The Balance between Acetylation and Deacetylation Controls Smad7 Stability*

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Transforming growth factor beta (TGFβ) regulates multiple cellular processes via activation of Smad signaling pathways. We have recently demonstrated that the inhibitory Smad7 interacts with the acetyl transferase p300 and that p300 acetylates Smad7 on two lysine residues. These lysine residues are critical for Smurf-mediated ubiquitination of Smad7, and acetylation protects Smad7 from TGFβ-induced degradation. In this study we demonstrate that Smad7 interacts with specific histone deacetylases (HDACs) and that the same HDACs are able to deacetylate Smad7. The interaction with HDACs is dependent on the C-terminal MH2 domain of Smad7. In addition, HDAC1-mediated deacetylation of Smad7 decreases the stability of Smad7 by enhancing its ubiquitination. Thus, our results demonstrate that the degradation of Smad7 is regulated by the balance between acetylation, deacetylation and ubiquitination, indicating that this could be a general mechanism to regulate the stability of cellular proteins.

Transforming growth factor beta (TGFβ)1 is a member of the TGFβ superfamily of cytokines that regulate multiple cellular processes including extracellular matrix production, cell growth, apoptosis, and differentiation. Dysfunction of TGFβ signaling has been implicated in various human disorders ranging from vascular diseases to cancer progression (for a recent review see Ref. 1). TGFβ exerts its cellular effects via formation of a heteromeric complex of type I and type II serine/threonine kinase receptors. The type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates and activates the receptor-activated Smads (Smad2 and Smad3) in their C-terminal SSXS motif. The activated Smads then interact with Smad4 and translocate into the nucleus where they act as transcription factors together with co-activators and co-repressors (2).

The subfamily of inhibitory Smads consists of Smad6 and Smad7, which are immediate early target genes of TGFβ (3–6). Smad7 is a nuclear protein in resting cells (7); after TGFβ stimulation, Smad7 translocates out of the nucleus to the plasma membrane in a manner that is dependent on the Smurf E3-ubiquitin ligases (8–10). At the cell membrane, the Smad7-Smurf complex interacts with the activated receptors and inhibits TGFβ signaling by blocking the interaction between the receptor-activated Smads and the activated receptors (3, 6). In addition, Smurf ubiquitinates both the receptors and Smad7, thereby inducing the degradation of both the receptors and Smad7 (8, 9).

We have recently shown that Smad7 interacts with the transcriptional co-activator p300, a protein acetyl transferase (11). p300-mediated acetylation transfers the acetyl moiety from acetyl coenzyme A to the amino group of a lysine residue of the acceptor protein. Acetylation is a dynamic process and the balance between acetylated and non-acetylated histones has major effects on chromatin structure and transcription (for a recent review see Ref. 12). Histones H3 and H4 are acetylated on specific lysine residues in their N-terminals, thereby relaxing the nucleosomal structure and allowing transcription. It has been demonstrated that non-histone proteins such as p53 (13), E2F (14), YY1 (15), NFκB (16), SREBP (17), and Smad7 (11) are acetylated. Acetylation can have multiple effects, including changes in protein-DNA interactions (18) or protein-protein interactions (19). The side chain of lysine residues is also targeted by another post-translational modification, ubiquitination. Polyubiquitination modulates protein function by inducing proteasome-dependent degradation. Protein acetylation can also affect protein stability, because it has been demonstrated that acetylation prevents ubiquitination of the same lysine residues (11, 20–22).

Acetylation is a reversible process, and removal of the acetyl group is catalyzed by histone deacetylases (HDACs) for their deacetylase activity (24) and are not affected by the well known HDAC inhibitor trichostatin A (TSA) (25). Based on its sequence, the most recently described HDAC, HDAC11 (26), has been placed in a separate group of HDACs (27). Class II HDACs have been shown to shuttle between the cytosol and the nucleus in response to extracellular stimulation (25, 28, 29), whereas class I HDACs are predominantly nuclear proteins. All classes of HDACs have been shown to deacetylate histones. In addition, it was recently shown that HDAC1 interacts with the ubiquitin ligase MDM2 and that HDAC1-mediated deacetylation of the tumor suppressor p53 promotes MDM2-mediated ubiquitination and degradation of p53 (20). Thus, HDAC-mediated deacetylation may be a novel mechanism to regulate the ubiquitination and degradation of some acetylated proteins.

