme E1, a heterodimer of Aos1 and Uba2, transfers activated SUMO to the E2-conjugating enzyme Ubc9 that directly catalyzes the isopeptide bond formation between the C-terminal glycine residue of the activated SUMO to Lys residue of the target. PIAS family members have been implicated as E3 SUMO ligases, although their roles in the sumoylation of the diverse substrates need to be further defined (8). For example, RanBP2 can serve as E3 ligase for the HDAC4 deacetylase and Sp100 (9, 10), whereas ARIP3 is involved in sumoylation of the androgen receptor (11, 12). As with other post-translational modifications, sumoylation is thought to be a reversible and dynamic process.

Sumoylation can regulate the function of a protein by changing its subcellular localization, protein-protein interactions, and its cellular localization, stability, and/or interactions with other macromolecules. SUMOylation can regulate the function of a protein by changing its subcellular localization, protein-protein interactions, and its cellular localization, stability, and/or interactions with other macromolecules.
and/or stability, depending on the identity of the protein. For example, SUMO-1 modification targets the PML protein to nuclear bodies (13), and the homeodomain-interacting protein kinase 2 into nuclear speckles (14). Sumoylation negatively impacts the transcription activities of the transcription factors Sp3 (15), LEF-1 (16), and androgen receptor (12), and may also affect their subnuclear distribution, whereas other studies indicate a positive effect of sumoylation on transcription factor activity such as heat shock transcription factor-1 (17). Signaling from the cell surface can also be directly impacted by sumoylation, as illustrated in the case of IκB, which sequesters NFκB in the cytoplasm. Tumor necrosis factor-α-induced phosphorylation of IκB allows for its ubiquitination, thus targeting it for proteasomal degradation and enabling nuclear translocation of NFκB, whereas sumoylation of the same Lys in IκB stabilizes it against degradation (18). This dynamic balance between sumoylation and ubiquitination of IκB can then regulate the gene expression of the cell response mediated by NFκB. Taken together, the effects of sumoylation on the function of the protein appear to depend on the substrate and, unlike ubiquitination, which frequently targets substrate for proteasomal degradation, are not predictable.

TGF-β and TGF-β-related proteins are key regulators of cell proliferation and differentiation. They regulate development from nematodes and flies to mammals (6), and play important roles in tumor progression (19). The central signaling pathway from TGF-β-activated cell surface receptors to alterations in gene expression involves a small class of signaling effectors, the Smads, as key mediators of a response of the cell to TGF-β (20). Thus, following ligand binding to type II/type I receptor complexes, the activated type I receptors phosphorylate the C-terminal two serines of receptor-activated Smads, e.g. Smad2 and Smad3 in response to TGF-β and Smad1, -5, and -8 upon stimulation by bone morphogenetic proteins. The phosphorylated Smads are then released from the receptors to form a heterotrimeric complex with Smad4 as a common component of all Smad signaling pathways, activated by TGF-β family members (20). The Smad complexes enter the nucleus where they interact at the promoter with other transcription factors and co-regulators to regulate gene expression. In this pathway, Smad4 acts as a central coactivator of all receptor-activated Smads, presumably through its ability to stabilize the interaction of receptor-activated Smads with the essential coactivator cAMP-response element-binding protein/p300 (21) and its ability to recruit yet another coactivator, SMIF1 (22). In addition to C-terminal phosphorylation, the activation of the receptor-activated Smads is also regulated by other phosphorylations, e.g. in response to mitogen-activated protein kinase signaling (23), and is counteracted by inhibitory Smad6 and -7 (24, 25).

Whereas phosphorylation plays a central role in the activation of the Smad pathway, recent studies have revealed an additional level of regulation of Smad signaling through ubiquitination. Smurf1 and Smurf2, two Hect family E3 ubiquitin ligases, target receptor-activated Smads for ubiquitination and proteasomal degradation, thus decreasing their availability (26, 27). In addition, ubiquitination and consequent degradation of ligand-activated Smad2 and Smad3 in the nucleus may be involved in the termination of their functions in transcriptional regulation (28). In contrast to the receptor-activated Smads, Smad4 levels are not regulated through ubiquitin-mediated degradation. However, some mutations, found in human tumors, destabilize Smad4 via ubiquitination and consequent degradation (29, 30). The role of Smad4 as the central mediator of signaling by all receptor-activated Smads in response to all TGF-β family members, and its shuttling between cytoplasm and nucleus (31) suggests that some mechanism may be in place to allow for stabilization and re-utilization during signaling.

In this report, we show that among the Smads known to be involved in TGF-β signaling, Smad4 is the only one that is preferentially subjected to sumoylation. We identified two sumoylation sites in Smad4, and sumoylation was enhanced in the presence of the conjugating enzyme Ubc9 and PIASy family E3 ligases. Sumoylation by the PIASy E3 ligase resulted in redistribution of Smad4 to subnuclear speckles that colocalized with SUMO-1 and PIASy. Replacement of the sumoylation target lysines with arginine, or increased sumoylation, enhanced the protein stability and increased transcription in mammalian cells and in Xenopus embryos. These results suggest a role for Smad4 sumoylation in the regulation of TGF-β family signaling through Smads.

