SUMO-1/Ube9 Promotes Nuclear Accumulation and Metabolic Stability of Tumor Suppressor Smad4*

Received for publication, March 13, 2003, and in revised form, June 13, 2003
Published, JBC Papers in Press, June 17, 2003, DOI 10.1074/jbc.C300112200

Xia Lin‡, Min Liang‡, Yao-Yun Liang‡, F. Charles Brunicki‡, and Xin-Hua Feng‡

From the ‡Michael E. DeBakey Department of Surgery, and ¶Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

Tumor suppressor Smad4/DPC4 is a central intracellular signal transducer for transforming growth factor-β (TGF-β) signaling. We recently reported that transcriptional potential of Smad4 was regulated by SUMOylation in transfected HeLa cells (1), but the precise mechanism and function of Smad4 SUMOylation in TGF-β signaling remain to be elucidated. Here, we describe the regulation of TGF-β signaling by SUMOylation through the control of Smad4 metabolic stability and subcellular localization. We found that SUMO-1 overexpression strongly increases Smad4 levels, while inhibition of SUMOylation by small interfering RNA (siRNA)-mediated knockdown of the E2 enzyme Ube9 reduces endogenous Smad4 levels. Concomitantly, SUMO-1 overexpression enhances and Ube9 knockdown reduces levels of intranuclear Smad4, growth inhibitory response, as well as transcriptional responses to TGF-β. Comparison of wild type and mutant forms of Smad4 for SUMOylation, ubiquitination, and half-life allows the conclusion that SUMO-1 modification serves to protect Smad4 from ubiquitin-dependent degradation and consequently enhances the growth inhibitory and transcriptional responses of Smad4.

The strength and intensity of TGF-β1 signaling require a tight control of the activity of each signaling component, including the central signal transducing Smad proteins. Tumor suppressor Smad4/DPC4 is the common mediator for TGF-β signaling by forming a complex with R-Smads in response to ligand stimulation (2–6). The heteromeric complexes of R-Smads and Smad4 are then translocated into the nucleus, where they exert ligand-induced changes in transcription of a variety of genes involved in cell responses, including cell proliferation, differentiation, and extracellular matrix remodeling (for reviews, see Refs. 2–7). The heteromeric Smad complex activates transcription through its ability to functionally cooperate with several promoter-specific transcription factors and/or to bind specific DNA sequences (7, 8).

Recent studies have shown R-Smads are regulated by the proteasome-mediated degradation system (9–13). Ubiquitination, the covalent attachment of ubiquitin to proteins, predominantly serves to target proteins for their degradation by proteasomes (14). Interestingly, a number of ubiquitin-related proteins are also present in eukaryotic cells (15). These proteins, including the small ubiquitin-like modifier-1 (SUMO-1), utilize a conjugation system that is similar to ubiquitination (16–18). In contrast to ubiquitination, SUMO-1 modifications of target proteins do not promote their degradation, but modulate the subcellular localization or biological activities of targets (19–25).

Smad4 is the central mediator for signaling of TGF-β superfamily, and thus it is important to study the regulation of Smad4. Recently, we and another group identified Smad4 as a substrate of SUMOylation pathway (1, 33). Here we further elucidate the mechanism of how the SUMOylation regulates Smad4 activity under physiological conditions. We have found that RNA interference (RNAi)-mediated silencing of the human Ube9 gene disrupts Smad4 SUMOylation, decreases Smad4 stability, reduces Smad4 accumulation in the nucleus, and consequently blocks TGF-β signaling. Thus, SUMOylation of tumor suppressor Smad4 provides a novel mechanism to control TGF-β antiproliferative signaling.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Transfections—Mammalian expression plasmids for epitope (HA, FLAG, and His)-tagged Smad4, Ube9, and SUMO were described previously (1, 26, 27). Human HeLa cells and mink lung epithelial Mv1Lu (RI-14 line) cells were grown and transfected as described previously (26, 27).

Ni-NTA Precipitation and Western Blot—Ni-NTA precipitation and Western blot analysis were essentially as described previously (1). Briefly, HeLa cells were transfected with expression plasmids for His-tagged Smad4 and FLAG-tagged SUMO1 and harvested in guanidine lysis buffer (6 M guanidine HCl, 0.1 M NaPO4, 0.01 M Tris Cl, pH 8). His-tagged Smad4 was retrieved from the lysates by using Ni-NTA beads (Qiagen), followed by standard gel electrophoresis and Western blotting analysis using anti-Smad4 (Santa Cruz Biotechnol-ogy) and anti-FLAG (Sigma) antibody.

