Control of Smad7 Stability by Competition between Acetylation and Ubiquitination

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Summary

Smad proteins regulate gene expression in response to TGFβ signaling. Here we present evidence that Smad7 interacts with the transcriptional coactivator p300, resulting in acetylation of Smad7 on two lysine residues in its N terminus. Acetylation or mutation of these lysine residues stabilizes Smad7 and protects it from TGFβ-induced degradation. Furthermore, we demonstrate that the acetylated residues in Smad7 also are targeted by ubiquitination and that acetylation of these lysine residues prevents subsequent ubiquitination. Specifically, acetylation of Smad7 protects it against ubiquitination and degradation mediated by the ubiquitin ligase Smurf1. Thus, our data suggest that competition between ubiquitination and acetylation of overlapping lysine residues constitutes a novel mechanism to regulate protein stability.

Introduction

TGFβ belongs to a superfamily of cytokines that regulate diverse biological functions, ranging from differentiation, motility, and apoptosis to the inhibition of cell growth (Massague and Wotton, 2000; Moustakas et al., 2001). Inappropriate regulation of TGFβ signaling has been implicated in multiple human diseases, such as fibrosis, rheumatoid arthritis, and carcinogenesis (Blobe et al., 2000; Piek and Roberts, 2001). TGFβ exerts its cellular effects via binding to type I and type II serine/threonine kinase receptors; within the heterotetrameric receptor complex, the type II receptor phosphorylates and activates the type I receptor, leading to activation of the Smad signaling pathway (Itoh et al., 2000b; Massague and Wotton, 2000; Miyazono et al., 2000; Moustakas et al., 2001). The TGFβ type I receptor phosphorylates the receptor-regulated Smads (R-Smads; Smad2 and Smad3), and subsequently, a heteromeric complex with the common-mediator Smad (Co-Smad; Smad4) is formed. The Smad complexes then translocate into the nucleus where they, in cooperation with coactivators and corepressors, act as transcription factors regulating gene expression.

The inhibitory Smads (I-Smads; Smad6 and Smad7) are induced by receptor signaling and act as negative regulators of the pathway. Smad7 resides in the nucleus in unstimulated cells and translocates to the plasma membrane following receptor activation (Itoh et al., 1998), where it binds the receptors and inhibits further signaling by at least two different mechanisms. First, Smad7 is able to interfere with TGFβ signaling by blocking the interactions between the R-Smads and the activated receptors (Hayashi et al., 1997; Nakao et al., 1997). Second, Smad7 interacts with the E3-ubiquitin ligases Smurf1 or Smurf2 in the nucleus; after TGFβ stimulation, the Smad7-Smurf complex translocates from the nucleus to the plasma membrane, where Smurf induces ubiquitination and degradation of the TGFβ receptors (Ebisawa et al., 2001; Kavsak et al., 2000).

Acetylation is a dynamic posttranslational modification of lysine residues. Proteins with intrinsic histone acetyltransferase (HAT) activity act as transcriptional coactivators by acetylating histones and thereby induce an open chromatin conformation, which allows the transcriptional machinery access to promoters (Roth et al., 2001). The best characterized HATs are p300, CBP, and P/CAF (Roth et al., 2001). These proteins interact with a large number of transcription factors, such as the Smads (Feng et al., 1998; Itoh et al., 1998, 2000a; Jancek et al., 1998; Nishihara et al., 1998; Pouponnot et al., 1998), as well as components of the basic transcriptional machinery. Thus, it is believed that HATs are central integrators of various signaling pathways in the nucleus.

Since the observation that p53 is a direct target for acetylation by the coactivators p300 and P/CAF (Gu and Roeder, 1997), a number of transcription factors and nuclear proteins have been found to be modified in this manner (Bannister and Miska, 2000; Kouzarides, 2000; Sterner and Berger, 2000). The functional consequences of acetylation are diverse and include increased DNA binding (p53 [Gu and Roeder, 1997]; GATA-1 [Boyes et al., 1998]; and MyoD [Polesskaya et al., 2000; Sartorelli et al., 1999]), decreased DNA binding (HMGI(Y) [Munshi et al., 1998]), increased stability (E2F-1 [Martinez-Balbas et al., 2000]), inhibition of nuclear export (importin-α and HNF4 [Bannister et al., 2000; Soutoglou et al., 2000]), and changes in protein-protein interactions (TCF [Waltzer and Bienz, 1998]; ACTR [Chen et al., 1999]; and EKLF [Zhang et al., 2001]).

In this paper, we demonstrate that Smad7 is a direct target of the transcriptional coactivator p300 in vivo and in vitro, and we identify the acetylated lysine residues. Acetylation of these residues enhances the stability of Smad7 by interfering with Smurf-mediated degradation. Our results identify a mechanism that regulates the activity of Smad7 and indicate that competition between ubiquitination and acetylation of common lysine residues controls the stability of Smad7.

