The DNA Binding Activities of Smad2 and Smad3 Are Regulated by Coactivator-mediated Acetylation

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Phosphorylation-dependent activation of the transcription factors Smad2 and Smad3 plays an important role in TGFβ-dependent signal transduction. Following phosphorylation of Smad2 and Smad3, these molecules are translocated to the nucleus where they interact with coactivators and/or corepressors, including p300, CBP, and P/CAF, and regulate the expression of TGFβ target genes. In the current study, we demonstrate that both Smad2 and Smad3 are acetylated by the coactivators p300 and CBP in a TGFβ-dependent manner. Smad2 is also acetylated by P/CAF. The acetylation of Smad2 was significantly higher than that of Smad3. Lys19 in the MH1 domain was identified as the major acetylated residue in both the long and short isoform of Smad2. Mutation of Lys19 also reduced the p300-mediated acetylation of Smad3. By generating acetyl-Lys19-specific antibodies, we demonstrate that endogenous Smad2 is acetylated on this residue in response to TGFβ signaling. Acetylation of the short isoform of Smad2 improves its DNA binding activity in vitro and enhances its association with target promoters in vivo, thereby augmenting its transcriptional activity. Acetylation of Lys19 also enhanced the DNA binding activity of Smad3. Our data indicate that acetylation of Lys19 induces a conformational change in the MH1 domain of the short isoform of Smad2, thereby making its DNA binding domain accessible for interactions with DNA. Thus, coactivator-mediated acetylation of receptor-activated Smad molecules could represent a novel way to regulate TGFβ signaling.

A large number of transcriptional coactivators, including CBP, p300, P/CAF, and GCN5, have intrinsic acetyltransferase activities that are important for their abilities to enhance transcription (5–12). Acetylation involves the transfer of 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 the acetylation and deacetylation of histones has major effects on chromatin structure and transcription (for a 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 (18) also are acetylated and that this modification affects their interactions with DNA and other proteins. Protein acetylation can also affect protein stability, because it has been demonstrated that acetylation prevents ubiquitination of the same lysine residues (18–21). We have previously found that the stability of Smad7, an inhibitory Smad molecule, is regulated by reversible acetylation (22).

Alternative splicing of exon 3 in the Smad2 gene gives rise to two distinct protein isoforms (23). The short isoform (Smad2(ΔE3)), unlike full-length Smad2 (Smad2(FL)), retains DNA-binding activity (24). The two isoforms of Smad2 are coexpressed throughout mouse development, but Smad2(FL) is the dominant isoform in most cell lines (23). It has been demonstrated that expression of Smad2(ΔE3), but not Smad2(FL), in Smad2-deficient mice results in viable and fertile animals (25). These results demonstrate that Smad2(ΔE3), but not Smad2(FL), has the ability to activate all essential target genes downstream of TGFβ during development.
In the current study, we demonstrate that both isoforms of Smad2, as well as Smad3, are acetylated on a specific lysine residue, Lys$^{19}$, in their MH1 domains in response to TGFβ signaling. Acetylation of the short isoform of Smad2 (Smad2($\Delta$E3)) augments its DNA binding activity in vitro and enhances its association with target promoters in vivo. Acetylation of Lys$^{19}$ also enhances the DNA binding of Smad3. Our data indicate that acetylation of Lys$^{19}$ induces a conformational change in the MH1 domain of Smad2($\Delta$E3), thereby making its DNA binding domain accessible for interactions with DNA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All tissue culture media and antibiotics were obtained from Invitrogen and Sigma. 293T, HepG2, HeLa, COS-1, and HaCaT cells were from the American Type Culture Collection. Smad2-deficient mouse embryonic fibroblasts were obtained from Anita Roberts (NCI) (26). 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 $\times$), 50 units/ml penicillin and 50 $\mu$g/ml streptomycin, in 5% CO2. For overnight starvation, cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with non-essential amino acids (1 $\times$), 50 units/ml penicillin, and 50 $\mu$g/ml streptomycin.

**TGFβ, Reagents, and Antibodies**—TGFβ was obtained from Peprotec EC. TSA was obtained from Sigma. Antibodies against Myc (9E10), HA (Y-11), p300 (N15), P/CAB (E8), and Gal4-DBD (RK5Cl) were from Santa Cruz Biotechnology. FLAG antibodies (M5) were from Sigma and anti-acetyl lysine antibodies were from Cell Signaling Technology and Upstate Biotechnology. The monoclonal Smad2/3 antibody (cat. no. 610843) was from BD Biosciences. Rabbit polyclonal anti-acetyl-Lys$^{19}$ Smad2 antisera was raised against an acetylated peptide corresponding to amino acids 15–24 in Smad2, and was affinity-purified as described (22). The rabbit polyclonal anti-phosphorylated Smad2 antibody has been described elsewhere (27). Secondary anti-mouse and anti-rabbit antibodies and protein-G Sepharose were from Amersham Biosciences.