In this study, we demonstrate that Smad7 interacts with spe-
cific HDACs. The HDACs that associate with Smad7 also deacetylate the protein. We also demonstrate that endogenous Smad7 interacts with HDAC1 and map the interaction to the C-terminal MH2 domain of Smad7. siRNA-mediated inactivation of endogenous HDAC1 enhances the acetylation and steady-state levels of endogenous Smad7. In addition, HDAC1-mediated deacetylation of Smad7 promotes the ubiquitination and degradation of Smad7. Thus, our results indicate that the balance between acetylation and deacetylation of Smad7 can control its degradation through the ubiquitin-proteasome pathway.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—*All tissue culture media and antibiotics were obtained from Invitrogen and Sigma Genosys. Human embryonic kidney epithelial 293, 293T, and HepG2 cells were from American Type Culture Collection. Cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, sodium pyruvate (1 mM), non-essential amino acids (1×), 50 units/ml penicillin, and 50 µg/ml streptomycin in 5% CO₂. For overnight starvation, cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal calf serum, sodium pyruvate (1 mM), non-essential amino acids (1×), penicillin, and 50 µg/ml streptomycin in 5% CO₂.

**TGFβ, Reagents, and Antibodies—**TGFβ was purchased from PeproTech EC Ltd. (London, UK). 14C-Acetyl coenzyme A (51.6 mCi/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Cycloheximide and TSA were obtained from Sigma Genosys. The following antibodies, anti-Myc (9E10), anti-HA (Y-11), anti-Smad7 (N19 and H79), anti-ubiquitin (P4D1), anti-HDAC1 (H-51), and anti-Gal4 DBD (RK5C1) were purchased from Santa Cruz Biotechnology. Anti-FLAG antibody (M5) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). Cycloheximide and antibiotics were purchased from Invitrogen and Sigma Genosys. Human embryonic kidney epithelial 293, 293T, and HepG2 cells were from American Type Culture Collection. Cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, sodium pyruvate (1 mM), non-essential amino acids (1×), penicillin, and 50 µg/ml streptomycin in 5% CO₂. For overnight starvation, cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal calf serum, sodium pyruvate (1 mM), non-essential amino acids (1×), penicillin, and 50 µg/ml streptomycin in 5% CO₂.

*Plasmids and DNA Transfections—*The expression vectors for FLAG- and Myc-tagged Smad7 in the mammalian expression vector pcDNA3 (Invitrogen) were generously provided by P. ten Dijke (The Netherlands Cancer Institute, Amsterdam). The expression vectors for HA-tagged p300, HDAC1, -2, and -3 were a kind gift from T. Kouzarides (Cambridge, UK). HDAC1, -2, and -3 were transiently transfected into HeLa cells using Comassie staining of SDS-PAGE gels, and equal amounts of protein were used in the binding assays. Proteins were in vitro translated using the T7 Tnt kit (Promega) and 35S-labeled methionine and cysteine (Promix, Amersham Biosciences) and incubated with GST fusion proteins prebound to glutathione beads at 4 °C for 3 h in the presence of bovine serum albumin (1 mg/ml) to avoid unspecific binding. The samples were treated as described for immunoprecipitates and resolved by SDS-PAGE. The gel was incubated in enhancer (Amplify, Amersham Biosciences) for 15 min, dried, and exposed to PhosphorImager overnight.

**RESULTS**

**Inhibition of HDAC Activity Increases the Acetylation of Smad7—**We have recently shown that Smad7 is acetylated on lysine residues 64 and 70 by p300 (11). During our studies we observed that activation of the TGFβ receptor resulted in a reduction in the acetylation of Smad7 (11), suggesting that Smad7 is deacetylated in response to TGFβ signaling. To test the possibility that Smad7 is a substrate for HDACs, 293T cells were transiently transfected with Smad7 either alone or together with p300 and incubated in the absence and presence of the HDAC inhibitor TSA. Following lysis of the cells, Smad7 was immunoprecipitated and resolved on SDS-polyacrylamide gel, and the acetylation of Smad7 was monitored by anti-acetyllysine antibodies. As previously reported, Smad7 was acetylated by p300 (Fig. 1A). Interestingly, the acetylation of Smad7 was enhanced in cells treated with TSA (Fig. 1A, compare lanes 2 and 4). The same result was obtained using an antibody specific for the major acetylation site in Smad7 (Fig. 1B). These results indicate that Smad7 is actively deacetylated in vivo. To further test the possibility that Smad7 is a HDAC substrate, Smad7 was expressed in 293T cells together with p300 and immunoprecipitated. The immunoprecipitated material was divided into three aliquots. One aliquot was left untreated, one aliquot was incubated with nuclear extract from HeLa cells, and the third aliquot was incubated with HeLa nuclear extract pretreated with TSA to inhibit endogenous HDACs. After incubation, the reactions were stoped by the addition of SDS sample buffer. The samples were resolved by SDS-PAGE, and the acetylation of Smad7 was detected by Western blotting. The incubation of Smad7 with HeLa nuclear extract significantly reduced the acetylation of Smad7 (Fig. 1C). Furthermore, pre-incubating the HeLa nuclear extracts with TSA blocked the effect on the acetylation of Smad7, indicating that the deacetylase of Smad7 is catalyzed by a TSA-sensitive process. Taken together, these results suggest that acetylated Smad7 is a potential HDAC substrate.