Results

Smad7 Interacts with p300

To analyze if Smad7 is directly associated with components of the transcriptional machinery, we performed coimmunoprecipitation experiments in transiently transfected 293T cells, using full-length p300 and three different Smad7 constructs. Full-length Smad7 and Smad7ΔN
The N Terminus of Smad7 Is Acetylated by p300

The fact that Smad7 interacted with the HAT domain of p300 suggested that Smad7 could be a substrate for the acetyltransferase activity of p300. In order to test this possibility, 293T cells were transfected with Smad7 alone or together with p300, CBP, or P/CAF, and the acetylation of Smad7 was monitored by anti-acetyl lysine antibodies (Figure 2A). Although CBP and p300 are homologous and have overlapping functions, p300 induced a robust acetylation of Smad7, whereas CBP and P/CAF failed to acetylate Smad7 (Figure 2A, lanes 1–4). CBP and P/CAF were able to acetylate p53, both in vivo and in vitro (data not shown), confirming their activity toward other substrates. To investigate whether acetylation of Smad7 is regulated in response to TGFβ signaling, the experiment was also performed in the presence of constitutively active TGFβ receptor (caALK5); coexpression of caALK5 decreased the acetylation of Smad7 by p300 (compare lanes 2 and 6 in Figure 2A). Smad7 was acetylated by endogenous acetyltransferases when expressed in HaCaT cells (Figure 2B). Interestingly, RNAi-mediated inactivation of endogenous p300 blocked this acetylation, demonstrating that p300 is required for the acetylation of Smad7 in vivo.

To map the acetylated lysines in Smad7, we performed in vitro acetylation assays using p300 and GST-Smad7, either full-length, Smad7ΔN, or Smad7ΔC. Full-length Smad7 and Smad7ΔC were acetylated by p300, while p300 failed to acetylate Smad7ΔN, indicating that the acetylated lysine residues reside in the N terminus of Smad7 (Figure 3A). Smad7ΔC interacts poorly with p300 (Figure 1A), and the fact that it is acetylated as efficiently as full-length Smad7 could indicate that the acetylated residues are not fully accessible in full-length Smad7 in vitro. In order to identify the acetylated residues, we analyzed tryptic fragments of in vitro acetylated GST-Smad7 by mass spectrometry. Compared

(amino acids 204–402) interacted strongly with p300, while Smad7ΔC (amino acids 2–261) failed to do so (Figure 1A), indicating that the C-terminal domain of Smad7 is required for binding to full-length p300.

To further map the interaction between Smad7 and p300, we performed GST pull-down experiments using GST-Smad7 together with four in vitro translated p300 fragments (amino acids 2–411, 412–1150, 1151–1673, and 1674–2414). Full-length Smad7 interacted with all p300 fragments except the region between amino acids 412–1150 (Figure 1B).

The intrinsic HAT activity of p300 is contained within one of the fragments used in Figure 1B (amino acids 1151–1673), indicating that Smad7 interacts with the HAT domain of p300. To further map this interaction, the HAT domain of p300 was in vitro translated and used in pull-down experiments with various fragments of Smad7. Both full-length and the N-terminal fragment of Smad7 interacted with the HAT domain of p300, whereas the C-terminal part of Smad7 was unable to interact with this domain of p300 (Figure 1C). This suggested that the N terminus of Smad7 could be a target for the acetyltransferase activity of p300.

To determine whether endogenous Smad7 and p300 form a complex in vivo, we used anti-Smad7 antibodies to immunoprecipitate Smad7 from HaCaT cell lysates. p300 was detected in the Smad7 immunoprecipitates, indicating that Smad7 and p300 interact in vivo (Figure 1D). In contrast, no p300 was detected when an unrelated antibody was used in the immunoprecipitation.

Figure 1. Smad7 Interacts with the Coactivator p300

(A) Myc-Smad7, either full-length or the indicated deletion mutants, was expressed in 293T cells in the absence or presence of p300-HA. Following immunoprecipitation with anti-HA antibodies, the samples were resolved by SDS-PAGE, and the presence of coimmunoprecipitated Smad7 was detected by Western blotting with anti-Myc antibodies. The levels of p300-HA and the different Smad7 proteins in the cell lysates were determined by Western blotting.

(B) The indicated fragments of p300 were in vitro translated and incubated with GST alone (lanes 2, 5, 8, and 11) or GST-Smad7 (lanes 3, 6, 9, and 12) coupled to glutathione beads. The complexes were washed, and the proteins separated by SDS-PAGE. Ten percent of the material used in each pull-down was loaded on the gel (lanes 1, 4, 7, and 10). The gel was dried and the 35S-labeled proteins detected by phosphorimage analysis.

(C) Poly-histidine p300-HAT (amino acids 1135–1810) was in vitro translated and incubated with GST alone (lane 2) or GST-Smad7, either full-length or the indicated deletion mutants (lanes 3–5). The glutathione beads were precipitated and washed, and the precipitated proteins were resolved by SDS-PAGE. Ten percent of the material used in each pull-down was loaded on the gel (lane 1). The gel was dried, and the 35S-labeled proteins detected by phosphorimage analysis.