MATERIALS AND METHODS

Expression plasmids for N-terminal FLAG-tagged Smad1, -2, -3, -4, -6, and -7 have been described (32, 33). Additionally, plasmids encoding Smad4 truncation mutants, i.e. pRK5-Smad4NL (aa 1–140), Smad4NL (aa 1–300), and Smad4C (aa 294–552) have also been described (32). Plasmids encoding Smad4 with amino acid substitutions were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions, and mutations were confirmed by nucleic acid sequencing. The pRK5-based expression plasmid for N-terminal FLAG-tagged SUMO-1 expressed the first 97 amino acids of SUMO-1 was kindly provided by Dr. Y. Zhang (Laboratory of Cellular and Molecular Biology, Center for Cancer Research, NCI, National Institutes of Health). The expression plasmid for Ub9, PIASy, and ARIP3, i.e. pcDNA3-Ub9, CMV-T7-PIASy, and pFlag-CMV2-ARIP3 (39, 62, 64) were generously provided by Dr. R. T. Hay (University of St. Andrews), Dr. R. Grosschedl (University of Munich), and Drs. O. A.Janne and J. J. Palvimo (University of Helsinki), respectively. Plasmids pRK5-β-gal and (SBE)12- luc were also previously described (34–36).

Cell Culture, Transfections, and Reporter Assays— COS-1, HeLa, and MBA-M46-88 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G. COS-1 and HeLa cells were transfected using LipofectAMINE (Invitrogen) and MBA-M46-88 cells were transfected with FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. In transcription reporter assays, MBA-M46-88 cells were seeded onto 6-well culture dishes and 2 μg of DNA, including 0.5 μg of (SBE)12-luc luciferase reporter plasmid, 0.1 μg of pRK5-β-gal, and other expression plasmids, as required, were used for each transfection. The total amount of DNA was kept constant by addition of pRK5 DNA. 12–18 h after transfection, cells were treated with or without 10 ng/ml TGF-β in Dulbecco’s modified Eagle’s medium containing 0.2% fetal bovine serum for 20 h. At the end of stimulation, cells were washed twice in cold phosphate-buffered saline and processed for luciferase β-galactosidase enzyme assays as described (37).

Cell Lysis, Immunoprecipitation, and Western Blotting Analysis— Cells were washed four times in phosphate-buffered saline and lysed in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), supplemented with 20 mM N-ethylmaleimide (Calbiochem, 34115), protease inhibitor mixture (Sigma, P8340), and 5% glycerol. In co-immunoprecipitation experiments cells were lysed in RIPA buffer without theionic detergents. Cell lysates were cleared by centrifugation at 20,000× g at 4 °C for 30 min. The resulting supernatant was subjected to immunoprecipitation with anti-FLAG M2-agarose (Sigma, A2220) overnight at 4 °C. The agarose beads were washed five times in lysis buffer and the bound proteins were eluted in SDS-PAGE loading buffer. The samples were separated to SDS-PAGE, followed by Western transfer and immunoblotting as described (38). Mouse monoclonal anti-FLAG M2 antibody (Sigma, F3165), anti-Myc tag 9E10 antibody (Covance, MMS-150P), anti-HA tag HA11 antibody (Covance, MMS-101R), anti-GMP-1 antibody (Zymed Laboratories Inc.), and rabbit polyclonal anti-Smad4 antibody H-552 (Santa Cruz, sc-7154) were used at 1 μg/ml in Western blotting. Peroxidase-linked anti-mouse (NA9340) secondary antibodies and ECL detection reagents (RP2106) were purchased and used according to the manufacturer’s instructions (Amersham Biosciences).

Pulse-Chase Analysis of Smad4 Stability— Transfected COS cells...
were starved in methionine- and cysteine-free medium containing 0.2% dialyzed fetal bovine serum for 2 h. The cells were then labeled with 0.25 μCi/ml [35S]EXPRESS protein labeling mix (PerkinElmer Life Sciences, NEG072) in the same medium for 1 h. For the labeling, the cells were washed three times in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum before incubating in the same medium for various times, as needed. Where indicated, lactacystin (Calbiochem, number 426100) was used at 30 μM during the pulse-chase. Cells were harvested and processed as above. The cell lysates were subjected to anti-FLAG immunoprecipitation for isolation of exogenous Smad4, and immunoprecipitates were analyzed by SDS-PAGE. Smad4 was visualized by autoradiography and analyzed by densitometry.

Immunofluorescence—HeLa cells were grown on coverslips and transfected with the indicated plasmid DNA. 24 h after transfection cells were kept in Dulbecco's modified Eagle's medium containing 0.2% fetal bovine serum for 3 h and subsequently treated with 5 ng/ml TGF-β for 40 min. Cells were then permeabilized with 0.5% Triton X-100 in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 2 mM EDTA) for 2 min on ice, and fixed in 3.7% paraformaldehyde in CSK buffer for 5 min at 37 °C (39). Cells were rinsed twice in TBS (20 mM Tris, pH 8, 150 mM NaCl) and then blocked in 3% bovine serum albumin in TBS containing 0.03% Triton X-100 for 1 h at room temperature. After incubation with a primary antibody in blocking solution, the cells were washed in blocking solution five times before incubating with 1:5000 diluted secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (A11029) and Alexa Fluor 594 goat anti-mouse IgG (A-11034) from Molecular Probes) for 1 h. After washings cells were stained in 300 nM 4,6-diamidino-2-phenylindole (Sigma, 32670) in TBS for 5 min, followed by brief washes in TBS. The samples were finally mounted in FluorSave Reagent (Calbiochem, 345798) and examined by fluorescence microscopy.