**Smad7 Interacts with Specific HDACs—**To analyze whether Smad7 is associated with specific members of the HDAC family (Santa Cruz Biotechnology). For reprobing, blots were incubated in stripping buffer (0.2 M NaOH, 0.5 M NaCl) at room temperature for 10 min and washed extensively with Tris-buffered saline before blocking. For Western blotting, proteins were resolved by SDS-PAGE (1% SDS, 1% PAG E, 0.5 mM EDTA, 0.5 mM Tris-HCl, pH 7.4) and blotted. The samples were boiled for 10 min and diluted 10 times with lysis buffer. After clearing by centrifugation, immunoprecipitations were performed as described.

**Generation of Recombinant Proteins and in Vitro Pull-down Assays—** GST-Smad7 and GST-HDAC1 fusion proteins were expressed in BL21 (DE3pLysS) and purified according to standard protocols. The amount of protein on the beads was estimated by Coomassie staining of SDS-PAGE gels, and equal amounts of protein were used in the binding assays. Proteins were in vitro translated using the T7 Tnt kit (Promega) and 35S-labeled methionine and cysteine (Promix, Amersham Biosciences) and incubated with GST fusion proteins prebound to glutathione beads at 4 °C for 3 h in the presence of bovine serum albumin (1 mg/ml) to avoid unspecific binding. The samples were treated as described for immunoprecipitates and resolved by SDS-PAGE. The gel was incubated in enhancer (Amplify, Amersham Biosciences) for 15 min, dried, and exposed to PhosphorImager overnight.

**Deacetylation Assay—**293T cells were transfected with Myc-Smad7 and HA-p300 using the reagents and methods described above. Acetylated Myc-Smad7 was immunoprecipitated, and the precipitates were washed extensively. After washing, HDAC buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1% (w/v) glycerol, 1% (w/v) Triton X-100) containing 10% (v/v) Triton X-100 was added, and the precipitates were aliquoted and used in deacetylation assays in the absence or presence of HeLa nuclear extract. To inactivate endogenous HDACs, HeLa nuclear extracts were preincubated 45 min on ice with TSA (final concentration 100 nM). The samples were incubated at 30 °C for 2 h, SDS sample buffer was added, and the samples were separated by SDS-PAGE followed by immunoblotting. The preparation of HeLa nuclear extracts has been described elsewhere (31).
of proteins, we performed co-immunoprecipitation experiments in transiently transfected 293T cells, using Myc-tagged Smad7 and FLAG-tagged HDACs (HDAC1–HDAC6). Among the class I HDACs, both HDAC1 and HDAC5 interacted well with Smad7, whereas a weak interaction was detected between Smad7 and HDAC2 (Fig. 2A). Among the class II HDACs, both HDAC5 and HDAC6 interacted with Smad7, whereas we were unable to detect any interaction between Smad7 and HDAC4 (Fig. 2A). The interactions between Smad7 and HDAC5 or HDAC6 were consistently weaker than the interaction between Smad7 and HDAC1 or HDAC3. This difference made us focus on the interaction between Smad7 and HDAC1. GST pull-down experiments using GST-HDAC1 and in vitro translated Smad7 showed that these two proteins interact in vitro and that the interaction most probably is direct (Fig. 2B). To verify that Smad7 and HDACs interact under physiological conditions, the interaction between endogenous proteins was investigated. As illustrated in Fig. 2C, Smad7 antibodies immunoprecipitated endogenous HDAC1 from 293T whole-cell lysates, whereas an unrelated antibody was unable to do so.