(D) HaCaT cell lysates were immunoprecipitated with anti-Gal4 (lane 1) or anti-Smad7 (lane 2) antibodies. The immunoprecipitates were washed, and the proteins separated by SDS-PAGE. Coimmunoprecipitated p300 was detected by Western blotting using anti-p300 antibodies.
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Figure 3. Lysine Residues 64 and 70 in Smad7 Are Targeted by p300-Mediated Acetylation

(A) GST-fusion proteins of full-length Smad7, Smad7ΔN, or Smad7ΔC were subjected to in vitro acetylation using p300. After separating the proteins by SDS-PAGE, the gel was dried and exposed to phosphorimage analysis.

(B) Amino acid sequence of the acetylated peptides in Smad7 identified by MALDI-TOF-MS. The positions of the acetylated lysines in mouse Smad7 are indicated below each residue. Trypsin cleavage sites are indicated by arrows.

Figure 2. Smad7 Is a Substrate for p300 In Vivo

(A) Flag-Smad7 was expressed in 293T cells in the absence or presence of the indicated acetyltransferases, either in the absence (lanes 1–4) or presence (lanes 5–8) of constitutively active ALK5 (caALK5). Following immunoprecipitation of Smad7, the 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-Flag antibodies (α-Flag). The levels of Gal4-p300, Gal4-CBP, Flag-P/CAF, and HA-caALK5 in the cell lysates were determined by Western blotting.

(B) Myc-Smad7 was expressed in HaCaT cells in the absence or presence of siRNA directed against either human p300 (p300 siRNA) or firefly luciferase (GL2 siRNA). Thirty-six hours following transfection, cell lysates were prepared, and Smad7 was immunoprecipitated with anti-Myc antibodies. The immunoprecipitates 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). The amount of p300 in the cell lysates was determined by Western blotting (α-p300).

To confirm that K64 and K70 are acetylated, GST-Smad7 in which lysine residues 64 and 70 were mutated to alanines, either alone (K64A and K70A) or together (K64/70A), were generated and used in in vitro acetylation assays. Mutation of K70 resulted in a minor decrease in acetylation (Figure 4A). However, mutation of K64 resulted in a significant decrease (50% reduction) in acetylation, and if both K64 and K70 were mutated, p300-mediated acetylation of Smad7 was almost completely prevented. All mutants retained their ability to interact with the HAT domain of p300 in GST pull-down experiments (data not shown).

To determine if K64 and K70 are acetylated in vivo, Smad7, either wild-type or the indicated mutants, was expressed in 293T cells alone or together with p300 (Figure 4B). Wild-type Smad7 was acetylated following coexpression with p300, and mutation of K64 significantly decreased the acetylation of Smad7, while mutation of K70 only had a limited effect (compare lanes 2, 4, and 6 in Figure 4B). However, mutation of both K64 and K70 prevented acetylation of Smad7 (Figure 4B, lane 8). When the assay was repeated in the presence of TSA, a histone deacetylase inhibitor, acetylation of Smad7 was increased, especially Smad7(K64A), demonstrating that K70 is acetylated and deacetylated in vivo (Figure 4B, lanes 9–16). However, even in the presence of TSA, no p300-mediated acetylation of Smad7(K64/70A) was seen. This lack of acetylation could not be explained by effects on the interaction between Smad7 and p300, since Smad7(K64/70A) still interacted with full-length...
p300 Acetylates Specific Lysine Residues in Smad7

Figure 4. p300 Acetylates Specific Lysine Residues in Smad7

(A) GST-fusion proteins of wild-type Smad7, Smad7(K64A), Smad7(K70A), or Smad7(K64/70A) were in vitro acetylated in the absence or presence of recombinant p300-HAT. After resolving the reaction products on SDS-PAGE, the gel was dried and exposed to phosphorimage analysis. The minor band seen in lane 9 originates from autoacetylated p300.

(B) Flag-Smad7, either wild-type or the indicated lysine mutants, was expressed in 293T cells in the absence or presence of p300-HA. Twenty-four hours post transfection, the cells were left untreated (lanes 1–8) or treated with TSA (200 ng/ml) (lanes 9–16) for 12 hr. Following immunoprecipitation of Smad7, samples were resolved by SDS-PAGE, and the acetylation of Smad7 was detected with anti-acetyl lysine antibodies (\(\alpha\)-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Flag antibodies (\(\alpha\)-Flag). The levels of p300-HA in the cell lysates were determined by Western blotting (\(\alpha\)-HA).

(C) Myc-Smad7, either wild-type or the K64/70A mutant, was expressed in 293T cells in the absence or presence of p300-HA. Following immunoprecipitation with anti-HA antibodies, the samples were resolved by SDS-PAGE, and coimmunoprecipitated Smad7 was detected by Western blotting (\(\alpha\)-Myc). The levels of p300-HA and the different Smad7 proteins in the cell lysates were determined by Western blotting. (D) Myc-Smad7 was expressed in 293T cells in the absence or presence of p300, either wild-type or HAT-deficient (p300-DY). Following immunoprecipitation, the samples were resolved by SDS-PAGE, and the acetylation of Smad7 was determined with anti-acetyl lysine antibodies (\(\alpha\)-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Myc antibodies (\(\alpha\)-Myc). The amount of p300 in the cell lysates was determined by Western blotting (\(\alpha\)-p300).