Mesoderm Marker Induction in Xenopus Embryos—cRNAs encoding Smad4 (wild-type and mutants) and SUMO-conjugating enzyme Ubc9 and β-catenin were synthesized in vitro, using Ambion mRNAMessage Machine kit. Capped RNAs were injected into both animal poles of two-cell stage embryos. The doses of cRNAs used were indicated in the legend to Fig. 7. The ectodermal explants (animal caps) of injected embryos were dissected at blastula stages (stage 9), and total RNA was extracted from these caps at gastrula stages (stage 11). For treatment with basic fibroblast growth factor (bFGF), the animal caps were dissected at stage 9 and incubated in buffer containing 100 ng/ml bFGF. The caps were harvested at stage 11 as other samples. Reverse transcription-PCR was performed using the primers as described previously (40).

RESULTS

Smad4 Is Sumoylated—Proteins that are substrates of ubiquitin modification are often modified by sumoylation, raising the possibility that the TGF-β Smads could be targeted for sumoylation. Furthermore, in a yeast two-hybrid screen for interaction proteins we found that Smad4, but not Smad3, had an affinity for SUMO-1 (data not shown). We therefore evaluated whether Smad3 and Smad4, expressed in transfected cells, were sumoylated. In the presence of coexpressed Myc-tagged SUMO-1, but not in its absence, a fraction of Smad4 reacted in Western blotting with the anti-Myc antibody (Fig. 1A, top panel). Its apparent size was compatible with the addition of a single SUMO-1 polypeptide to Smad4, and corresponded to the upper edge of the total Smad4 band, detected by Western blotting (Fig. 1B, lower panel). Smad3 did not display reactivity for Myc-tagged SUMO-1 under these conditions.

Although many gene expression responses of TGF-β are mediated by Smad3 and Smad4 (41), Smad2 and Smad1 can also be activated by TGF-β (42, 43), and Smad6 and Smad7 act as inhibitory Smads for the TGF-β response (25, 41). We therefore evaluated whether any of these Smads are targeted by sumoylation. As shown in Fig. 1B, only Smad4, and not the other Smads tested, was sumoylated under this condition.

Lysines 159 and 113 Are Major Sumoylation Sites in Smad4—To localize the site of sumoylation within Smad4, we expressed several Smad4 segments, either in the presence or absence of coexpressed SUMO-1. Immunoprecipitation followed by Western detection of SUMO-1 indicated that the NL segment, corresponding to amino acids 1–300 and containing the MH1 domain and linker segment, was sumoylated (Fig. 2A). Smad4C, i.e. amino acids 266–552 containing the MH2 domain, was not detectably sumoylated. Smad4N, i.e. amino acids 1–140 corresponding to the MH1 domain, showed only a minimal level of immunoreactivity for Myc-SUMO-1 (Fig. 2A).
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Fig. 2. Lysine 159 and 113 are the major in vivo sumoylation sites in Smad4. A, Smad4 sumoylation is localized to the NL segment. HEla cells transfected to express FLAG-tagged full-length Smad4 (FL, aa 1–552), Smad4 N (aa 1–140), Smad4 NL (aa 1–300), or Smad4 C (aa 266–552), and Myc-tagged SUMO-1 were subjected to anti-FLAG immunoprecipitation and analyzed by immunoblotting. The upper panel shows the immunoblot probed with anti-Myc antibody to detect sumoylated proteins, and the lower panel shows the blot, after stripping and reprobing with anti-FLAG antibody to detect the Smad segments. Arrows indicate the sumoylated Smad4 bands. B, lysine 159 is a major sumoylation site in Smad4 NL. HEla cells were transfected to express, in the presence of Myc-tagged SUMO-1, FLAG-tagged Smad4 NL or the Smad4 NL lysine to arginine mutants (K51R, K106R, K159R, or K51R/K106R/K159R). Sumoylation of these Smad4 NL versions were analyzed as in A. C, lysine 159 is a major sumoylation site in full-length Smad4. HEla cells expressed FLAG-tagged Smad4 or Smad4 mutants with single lysine to arginine mutation at positions 51, 106, 122, 159, or 392, and double lysines to arginines mutants 51/159 and 122/159, in the presence of Myc-tagged SUMO-1. Smad4 sumoylation was examined as in A. Single mutations at Lys-51, -106, -122, or -392 did not affect Smad4 sumoylation, whereas the Lys-159 mutants showed significant reduction in Smad4 sumoylation. D, lysine 113 is a sumoylation site in Smad4. HEla cells expressing different FLAG-tagged Smad4 species were analyzed for sumoylation in the presence or absence of SUMO-1 and/or SUMO-conjugating enzyme Ubc9 as in A.

Direct Western blotting of the cell lysates for Smad4 detected small fractions of Smad4, Smad4N, and Smad4NL (Fig. 2A, lower panel) with the same mobilities as the sumoylated forms that were detected using the anti-Myc antibody for SUMO-1 (Fig. 2A, upper panel).

SUMO conjugation occurs on lysine (K) residues within a general minimal consensus sequence ψKXE, in which ψ is a large hydrophobic residue (T). Only 1 lysine within such sequence context, i.e. Lys-159 within the VKDE sequence, is present in Smad4 and is located within the Smad4NL segment, but not the Smad4N segment. Within the Smad4NL sequence are two other lysines, i.e. Lys-51 and Lys-106, that are located within a KXE sequence that lack a preceding hydrophobic residue. To examine which lysine is sumoylated, we individually replaced each of these three lysines within Smad4NL by an arginine (R). As shown in Fig. 2B, the K51R and K106R mutants were sumoylated to a similar extent as the normal Smad4NL. In contrast, the K159R mutation strongly reduced, yet did not abolish the sumoylation. This result suggests that Lys-159 is a major sumoylation site. The triple mutation of Smad4NL, resulting in replacement of all three lysines with arginines further reduced the reactivity in Western blotting for SUMO to background level. This result suggests that Lys-51 and/or Lys-106 can serve as targets for sumoylation enzymes, albeit with a much lower efficiency than Lys-159.