**The C-terminal Domain of Smad7 Interacts with HDAC1—Smad7 contains an N-terminal domain, which is conserved between the I-Smads and a C-terminal MH2 domain, which is conserved in all members of the Smad family. Smad7 also contains a linker region, which, via its PY motif, is critical for the interaction with the Smurf family of ubiquitin ligases (8, 32). The acetylated lysine residues are located within the N-terminal domain of Smad7 (11). To determine the domain(s) in Smad7 that are responsible for its interaction with HDAC1, we used deletion mutants of Smad7 together with full-length HDAC1 in co-immunoprecipitation experiments. As seen in Fig. 3A, full-length Smad7 and the isolated MH2 domain (amino acids 261–246) interacted strongly with HDAC1, whereas no interaction was detected between HDAC1 and the N-terminal domain (amino acids 1–126) or linker region (amino acids 127–260) of Smad7. To further map the interaction between Smad7 and HDAC1, we performed co-immunoprecipita-

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**Fig. 1.** Smad7 acetylation is enhanced in the presence of TSA. A, Myc-tagged Smad7 was expressed in 293T cells in the absence or presence of Ga4-p300. Twenty-four hours posttransfection, cells were either left untreated or treated with TSA (200 ng/ml) for 12 h. Following immunoprecipitation (IP) of Smad7, samples were resolved by SDS-PAGE, and the acetylation of Smad7 was determined with anti-acetyl lysine antibodies (α-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Myc antibodies (α-Myc). B, FLAG-tagged, wild-type, or mutant (K64A) Smad7 was expressed in 293T cells in the absence or presence of HA-p300. Twenty-four hours posttransfection, cells were either left untreated or treated with TSA (200 ng/ml) for 12 h. Following immunoprecipitation of Smad7, samples were resolved by SDS-PAGE, and the acetylation of Smad7 was detected with anti-acetyl lysine antibodies directed against the major acetylation site in Smad7 (α-acetyl K64). The amount of Smad7 in the immunoprecipitates was determined with anti-FLAG antibodies (α-Flag). C, 6Myc-tagged Smad7 was expressed together with HA-tagged p300. Following immunoprecipitation of Smad7 with anti-Myc antibodies, the sample was divided and incubated alone, with HeLa nuclear extracts, or with HeLa nuclear extract preincubated with TSA (100 nm). After incubation, the samples were resolved by SDS-PAGE, and the amount of acetylated Smad7 was determined by Western blotting using anti-acetyl lysine antibodies (α-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Myc antibodies (α-Myc).

**Fig. 2.** Smad7 interacts with specific deacetylases. A, 6Myc-tagged Smad7 was expressed in the absence or presence of the indicated FLAG-tagged HDACs. Following immunoprecipitation of the HDACs with anti-FLAG antibodies, the samples were resolved by SDS-PAGE, and the amount of co-precipitating Smad7 was determined with anti-Myc antibodies (α-Myc). The levels of Flag-HDAC1, -2, -3, -4, -5, and -6 in the immunoprecipitates and 6Myc-Smad7 in total cell lysates were determined by Western blotting. B, Smad7 was in vitro translated and used in GST pull-down assays with GST alone or GST-HDAC1. The amount of Smad7 was analyzed by phosphorimage analysis. C, 293T cell lysates were immunoprecipitated with an unrelated polyclonal antiserum (lane 1) or an antiserum directed against Smad7 (lane 2). The immunocomplexes were washed, and the proteins were separated by SDS-PAGE. Co-immunoprecipitating HDAC1 was detected by Western blotting using anti-HDAC1 antibodies. The amount of Smad7 in the immunoprecipitates was determined with anti-Smad7 antibodies (α-Smad7).
HDACs Control Smad7 Stability

Smad7 is a HDAC Substrate—To investigate whether Smad7 is a substrate for the deacetylase activity of HDACs, 293T cells were transiently transfected with p300 and Smad7, either in the absence or presence of various HDACs (HDAC1–6). After immunoprecipitation, the acetylation of Smad7 was monitored with anti-acetyl lysine antibodies. The acetylation of Smad7 was drastically reduced in the presence of HDAC1, -3, and -6 (Fig. 4A), indicating that endogenous HDAC1 affects the acetylation of Smad7. To further confirm that the acetylation of Smad7 is regulated by endogenous HDACs, HepG2 cells were transfected with a vector expressing short hairpin RNA for HDAC1. Twenty-four hours after transfection, TGFβ was added to the cells to stimulate the expression of Smad7, and the levels of acetylated Smad7 were determined by Western blotting with anti-acetyl lysine antibodies (α-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Myc antibodies (α-Myc). The amounts of HDAC1 in the immunoprecipitates were resolved by SDS-PAGE, and the levels of acetylated Smad7 were determined with anti-Myc antibodies (α-Myc).