(E) p300, either wild-type (WT) or acetyltransferase-deficient (DY), was expressed in 293T cells. Total cell lysates were used in pull-down experiments with GST alone (lanes 2 and 5) or GST-Smad7 (lanes 3 and 6) coupled to glutathione beads. The complexes were washed, and the proteins separated by SDS-PAGE. Ten percent of the material used in each pull-down was loaded on the gel (lanes 1 and 4).

(F) HaCaT cell lysates were immunoprecipitated with anti-Gal4 (lane 1) or anti-Smad7 (lane 2) antibodies. The immunoprecipitates were washed, the proteins were separated by SDS-PAGE, and the acetylation of Smad7 was detected with anti-acetyl lysine antibodies (\(\alpha\)-AcK). The amount of Smad7 in the immunoprecipitates was determined with anti-Smad7 antibodies.

p300 in vivo (Figure 4C). In addition, the interaction between Smad7 and p300 was not dependent on the acetyltransferase activity of p300, since an acetyltransferase-deficient mutant form of p300 (p300-DY) (Ito et al., 2001) retained its ability to interact with Smad7 (Figure 4E).

To confirm that Smad7 is acetylated in vivo, endogenous Smad7 was immunoprecipitated from HaCaT cell lysates. The anti-Smad7 antibody immunoprecipitated acetylated Smad7, while an unrelated antibody was unable to do so (Figure 4F).

Acetylation of Smad7 Inhibits Its Degradation

Theoretically, acetylation of specific lysines could increase the stability of a protein, since acetylation would prevent ubiquitination of the same lysine residues. In order to determine if p300 could affect the expression of Smad7, 293T cells were transfected with Smad7, either wild-type or the indicated lysine mutants, in the absence or presence of cotransfected p300 (Figure 5A). The expression of Smad7(K64A) was higher than that of wild-type Smad7 (Figure 5A, lanes 1 and 2 in Figure 5A). The expression of Smad7(K64/70A) was higher than that of wild-type Smad7 (Figure 5A, lanes 1 and 3), and only a weak induction in response to p300 could be observed (lanes 3 and 4). The levels of Smad7(K70A) and the double mutant (K64/70A) were very high in the absence of p300, and no significant increase was observed in response to p300 expression (Figure 5A, lanes 5–8). These results indicate that acetylation by p300 or mutation of K64 and K70 increases the stability of Smad7. In support of this notion, the p300-mediated stabilization of Smad7 was dependent on the acetyltransferase activity of p300, since an acetyltransferase-deficient mutant form of p300 (p300-DY) was unable to stabilize Smad7 (Figure 5B). As shown
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Figure 5. Acetylation of Smad7 Protects It from caALK5-Induced Degradation

(A) Flag-Smad7, either wild-type or the indicated mutants, was expressed in 293T cells in the absence or presence of p300-HA. Thirty-six hours following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of Smad7 were detected by Western blotting (α-Flag).

(B) Flag-Smad7 was expressed in 293T cells in the absence or presence of p300, either wild-type or acetyltransferase-deficient (p300-DY). Thirty-six hours following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of Smad7 and p300 were determined by Western blotting using anti-Flag (α-Flag) and anti-p300 (α-p300) antibodies, respectively.

(C) Flag-Smad7, either wild-type or the indicated mutants, was expressed in 293T cells in the absence or presence of HA-caALK5. Thirty-six hours following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of Smad7 and caALK5 in the cell lysates were determined by Western blotting using anti-Flag (α-Flag) and anti-HA (α-HA) antibodies, respectively. To ensure that equal amounts of protein were loaded in each well, the levels of tubulin in the samples were estimated by Western blotting using anti-tubulin antibodies (α-Tub).

(D) Flag-Smad7, either wild-type or Smad7(K64A), was expressed in 293T cells in the absence or presence of HA-caALK5 and p300-HA. Thirty-six hours following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of p300-HA and HA-caALK5 in the cell lysates were determined by Western blotting (α-HA). To ensure that equal amounts of protein were loaded in each well, the levels of tubulin in the samples were estimated by Western blotting using anti-tubulin antibodies (α-Tub).

(E) Flag-Smad7 was expressed in 293T cells in the absence or presence of p300, either wild-type or acetyltransferase-deficient (p300-DY). Thirty-six hours following transfection, cells were treated with TGFβ for 4 hr. Total cell lysates were prepared and resolved by SDS-PAGE. The levels of Smad7 and p300 were determined by Western blotting using anti-Flag (α-Flag) and anti-p300 (α-p300) antibodies, respectively. To ensure that equal amounts of protein were loaded in each well, the levels of tubulin in the samples were estimated by Western blotting using anti-tubulin antibodies (α-Tub).

(F) GST-Smad7, either wild-type or the K64/70A mutant, was used as substrate in in vitro ubiquitination reactions in the absence or presence of rabbit reticulocyte lysate (RRL). Where indicated, GST-Smad7 was acetylated prior to the ubiquitination reaction. The samples were resolved by SDS-PAGE, and the ubiquitination of Smad7 was determined with anti-ubiquitin antibodies (α-Ub). The amount of Smad7 and its acetylation were determined with anti-Smad7 (α-Smad7) and anti-acetyl lysine (α-AcK) antibodies, respectively.

above, p300-DY is able to interact with Smad7 (Figure 4E), demonstrating that the interaction between Smad7 and p300 is not sufficient to stabilize Smad7.