**Plasmids and DNA Transfections**—The expression vectors for FLAG- and FLAG-tagged Smad2(FL) and Smad3 in the mammalian expression vector pcDNA3 (Invitrogen) were generously provided by P. ten Dijke (The Netherlands Cancer Institute, Amsterdam). The short isoform of Smad2 (Smad2($\Delta$E3)) was generated from the corresponding Smad2(FL) construct by PCR. The expression vectors for p300, CBP and P/CAB have been described (28). Point mutants in Smad2(FL), Smad2($\Delta$E3), and Smad3 were generated by site-directed mutagenesis (QuikChange, Stratagene). The Smad-responsive ARE-Luc and 12xCAGA-Luc promoter-reporter constructs have been described (28). Transient transfections were performed using the MB8 transfection kit (Stratagene).

**Immunoprecipitations and Immunoblotting**—Cells were lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate, and 1% aprotinin) and cleared by centrifugation. Immunoprecipitations were performed by adding the appropriate antibodies plus protein G-Sepharose beads, followed by incubation for 3 h at 4 °C. The immune complexes were washed three times with lysis buffer, once with 0.5 M NaCl and once with water. The samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). After blocking in phosphate-buffered saline with the addition of 5% bovine serum albumin, the membranes were incubated with the appropriate antibodies, washed with phosphate-buffered saline containing 0.05% Triton X-100 and incubated with horseradish peroxidase-coupled secondary antibodies. After washing, the blots were visualized with Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology).

**Luciferase and β-Galactosidase Assays**—Cells were transiently transfected with promoter-reporter genes in the absence or presence of expression vectors for the indicated Smad protein, either wild-type or the indicated mutants. 24-h post-transfection, the medium was replaced with medium containing 0.5% fetal calf serum and treated in the absence or presence of TGFβ (5 ng/ml). After 36 h, luciferase activities were determined in duplicate samples as described by the manufacturer (Promega). The pCH110 vector encoding the β-galactosidase reporter gene (Amersham Biosciences) was used as an internal control for transfection efficiency. Luciferase values (relative light units, RLU) were calculated by dividing the luciferase activity by the β-galactosidase activity. The data represent the average ± S.D. of three independent experiments performed in duplicates.

**Electrophoresis Shift Assay**—Total cell extracts were prepared from transiently transfected COS-1 cells using hyperionic lysis buffer (20 mM Hepes, pH 7.6, 20% (w/v) glycerol, 500 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1% (w/v) Triton X-100, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 1% aprotinin). The transfected proteins were visualized by Western blotting using anti-FLAG antibodies, followed by quantitation with a charge-coupled device camera (Fuji) and image analysis software (Aida Image Analyzer, version 3.10). Equal amounts of proteins were incubated with 1 μg of poly-dIdC and a $^{32}$P-labeled oligonucleotide probe containing four Smad-binding sites (4xCAGA) in hypotonic lysis buffer (20 mM Hepes, pH 7.6, 20% (w/v) glycerol, 20 mM NaCl, 10 mM MgCl$_2$, 0.2 mM EDTA, 1% (w/v) Triton X-100, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and 1% aprotinin). The samples were incubated for 15 min on ice and run on 5% polyacrylamide gels. The gels were analyzed by PhosphorImager analysis. For EMSAs with purified GST-Smad2($\Delta$E3) and in vitro translated Smad2($\Delta$E3)-MH1, the proteins were visualized by Western blotting, followed by quantitation with a charge-coupled device camera (Fuji) and image analysis software (Aida Image Analyzer, version 3.10). Equal amounts of proteins were incubated with 1 μg of poly(dIdC) and $^{32}$P-labeled 4xCAGA probe in binding buffer (50 mM Hepes, pH 7.9, 15% (w/v) glycerol, 75 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, and 10 mM spermine). The reaction products were separated and analyzed as described above. Where indicated, GST-Smad2($\Delta$E3) was incubated in the presence of purified GST-P/CAB in the absence or presence of 1 mM acetyl coenzyme A in acetylation buffer (50 mM Tris-HCl, pH 8.0, 10% (w/v) glycerol, 1 mM dithiothreitol,
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1 mM MgCl₂, and 20 mM sodium butyrate) for 2 h prior to the EMSA.