Effects on the acetylation of Smad7 were seen when the protein levels of endogenous HDAC1 were reduced, indicating that endogenous HDAC1 affects the acetylation of Smad7. To further confirm that the acetylation of Smad7 is regulated by endogenous HDACs, HepG2 cells were transfected with Flag-Smad7 in the absence or presence of HA-HDAC1. 293T cells were transfected with Smad7 with or without p300. As seen in Fig. 4A, the acetylation of Smad7 was significantly increased when the protein levels of endogenous HDAC1 were reduced, indicating that endogenous HDAC1 affects the acetylation of Smad7. To further confirm that the acetylation of Smad7 is regulated by endogenous HDACs, HepG2 cells were transfected with Flag-Smad7 in the absence or presence of a vector expressing short hairpin RNA for HDAC1. Twenty-four hours after transfection, TGFβ was added to the cells to stimulate the expression of Smad7, and the levels of acetylated Smad7 were determined by Western blotting with anti-acetyl lysine antibodies (α-AcK).

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HDAC1 regulates the stability and ubiquitination of Smad7. A, FLAG-tagged Smad7 was expressed in 293 cells in the absence or presence of FLAG-tagged HDAC1. Twenty-four hours after transfection, cells were starved overnight. The following day, fresh starvation medium supplemented with cycloheximide (100 ng/ml) and TGFβ (5 ng/ml) was added to the cells. Where indicated, TSA (500 ng/ml) was also added to the cells. After incubation for the indicated times, cell lysates were prepared and analyzed by SDS-PAGE. The levels of Smad7 were detected by Western blotting with anti-FLAG antibodies. As a control for equal loading, the filters were probed for tubulin (lower panels). B, HepG2 cells were transfected with FLAG-tagged Smad7 in the absence or presence of pSUPER-control (lane 1) or pSUPER-HDAC1 (lane 2). Following lysis, the samples were resolved by SDS-PAGE, and the protein levels of Smad7 were detected by Western blotting with anti-FLAG antibodies (α-Flag). The amount of HDAC1 in the lysates was determined with anti-HDAC1 antibodies. The filters were probed for tubulin as a control for equal loading (lower panel). C, Myc-tagged Smad7 was expressed in 293T together with HA-tagged ubiquitin in the absence or presence of FLAG-tagged HDAC1. Thirty-six hours after transfection, cell lysates were prepared. Following immunoprecipitation (IP) of Smad7 with anti-Myc antibodies, the samples were resolved by SDS-PAGE, and the presence of ubiquitinated (Ub) Smad7 was detected with anti-Ub antibodies (α-Ub). The levels of Smad7 in the immunoprecipitates and of HDAC1 in the cell lysates were determined with anti-Myc (α-Myc) and anti-FLAG (α-Flag) antibodies, respectively. The migration of an antibody-related band is indicated by an asterisk.

Smad7 turnover. Following immunoprecipitation of the transfected Smad7, the acetylation of the protein was analyzed with anti-acetyl lysine antibodies. Inactivation of endogenous HDAC1 enhanced the acetylation of Smad7 in HepG2 (Fig. 4C). Similar results were also observed in HeLa cells (data not shown).

HDAC1 Decreases the Half-life of Smad7 by Promoting Its Ubiquitination—For some proteins, the acetylation of specific lysine residues is able to prevent ubiquitination of the same residues, thereby preventing protein degradation (11, 20, 21). To determine whether HDAC1 could affect the half-life of Smad7, 293 cells were transfected with FLAG-tagged Smad7 in the absence or presence of co-transfected FLAG-tagged HDAC1. The cells were starved overnight and stimulated with TGFβ for the indicated time periods in the presence of cycloheximide. In a separate experiment, TSA was added to cells expressing Flag-Smad7 to inhibit the activity of endogenous HDACs. Following lysis of the cells, the levels of Smad7 were determined by Western blotting. Co-expression of HDAC1 significantly decreased the half-life of Smad7 (Fig. 5A, compare the top and middle panels). Interestingly, the degradation of transiently transfected Smad7 was blocked in cells treated with TSA, indicating that endogenous HDACs regulate the stability of Smad7 (Fig. 5A, lower panel). To determine whether endogenous HDAC1 could influence the stability of Smad7, HepG2 cells were transfected with low amounts of Flag-Smad7 in the absence or presence of a vector expressing short hairpin RNA for HDAC1. As illustrated in Fig. 5B, the steady-state levels of Flag-Smad7 were enhanced in response to inactivation of endogenous HDAC1, suggesting that HDAC1 regulates the stability of Smad7. Similar results were obtained when the experiment was repeated in HeLa cells (data not shown). To determine whether HDAC1 could also affect the ubiquitination of Smad7, 293T cells were transfected with FLAG-tagged Smad7 and HA-tagged ubiquitin in the absence or presence of co-transfected HDAC1. After lysis of the cells, Smad7 was immunoprecipitated and the ubiquitination of Smad7 was determined by Western blotting. Co-expression of HDAC1 significantly increased the ubiquitination of Smad7 (Fig. 5C), indicating that deacetylation of Smad7 increases the amount of lysine residues available for subsequent ubiquitination.