To determine if K64 and K70 are involved in regulating the stability of Smad7 in response to TGFβ signaling, wild-type Smad7 and the different lysine mutants were expressed in 293T cells in the absence or presence of constitutively active TGFβ type I receptor (caALK5). Expression of caALK5 drastically reduced the amounts of wild-type Smad7 (compare lanes 1 and 2 in Figure 5C), while all three lysine mutants were resistant to the effects of caALK5 (Figure 5C, lanes 3–8). To determine if p300-mediated acetylation of these lysine residues is able to protect Smad7 from caALK5-mediated degradation, the experiment described in Figure 5C was repeated in the absence or presence of cotransfected p300 (Figure 5D). Again, cotransfection of caALK5 decreased the levels of Smad7 in the absence of p300 (compare lanes 1 and 2 in Figure 5D). However, the enhanced level of Smad7 in the presence of p300 was
not reduced by caALK5 (Figure 5D, lanes 3 and 4). The expression of Smad7(K64A) was higher in the absence of p300 compared to wild-type Smad7, and coexpression of caALK5 or p300 had no major effects on the expression of this mutant (Figure 5D, lanes 5–8). Transfected Smad7 was degraded in response to TGFβ treatment (Figure 5E, lanes 1 and 2). Interestingly, expression of p300 blocked TGFβ-dependent degradation of Smad7, and this effect was dependent on the acetyltransferase activity of p300, since the acetyltransferase-deficient p300-DY was unable to stabilize Smad7 (Figure 5E, lanes 3–6).

Our results suggest that acetylation of Smad7 enhances its expression, potentially by interfering with the ubiquitin-proteasome pathway. To test this hypothesis, we used GST-Smad7, either wild-type or K64/70A, in a reconstituted in vitro ubiquitination assay (Figure 5F). Wild-type Smad7 was ubiquitinated in the presence of rabbit reticulocyte lysate, while a similar amount of Smad7(K64/70A) was significantly less ubiquitinated (compare lanes 2 and 4 in Figure 5F). To determine if p300-mediated acetylation is able to affect the ubiquitination of Smad7, the experiment was repeated with acetylated and nonacetylated GST-Smad7 (Figure 5F, lanes 5–10). Interestingly, acetylation of wild-type Smad7 prevented its ubiquitination (compare lanes 6 and 7 in Figure 5F), while no such effect was seen with Smad7(K64/70A) (Figure 5F, lanes 9 and 10). These results suggest that K64 and K70 are targeted by ubiquitination and that p300-mediated acetylation of these residues prevents this process.

The results presented in Figure 5 demonstrate that p300 is able to enhance the expression of Smad7, especially during caALK5-induced signaling. Based on this observation, we hypothesized that acetylation of lysine residues 64 and 70 in Smad7 is important for its stability. To address this possibility, we determined the half-life of wild-type and acetylation-deficient (K64A and K70A) Smad7, both in the absence and presence of caALK5. Both wild-type and mutant Smad7 were relatively stable in the absence of caALK5 expression (Figure 6). However, the degradation of wild-type Smad7 was enhanced following caALK5 expression, while K64A and K70A were insensitive to caALK5-induced degradation.

Smad7 was acetylated by endogenous acetyltransferases when expressed in HeLa cells (Figure 7A). Interestingly, the acetylation of Smad7 was prevented in cells expressing a HAT-deficient version of p300 (p300-DY), indicating that this mutant interferes with p300-mediated acetylation of Smad7 in a dominant-negative manner. The dominant-negative p300-DY decreased the expression of Smad7 when the two proteins were coexpressed in HeLa cells (Figure 7B). Interestingly, this effect was dependent on the acetylated lysine residues

![Figure 6](image6.png)

**Figure 6.** The Acetylated Lysine Residues in Smad7 Control Its Stability

Cos7 cells, transfected with wild-type or mutant Flag-Smad7, were pulse-labeled with [35S]methionine/cysteine and chased for the indicated time periods. [35S]labeled Smad7 was immunoprecipitated, resolved by SDS-PAGE, and quantified by phosphorimage analysis. The experiment was performed both in the absence (left panel) and presence (right panel) of caALK5. The amount of [35S]labeled Flag-Smad7 at each time point is plotted as percent of the amount at the start of the chase. The data represent the average ± SEM of three independent experiments.

![Figure 7](image7.png)

**Figure 7.** Inhibition of p300 Negatively Affects the Acetylation and Stability of Smad7

(A) Myc-Smad7 was expressed in HeLa cells in the absence or presence of HAT-deficient p300-DY. Following immunoprecipitation of Smad7, the 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). The amount of p300 in the cell lysates was determined by Western blotting (α-p300).