**DNAP Assays**—Cell lysates from transiently transfected 293T cells were precleared with streptavidin-agarose (Sigma) and subsequently used in DNA precipitation (DNAP) assays. The biotinylated double-stranded DNA was composed of a multimerized Smad-binding element (4xCAGA). DNA-bound proteins were precipitated with streptavidin-agarose for 60 min at 4°C, washed, and detected by Western blot analysis.

**Chromatin Immunoprecipitation**—The chromatin immunoprecipitation assays were performed as described previously (29). For the analysis of transfected material, 1 × 10⁶ COS-1 cells were transfected with 1 µg of FLAG-tagged Smad constructs, with or without constitutively active ALK5. After transfection, cells were fixed with 1% formaldehyde, sonicated, and one-fourth of the material was immunoprecipitated with 5 µg of the indicated antibody. The cross-link was reversed at 65°C overnight, followed by proteinase K treatment. The DNA was extracted using phenol:chloroform. For analysis of endogenous Smad2, two 15-cm dishes of HeLa cells (10 × 10⁶ cells) were used per immunoprecipitation. The PCR conditions for each target gene were optimized to remain in the linear range of amplification. The primers used to amplify the PAL-1 promoter have been described (29). The primers used for the p21 promoter were (forward primer) 5′-CAT TGT GAA GCT CAG TAC CAC AA-3′ and (reverse primer) 5′-TGC TTT CAG GCA TTT CAA ATA GAC-3′. The PCR primers used for 12xCAGA-Luc reporter gene were (forward primer) 5′-ACT GCA GGT GCC AGA AC ATT-3′ and (reverse primer) 5′-GTT CCA TCT TCC AGC GGA TA-3′. The PCR products were separated by electrophoresis in 6% polyacrylamide gels, and stained by ethidium bromide.

**RESULTS**

Smad2(FL), Smad2(ΔE3), and Smad3 Are Acetylated—The histone acetyltransferases CBP/p300 are able to interact with Smad2, -3, and -4, and they function as coactivators of TGFβ-induced transcription in a Smad4-dependent fashion (30–34). In addition, P/CAB and GCN5 have been shown to potentiage TGFβ signaling (28, 35). These observations prompted us to determine if Smad2(FL) and Smad3 were acetylated in vivo. When Smad2(FL) or Smad3 were transiently expressed in 293T cells, both proteins were acetylated in response to coexpression of either p300 or CBP (Fig. 1A). The acetylation of Smad2(FL) was more pronounced than that of Smad3, indicating that Smad2(FL) is a better substrate for these acetyltransferases.

Smad2(FL) and Smad3 are highly similar proteins, but whereas Smad3 can bind DNA directly, Smad2(FL) is dependent on coactivators to associate with DNA. This difference between Smad2(FL) and Smad3 is caused by an additional exon (exon 3) that is inserted in front of the DNA binding domain in Smad2(FL) (24). A splice variant of Smad2, which lacks exon 3 (Smad2(ΔE3)) has been identified (23). This isoform of Smad2 plays an important role during embryonal development (25). To determine if Smad2(ΔE3) was acetylated and establish if the acetylation of the R-Smads was regulated by TGFβ signaling, Smad2(FL), Smad2(ΔE3), and Smad3 were transfected together with p300 in the absence or presence of constitutively active ALK5 receptor. As illustrated in Fig. 1B, expression of ALK5 enhanced the acetylation of all the Smads tested.

In vitro acetylation assays using deletion mutants of GST-Smad2(FL) and immunoprecipitated p300, indicated that the major acetylation site in Smad2(FL) resided in the N-terminal MH1 domain (data not shown). To identify the lysine residues...
targeted by p300-mediated acetylation, all lysine residues in the MH1 domain of Smad2(FL) were mutated to arginine, either individually or in sets of two residues, and the mutant proteins were expressed in 293T cells in the absence or presence of p300. Mutation of Lys\(^{19}\) and Lys\(^{20}\) (K19R/K20R), but not mutation of other lysine residues, blocked the acetylation of Smad2(FL) (Fig. 1C).

