Activation of Transforming Growth Factor-β Signaling by SUMO-1 Modification of Tumor Suppressor Smad4/DPC4

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Smads are important intracellular effectors in signaling pathways of the transforming growth factor-β (TGF-β) superfamily. Upon activation by TGF-β, receptor-phosphorylated Smads form a complex with tumor suppressor Smad4/DPC4, and the Smad complexes then are imported into the nucleus. Although diverse pathways regulate the activity and expression of receptor-phosphorylated and inhibitory Smads, cellular factors modulating the activity of the common Smad4 remain unidentified. Here we describe the involvement of the small ubiquitin-like modifier-1 (SUMO-1) conjugation pathway in regulating the growth inhibitory and transcriptional responses of Smad4. The MH1 domain of Smad4 was shown to associate physically with Ubc9, the ubiquitin carrier protein (E2) conjugating enzyme in sumoylation. In cultured cells, Smad4 is modified by SUMO-1 at the endogenous level. The sumoylation sites were identified as two evolutionarily conserved lysine residues, Lys-113 and Lys-159, in the MH1 domain. We found that the mutations at Lys-113 and Lys-159 did not alter the ability of Smad4 to form a complex with Smad2 and FAST on the Mix.2 promoter. Importantly, SUMO-1 overexpression enhances TGF-β-induced transcriptional responses. These findings identify sumoylation as a unique mechanism to modulate Smad4-dependent cellular responses.

Transforming growth factor-β (TGF-β)1 is a secreted multifunctional protein that exhibits a diverse set of cellular responses including cell proliferation and differentiation. TGF-β functions as a potent growth inhibitor and induces the expression of a variety of genes during growth and development (1–4).

1 The abbreviations used are: TGF-β, transforming growth factor-β; R-Smad, receptor-phosphorylated Smad; SUMO, small ubiquitin-like modifier; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Ni-NTA, nickel-nitrilotriacetic acid; HA, hemagglutinin; SBE, Smad-binding element(s); ARF, activin-responsive factor.

TGF-β signals are transduced by transmembrane serine/threonine kinase receptors and intracellular effectors called Smads (2, 5, 6). Upon TGF-β stimulation, Smad2 and/or Smad3 are phosphorylated by the activated type I receptor and then form complexes with Smad4. The heteromeric complexes of R-Smads and Smad4 then are translocated into the nucleus, where they exert ligand-induced changes in the transcription of a variety of genes (2, 6, 7). The heteromeric Smad complex activates transcription through its ability to cooperate functionally with several promoter-specific transcription factors and/or to bind specific DNA sequences (7, 8).

Proper TGF-β signaling requires precise control of Smad functions. Recent studies have shown that R-Smads are modified post-translationally by ubiquitin and can be removed irreversibly by the proteasome-mediated degradation system (9–13). A number of ubiquitin-related proteins also are present in eukaryotic cells. These proteins, including the small ubiquitin-like modifier-1 (SUMO-1), utilize a conjugation system that is similar to ubiquitination (14–16). SUMO-1 uses Aos1/Uba2 as like modifier-1 (SUMO-1), utilize a conjugation system that is similar to ubiquitination (14–16). SUMO-1 uses Aos1/Uba2 as E1 activating enzymes (17, 18) and Ubc9 as an E2 conjugating enzyme (19, 20). Unlike ubiquitination, SUMO-1 modifications of target proteins do not promote their degradation. In contrast, SUMO-1 modifications of IκB preclude its ubiquitination (21). Functional consequences of SUMO-1 modification vary depending on the target. For example, SUMO-1 conjugation of RanGAP1 is necessary for its localization to the nuclear pore complex (22, 23), whereas SUMO-1 conjugation of PML protein is necessary for its localization to the nuclear bodies (24, 25). For other proteins, their covalent modifications by SUMO-1 modulate their biological activities. Recent reports demonstrate that the transcriptional potentials of p53 increase upon SUMO-1 conjugation (26, 27).