(B) Flag-Smad7, either wild-type or the K64/70A mutant, was expressed in HeLa cells in the absence or presence of p300-DY. Thirty-six hours following transfection, cell lysates were prepared and resolved by SDS-PAGE. The levels of Smad7 and p300 were determined by Western blotting using anti-Flag (α-Flag) and anti-p300 (α-p300) antibodies, respectively. To ensure that equal amounts of protein were loaded in each well, the levels of tubulin in the samples were estimated by Western blotting using anti-tubulin antibodies (α-Tub).
in Smad7, since the K64/70A mutant was insensitive to the destabilizing activity of p300-DY. These results confirm that p300 is required for the acetylation of Smad7 in vivo and indicate that Smad7 is destabilized and degraded when the activity of this acetyltransferase is compromised in cells.

Acetylation Protects Smad7 against Smurf-Mediated Degradation

Smad7 interacts with the ubiquitin ligases Smurf1 and Smurf2, and this interaction furthers the degradation of Smad7. To address the question whether K64 and K70 are involved in Smurf-mediated degradation of Smad7, wild-type and mutant Smad7 were expressed in the absence or presence of Smurf1 (Figure 8A). The levels of Smad7 were decreased in response to Smurf1 expression (compare lanes 1 and 2 in Figure 8A). In contrast, Smad7(K64A), Smad7(K70A), and the double mutant were protected against Smurf-dependent decreases in expression (Figure 8A, lanes 3–8), although all mutants tested still interacted with Smurf1 (data not shown). To investigate if p300 is able to protect Smad7 from Smurf-mediated degradation, the experiment described in Figure 8A was repeated in the absence or presence of cotransfected p300 (Figure 8B). Again, cotransfection of Smurf1 decreased the levels of Smad7 in the absence of p300. In the presence of cotransfected p300, Smurf1 was unable to reduce the levels of Smad7 (compare lanes 3 and 4 in Figure 8B). Transfected Smad7 was ubiquitinated in response to Smurf1 expression in 293T cells (Figure 8C). Interestingly, the Smurf-mediated polyubiquitination of Smad7 was blocked when K64 and K70 were mutated. Taken together with the results presented in Figures 5–7, our results suggest that K64 and K70 in Smad7 are targeted by Smurf-mediated ubiquitination and that acetylation prevents this process.

Discussion

Here we present evidence that Smad7 interacts with the transcriptional coactivator p300, resulting in acetylation of Smad7 on two lysine residues in its N terminus. Acetylation of these residues stabilizes Smad7 and protects it against TGFβ- and Smurf-dependent degradation, indicating that competition between acetylation and ubiquitination controls the stability of Smad7.

We demonstrate that Smad7 interacts with several domains of the coactivator p300. The C-terminal part of Smad7, containing the MH2 domain, interacted strongly with full-length p300 in vivo (Figure 1). This is similar to Smad2 and Smad3, which also interact with p300 and/or CBP through their MH2 domains (Feng et al., 1998; Janknecht et al., 1998; Nishihara et al., 1998). In contrast, Smad4 has been shown to interact with p300 through a sequence in its linker region (de Caestecker et al., 2000). The R- and Co-Smads interact with the C terminus of p300 and/or CBP, and we found that this was also true for Smad7. However, Smad7 demonstrated a much broader binding specificity and also interacted with N-terminal domains of p300. Acetylation of Smad7 occurs at the N terminus of the protein, both in vitro and in vivo (Figures 2 and 3). In vitro, acetylation of Smad7 required only the N terminus of Smad7 and the HAT domain of p300, indicating that the acetyltransferase activity of p300-DY is required for the acetylation of Smad7 in vivo.
domain of p300, suggesting that the interaction of Smad7 with other domains of p300 may serve other functions. We and others have been unable to demonstrate acetylation of R- or Co-Smads (Fukuchi et al., 2001; Itoh et al., 2000a; E.G. and J.E., unpublished data), making Smad7 the first member of the Smad family found to be modified by acetylation. Interestingly, CBP and P/CAF were unable to acetylate Smad7 in vivo. We were able to reproduce the specificity in acetylation using metabolic labeling of cells with [3H]acetate; incorporation of [3H]acetate into Smad7 was only detected in the presence of coexpressed p300 (Figure 2A, lower panel). In addition, RNAi-mediated inactivation of endogenous p300 in HaCaT cells prevented acetylation of Smad7, confirming the role of p300 in the acetylation of Smad7 (Figure 2B). A similar pattern was recently reported for the acetylation of p73 following DNA damage (Costanzo et al., 2002).

The acetylation of Smad7 was significantly decreased following ectopic expression of constitutively active ALK5, indicating that the acetylation of Smad7 is negatively regulated by TGFβ signaling (Figure 2A). The interaction between R- and Co-Smads and p300/CBP is enhanced following TGFβ treatment, and it is possible that the interaction between Smad7 and p300 is disrupted following activation of the TGFβ pathway, since Smad7 is translocated out of the nucleus following activation of the TGFβ pathway.

Using a combination of in vitro acetylation assays followed by mass spectrometry analyses and mutations of specific Smad7 lysines in vivo, we could map the acetylated residues in Smad7 to K64 and K70 in the N terminus of the protein (Figures 3 and 4). One of these lysine residues, K64, conforms to the consensus site for acetylation, i.e., a lysine preceded by a glycine or a serine residue. Both sites were also acetylated in vivo, with K64 being the major acceptor site. Interestingly, the acetylation of Smad7, and in particular K70, was greatly enhanced following treatment with the deacetylase inhibitor TSA. This indicates that the acetylation of Smad7 is a dynamic process that is regulated by both acetyltransferases and deacetylases.