We also tested the effect of these lysine mutations on sumoylation of full-length Smad4 (Fig. 2C). These results confirmed Lys-159 as the major sumoylation site, because the K159R mutation nearly abolished sumoylation. In contrast, mutation of Lys-106 exerted only a minor decrease, and mutation of Lys-51 had no effect. We also replaced Lys-392, which is located within the CKGE sequence in the carboxyl MH2 domain of Smad4, by Arg. This mutation also had only a minimal, if any, effect on Smad4 sumoylation (Fig. 2C), consistent with the lack of detectable sumoylation of Smad4C (Fig. 2A). Finally, an alternate, less common sumoylation target sequence, ψKXD, has also been described (44). Lys-122, within an LKCD sequence, was therefore mutated and the effect on Smad4 sumoylation was assessed. As shown in Fig. 2C, the K122R mutation did not detectably decrease the level of Smad4 sumoylation. We also examined the sumoylation status of double lysine mutants, K51R/L159R and K122R/L159R and found that they had a low level of sumoylation like K159R (Fig. 2C).

While this paper was under review, Lin et al. (45) reported that Smad4 is sumoylated on Lys-113, in addition to Lys-159. We therefore verified the possible sumoylation of this site (Fig. 2D). As evaluated in the presence of SUMO-1, mutation of Lys-113 to Arg reduced sumoylation of Smad4. Mutation of both Lys-113 and Lys-159 to arginines almost abolished Smad4 sumoylation.

Ubc9 and PIAS E3 Ligases Enhance Smad4 Sumoylation—Sumoylation involves the E2-conjugating enzyme Ubc9 (46) and the recently identified E3 ligases of the PIAS family, such as PIASy or ARIP3 (47). We therefore examined the effect of coexpression of Ubc9 in the absence or presence of PIASy or ARIP3 on Smad4 sumoylation. As shown in Figs. 2D and 3A, coexpression of Ubc9 with SUMO-1 strongly enhanced the sumoylation of Smad4. Under these conditions, we also detected, in addition to the singly conjugated Smad4 species, a low level of a higher molecular weight form that is likely to correspond to doubly sumoylated Smad4. Mutating either Lys-113 or Lys-159 resulted in mostly singly sumoylated Smad4 species while the K113R/L159R mutant showed almost no sumoylation (Figs. 2D and 3A).

Overexpression of PIASy or ARIP3 in the absence or presence of coexpression of Ubc9 markedly increased Smad4 sumoylation. In these experiments, we additionally detected higher molecular weight forms of Smad4 (Fig. 3A) that likely result from sumoylation at multiple lysines in addition to Lys-113 and Lys-159. The K113R/K159R mutant of Smad4 showed no detectable sumoylation even when both Ubc9 and PIASy were expressed. Nevertheless, coexpression of Ubc9 and ARIP3 resulted in polysumoylation of this double mutant (Fig. 3A), consistent with the use of alternative lysines as substrates for sumoylation under these conditions.

Because Smad4 can be sumoylated at more than one site, particularly when Ubc9 was coexpressed, we tested the effect of overexpressed Ubc9 on Smad1, -2, -3, -6, and -7 sumoylation,
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Increased expression of SUMO-1 and PIASy, a condition that enhances the sumoylation of Smad4 (Fig. 3), strongly affected the subnuclear localization of Smad4. Indeed, in contrast to its normally diffuse distribution (Fig. 4A, a), coexpression of SUMO-1 and PIASy, and consequent Smad4 sumoylation, correlated with a recruitment of Smad4 into a punctate subnuclear pattern (Fig. 4B, a), similarly to that of PIASy (Fig. 4, B, b, and A, b). In fact, under these conditions, the Smad4 and PIASy staining largely overlapped (Fig. 4B, c). Additionally, when SUMO-1, Ubc9, and PIASy were coexpressed with Smad4, we observed that Smad4 colocalized with SUMO-1 in a punctated pattern (Fig. 4C, a–c). In the absence of the E3 ligase, however, the immunofluorescence staining of Smad4 was more diffuse (data not shown). Together, these results suggest that the interaction with the E3 ligase and concomitant sumoylation enhancement targeting of Smad4 to the SUMO-1 containing subnuclear bodies that also define the localization of PIASy.

To further address the role of sumoylation in subnuclear targeting of Smad4, we examined the effect of increased expression of SUMO-1, Ubc9, and PIASy on the subnuclear localization of the K122R/K159R mutant of Smad4 that lacks one of the major sumoylation sites (Fig. 4C, c–h). Unexpectedly, the K122R/K159R mutant showed similarly punctuated subnuclear localization as wild-type Smad4 under these conditions. This is consistent with the observation that PIASy can target LEF-1 into subnuclear bodies, independently from sumoylation of LEF-1 (16). This targeting of the K122R/K159R mutant of Smad4 in the presence of PIASy may be because of residual sumoylation of this mutant, but could also be explained by a sufficiently strong physical interaction of PIASy with Smad4. Indeed, we found that PIASy interacted equally well with wild-type Smad4 and with the K122R/K159R or K133/159R mutants in coimmunoprecipitation assays (Fig. 4D, data not shown). Thus, the coexpression of PIASy, and consequent physical interaction with Smad4, rather than Smad4 sumoylation per se, may be sufficient to target Smad4 into a punctate pattern of subnuclear bodies.