Transcription Reporter Assays—Transfections, TGF-β treatment, and reporter assays were carried out as described previously (27). Generally, HeLa cells at 25–30% confluence were transfected with expression plasmids for Smad4 and/or reporter plasmids. Reporter plasmid SBE-Luc contains the luciferase gene under control of the Smad-binding elements (SBE) (28). The amount of transfected DNA was always the same by adding 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 triplicate and all values were normalized for transfection efficiency against β-galactosidase activity.

Immunofluorescence—HeLa and RI-14 cells, untransfected or trans-
SUMOylation Stabilizes Smad4

For precipitation-Western analysis, HeLa cells were first transfected with plasmid DNA, as specified in the text. Twenty-four hours upon DNA transfection, the same cells were transfected with Ubch9 siRNA. Forty-eight hours later, cells were harvested, and cell lysates were used to detect Ubch9 protein in anti-Ubch9 Western blot or further processed to analyze Smad4 SUMOylation.

**Cell Proliferation**—HeLa cells were first transfected with siRNA as described above. After 24 h, 1 × 10⁵ cells were then replated into each well of a 96-well flat-bottom plate. Upon attachment to the well (3 h), cells were treated with or without TGF-β for 2 or 3 days. Measurement of cell proliferation was then carried out using MTS (3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) based CellTiter96 Aqueous assay (Promega). Tetrazolium compound MTS was added to the cells, which were then returned to the cell culture incubator (37 °C, 5%CO₂) for 1.5 h. The absorbance of cells was read at 590 nm in a microplate reader. The TGF-β-induced inhibition of cell proliferation was calculated by the following formula: % inhibition = [(OD without TGF-β) − (OD TGF-β)]/(OD without TGF-β) × 100.

**RESULTS AND DISCUSSION**

SUMOylation of Smad4 Occurs in the Nucleus and Is Regulated by TGF-β—Our recent investigation of Smad4 signaling revealed that Smad4 is SUMOylated in cells (1). The modification sites occur on two lysine residues in the MH1 domain of Smad4, i.e. Lys₁³¹ and Lys₁⁶⁹ (Fig. 1A; Ref. 1). To investigate the subcellular location of Smad4 SUMOylation, we examined the in vivo SUMOylation of two Smad4 mutants carrying mutation in the nuclear export sequence (NES) or the nuclear export sequence (NES) or the nuclear import sequence (NLS). For precipitation-Western analysis, HeLa cells were first transfected with plasmid DNA, as specified in the text. Twenty-four hours upon DNA transfection, the same cells were transfected with Ubch9 siRNA. Forty-eight hours later, cells were harvested, and cell lysates were used to detect Ubch9 protein in anti-Ubch9 Western blot or further processed to analyze Smad4 SUMOylation. For precipitation-Western analysis, HeLa cells were first transfected with plasmid DNA, as specified in the text. Twenty-four hours upon DNA transfection, the same cells were transfected with Ubch9 siRNA. Forty-eight hours later, cells were harvested, and cell lysates were used to detect Ubch9 protein in anti-Ubch9 Western blot or further processed to analyze Smad4 SUMOylation.
SUMOylation Stabilizes Smad4

SUMOylation stabilizes Smad4. A, metabolic stability of Smad4 is enhanced in the K113R/K159R mutant, but reduced in a SUMOylation-deficient R100T mutant. Pulse-chase conditions are described under “Experimental Procedures.” Three experiments were carried out. A representative is shown. WT, wild type.

B, ubiquitination of Smad4 and its mutants. HeLa cells were transfected with His-tagged wild type Smad4, Smad4(K113R/K159R), or Smad4(R100T), together with HA-ubiquitin. Cytosol was then incubated with Ni-NTA beads and treated with HA-ubiquitin. Smad4 and its mutants. HeLa cells were transfected with His-tagged Smad4 and its mutants. HeLa cells were transfected with His-tagged wild type Smad4, Smad4(K113R/K159R), or Smad4(R100T), together with HA-ubiquitin. Cytosol was then incubated with Ni-NTA beads and treated with anti-HA antibody (top) or anti-Smad4 (bottom). WCL, whole cell lysates; IP, immunoprecipitation.

C, loss of SUMOylation on R100T. HeLa cells were transfected with the indicated plasmids, and Ni-NTA precipitation and Western blotting (IB) were the same as described in the legend to Fig. 1B. WT, Smad4 wild type. D, stability status of R100T/K113R/K159R triple mutant. HeLa cells were transfected with His-tagged Smad4(R100T) and Smad4(R100T/K113R/K159R) and treated with cycloheximide (CHX) (100 μM) for the indicated time period. The level of remaining Smad4 (undegraded) was analyzed by anti-His Western blotting.