Mutation or acetylation of K64 and/or K70 blocked the ALK5- and TGFβ-dependent decrease in Smad7 expression, indicating that these residues are involved in TGFβ-induced degradation of Smad7 (Figure 5). Indeed, when we determined the half-life of Smad7, we found that the degradation of the wild-type protein increased significantly following ALK5 expression, while the mutants were insensitive to ALK5 expression and remained stable (Figure 6). One interpretation of our results is that K64 and K70 confer instability to Smad7, presumably because they are also targeted by ubiquitination. The latter hypothesis gained support from our observation that mutation or acetylation of K64 and K70 prevented ubiquitination of Smad7 in vitro. Martinez-Balbón et al. (2000) demonstrated that the expression levels of E2F1 are enhanced following coexpression with P/CAF. This enhanced expression was dependent on the HAT domain of P/CAF, indicating that acetylation of E2F1 is important for this effect. However, when the acetylated lysines in E2F1 were mutated, the mutant protein did not accumulate. Rather, and in contrast to the case for Smad7, the lysine-mutated version of E2F1 was rapidly degraded. Further studies are, therefore, necessary to determine if competition between ubiquitination and acetylation of common lysine residues is responsible for the stabilization of E2F1 in response to P/CAF expression. There have also been reports of a potential correlation between the acetylation and stability of the p53 tumor suppressor protein (Ito et al., 2001; Nakamura et al., 2000; Rodriguez et al., 2000). However, the stabilization was not linked to individual lysine residues in p53, and it is unclear if acetylation of p53 is the only factor contributing to the observed effects. The issue is further complicated by the fact that p53 is destabilized through its interaction with p300/CBP under certain conditions (Grossman et al., 1998).

Further evidence for a link between acetylation and ubiquitination of Smad7 comes from our observation that mutation or acetylation of K64 and K70 protected Smad7 against Smurf1-mediated degradation (Figure 8). Members of the Smurf family of E3 ubiquitin ligases interact with Smads, including Smad7. In the case of Smad7, translocation of the Smad7-Smurf complex from the nucleus to the plasma membrane induces ubiquitination and degradation of the TGFβ receptors. At the plasma membrane, Smad7 is ubiquitinated and degraded, while the nuclear Smad7-Smurf complex is stable (Kavsak et al., 2000). Interestingly, we found that Smurf-mediated poly-ubiquitination of Smad7 was blocked when K64 and K70 were mutated (Figure 8C). One possibility is that mutation or acetylation of K64 and K70 affects the localization of Smad7 and, thereby, its stability. However, we were unable to detect any effects on the localization of Smad7 in response to mutation of the acetylated lysine residues or as a result of coexpression of p300 (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/10/3/483/DC1). Rather, our results suggest that p300-mediated acetylation of Smad7 protects it against premature Smurf-mediated degradation while in the nucleus. In support of this model, we found that both the acetylation and stability of Smad7 was decreased in HeLa cells expressing an acetyltransferase-deficient p300 mutant (Figure 7).

The potential link between acetylation/deacetylation and ubiquitination received further support from the recent identification of the components of the murine HDAC6 complex (Seigneurin-Berny et al., 2001). It was demonstrated that a number of the proteins in this complex are involved in the ubiquitin signaling pathway. Active deacetylation of p53 by HDAC1 and Sir2 plays important roles in the regulation of p53-dependent processes, including transcription, cell cycle regulation, and apoptosis (Luo et al., 2000, 2001; Vaziri et al., 2001). Future studies will address whether acetylated Smad7 is a substrate of HDACs.

It was recently demonstrated that TGFβ stimulation induces the assembly of Smad2-Smurf2 and Smad3-APC ubiquitin ligase complexes that target SnoN for degradation (Bonni et al., 2001; Stroschein et al., 2001; Wan et al., 2001). These observations indicate that certain proteins are targeted for degradation through their interactions with Smad proteins. It is striking that Smad7 undergoes two different modifications which both target the same lysine residues. We propose that acetylation of specific lysines in Smad7 prevents subsequent ubiquitination.
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utilization of the same residues, thereby blocking protease-
osome-mediated degradation of Smad7. The ubiquitin-
proteasome pathway regulates a large number of nu-
clear proteins, and many of these are also acetylated.
Therefore, it will be of utmost importance to determine
if competition between acetylation and ubiquitinization
is a general mechanism to regulate protein stability.

Experimental Procedures

Cell Culture

All tissue culture media and antibiotics were from Invitrogen and Sigma. Cells were from ATCC and maintained at 37°C in DMEM supplemented with 10% fetal calf serum, sodium pyruvate, nones-
sential amino acids, 50 μg/ml penicillin, and 50 μg/ml streptomycin, in 5% CO2.