Lys\(^{19}\) and Lys\(^{20}\) are conserved between Smad2 and Smad3 and are located just in front of a specific insert in Smad2 (Fig. 2A). To identify the specific lysine residue acetylated by p300, Lys\(^{19}\), and Lys\(^{20}\) in Smad2(FL) were mutated individually and subjected to p300-mediated acetylation following expression in 293T cells. Mutation of Lys\(^{19}\) blocked the p300-mediated acetylation of Smad2(FL), whereas mutation of Lys\(^{20}\) had no effect (Fig. 2B), suggesting that Lys\(^{19}\) is the preferred site for p300-mediated acetylation of Smad2(FL). Mutation of Lys\(^{19}\) also blocked the p300-dependent acetylation of Smad2(ΔE3) (Fig. 2C, left panel). Mutation of Lys\(^{19}\) also reduced the acetylation of Smad2(FL) in HepG2, COS-1, and HeLa cells (supplemental Fig. S1), suggesting that Lys\(^{19}\) is the major acetylated residue in these cell lines. We were unable to detect any differences in the phosphorylation or interaction with p300 between wild-type Smad2(FL) or Smad2(ΔE3) and the corresponding K19R mutants (data not shown). The p300-dependent acetylation of Smad3 was attenuated when Lys\(^{19}\) was mutated, whereas mutation of Lys\(^{20}\) had no effect (Fig. 2C, right panel), suggesting that Lys\(^{19}\) is acetylated also in Smad3. Smad2(FL) and Smad2(ΔE3) were also acetylated by P/CAF in vivo and the acetylation of both proteins was lost following mutation of Lys\(^{19}\) (Fig. 2D), suggesting that Lys\(^{19}\) in Smad2 is also targeted by P/CAF. We were unable to detect any acetylation of Smad3 by P/CAF under these conditions. Because Smad2(FL) and Smad2(ΔE3) were better substrates for p300, CBP, and P/CAF when compared with Smad3, we focused on Smad2 in our attempts to elucidate the functional role of acetylation of R-Smads.

Lys\(^{19}\) in Smad2 Is Acetylated in Vivo—To determine if endogenous Smad2 was acetylated on Lys\(^{19}\), we generated an acetyl-Lys\(^{19}\)-specific Smad2 antisera (AcK19). The affinity-purified antibody recognized wild-type Smad2(FL) following expression in 293T cells together with p300, whereas it failed to recognize the K19R mutant (Fig. 3A). The acetylation of Lys\(^{19}\) in endogenous Smad2 in HaCaT cells was enhanced following TGFβ stimulation (Fig. 3B). The acetylation was further enhanced when cells were treated with the deacetylase inhibitor TSA (compare lanes 2 and 4 in Fig. 3B), suggesting that the acetylation of Lys\(^{19}\) is a dynamic process regulated by deacetylases. Smad2 was also acetylated on Lys\(^{19}\) in response to TGFβ stimulation in HeLa cells (Fig. 3C). The acetylation of endogenous Smad2 was inhibited by the acetyltransferase inhibitor anacardic acid (Fig. 3D), confirming that the acetylation of Lys\(^{19}\) in Smad2 is dependent on cellular acetyltransferases. The Lys\(^{19}\)-specific antibody also recognized Smad3 in response to p300-mediated acetylation (Fig. S2), confirming that Lys\(^{19}\) is acetylated in Smad3. However, we were unable to detect any acetylation of endogenous Smad3 on Lys\(^{19}\), confirming our observation that the acetylation of Smad3 is low compared with Smad2.

Following receptor-mediated phosphorylation, Smad2 functions as a transcription factor by interacting with the promoters of Smad target genes. We used the Ack1 antibody in chromatin immunoprecipitation (ChIP) assays to determine if acety-
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FIGURE 3. Lys19 in endogenous Smad2 is acetylated in response to TGFβ signaling. A, FLAG-Smad2(FL), either wild-type or the K19R mutant, was expressed in 293T cells in the absence or presence of p300-HA. Following immunoprecipitation of the FLAG-tagged proteins, samples were resolved by SDS-PAGE, and the acetylation of Smad2(FL) was determined by Western blotting with an anti-acetyl lysine antibody directed against the major acetylation site in Smad2 (AcK19). The levels of Smad2(FL) in the immunoprecipitates were determined by Western blotting with an anti-FLAG antibody. B, HaCaT cells were treated in the absence or presence of TGFβ (5 ng/ml) and TSA (100 ng/ml) for 4 h. Smad2 and Smad3 were immunoprecipitated from cell lysates, and the samples were resolved by SDS-PAGE. The acetylation of Lys19 in Smad2 and Smad3 (AcK19), and the levels of Smad2 and Smad3 were determined by Western blotting. C, HeLa cells were treated in the absence or presence of TGFβ (5 ng/ml) for 4 h. Smad2 and Smad3 were immunoprecipitated from cell lysates and the samples were resolved by SDS-PAGE. The acetylation of Lys19 in Smad2 and Smad3 (AcK19), and the levels of Smad2 and Smad3 were determined by Western blotting. D, HaCaT cells were treated in the absence or presence of TGFβ (5 ng/ml) and anacardic acid (10 μM) for 4 h. Smad2 and Smad3 were immunoprecipitated from cell lysates, and the samples were resolved by SDS-PAGE. The acetylation of Lys19 in Smad2 and Smad3 (AcK19), and the levels of Smad2 and Smad3 were determined by Western blotting. E, HaCaT cells were treated in the absence or presence of TGFβ (5 ng/ml) for 4 h. Smad2 and Smad3 were immunoprecipitated from cell lysates and the samples were resolved by SDS-PAGE. The acetylation of Lys19 in Smad2 and Smad3 (AcK19), and the levels of Smad2 and Smad3 were determined by Western blotting. F, HaCaT cells were treated in the absence or presence of TGFβ (5 ng/ml) for the indicated times (min) and processed for ChIP analysis of the PAI-1 promoter, using Smad2 and AcK19 antibodies for immunoprecipitation. G, HaCaT cells were treated in the absence or presence of TGFβ (5 ng/ml) for 60 min and processed for ChIP analysis of the PAI-1 promoter, using p300 and P/CAF antibodies for immunoprecipitation. —Ab, no antibody.