A growing body of work provides evidence demonstrating the regulation of R-Smads (2). On the other hand, as a common partner for TGF-β signaling, Smad4 does not appear to be regulated directly by TGF-β receptors or any other factors. Thus, given the key role of Smad4 in TGF-β responses, it is important to study the regulation of Smad4 by other cellular pathways. In this study, we elucidate the involvement of the SUMO-1 conjugation pathway in regulating Smad4 activity. Smad4 is modified by SUMO-1 in vivo. SUMO-1 modification greatly enhances TGF-β signaling. Thus, we propose that post-translational modification of Smad4 by SUMO-1 conjugation provides a unique mechanism to modulate the biological activity of Smad4.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Transfections—Mammalian expression plasmids for epitope (HA or FLAG)-tagged Smad and Gal4-Smad proteins were described previously (28, 29). Ubc9 and SUMO-1 were obtained by PCR and cloned as amino-terminal FLAG-tagged or HA-
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tagged proteins in pXF2F or pXF2H, which also are derived from the pRSK vector.

Human HeLa cells, HEK 293 cells, and mink lung epithelial Mv1Lu (RI-14 line) cells were grown and transfected as described previously (28, 29).

Yeast Two-hybrid Analysis—A LexA-based yeast two-hybrid assay (30) was used to search for Smad4-interacting proteins and to analyze interactions between the domains of Smad4 in bait plasmid pEG202 and of Ubc9 in prey plasmid pJG4-5. pEG202-Smads were described previously (28). pJG4-5-Ubc9 was obtained by releasing the Ubc9 cDNA insert from pXF2F-Ubc9 using EcoRI and SalI and subcloning it in EcoRI-XhoI sites of pJG4-5. Plasmids were transformed into yeast EGY48 using Alkali cation (BIO 101, Inc.), and protein-protein interactions were assessed by scoring β-galactosidase activity as reporter.

Immunoprecipitation, Ni-NTA Precipitation, and Western Blot—For Ni-NTA precipitation, HeLa cells were transfected with His-tagged Smad4 and FLAG-tagged SUMO-1. Forty-eight hours after transfection, the cells were sonicated in guanidine lysis buffer (6 M guanidine HCl, 0.1 M NaNO3, 0.01 mM Tris-HCl, pH 8). His-tagged Smad4 was immobilized on Ni-NTA beads (Qiagen) and eluted in elution buffer (200 mM imidazole, 5% SDS, 0.15% Trit, pH 6.7, 30% glycerol, 0.72% β-mercaptoethanol). Eluted proteins were analyzed by Western blot using anti-His or anti-Smad4 antibody (Santa Cruz Biotechnology) for Smad4 and anti-FLAG (Sigma) or anti-SUMO-1 antibody (Zymed Laboratories Inc.) for SUMO-conjugated Smad4 protein. Antibody-bound proteins were visualized by chemiluminescence (Pierce).

Immunoprecipitations using anti-FLAG or anti-HA antibody essentially were carried out as described (28, 31). To detect sumoylation of endogenous Smad4, cells were subjected to lysis in a buffer containing FLAG lysis buffer (31) supplemented with 5% SDS and 20 mM isopeptidase inhibitor N-ethylmaleimide. Cell lysates then were diluted with the same buffer without SDS to achieve an SDS concentration of 0.5%.

Immunoprecipitation of Smad4 was performed using anti-Smad4 antibody (B8, Santa Cruz Biotechnology) as described (31).

Gel Shift Assays—Gel shift assays using nuclear extract were performed as described previously (28, 29). A 10-μl reaction contained nuclear extract, as specified in the text, in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM MgCl2, 50 mM NaCl, 0.5 mM dithiothreitol, 4% glycerol, and 0.05 mg/ml poly(dI-dC). The reaction was incubated with 100,000 dpm of 32P-labeled probe at room temperature for 30 min. A DNA probe containing the Mix.2 activin-responsive element (32) was used. DNA-protein complexes were separated in a 5% polyacrylamide gel in 0.5 TBE (Tris borate/EDTA) buffer and visualized by autoradiography.

Transcription Reporter Assays—Plasmid SBE-luc, which contains the luciferase gene under control of the Sma(l)-binding elements (SBE) (35), was used to measure TGF-β- and Smad-induced transcription. Transfections, TGF-β treatment, and reporter assays were carried out as described (29, 31). Generally, exponentially grown cells at 25–30% confluency were transfected with expression plasmids for Smads and/or reporter plasmids. 40–45 h after transfection, cells were treated with 200 μM TGFB-β for 12 h. Cells then were harvested for measurement of luciferase and β-galactosidase activities. All assays were done in triplicate, and all values were normalized for transfection efficiency against β-galactosidase activity.