TGFβ, Reagents, and Antibodies

TGFβ3 was a generous gift from N. Ferrara (Genentech). [3H]Acetyl-
CoA (51.6 μCi/mmol) was from ICN. Promix (14 μCl/μl), peroxidase-
conjugated sheep anti-mouse and donkey anti-rabbit IgG were from Amersham-Pharmacia Biotech. Trichostatin A (TSA), acetyl-CoA, ubiquitin, and anti-Flag antibody (M5) were from Sigma. Monoclonal anti-Myc (9E10), anti-ubiquitin (P4D1), goat and rabbit anti-Smad7 (N-19 and H-79), anti-HA (Y-11), anti-p300 (N-15), and anti-Gal4 DBD (RK51C) antibodies were from Santa Cruz. Monoclonal anti-
HA (12CA5) antibodies were from Roche. Anti-acetyl lysine antibod-
ies were from Cell Signaling Technology and Upstate Biotechnology.
Protein A was obtained from Zymed.

Plasmids and DNA Transfections

The expression vectors for Flag- and Myc-tagged Smad7, Smad7ΔN (amino acids 204–402) and Smad7ΔC (amino acids 2–261) in the mammalian expression vector pcDNA3 (Invitrogen) were kind gifts from P. ten Dijke (The Netherlands Cancer Institute, Amsterdam). The corresponding GST-tagged Smad7 constructs were generated by subcloning into pGEX4T-1 (Amersham-Pharmacia Biotech). Smad7 point mutants were generated by site-directed mutagenesis (QuickChange, Stratagene). The various fragments of human p300 (amino acids 2–411, 412–1150, 1151–1673, and 1674–2154) were generated by PCR and cloned into HA-pCDNA3. The expression vector for HA-tagged constitutively active ALK5 has been described previously (Nakao et al., 1997). The expression vectors for p300-HA and Flag-FCαF were kind gifts from T. Kouzarides (Wellcome/CRC Institute, Cambridge, UK). Gal4-tagged p300 and CBP in the pVR1012 vector (Vical) were from N. Perkins (University of Dundee, Dundee, UK). The bacterial expression vector for the poly-histidine-
tagged p300 HAT domain (amino acids 1135–1810) was a kind gift from P. ten Dijke (The Netherlands Cancer Institute, Amsterdam).

Generation of Recombinant Proteins

Cos7 cells were transfected using the reagents and methods de-
dscribed above. 24 hr after transfection, cells were incubated for 1 hr with [35S]-labeled cysteine and methionine (Promix, 20 μCi/ml) in methionine- and cysteine-free MCDB medium. The cells were washed and incubated in MCDB medium supplemented with 50 μg/ml methionine and cysteine for the indicated time periods. The immunoprecipitated samples were separated by SDS-PAGE. The gels were dried and analyzed by phosphorimage analysis (Fuji).

In Vitro Ubiquitination Assay

GST-Smad7, either wild-type or K64/70A, was used as substrate in
in vitro ubiquitination reactions in the absence or presence of 5 μl crude rabbit reticulocyte lysate (Promega) as described (Shenoy et al., 2001). Where indicated, GST-Smad7 was acetylated prior to the ubiquitination reaction, using recombinant p300-HAT and unlabeled acetyl-CoA. Reaction mixtures were incubated for 1 hr at 30°C. GST-
Smad7 was captured on glutathione beads and washed extensively. The samples were resolved by SDS-PAGE and transferred to nitro-
cellulose membranes, and the ubiquitination of Smad7 was deter-
mained by Western blotting.

Determination of Protein Half-Life

Cos7 cells were transfected using the reagents and methods de-
dscribed above. 24 hr after transfection, cells were incubated for 1 hr with [3S]-labeled cysteine and methionine (Promix, 20 μCi/ml) in methionine- and cysteine-free MCDB medium. The cells were washed and incubated in MCDB medium supplemented with 50 μg/ml methionine and cysteine for the indicated time periods. The immunoprecipitated samples were separated by SDS-PAGE. The gels were dried and analyzed by phosphorimage analysis.

In Vitro Acetylation Assay

GST-Smad7 (1 μg) was incubated for 2 hr at 30°C in acetylation buffer (50 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM DTT, 1 mM MgCl2, 1 mM PMSF, 20 mM sodium butyrate, and 10 nCi [3H]Acetyl-
CoA) in the absence or presence of the HAT domain of p300 (50 ng). To avoid unspecific interactions, the samples were incubated with unlabeled acetyl-CoA (final concentration, 1 mM) for an addi-
tional 15 min. SDS sample buffer was added, and the samples were boiled and separated by SDS-PAGE. The gels were dried and ana-
yzed by phosphorimage analysis (Fuji).

Mass Spectrometry and Analysis of Acetylated Lysine Residues

The Coomassie stained band was excised and treated for in-gel digestion as described (Heilmann, 2000). The peptide mixture was analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) on a Bruker Biflex III instru-
ment (Bruker) using standard settings for peptide mass fingerprint-
ing (PMF). The identity of Smad7 was confirmed by PMF using ProFound. To identify acetylated lysine residues, the peptide mass list was imported into GPMaw (version 4.22, Lighthouse Data) where the sequence of Smad7 was scanned for the addition of 42 Da on a lysine residue. A similar search for acetylated lysines was also done in ProFound, long form.

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