Acetylation of Smad2 was associated with the promoters of target genes in vivo. Smad2 acetylated on Lys19 was bound to the promoters of both the PAI-1 and p21 genes in HeLa cells and the occupancy increased in response to TGFβ stimulation (Fig. 3F). The increased association of acetylated Smad2 with the PAI-1 promoter in response to TGFβ stimulation followed the same time course as the recruitment of total Smad2 (Fig. 3F). The increased association of Smad2 with the PAI-1 promoter in response to TGFβ stimulation coincided with an increased recruitment of p300 and P/CAF to the promoter (Fig. 3G). Acetylated Smad2 was also recruited to the endogenous PAI-1 and p21 promoters in HaCaT cells in response to TGFβ stimulation (supplemental Fig. S3). Thus, our results demonstrate that endogenous Smad2 is acetylated on Lys19 in response to TGFβ signaling and that the acetylated molecules are associated with the promoters of target genes in vivo. Because the AcK19 antibody also recognizes acetylated Smad3, it is possible that Smad3 contributes to the positive signals observed in the ChIP assays in Fig. 3. However, HeLa cells express very low levels of Smad3 compared with Smad2 (Fig. 3C). In addition, the acetylation of Smad3 is low compared with Smad2, suggesting that acetylated Smad2 is the major contributor to the positive ChIP signals in our experiments. Further work is required to analyze the recruitment of acetylated Smad3 to target genes in vivo.

Acetylation of Lys19 Enhances the Transcriptional Activity of Smad2(ΔE3)—Smad3 and the short form of Smad2 (Smad2(ΔE3)) function as transcription factors by directly binding to DNA. However, the long form of Smad2 is unable to bind DNA and is, therefore, dependent on interactions with other DNA binding factors to function as a transcription factor. To test if the acetylation of Lys19 affects the transcriptional activity of Smad2, HepG2 cells were transfected with Smad-responsive promoter-reporter genes and Smad2(FL) or Smad2(ΔE3), either wild-type or the corresponding K19R mutants, in the absence or presence of p300. We were unable to detect any difference in transcriptional activity between wild-type Smad2(FL) and the K19R mutant on the ARE-Luc promoter-reporter gene (data not shown), indicating
Acetylation of Lys^{19} enhances the DNA binding activity of Smad2(ΔE3) and Smad3—Our data suggest that the acetylation of Lys^{19} enhances the transcriptional activity of Smad2(ΔE3), whereas the same modification only has a limited effect on the transcriptional activity of Smad2(FL). One possible explanation for this difference could be that the acetylation of Lys^{19} has a positive effect on the DNA binding activity of Smad2(ΔE3). To test this hypothesis, whole cell extracts from COS-1 cells transfected with Smad2(ΔE3), either wild-type or the K19R mutant, and P/CAF, were used in electromobility shift assays (EMSAs). In the absence of p300, wild-type Smad2(ΔE3) bound DNA only weakly, even in the presence of ALK5 (Fig. 5A). However, the DNA binding activity of wild-type Smad2(ΔE3) was greatly enhanced in response to p300 expression. The increase in DNA binding in response to p300 was dependent on Lys^{19}, because the K19R mutant displayed low DNA binding activity both in the absence and presence of p300. Similar results were also obtained when the DNA binding activity of Smad2(ΔE3) was analyzed in DNA precipitation (DNAP) assays, using a biotinylated oligonucleotide containing a multimerized Smad-binding element (4xCAGA) (data not shown). The DNA binding activity of wild-type Smad2(ΔE3) was also enhanced in response to P/CAF expression, whereas the K19R mutant failed to bind DNA both in the absence and presence of P/CAF (Fig. 5B), suggesting that P/CAF-mediated acetylation of Lys^{19} in Smad2(ΔE3) enhances its DNA binding activity. The addition of either FLAG or AcK19 antibodies to the EMSA disrupted the shifted complex, suggesting that both antibodies inhibit the DNA binding activity of Smad2(ΔE3). The hypothesis that acetylation enhances the DNA binding activity of Smad2(ΔE3) was supported when purified P/CAF was used to acetylate GST-Smad2(ΔE3) in vitro. P/CAF-mediated acetylation of wild-type Smad2(ΔE3) enhanced its DNA binding activity, whereas it failed to promote the DNA binding of the K19R mutant (Fig. 5C). When Smad3 was analyzed in DNAP assays, we found that the DNA binding activity of wild-type Smad3 was enhanced in response to p300 expression, whereas the activity of the K19R mutant was unaffected by p300 (Fig. 5D), suggesting that acetylation of Lys^{19} enhances the DNA binding of Smad3.