To establish that endogenous HDACs regulate the deacetylation of endogenous Smad7, HepG2 cells were treated with or without TSA for 8 h. After lysis, Smad7 was immunoprecipitated and resolved by SDS-PAGE. As seen in Fig. 6A, the acetylation of endogenous Smad7 was increased after TSA treatment, suggesting that the acetylation of Smad7 is regulated by endogenous HDACs. To investigate the effect of en-

FIG. 5. HDAC1 regulates the stability and ubiquitination of Smad7. A, FLAG-tagged Smad7 was expressed in 293 cells in the absence or presence of FLAG-tagged HDAC1. Twenty-four hours after transfection, cells were starved overnight. The following day, fresh starvation medium supplemented with cycloheximide (100 μg/ml) and TGFβ (5 ng/ml) was added to the cells. Where indicated, TSA (500 ng/ml) was also added to the cells. After incubation for the indicated times, cell lysates were prepared and analyzed by SDS-PAGE. The levels of Smad7 were detected by Western blotting with anti-FLAG antibodies. As a control for equal loading, the filters were probed for tubulin (lower panels). B, HepG2 cells were transfected with FLAG-tagged Smad7 in the absence or presence of pSUPER-control (lane 1) or pSUPER-HDAC1 (lane 2). Following lysis, the samples were resolved by SDS-PAGE, and the protein levels of Smad7 were detected by Western blotting with anti-FLAG antibodies (α-Flag). The amount of HDAC1 in the lysates was determined with anti-HDAC1 antibodies. The filters were probed for tubulin as a control for equal loading (lower panel). C, Myc-tagged Smad7 was expressed in 293T together with HA-tagged ubiquitin in the absence or presence of FLAG-tagged HDAC1. Thirty-six hours after transfection, cell lysates were prepared. Following immunoprecipitation (IP) of Smad7 with anti-Myc antibodies, the samples were resolved by SDS-PAGE, and the presence of ubiquitinated (Ub) Smad7 was detected with anti-Ub antibodies (α-Ub). The levels of Smad7 in the immunoprecipitates and of HDAC1 in the cell lysates were determined with anti-Myc (α-Myc) and anti-FLAG (α-Flag) antibodies, respectively. The migration of an antibody-related band is indicated by an asterisk.

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Smad7 is acetylated by p300, which protects it from Smurf-mediated ubiquitination (UB) and degradation. In contrast, HDAC1-mediated deacetylation increases Smad7 ubiquitination and subsequent degradation.

**DISCUSSION**

In this study we presented evidence that Smad7 interacts with multiple HDACs, resulting in the deacetylation of Smad7, which enhances its ubiquitination and degradation, further strengthening the hypothesis that the acetylation of Smad7 is critical for its stability. We demonstrated that the acetylation of Smad7 is increased in the presence of the deacetylase inhibitor TSA 	extit{in vivo}, indicating that Smad7 is actively deacetylated by HDACs (Figs. 1A and 6A). An additional indication that Smad7 is a substrate for HDACs came from the observation that HeLa nuclear extracts contained an activity that could deacetylate Smad7 and that this activity was TSA-sensitive (Fig. 1C). TSA has been shown to inhibit both class I and class II HDACs, whereas class III HDACs are insensitive to TSA (25). Thus, our results indicate that class III HDACs are not involved in the deacetylation of Smad7; in accordance, treatment of cells with nicotinamide, an inhibitor of the class III HDACs, had no effect on Smad7 acetylation (data not shown).

Smad7 interacted with both HDAC1 and -3, whereas the interaction with HDAC2 was weak (Fig. 2A). Although HDAC1 and -2 have 85% amino acid sequence similarity (34) and are found in the same repressor complexes, it was recently shown that in the DT40 chicken B-cell line, disruption of either HDAC1 or -2 resulted in different protein expression patterns (35). This observation indicates that HDAC1 and -2 may have both specific and common interaction partners, which is consistent with the data on the differential interaction with Smad7 described in the present study.