RESULTS AND DISCUSSION

Identification of Ubc9 as a Candidate Smad4-interacting Protein—To search for regulators of Smad4, we performed a yeast two-hybrid screen using Smad4 as bait. A cDNA library derived from human ovarian epithelial HeLa cells was screened. Results showed three clones isolated in the screen were identical to Ubc9. Ubc9 is an E2 conjugating enzyme required for covalent modification of the SUMO proteins (19, 20).

We next analyzed whether Ubc9 could interact with various Smads. In a two-hybrid analysis, two R-Smads, i.e. Smad1 (bone morphogenetic protein-specific) and Smad3 (TGF-β/activin-specific), were included for their interactions with Ubc9. Smad4 exhibited a strong interaction with Ubc9, whereas Smad1 or Smad3 was unable to interact with Ubc9 in yeast cells (Fig. 1A). Thus, analysis of the results showed a specific interaction of Ubc9 with Smad4.

Smad4 has two conserved domains, MH1 at the amino terminus and MH2 at the carboxyl terminus, which are linked by a divergent linker region (Fig. 1C). To map the Ubc9-interacting domain of Smad4, we created a series of Smad4 deletion mutants. Deletions of the MH2 domain or of MH2 together with the linker region did not alter the ability of Smad4 to bind to Ubc9, whereas deletion of the MH1 domain and part of the linker region completely abolished the interaction between Smad4 and Ubc9 (Fig. 1B). Further deletion analysis narrowed the Ubc9-interacting domain of Smad4 down to the region of amino acids 2–186 (Fig. 1B).

Smad4 Is Sumoylated at Two Evolutionarily Conserved Lysine Residues, Lys-113 and Lys-159—Because Ubc9 is the E2 enzyme for sumoylation, we sought to determine whether Smad4 is a substrate for the SUMO-1 conjugation. We first tested Smad4-SUMO conjugation in transfected cells. Extracts from HeLa cells that were transfected with expression plasmids for His-tagged Smad4 and FLAG-tagged SUMO-1 were subjected to precipitation with Ni-NTA-agarose beads in a lysis buffer containing 6 M guanidine HCl. The precipitates then were analyzed by immunoblotting with antibodies to SUMO-1. Two protein bands in the range of 80 to 100 kDa were detected by anti-SUMO antibody in Ni-NTA-agarose complexes only when Smad4 and SUMO-1 were cotransfected (Fig. 2A, top). To verify whether these bands represent SUMO modification on Smad4, we stripped the same membrane and reblotted it with Smad4 antibody (Fig. 2A, bottom). Because SUMO-1 often migrates at 20 kDa, the 80–100-kDa bands represent the addition of one to two SUMO-1 molecules to Smad4. Furthermore, an estimated 30% of Smad4 was sumoylated under these conditions.

SUMO-1 is attached to the lysine residues of target proteins. We next mapped the lysine residues of Smad4 that were modified by SUMO-1. Deletion analysis of Smad4 showed that sumoylation could be observed on the amino acid 2–186 fragment that spanned both the MH1 domain (amino acids 2–154)
and part of the linker region (amino acids 154–184) but not on the amino acid 184–552 fragment containing the linker MH2 domain, indicating that sumoylation sites were located in the first 186 amino acids (Fig. 2B), which coincides with the Ubc9-interacting region on Smad4 (Fig. 1B). In the amino acid 2–186 region of Smad4, the sequence around Lys-159 (amino acids 157–161, MVKDE) matched perfectly with the consensus sumoylation motif (ΨH9023KXE, where “Ψ” represents a hydrophobic amino acid and “X” represents any amino acid). We created a mutant named Smad4 (K159R), in which Lys-159 was changed into a similarly charged Arg residue. Our data showed that Smad4 (K159R) only abolished the slower migrating SUMO-1-conjugated form, suggesting that Lys-159 represents only one of the SUMO-1 modification sites on Smad4 (Fig. 2C). Surprisingly, another consensus sumoylation motif at Lys-122 (amino acids 120–124, DLKCD) was not sumoylated, as the K122R mutant behaved like wild-type Smad4 (Fig. 2C). To identify the other site, we mutated each individual Lys residue in the MH1 domain and analyzed the sumoylation profile of these mutants (data not shown). We found that only mutation at Lys-113 affected the modification of Smad4. Also noteworthy is that Smad4 (K113R) abolished only the faster migrating form (Fig. 2D). Simultaneous mutation at both sites, as in Smad4 (K113R/K159R), completely prevented the sumoylation of Smad4 (Fig. 2D).