The DNA binding domains of Smad2(ΔE3) and Smad3 are localized to their N-terminal MH1 domains. It has been suggested that the DNA binding activity of the MH1 domain is short isoform of Smad2. Similar results were obtained following coexpression of Smad2(ΔE3) and P/CAF (Fig. 4B).

Our data indicate that the acetylation of Lys^{19} in Smad2(ΔE3) is important for its ability to transactivate promoter-reporter genes. To test if this was also true for endogenous target genes, Smad2-deficient mouse embryonic fibroblasts were transfected with Smad2(ΔE3), either wild-type or the K19R mutant, in the absence or presence of p300 and the expression of the p21 gene was analyzed by RT-PCR. The expression of the p21 gene was greatly increased in the presence of Smad2(ΔE3) and p300, whereas p300 failed to enhance the expression of the p21 gene in the presence of the K19R mutant (Fig. 4C). Taken together, our results suggest that the acetylation of Lys^{19} in Smad2(ΔE3) enhances its transcriptional activity.

Acetylation of Lys^{19} stimulates the transcriptional activity of Smad2(ΔE3) and p300—Our data suggest that the acetylation of Lys^{19} stimulates the transcriptional activity of Smad2(ΔE3), whereas the same modification only has a limited effect on the transcriptional activity of Smad2(FL). One possible explanation for this difference could be that the acetylation of Lys^{19} has a positive effect on the transcriptional activity of Smad2(ΔE3). To test this hypothesis, whole cell extracts from COS-1 cells transfected with Smad2(ΔE3), either wild-type or the K19R mutant, and p300, were used in electromobility shift assays (EMSAs). In the absence of p300, wild-type Smad2(ΔE3) was also enhanced in transcription in response to p300 expression. Similar results were also obtained when the DNA binding activity of Smad2(ΔE3) was analyzed in DNA precipitation (DNAP) assays, using a biotinylated oligonucleotide containing a multimerized Smad-binding element (4xCAGA) (data not shown). The DNA binding activity of wild-type Smad2(ΔE3) was also enhanced in response to P/CAF expression, whereas the K19R mutant failed to bind DNA both in the absence and presence of P/CAF (Fig. 5B), suggesting that P/CAF-mediated acetylation of Lys^{19} in Smad2(ΔE3) enhances its DNA binding activity. The addition of either FLAG or AcK19 antibodies to the EMSA disrupted the shifted complex, suggesting that both antibodies inhibit the DNA binding activity of Smad2(ΔE3). The hypothesis that acetylation enhances the DNA binding activity of Smad2(ΔE3) was supported when purified P/CAF was used to acetylate GST-Smad2(ΔE3) in vitro. P/CAF-mediated acetylation of wild-type Smad2(ΔE3) enhanced its DNA binding activity, whereas it failed to promote the DNA binding of the K19R mutant (Fig. 5C). When Smad3 was analyzed in DNAP assays, we found that the DNA binding activity of wild-type Smad3 was enhanced in response to p300 expression, whereas the activity of the K19R mutant was unaffected by p300 (Fig. 5D), suggesting that acetylation of Lys^{19} enhances the DNA binding of Smad3.
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A. COS-1 cells were transfected with FLAG-tagged Smad2(ΔE3), either wild-type or the K19R mutant, in the absence or presence of ALK5 and p300. The amount of Smad2(ΔE3) in whole cell lysates was determined, and equal amounts of proteins were used in EMSAs with a 32P-labeled oligonucleotide probe containing a multimerized Smad-binding element (4xCAGA). In lane 10, a 100-fold excess of unlabeled probe was added to the reaction. Where indicated, FLAG (lane 11) or AcK19 (lane 12) antibodies were included in the assay.