Sumoylation Enhances the Stability of Smad4—In contrast to ubiquitylation, SUMO conjugation does not appear to target proteins for degradation (48, 49). Furthermore, in the case of IκBa, sumoylation has been shown to protect against ubiquitination of the same lysine residue, and thereby protect IκBa against ubiquitin-mediated degradation (18). We thus tested the role of sumoylation on the stability of Smad4 using pulse-chase experiments, in which proteins were 35S-labeled, and the stability of 35S-labeled Smad4 was followed over time.

We first compared the stability of wild-type Smad4 with the K113R/K159R mutant that has the major sumoylation target Lys replaced by Arg. As shown in Fig. 5A, the K113R/K159R mutant, as well as the K159R mutant (data not shown), had a higher stability than wild-type Smad4. Whereas wild-type Smad4 is a relatively stable protein, replacement of Arg-100 by Thr (R100T), a mutation observed in tumor specimens, results in increased ubiquitination and a shorter half-life because of increased degradation (29). As reported previously (29), we also observed that Smad4 R100T has a shorter half-life than wild-type Smad4 and the protein was stabilized by treatment of cells with proteasomal inhibitor lactacystin (Fig. 5B). The K159R

which are normally not sumoylated (Fig. 1B). As shown in Fig. 3B, Ubc9 overexpression facilitates sumoylation of Smad1, -2 and -3, albeit at a much lower level than Smad4. As in Fig. 1, only Smad4 was sumoylated at endogenous levels of Ubc9.

Sumoylation Alters the Subnuclear Distribution of Smad4—Sumoylation of some proteins has been shown to correlate with targeting of the modified proteins to a specific subcellular localization. For example, sumoylation of the PML protein is required for its localization to subnuclear bodies (13). Furthermore, the E3 ligase PIASy itself is localized in a punctated pattern within the nucleus and can target LEF-1 to these subnuclear bodies (16). Because Smad4 is translocated into the nucleus upon TGF-β treatment, we examined the possible role

of sumoylation in the subnuclear localization of Smad4 in transfected HeLa cells by immunofluorescence staining. Without increased SUMO-1 or PIASy expression, Smad4 was visualized as a diffuse nuclear staining in TGF-β-treated cells (Fig. 4A, a). PIASy by itself exhibited a punctated, subnuclear staining pattern (Fig. 4A, b), whereas SUMO-1 alone also exhibited a somewhat punctuated staining in the nucleus (Fig. 4A, c).

Fig. 3. Enhancement of Smad sumoylation by SUMO modification enzymes. A, effect of Ubc9 and E3 ligases on sumoylation of wild type Smad4 and Smad4 K113R/K159R mutant. Expression of Ubc9 and E3 ligases enhances Smad4 sumoylation. HeLa cells were transfected to express FLAG-tagged Smad4 and Myc-tagged SUMO-1 in the absence or presence of coexpressed Ubc9 and/or PIASy or ARIP3. Anti-FLAG immunoprecipitates of cell lysates were subjected to SDS-PAGE and anti-Myc immunoblotting, thus revealing increased levels of Smad4 sumoylation (upper panel). The membrane was stripped and reprobed with anti-FLAG antibody to visualize tagged Smad4 (lower panel). A white arrow indicates the position of unmodified Smad4, whereas the black arrows point to the positions of mono-, di-, and tri-sumoylated Smad4, and the semi-open bracket delimits the positions of the psumoylated Smad4 forms. B, Smad1, -2, and -3 are also sumoylated, albeit at lower levels than Smad4, when co-expressed with SUMO-1 and Ubc9. HeLa cells were transfected to express various FLAG-tagged Smads, in the absence or presence of Myc-tagged SUMO-1 or Ubc9. Sumoylated Smads were visualized by anti-FLAG immunoprecipitation of cell lysates, followed by SDS-PAGE and immunoblotting with Myc antibody (upper panel, black arrows). Anti-FLAG immunoblots revealed the total levels of tagged Smads (lower panel).
Fig. 4. Roles of sumoylation and PIASy in subnuclear localization of Smad4. Panels A–C show changes in subnuclear localization of Smad4 upon coexpression of SUMO-1 and sumoylation enzymes. A, SUMO-1 and PIASy showed punctuated nuclear staining patterns whereas Smad4 exhibited a diffuse nuclear staining. Transfected HeLa cells overexpressing (a) Smad4, (b) PIASy, or (c) SUMO-1 were processed for sumoylation of Smad4.
Sumoylation of Smad4

Fig. 5. Effects of sumoylation on Smad4 protein stability. A, Smad4 K113R/K159R protein is more stable than wild-type Smad4. HeLa cells expressing FLAG-tagged wild-type Smad4 or Smad4 K113R/K159R, were pulse-labeled with [35S]Met/Cys and chased for various times, as shown. Smad4 was immunoprecipitated using anti-FLAG antibody and subjected to SDS-PAGE analysis. The gel was dried and processed for autoradiography (left panel). The right panel shows the plot of relative intensity of the labeled Smad4 bands by densitometry. B, the K159R mutation also stabilized Smad4 R100T mutant. HeLa cells were transfected to express FLAG-tagged Smad4 R100T and analyzed by pulse-chase analysis as in A, either in the absence or presence of proteasomal inhibitor lactacystin. C, coexpression of SUMO-1, Ubc9, and PIASy enhanced the stability of Smad4 R100T. FLAG-tagged Smad4 R100T, either alone or together with SUMO-1, Ubc9, and PIASy (i.e. Enz), were expressed in HeLa cells and processed for pulse-chase analysis as above.

mutation introduced into Smad4 R100T similarly conferred a higher stability (Fig. 5B), as observed for wild-type Smad4. Finally, we evaluated the effect of increased sumoylation on the stability of Smad4 R100T. We found that coexpression of SUMO-1, Ubc9, and PIASy enhanced the stability of the Smad4 R100T mutant (Fig. 5C). These results suggest that sumoylation enhances the stability of Smad4, but that replacement of the targeted lysine 159 also confers increased stability.