Smad4 is an evolutionarily conserved common Smad partner for TGF-β signaling. A comparison of the sumoylation sites of human Smad4 with the same region from other organisms revealed that the positions of the two sumoylated lysines are highly conserved (Fig. 2E). The two conserved lysine residues are absent from other members of the Smad family, i.e. R-Smads and inhibitory Smads (data not shown). This suggests that sumoylation of Smad4 is specific and remains highly conserved throughout evolution.

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We next examined endogenous Smad4-SUMO conjugation in vivo. Epithelial HeLa, HM3, and Chinese hamster ovary (CHO) cells were lysed in a buffer containing SDS and isopeptidase inhibitor N-ethylmaleimide. The cytosols were subjected to immunoprecipitation with anti-Smad4 antibody, and the immunoprecipitates were analyzed by immunoblotting with antibodies to SUMO-1 or Smad4. Results showed that two bands of 80–100 kDa representing Smad4-SUMO conjugates were detected by anti-SUMO-1 or anti-Smad4 antibody in anti-Smad4 immunoblotting (Fig. 2F).
precipitates from HeLa cells (Fig. 3, left). In contrast, control antibody (IgG) did not retrieve Smad4 or sumoylated Smad4 proteins. The estimated amount of sumoylated Smad4 occupied about 20% of total Smad4 level. Transfection of SUMO-1 increased the level of sumoylated Smad4, reaching ~40% of the sumoylated form (Fig. 3, left, lane 1). The same two endogenous Smad4-SUMO conjugates also were observed in HM3 and Chinese hamster ovary cells (Fig. 3, right) and in HaCaT cells (data not shown). Thus, our data suggest that Smad4 is sumoylated in vivo under physiological conditions.

Sumoylation of Smad4 Has No Effects on the Formation of a Smad4-dependent Transcription Activator Complex—To determine whether SUMO/Ubc9 influences TGF-β signaling, we examined the effect of Smad4 sumoylation on Smad complex formation. In RI-14 mink epithelial cells, TGF-β induced endogenous Smad2-Smad4 interaction (Fig. 4A, compare lanes 1 and 3). Expression of SUMO-1 did not alter the TGF-β-induced Smad complex formation (Fig. 4A, compare lanes 2 and 4 with lanes 1 and 3). We also tested the ability of the Smad4 (K113R/K159R) mutant to form a complex with Smad2. The results showed that in 293 cells, TGF-β (activated TGF-β receptor type I) induced Smad2 interaction with either wild-type Smad4 or mutant Smad4 with no detectable differences (Fig. 4B).

We then investigated whether SUMO-1 or K113R/K159R mutation could influence the formation of a Smad activator complex on the TGF-β-responsive promoter. In the activin-responsive Mix.2 promoter, Smad4 is essential in the assembly of activin-responsive factor (ARF) complex, which also contains Smad2 and the forkhead protein FAST1 (32). To examine ARF formation, we used SW480.7 cells, which lack expression of endogenous Smad4. As shown in Fig. 4C, in the absence of FAST1 or Smad2/4, no ARF was formed (lanes 1–4). TGF-β receptor activation stimulated the formation of ARF (Fig. 4C, lane 8). Mutation of K113R/K159R did not affect the ability of Smad4 to form ARF complex (Fig. 4C, lane 10). Furthermore, SUMO-1 expression in SW480.7 cells did not produce any effects on the formation of ARF complex (Fig. 4C, compare lanes 11 and 12 and lanes 13 and 14).