SUMOylation Enhances TGF-β-induced Nuclear Accumulation of Smad4—One key regulation of Smad4 activity is its nuclear translocation in response to TGF-β. However, Smad4 also has NES (see Fig. 1A), and thus, is frequently shuttled between the nucleus and cytoplasm (30). In our work, we examined whether SUMOylation influenced Smad4 subcellular accumulation in RI-14 cells. Results from immunofluorescence microscopy are shown in Fig. 1E. Since Smad4 is a shuttling protein, we divided the subcellular localization of Smad4 into two categories: exclusive nuclear (N) or non-exclusive nuclear staining (N/C + C); the latter included cytoplasmic staining (C) or a mixed distribution between the nucleus and cytoplasm (N/C). In the absence of TGF-β stimulation, there were about 30% (63/213) of cells that expressed wild type Smad4 exclusively in the nucleus and 70% of cells in the cytoplasm or in both compartments. With TGF-β stimulation, 60% (106/177) of cells expressed Smad4 exclusively in the nucleus, while 40% were in the cytoplasm or both, supporting the notion that TGF-β causes a nuclear shift of Smad4 protein. We also examined the effect of SUMO1 expression on subcellular distribution of Smad4. In the absence of TGF-β stimulation, SUMO1 ex-
experiments, we observed that the steady state level of Smad4 is low in the absence of SUMO1, but overexpression of SUMO1 can increase the steady state level of Smad4 (1).

To examine the metabolic stability of Smad4, we performed pulse-chase experiment. We compared the stability of wild type, K113R/K159R mutant, and a cancer-derived Smad4 mutant with Arg → Thr mutation (R100T) that has previously been shown to undergo faster degradation (11). The half-life of wild type Smad4 was found to be around 9 h, whereas the level of the K113/159R mutant remained 50% upon 24 h of chase (Fig. 2A). The R100T mutant had only a half-life of 3 h. Similar results were obtained in cycloheximide chase experiments (data not shown). These results suggest that Lys113, Lys159, or both, could also serve as a negative element for regulating Smad4 degradation. Thus, the SUMOylation on Lys113/159 residues or mutation of the same sites simply prevent Smad4 from degradation. This conclusion is consistent with the reduced ubiquitination on the K113R/K159R mutant (Fig. 2B; Ref. 1) and increased ubiquitination on the R100T mutant (Fig. 2B; Ref. 1). This observation is reminiscent of IκB, of which SUMOylation antagonizes ubiquitination through the lysine residue Lys21 (19). Jab1, a component of CIB9 signalosome, was found to promote Smad4 degradation (32). However, over-
expression of Jab1 still induced the degradation of K113R/K159R mutant (data not shown), suggesting that additional ubiquitin E3 ligases or factors (other than Jab1 pathway) are responsible for Smad4 degradation through the Lys113 and/or Lys159 residues.

Since our data suggest a role of SUMOylation in Smad4 stability, we set out to determine whether the R100T mutant might have a loss or reduction in SUMOylation. As shown in Fig. 2C, the R100T mutant failed to be SUMOylated, while the wild type was efficiently SUMOylated in HeLa cells (Fig. 2C), suggesting that the rapid proteolysis of this mutant could be responsible for its inability to be SUMOylated. In light of the observation that the R100T mutant has normal nuclear localization with Smad2 in response to TGF-β (11), its loss of SUMOylation may be attributed to a conformational change such that efficient SUMO modification cannot occur on the Lys113/159 residues. As SUMOylation competes or blocks ubiquitination on Smad4, the lack of SUMOylation thus contributes to the enhanced ubiquitination and faster degradation of the R100T mutant (Fig. 2, A and B). More importantly, mutations of ubiquitination sites at Lys113 and Lys159 greatly enhanced the metabolic stability of R100T; the cycloheximide experiment clearly showed that the triple mutant (R100T/K113R/K159R) had a much longer half-life than the unstable R100T mutant (Fig. 2D). A similar observation was reported recently (33). Therefore, insufficient SUMOylation may serve as one mechanism for cells to escape TGF-β growth inhibitory control during cancer development.

Reduction in Smad4 SUMOylation Decreases the Steady State Level of Endogenous Smad4—Taken together, data described above would suggest that SUMO modification plays a positive role in regulating Smad4 activity. Indeed, reporter assays demonstrated that overexpression of SUMO1 and Ubc9 stimulated TGF-β-induced transcriptional responses (1). To test this more directly in a physiologically relevant context, we sought to assess the impacts of disrupting endogenous Ubc9 expression using RNAi. Since Ubc9 is the only E2 enzyme for modification by members of SUMO family, inhibition of its expression should disrupt SUMOylation of its substrates.