B. COS-1 cells were transfected with FLAG-Smad2(ΔE3), either wild-type or the K19R mutant, in the absence or presence of ALK5 and P/CAF. The amount of Smad2(ΔE3) in whole cell lysates was determined and equal amounts of proteins were used in EMSAs with the 32P-labeled 4xCAGA probe. In lane 9, a 100-fold excess of unlabeled probe was added to the reaction. In lane 10, FLAG antibodies were included in the assay.

C. Equal amounts of purified GST-Smad2(ΔE3), either wild-type or the K19R mutant, was incubated with GST-P/CAF in the absence or presence of acetyl coenzyme-A. Following a 2-h incubation, a portion of the reactions were used in EMSAs with the 32P-labeled 4xCAGA probe. D. 293T cells were transfected with FLAG-Smad3, either wild-type or the K19R mutant, and ALK5 in the absence or presence of p300. Lysates from the transfected cells were used in DNAP assays, using a biotinylated oligonucleotide containing a multimerized Smad-binding element (4xCAGA). The amount of Smad3 associated with DNA, and the amounts of Smad3 and p300 in cell lysates were determined by Western blotting.

E. Equal amounts of purified GST-Smad2(ΔE3), either wild-type or the K19Q or K19R mutants, were used in EMSAs with the 32P-labeled 4xCAGA probe. F. Equal amounts of in vitro translated Smad2(ΔE3)-MH1, either wild-type or the K19Q and K19R mutants, was used in EMSAs with the 32P-labeled 4xCAGA probe. Where indicated, a 100-fold excess of unlabeled probe, either nonspecific (NS) or specific (SP), was added to the reaction.

G. Smad2(ΔE3), either wild-type or the K19R and K19Q mutants, was in vitro translated in the presence of 35S-labeled cysteine and methionine and incubated in the absence (Input) or presence of thrombin (0.2 units) at 30 °C for 1 h. The reaction products were resolved by SDS-PAGE and analyzed by PhosphorImager analysis.

FIGURE 5. Acetylation of Lys19 stimulates the DNA binding activities of Smad2(ΔE3) and Smad3. A, COS-1 cells were transfected with FLAG-tagged Smad2(ΔE3), either wild-type or the K19R mutant, in the absence or presence of ALK5 and p300. The amount of Smad2(ΔE3) in whole cell lysates was determined, and equal amounts of proteins were used in EMSAs with a 32P-labeled oligonucleotide probe containing a multimerized Smad-binding element (4xCAGA). In lane 10, a 100-fold excess of unlabeled probe was added to the reaction. Where indicated, FLAG (lane 11) or AcK19 (lane 12) antibodies were included in the assay. B, COS-1 cells were transfected with FLAG-Smad2(ΔE3), either wild-type or the K19R mutant, in the absence or presence of ALK5 and P/CAF. The amount of Smad2(ΔE3) in whole cell lysates was determined and equal amounts of proteins were used in EMSAs with the 32P-labeled 4xCAGA probe. In lane 9, a 100-fold excess of unlabeled probe was added to the reaction. In lane 10, FLAG antibodies were included in the assay. C, Equal amounts of purified GST-Smad2(ΔE3), either wild-type or the K19R mutant, was incubated with GST-P/CAF in the absence or presence of acetyl coenzyme-A. Following a 2-h incubation, a portion of the reactions were used in EMSAs with the 32P-labeled 4xCAGA probe. D, 293T cells were transfected with FLAG-Smad3, either wild-type or the K19R mutant, and ALK5 in the absence or presence of p300. Lysates from the transfected cells were used in DNAP assays, using a biotinylated oligonucleotide containing a multimerized Smad-binding element (4xCAGA). The amount of Smad3 associated with DNA, and the amounts of Smad3 and p300 in cell lysates were determined by Western blotting. E, Equal amounts of purified GST-Smad2(ΔE3), either wild-type or the K19Q or K19R mutants, were used in EMSAs with the 32P-labeled 4xCAGA probe. F, Equal amounts of in vitro translated Smad2(ΔE3)-MH1, either wild-type or the K19Q and K19R mutants, was used in EMSAs with the 32P-labeled 4xCAGA probe. Where indicated, a 100-fold excess of unlabeled probe, either nonspecific (NS) or specific (SP), was added to the reaction. G, Smad2(ΔE3), either wild-type or the K19R and K19Q mutants, was in vitro translated in the presence of 35S-labeled cysteine and methionine and incubated in the absence (Input) or presence of thrombin (0.2 units) at 30 °C for 1 h. The reaction products were resolved by SDS-PAGE and analyzed by PhosphorImager analysis.