Stimulation of TGF-β Signaling Activity by SUMO through Smad4 Stabilization—To investigate the functional consequence of Smad4 sumoylation in TGF-β signaling, we tested whether overexpression of SUMO-1 and Ubc9 exerted any stimulatory or inhibitory effects on TGF-β-induced transcription. To avoid any interference of other DNA-binding cofactors, we examined the TGF-β-dependent induction by utilizing SBE-luc, a Smad-responsive synthetic reporter gene that depends solely on Smad activation. SBE-luc contains four copies of SBE linked to a luciferase reporter gene (35). TGF-β normally induced a 5–10-fold increase in the SBE-luc reporter gene activity in RI-14 cells (Fig. 5A). Interestingly, cotransfection of SUMO-1 increased the reporter gene activity in response to TGF-β. Additionally, the combination of SUMO-1 and Ubc9 was the most potent in stimulating SBE reporter activity. Even in the presence of overexpressed Smad3 and Smad4, SUMO-1 and Ubc9 exerted additional activation on the SBE reporter response (Fig. 5A).

We then determined whether stimulation of TGF-β signaling by SUMO-1 was Smad4-dependent by using Smad4-deficient SW480.7 cells. As shown in Fig. 5B, activation of the TGF-β receptor did not stimulate the activity of SBE-luc in SW480.7 cells without Smad4, and SUMO-1 did not exhibit any effects on the transcription. In the presence of Smad4, TGF-β induced an approximately 2.5-fold increase in the SBE-luc activity (Fig. 5B). Importantly, the addition of SUMO-1 further increased the TGF-β-induced reporter gene activity (Fig. 5B). In contrast, transcriptional activity of Smad4 (K113R/K159R) remained unaffected by SUMO-1 (Fig. 5B). Interestingly, Smad4 (K113R/K159R) had high basal transcription activity. One possibility is that SUMO-deficient mutant K113R/K159R may represent a gain-of-function mutant through antagonizing Smad4 degradation.

To investigate the effect of SUMO-1 on Smad4 stability, we...
Fig. 5. Activation of TGF-β signaling by Smad4 sumoylation. A, SUMO/UbC activates SBE-luc activity. Mink lung epithelial RI-14 cells were transfected with the indicated plasmids. TGF-β treatment and the luciferase reporter assay are described under “Experimental Procedures.” B, stimulatory effect of SUMO requires Smad4. The assay was performed as described for panel A except that Smad4-deficient SW480.7 cells were used. C, dosage-dependent effect of SUMO on Smad4 stability. HeLa cells were transfected with 1 μg of His-tagged wild-type (WT) Smad4 or K113R/K159R mutant, together with increasing the amount (μg) of FLAG-SUMO-1 cDNA. NTA precipitation (Ni-NTA ppt) and Western blotting were as described for Fig. 2. An asterisk indicates a nonspecific band. D, ubiquitination of Smad4 and its KR mutant. HeLa cells were transfected both with His-tagged wild-type Smad4 or Smad4 (K113R/K159R) and with HA-ubiquitin. Cytosol was incubated with Ni-NTA beads and blotted with anti-HA antibody.

Compared the K113R/K159R mutant with wild-type Smad4. Increasing the amount of SUMO-1 transfected into HeLa cells yielded an increased level of free Smad4 in addition to the increase in Smad4-SUMO conjugates (Fig. 5C). In sharp contrast, the level of the mutant was already high and remained constant with increasing SUMO expression (Fig. 5C). In sharp contrast, the level of the mutant was already high and remained constant with increasing SUMO expression (Fig. 5C). We next determined the ubiquitination of the wild-type and mutant Smad4 proteins. In agreement with their degradation profile, moderate ubiquitination of the wild type and weak ubiquitination of the K113R/K159R mutant were detected (Fig. 5D). These results suggest that Lys-113, Lys-159, or both also could serve as negative elements for regulating Smad4 degradation. Thus, the sumoylation on Lys-113 and/or Lys-159 residues or the mutation of these sites simply prevents Smad4 from ubiquitination-dependent degradation.

In summary, our study described the post-translational modification of tumor suppressor Smad4/DPC4. Smad4 is the common mediator for TGF-β signaling, forming a complex with R-Smads in response to ligand stimulation. Because the strength and intensity of TGF-β signaling is controlled by the activity of each signaling component, R-Smads often serve as regulatory targets for phosphorylation and ubiquitination (2). In this study, we provide the first evidence that Smad4 is subjected to regulation through SUMO-1 modifications. Sumoylation occurs on two conserved lysine residues and positively stimulates the transcriptional functions of Smad4 through stabilization of Smad4 protein. Further investigation of Smad4 sumoylation through identification of its specific SUMO E3 ligase should shed light on the physiological functions of sumoylation in TGF-β signaling.

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