We used a 21-nucleotide siRNA that specifically targets to the human Ubc9 coding region and examined its effectiveness in silencing Ubc9 expression in HeLa cells. 48 h after transfection with the siRNA, HeLa cells expressed a significantly low level of endogenous Ubc9 protein, in comparison with the mock-transfection control (Figs. 3, A and B, and 4B). In contrast, expression of β-actin remained unchanged (Figs. 3, A and B, and 4B). Treatment of HeLa cells with unrelated RNAi oligonucleotides did not reduce the level of Ubc9 protein (data not shown).

We then determined whether diminished Ubc9 expression could affect the SUMOylation of Smad4 and reverse the effects of SUMOylation on Smad4 stability. Our results showed that increasing doses of Ubc9 siRNA caused gradual reduction in the steady state level of SUMO-conjugated Smad4 in transfected HeLa cells (Fig. 3A). Accordingly, the steady state level of endogenous Smad4 decreased upon siRNA transfection (Fig. 3, B and C). Smad4 was present in both the nucleus and cytoplasm and displayed a diffused and weak staining (Fig. 3C). TGF-β treatment induced a concentration of Smad4 in the nucleus, causing a brighter image under microscope. Inhibition of Ubc9 expression with siRNA reversed the effect of TGF-β on Smad4 accumulation. Levels of Smad2 and Smad3 (controls) were the same with or without the siRNA (Fig. 3B).

Ubc9 Is Required for TGF-β-Induced Growth Inhibition and Endogenous Plasminogen Activator Inhibitor-1 (PAI-1) Expression—TGF-β exerts its responses by regulating gene transcrip-

SUMOylation Stabilizes Smad4

Reduction in Smad4 SUMOylation Decreases the Steady State Level of Endogenous Smad4—Taken together, data described above would suggest that SUMO modification plays a positive role in regulating Smad4 activity. Indeed, reporter assays demonstrated that overexpression of SUMO1 and Ubc9 stimulated TGF-β-induced transcriptional responses (1). To test this more directly in a physiologically relevant context, we sought to assess the impacts of disrupting endogenous Ubc9 expression using RNAi. Since Ubc9 is the only E2 enzyme for modification by members of SUMO family, inhibition of its expression should disrupt SUMOylation of its substrates.

We used a 21-nucleotide siRNA that specifically targets to the human Ubc9 coding region and examined its effectiveness in silencing Ubc9 expression in HeLa cells. 48 h after transfection with the siRNA, HeLa cells expressed a significantly low level of endogenous Ubc9 protein, in comparison with the mock-transfection control (Figs. 3, A and B, and 4B). In contrast, expression of β-actin remained unchanged (Figs. 3, A and B, and 4B). Treatment of HeLa cells with unrelated RNAi oligonucleotides did not reduce the level of Ubc9 protein (data not shown).

We then determined whether diminished Ubc9 expression could affect the SUMOylation of Smad4 and reverse the effects of SUMOylation on Smad4 stability. Our results showed that increasing doses of Ubc9 siRNA caused gradual reduction in the steady state level of SUMO-conjugated Smad4 in transfected HeLa cells (Fig. 3A). Accordingly, the steady state level of endogenous Smad4 decreased upon siRNA transfection (Fig. 3, B and C). Smad4 was present in both the nucleus and cytoplasm and displayed a diffused and weak staining (Fig. 3C). TGF-β treatment induced a concentration of Smad4 in the nucleus, causing a brighter image under microscope. Inhibition of Ubc9 expression with siRNA reversed the effect of TGF-β on Smad4 accumulation. Levels of Smad2 and Smad3 (controls) were the same with or without the siRNA (Fig. 3B).

Ubc9 Is Required for TGF-β-Induced Growth Inhibition and Endogenous Plasminogen Activator Inhibitor-1 (PAI-1) Expression—TGF-β exerts its responses by regulating gene transcrip-
ylation may be an important gatekeeper that prevents Smad4 from degradation in normal cells. SUMOylation of Smad4 occurs in the nucleus. The stimulatory effects of Smad4 SUMOylation on TGF-β signaling may also be connected to an inhibition of its nuclear export by SUMOylation. The SUMOylation on Lys113 and Lys159, which spans the NES (amino acids 143–148) of Smad4, may block the interaction of the NES with the export machinery. Fig. 5C illustrates our working model on the relationship of SUMOylation, stability, and nuclear retention of Smad4. Further delineation on the mechanism of Smad4 nuclear import and export will provide more insights into the functions of SUMOylation in Smad4-dependent signaling events.