The class II HDACs, HDAC5 and -6, were also able to interact with (Fig. 2A) and deacetylate (Fig. 4A) Smad7, although to a lesser extent than the class I HDACs. In contrast, Bai and Cao (36) showed that Smad6, which interacts with HDAC1 and -3, failed to interact with any member of the class II HDACs tested.

The C-terminal MH2 domain of Smad7 interacted strongly with full-length HDAC1 (Fig. 3A), whereas no interaction was seen with the N-terminal domain or linker region. This is similar to Smad6, which also interacts with HDAC1 through its MH2 domain (36). The interaction between Smad7 and HDAC1 is most probably direct as GST-HDAC1 was able to pull down Smad7 (Fig. 2B). Interestingly, GST-Smad7 failed to interact with any of the HDACs tested (data not shown), indicating that Smad7 needs to be modified to interact with HDACs. Smad7 has been shown to be both acetylated (11) and phosphorylated (37). Acetylation of Smad7 is mediated by p300 and Smad7 is readily acetylated 	extit{in vitro}. However, 	extit{in vitro} acetylated GST-Smad7 still failed to associate with any of the HDACs tested indicating that this modification does not suffice for HDAC interaction (data not shown). The kinase(s) phosphorylating Smad7 is currently unknown preventing us from testing if this modification is critical for the interaction with HDACs 	extit{in vitro}. It was recently shown that the interaction between BCL-3 and HDAC1 is regulated by phosphorylation of BCL-3 and that HDAC1 negatively regulates BCL-3 stability (38). It is tempting to speculate that a similar mechanism regulates the interaction between HDAC1 and Smad7. The observation that Smad7 interacts with both transcriptional co-activators (p300) and co-repressors (HDACs) indicate that Smad7 could have a direct role in transcriptional regulation. Further studies will be necessary to analyze this possibility.

HDAC6 has been shown to associate with proteins involved in the ubiquitin signaling pathway (39). The link between acetylation and ubiquitination gained further support from the observations that the ubiquitination and degradation of various proteins such as Smad7 (11), SREBP (17), and c-Myc (40) were reduced following acetylation. In Smad7, mutation of the two acetylated residues prevented its polyubiquitination and degradation (11), suggesting that acetylation protects Smad7 from ubiquitination. In support of this hypothesis, co-expression of HDAC1 and Smad7 enhanced the ubiquitination of Smad7 and increased its degradation (Fig. 5). In addition, the acetylation as well as the protein levels of endogenous Smad7 were increased after siRNA-mediated inactivation of endogenous HDAC1 (Fig. 6B). We have shown previously that the acetylation of Smad7 is decreased in response to TGFβ-signaling (11). The reduction in acetylation is most probably the result of multiple, separate events. TGFβ induces nuclear export of Smad7, thereby preventing p300-mediated acetylation. It is also possible that TGFβ signaling enhances HDAC1-mediated deacetylation of Smad7. However, we were unable to detect any TGFβ dependence in the interaction between Smad7 and HDAC1 (data not shown). Thus, further investigations regarding the mechanisms involved in TGFβ-dependent regulation of the acetylation and deacetylation of Smad7 are warranted. Together with our previous findings, the data presented in the current report support a model where the activity of p300 and one or more HDACs determine the stability of Smad7 (Fig. 7). It is an interesting possibility that the stability of other proteins is also regulated by the balance between acetylation, deacetylation, and ubiquitination.