Effect of Sumoylation on Smad4-mediated Transcriptional Activation—Sumoylation has the potential to enhance the responses of all receptor-activated Smads, induces expression of ventral mesoderm marker expression (55). Smad4, which serves as coactivator for all receptor-activated Smads, induces expression of the pan-mesodermal marker Brachyury (Xbra) (56), yet also has the potential to enhance the responses of all receptor-activated Smads. We therefore addressed the role of Smad4 sumoylation in these Xenopus explant assays.

In one set of experiments, we compared the activities of Smad4 and the mutant Smad4 K159R, in which the major sumoylation target Lys-159 was replaced by Arg, for their abilities to induce Xbra expression. As shown in Fig. 7A, Smad4 induced the expression of Xbra mRNA, while Smad4 K159R, translated from an equal quantity of injected cRNA, induced a higher expression of Xbra (compare lanes 2 and 4). This result is in agreement with the data obtained from the transcriptional reporter assay shown in Fig. 6A and may reflect the increased responsiveness of the Smad4 K159R mutant over wild-type Smad4 (data not shown). A similar effect of the K159R mutation was also observed in the Smad4 mutant, in which Arg-100 was replaced by Thr (compare lanes 3 and 5). These results suggest that...
substitution of Lys-159 by Arg can alter the activity of Smad4 in mesoderm induction.

In another set of experiments, we also compared the effects of the K113R, K159R, and K113R/K159R Smad4 mutants on their ability to regulate gene expression (Fig. 7B). In these experiments we used a more sensitive assay for Smad4 activity, based on the ability of Smad4 to enhance Smad2-mediated mesodermal marker expression. Similarly to activin, Smad2 induces the expression of various mesodermal and endodermal markers in a dose-dependent manner. At low doses, Smad2 induces ventral and ventrolateral mesodermal markers, such as Xwnt8. With increasing Smad2 levels, the expression of dorsal mesodermal and endodermal marker genes, such as chordin and mixer, is turned on (Fig. 7B (54, 55)). Smad4 enhances the mesodermal marker gene expression by Smad2, so that, even at low levels of Smad2, dorsal mesoderm marker expression is turned on efficiently (57). In our experiments, Smad2 induced the pan-mesodermal marker Xbra and the ventrolateral marker Xvent2, whereas coexpression of Smad4 with Smad2 not only enhanced the expression of these markers, but also induced stronger expression of chordin and mixer (Fig. 7B, lanes 3 versus 2). Either K113R or K159R mutation further increased gene activation by Smad4, which is evident in the levels of transcription of the dorsal mesodermal marker chordin and the endodermal marker mixer (Fig. 7B, lanes 4 and 5). The K159R mutant is slightly more active than the K113R mutant whereas the K113R/K159R double mutant has a comparable effect on activation of gene expression as the K113R mutant (Fig. 7B, lane 6).

To further address the role of Smad4 sumoylation on gene regulation, we examined the effect of Ubc9, which strongly enhances Smad4 sumoylation (Figs. 2D and 3A), on the ability of Smad4 to induce mesoderm marker gene expression (Fig. 7C). Again, we used the sensitive assay of enhancement of
Smad2-dependent gene activation by Smad4. In our experiments, coexpression of Smad4 with Smad2 induced mixer, chordin, and Xbra expression to a higher level than Smad2 by itself (i.e. with endogenous Smad4), as illustrated in lanes 4 versus 2 in Fig. 7C. Coexpression of Ubc9 strongly enhanced the expression of the endodermal and dorsal mesoderm markers mixer and chordin, as well as the ventral mesoderm marker Xvent8 and the pan-mesoderm marker Xbra (Fig. 7C, lanes 5 versus 4). Ubc9 also enhanced the marker induction by Smad2 (Fig. 7C, lanes 3 versus 2), possibly through its ability to drive sumoylation of endogenous Smad4, or of Smad2 itself.

It is conceivable, if not likely, that sumoylation has a general effect on transcription through its effects on the general transcription machinery, and thus may affect signaling mediated by other growth factors. We therefore analyzed the effect of Ubc9 on marker induction by two unrelated signaling molecules (Fig. 7D). bFGF induced the mesodermal gene Xbra in Xenopus animal caps, and coexpression of Ubc9 had only minimal effect on this marker induction (Fig. 7D, compare lanes 4 with 3). In contrast, the activity of β-catenin, a downstream signaling effector of the Wnt pathway, which stimulates Siamois expression, was reduced by Ubc9 (Fig. 7D, lanes 6 versus 5). Our results therefore suggest that the enhancement of Smad4-mediated transcription by sumoylation is primarily an effect of Ubc9 on Smad4, and is not because of a general transcription activation by this sumoylation enzyme.