Acknowledgments—We thank Frauke Melchior for stimulatory discussion. We are grateful to Caroline Hill for Smad4 NES/NLS mutants, Bert Vogelstein for SBE-luc, Joan Massague for the original Mv1Lu-L17 cell line, and Mary Moore for leptomycin B.

REFERENCES
1. Lin, X., Liang, M., Liang, Y.-Y., Brunicardi, F. C., Melchior, F., and Feng, X.-H. (2003) J. Biol. Chem. 278, 13714–13719
2. Dennler, S., Goumans, M. J., and Ten Dijke, P. (2002) J. Leukoc. Biol. 71, 731–740
3. Massagué, J. (2000) Nat. Rev. Mol. Cell. Biol. 1, 169–176
4. Miyazono, K., ten Duke, P., and Heldin, C. H. (2000) Adv. Immunol. 75, 115–157
5. Moustakas, A., Souchelnytskyi, S., and Heldin, C. H. (2001) J. Cell Sci. 114, 4359–4369
6. Wrana, J. L. (2000) Cell 100, 189–192
7. Derynck, R., Zhang, Y., and Feng, X.-H. (1998) Cell 95, 737–749
8. Massagué, J., and Wotton, D. (2000) EMBO J. 19, 1745–1754
9. Lin, X., Liang, M., and Feng, X.-H. (2000) J. Biol. Chem. 275, 36818–36822
10. Lo, R. S., and Massague, J. (1999) Nat. Cell Biol. 1, 472–478
11. Xu, J., and Attisano, L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4820–4825
12. Zhang, Y., Chang, C., Gehling, D. J., Hemmati-Brivanlou, A., and Derynck, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 974–979
13. Zhu, H., Kavak, P., Abdollah, S., Wrana, J. L., and Thomsen, G. H. (1999) Nature 400, 687–693
14. Ciechanover, A., Orian, A., and Schwartz, A. L. (2000) Bioessays 22, 442–451
15. Hochstrasser, M. (2000) EMBO J. 19, 18714–18719
16. Hay, R. T. (2001) EMBO J. 20, 8826–8830
17. Melchior, F. (2000) Annu. Rev. Cell Dev. Biol. 16, 591–626
18. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Gene 248, 1–14
19. Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) Mol. Cell 2, 233–239
20. Mahajan, R., Delphin, C., Guan, T., Georg, L., and Melchior, F. (1997) Cell 88, 97–107
21. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 135, 1457–1470
22. Kamitani, T., Kito, K., Nguyen, H. P., Wada, H., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 26675–26682
23. Lin, X., Sun, B., Liang, M., Liang, Y.-Y., Gast, A., Liang, M., Zhai, W., and Lin, X. (2002) Mol. Cell 9, 133–143
24. Freiman, R., and Tjian, R. (2003) Cell 112, 11–17
25. Verger, A., Perdomo, J., and Crossley, M. (2003) EMBO Rep. 4, 137–142
26. Feng, X.-H., Liang, Y.-Y., Liang, M., Zhai, W., and Lin, X. (2002) Mol. Cell 9, 133–143
27. Feng, X.-H., Lin, X., and Derynck, R. (2000) EMBO J. 19, 5178–5193
28. Zawel, L., Dut, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
29. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494–498
30. Parreux, C. R., Nicolas, F. J., and Hill, C. S. (2000) Mol. Cell. Biol. 20, 9041–9054
31. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) J. Biol. Chem. 276, 12654–12659
32. Wan, M., Cao, X., Wu, Y., Bai, S., Wu, L., Shi, X., and Wang, N. (2002) EMBO Rep. 3, 171–176
33. Lee, P. S., Chang, C., Liu, D., and Derynck, R. (2003) J. Biol. Chem. 278, 27853–27863

Sumoylation Stabilizes Smad4
SUMO-1/Ubc9 Promotes Nuclear Accumulation and Metabolic Stability of Tumor Suppressor Smad4
Xia Lin, Min Liang, Yao-Yun Liang, F. Charles Brunicardi and Xin-Hua Feng

J. Biol. Chem. 2003, 278:31043-31048.
doi: 10.1074/jbc.C300112200 originally published online June 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300112200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 33 references, 13 of which can be accessed free at
http://www.jbc.org/content/278/33/31043.full.html#ref-list-1