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REFERENCES

1. Massague, J., Blain, S. W., and Lo, R. S. (2000) Cell 103, 295–309
2. Shi, Y., and Massague, J. (2003) Cell 113, 685–708
3. Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. (1997) Nature 389, 631–635
4. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997) Nature 389, 622–626
5. Topper, J. N., Cai, J., Qi, Y., Anderson, K. R., Xu, Y. Y., Deeds, J. D., Feeley, R., Gimeno, C. J., Woolf, E. A., Tayber, O., Mays, G. G., Sampson, B. A., Schoen, F. J., Gimbrone, M. A., Jr., and Falb, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9314–9319
6. Hayashi, H., Abdullah, S., Qi, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Wrana, J. L., and Falb, D. (1997) Cell 89, 1165–1173
7. Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C. H., Heldin, N. E., and ten Dijke, P. (1996) J. Biol. Chem. 271, 29195–29201
8. Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001) J. Biol. Chem. 276, 12477–12480
9. Kavak, P., Rasmussen, R. K., Causin, G., Bonni, S., Zhu, H., Thomsen, G. H., and Wrana, J. L. (2000) Mol. Cell 6, 1369–1373
10. Tajima, Y., Goto, K., Yoshida, M., Shinomiya, K., Sekimoto, T., Yoneda, Y., Miyazono, K., and Imamura, T. (2003) J. Biol. Chem. 278, 10716–10721
11. Gronroos, R., Hellman, U., Heldin, C. H., and Ericsson, J. (2000) Mol. Cell 5, 483–493
12. Grewal, S. I., and Moazed, D. (2003) Science 301, 798–802
13. Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001) EMBO J. 20, 1331–1349
14. Martinez-Balbas, M. A., Bauer, U. M., Nielsen, S. J., Brehm, A., and Keuzenerides, T. (2000) EMBO J. 19, 662–671
15. Viatour, P., Dejardin, E., Marchi, W., and Keuzenerides, T. (2001) Mol. Cell. Biol. 21, 5979–5991
16. Chen, L., Fischle, W., Verdin, E., and Greene, W. C. (2001) Science 293, 1653–1657
17. Gaudemar, V., Simonsson, M., Gronroos, K., and Ericsson, J. (2003) Mol. Cell. Biol. 23, 2587–2599
18. Gu, W., and Roeder, R. G. (1997) Cell 90, 595–606
19. Chen, L. F., Mu, Y., and Greene, W. C. (2000) EMBO J. 19, 6539–6548
20. Ita, A., Kawaguchi, Y., Lai, C. H., Koizumi, Y., Higashimoto, Y., Appella, E., and Yao, T. P. (2002) EMBO J. 21, 6236–6245
21. Zhao, Q., Cumming, H., Cerruti, L., Cunningham, J. M., and Jane, S. M. (2004) J. Biol. Chem. 279, 41477–41486
22. Caron, C., Boyault, C., and Khochbin, S. (2005) BioEssays 27, 408–415
23. Thiangalingam, S., Cheng, K. H., Lee, H. J., Mineva, N., Thiangalingam, A., and Ponte, J. F. (2003) Ann. N. Y. Acad. Sci. 983, 84–100
24. Imai, S., Armstrong, C. M., Kaelberlein, M., and Guarente, L. (2000) Nature 403, 795–800
25. Barlow, A. L., van Brune, C. M., Johnson, C. A., Tweedie, S., Bird, A., and Turner, B. M. (2001) Exp. Cell Res. 265, 90–101.
26. Gao, L., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002) J. Biol. Chem. 277, 25748–25755
27. Gregoretti, I. V., Lee, Y. M., Goodson, H. V., Gao, L., Cueto, M. A., Asselbergs, F., and Atadja, P. (2004) J. Mol. Biol. 338, 17–31
28. Grozinger, C. M., Hassig, C. A., and Schreiber, S. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4868–4873
29. Chawla, S., Vanhoutte, P., Arnold, F. J., Huang, C. L., and Bading, H. (2003) J. Neurochem. 85, 151–159
30. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Science 296, 550–553
31. Kanopka, A., Muhlemann, O., and Akusjarvi, G. (1995) Nature 375, 431–438
32. Zhang, Y., Chang, C., Gehling, D. J., Hemmati-Brivanlou, A., and Derynck, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 974–979
33. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1998) Science 282, 408–411
34. Yang, W. M., Iouye, C., Zeng, Y., Bearss, D., and Seto, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12845–12850
35. Takami, Y., Rikuchi, H., and Nakayama, T. (1999) J. Biol. Chem. 274, 23977–23990
36. 2003 BioEssays 25, 416–421
37. Palacik, L., Landstreen, M., Heldin, C. H., and Souchelnytskyi, S. (2001) J. Biol. Chem. 276, 14344–14349
38. Viator, P., Dejardin, E., Warnier, M., Lair, F., Claudio, E., Bureau, F., Marine, J. C., Merville, M. P., Maurer, U., Green, D., Piette, J., Siebenlist, U., Bouras, V., and Charist, A. (2004) Mol. Cell 16, 35–45
39. Seigneurin-Berny, D., Verdel, A., Curtet, S., Lemercier, C., Garin, J., Rousseau, S., and Khochbin, S. (2001) Mol. Cell. Biol. 21, 8035–8044
40. Vervoorts, J., Luscher-Firzlaff, J. M., Rottmann, S., Liobschik, R., Walsemann, G., Dohmann, K., Austen, M., and Luscher, B. (2003) EMBO Rep. 4, 1–7