**DISCUSSION**

Numerous studies have established roles of post-translational modifications in a variety of signal transduction pathways. Among these, phosphorylation of signal transducers plays the predominant role in information transfer from the cell surface into the nucleus. Whereas ubiquitylation, and consequent protein degradation, has been shown to regulate the levels of some signaling effectors, a role of the biochemically related mechanism of sumoylation in signal transduction has only recently been recognized. In fact, sumoylation has only been shown to help define ligand-induced signaling mediated by NFκB/IκB (18) and by the intracellular androgen receptor (51) in mammalian cells. We have now demonstrated the sumoylation of Smad4 and evaluated its role in TGF-β family signaling.

Smads act as central effectors of TGF-β family-induced gene expression (58, 59). The receptor-activated Smads relay the information from the ligand-activated receptors, while Smad4 acts as a common Smad that is required for ligand-induced activation of gene expression. The receptor-activated Smads are activated through direct C-terminal phosphorylation by the type I receptors (60, 61), whereas additional phosphorylation events by Erk mitogen-activated protein kinase, c-Jun N-terminal kinase, CamKII, and protein kinase C further define their activation state (62–66). Furthermore, ubiquitin-mediated degradation regulates the levels of receptor-activated Smads, thus providing an additional level of control of signaling (26, 27). In contrast to this extensive post-translational regulation of the receptor-activated Smads, Smad4 is not normally subject to regulation by phosphorylation or ubiquitylation. Because of its central role as a common Smad, one would intuitively expect that Smad4 needs to be stably available, a concept that would be consistent with the apparent ability of Smad4 to shuttle between the cytoplasmic and nuclear compartments (91). Remarkably, sumoylation confers a unique modification to the common Smad4, not present in the receptor-activated and inhibitory Smads.

Biochemical and mutation analyses have identified Lys-159 and Lys-113 as major sumoylation sites in Smad4. The lysine 159 is located in the consensus sequence VXE/D (49), present in Smad4 but not in the other Smads tested, i.e., Smad1, -2, -3, -6, and -7. In agreement with the role of this sequence in recognition and binding of E2-conjugating enzyme Ubc9 (67), sumoylation of Smad4 was increased when Ubc9 was coexpressed. This effect was further enhanced by coexpression with PIASy or ARIP3, members of the recently identified PIAS family of SUMO E3 ligases (11, 16), suggesting that a PIAS E3 ligase is naturally involved in Smad4 sumoylation. Contrary to Lys-159, the sequence around Lys-113 does not conform to the consensus sequence suggesting that other factors, perhaps secondary structure features in the surrounding sequence, may be involved in determining the choice of target lysine. Interestingly the Lys-113 is flanked by two hydrophobic amino acids. The hydrophobic amino acid preceding the lysine is apparently required for sumoylation, because Lys-51, -106, and -392, which are located in a XXE sequence lacking a preceding hydrophobic amino acid, were only minimally if at all targeted for sumoylation. Additionally, we did not have evidence that Lys-122, located in the VXE/KXD sequence, was sumoylated. These potential secondary sumoylation sites likely account for the low level sumoylation, when Lys-159 and Lys-113 are mutated, and the sumoylation of the lysine double mutant, when Ubc9 and E3 ligases are overexpressed.

Fig. 7. Enhancement of Smad4-mediated gene expression by sumoylation in Xenopus animal caps. A, mutation of a major sumoylation site, Lys-159, enhanced the ability of Smad4 to induce mesodermal marker expression. A destabilizing mutation at Arg-100 reduced the Smad4 activity. RNA doses used in this experiment were 8 ng for all the constructs. Gene expression was determined by reverse transcriptase-PCR. B, the K113R and K159R mutations enhanced the ability of Smad4 to stimulate Smad2-dependent gene expression. The doses of RNAs used were 0.25 ng for Smad2 and 0.25 ng for the wild-type and the mutants Smad4. C, the sumoylation enzyme Ubc9 enhanced the marker gene induction by Smad4. The following doses of transcriptase-PCR. D, the sumoylation enzyme Ubc9 only minimally enhanced the marker gene induction by bFGF, and did not increase the activity of β-catenin. The amount of bFGF used was 100 ng/ml, and β-catenin and Ubc9 were injected at the RNA doses of 0.5 and 1 ng, respectively.
mutated lysine 159, a major sumoylation site, either alone or in combination with other secondary lysine modification sites, to arginine and compared the functions of these mutants with wild-type Smad4. Arginine substitution preserves the charge while prohibiting the isopeptide bond formation with SUMO-1. Additionally, we examined the effects of increased sumoylation by E2 and/or E3 enzymes.

Without ectopic coexpression of SUMO-1 and PIASy, Smad4 was diffusely localized in the nucleus. Coexpression of the sumoylation enzymes relocated Smad4 into speckles, which colocalized with PIASy. A similar, punctated redistribution of the sumoylated proteins in the presence of SUMO-1 or sumoylation enzymes has also been observed in the case of the nuclear proteins LEF-1, PML, and HIPK2 (14, 16, 68). Under similar conditions, the Smad4 K122R/K159R mutant also displayed a speckled nuclear distribution. This redistribution of Smad4 is likely because of the ability of PIASy to interact with Smad4, independent of the presence of Lys-159 or Lys-113, and the punctated, subnuclear localization of PIASy. Whereas some protein interactions have been shown to help define the distribution of transcription factors inside the nucleus (16), the subnuclear distribution of receptor-activated Smads is so far only known to be affected by Runx proteins, which can directly interact with Smads (69). Such an effect has not been reported for Smad4. The ability of PIASy to define the subnuclear localization of Smad4 in speckles thus represents the first example of intranuclear redistribution of Smad4. The role of the punctated sequestration of Smad4, or any other protein with similar subnuclear distribution, is as yet unclear, but is likely related to the regulation of the transcriptional activity and/or nuclear metabolism (70, 71).

Sumoylation also modestly enhanced the stability of Smad4, as assessed using pulse-chase analyses. The effect of sumoylation on protein stability was better appreciated using the Smad4 R100T mutant than using the more stable, wild-type Smad4. Smad4 R100T is one of the few Smad4 mutants found in cancer cells that predisposes Smad4 to increased ubiquitin-mediated degradation, thus significantly decreasing its stability (29). Coexpression of SUMO-1 and sumoylation enzymes enhanced the stability of Smad4 R100T, suggesting that it may offset ubiquitin-mediated degradation. As both sumoylation and ubiquitylation occur at Lys residues, it is conceivable that sumoylation could compete with ubiquitylation at the same Lys, thereby preventing degradation. Such a mechanism has been implicated in the regulation of the IkBα stability by sumoylation and ubiquitylation (18). In the case of Smad4 R100T, however, Smad4 ubiquitylation was as efficient as Smad4 R100T/K159R (data not shown), suggesting that a mechanism other than competition is involved. Similarly, sumoylation has been shown to enhance the stability of c-Myc independent of an effect on ubiquitin-mediated degradation (72). The overall higher stability of Smad4, even though at any time only a fraction is sumoylated, is consistent with the dynamic and reversible nature of the sumoylation process. Remarkably, replacement of Lys-159 and Lys-113 with Arg also enhanced the stability of Smad4 independent of sumoylation at these residues, supporting the critical nature of these Lys residues in Smad4 stability and that sumoylation may alter protein stability indirectly such as via a conformational change. Further study is required to identify the detailed molecular basis.

Finally, we also evaluated the role of Smad4 sumoylation in Smad-mediated transcription. Smad4 is known to function as a coactivator of receptor-activated Smads, thereby enhancing Smad-mediated transcription. In one set of experiments, Smad4 enhanced Smad-mediated transcription in transfected mammalian cells that lack endogenous Smad4. In the second system, i.e. Xenopus embryo explant assays, defined amounts of miRNA were injected, thus overcoming any possible effects of sumoylation on transcription from the transfected plasmid. Additionally, the gene expression monitored in Xenopus assays is exquisitely sensitive to quantitative differences in Smad signaling. In both systems we found that replacement of Lys-159 by Arg enhanced the coactivator function of Smad4. In addition, increased sumoylation, resulting from Ubc9 or Ubc9 and SUMO-1 coexpression, also enhanced transcription.

These results need to be interpreted with great caution. Indeed, substitution of Lys-159 by Arg is not necessarily equivalent to lack of sumoylation of Lys-159, and may result in a conformational change. On the other hand, increased Ubc9 expression may affect other components of the transcription machinery, and result in low level sumoylation of receptor-activated Smads. Nevertheless, ectopic Ubc9 expression either did not affect or decreased non-Smad-mediated signaling in Xenopus, in contrast to the enhanced Smad signaling (Fig. 7). Also, increased sumoylation by Ubc9 and SUMO conferred increased Smad4-dependent transcription in luciferase reporter assays that were normalized for nonspecific effects on general transcription. The basis for the increased transcription may be related to the increased Smad4 stability, which was observed when sumoylation enzymes were overexpressed or when Lys-159 was replaced with Arg.

Additionally, the redistribution of Smad4 into speckles may also affect its function. However, in the cases of other transcription factors studied, sumoylation and redistribution into speckles correlate frequently, but not always, with decreased transcription (12, 16, 73). An additional possibility would be that sumoylation of Lys-159 and Lys-113 affect, through conformational changes or protein-protein interactions, the function of Smad4. A previously identified short sequence, the “synergy control” motif, that is found within negative regulatory regions of several transcription factor such as glucocorticoid receptor and C/EBP transcription factors, contains the YKXE sumoylation consensus sequence identical to the one at Lys-159 in Smad4 (74). The lysine residue within this motif in the C/EBP-transcription factors has been shown recently to be sumoylated (75, 76). Mutations of this sequence in the synergy control motif selectively increases the transcription activity of glucocorticoid receptor (74) and C/EBP transcription factors (75, 76) at promoter, but not in single response element, suggesting a regulatory role of sumoylation in higher order interactions among transcription regulators. In addition, sumoylation at Lys-159, which resides in the linker segment (aa 136–322) of Smad4, may somehow affect the function of the SAD domain (Smad4 activation domain, aa 274–322) that is also located in the linker segment of Smad4 (52, 77). This SAD domain is required for efficient function of Smad4 as coactivator, presumably through its ability to recruit the SMIF1 coactivator (22) and CAMP-response element-binding protein/p300 (77). Further studies will be required to define the effect of sumoylation on the intrinsic activity of Smad4.

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1. Pawson, T., and Saxton, T. M. (1999) Cell 97, 675–678
2. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
3. Parekh, R. B., and Rohlf, C. (1997) Curr. Opin. Biotechnol. 8, 718–723
4. Bretonneau, A. H., and Darbess, J. E., Jr. (2000) Science 285, 813–818
5. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Gene (Amst.) 245, 1–14
6. Glickman, M. H., and Ciechanover, A. (2002) Physiol. Rev. 82, 373–428
7. Hay, R. T. (2001) Trends Biochem. Sci. 26, 322–333
8. Jackson, P. K. (2001) Genes Dev. 15, 3053–3058
9. Kirsh, O., Seeler, J. S., Pichler, A., Gaet, A., Muller, S., Miska, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F., and Dejean, A. (2002) EMBO