blocked by intramolecular interactions with the MH2 domain (2, 36–38). Receptor-mediated phosphorylation of the C terminus of Smad2(ΔE3) and Smad3 is thought to counteract these intramolecular interactions, thereby making the MH1 domain accessible for DNA binding. Thus, one possible interpretation of our results is that the acetylation of Lys19 affects the structure of Smad2(ΔE3), allowing the MH1 domain to interact with DNA. This hypothesis was supported by our observation that purified GST-Smad2(ΔE3) containing a mutation that mimics acetylation of Lys19 (K19Q) was able to bind DNA, whereas the corresponding wild-type and K19R proteins did not (Fig. 5E). This result is compatible with the possibility that the acetylation of Lys19 in Smad2(ΔE3) induces a conformational change that relieves the MH2-dependent inhibition of DNA binding. An alternative possibility is that the acetylation of Lys19 directly affects the DNA binding activity of the MH1 domain. To test this possibility, the isolated MH1 domain of Smad2(ΔE3), either wild-type or the K19R and K19Q mutants, was used in EMSAs. The DNA binding activity of the isolated MH1 domain of Smad2(ΔE3) was unaffected by mutation of Lys19 (Fig. 5F),...
Acetylation of Smad2 and Smad3

A Acetylation of Smad2 and Smad3

B Acetylation of Smad2 and Smad3

C Acetylation of Smad2 and Smad3

**FIGURE 6.** Acetylation of Ly**s** enhances the recruitment of Smad2(D3E3) to target promoters in vivo. A, COS-1 cells were transfected with 12xCAGA-Luc in the absence or presence of FLAG-Smad2(D3E3), either wild-type or the K19R mutant, p300, and ALK5. 36 h following transfection, cells were processed for ChIP analysis of the 12xCAGA promoter, using FLAG antibodies for immunoprecipitation. B, COS-1 cells were transfected with FLAG-Smad2(D3E3), either wild-type or the K19R mutant, p300 and ALK5. 36 h following transfection, cells were processed for ChIP analysis of the PAI-1 promoter, using FLAG antibodies for immunoprecipitation. C, COS-1 cells were transfected with FLAG-Smad2(D3E3), either wild-type or the K19Q mutant, and ALK5. 36 h following transfection, the cells were treated in the absence or presence of anacardic acid (5 μM) for 5 h and processed for ChIP analysis of the p21 promoter, using FLAG antibodies for immunoprecipitation. -- Ab, no antibody.

**DISCUSSION**

Acetylation of lysine residues is a reversible post-translational modification that has been shown to regulate the activity
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of a large number of transcription factors by a variety of mechanisms, including effects on protein stability, DNA binding and protein-protein interactions (5). In the current study, we demonstrate that both isoforms of Smad2 are acetylated on a specific lysine residue, Lys19, in their MH1 domains. Acetylation of the short isoform of Smad2 (Smad2(ΔE3)) improves its DNA binding activity in vitro and enhances its association with target promoters in vivo. Our data indicate that acetylation of Lys19 induces a conformational change in the MH1 domain of Smad2(ΔE3), thereby making its DNA binding domain accessible for interactions with DNA.

The histone acetyltransferases CBP/p300 interact with Smad2, -3, and -4, and they function as coactivators of TGFβ-induced transcription in a Smad4-dependent fashion (30–34). Both Smad2 and Smad3 interact with CBP/p300 through their C-terminal MH2 domains (30–32). However, deletion of the MH2 domain in Smad2(FL) did not affect the acetylation of Smad2 in vitro (data not shown), indicating that the major acetylated lysine residue(s) did not reside in this domain of the protein. Indeed, mutation of Lys19 in the MH1 domain of Smad2(FL) and Smad2(ΔE3) abolished the acetylation of both proteins in vivo in response to p300 expression, indicating that Lys19 is the major acetylation site in Smad2. By generating an antibody that recognizes Smad2 following acetylation of Lys19, we could confirm that endogenous Smad2 is acetylated on this residue in response to TGFβ signaling.

We have previously demonstrated that the inhibitory Smad7 is acetylated by p300 (18). The acetylation of Smad7 is reversible and acetylated Smad7 is a substrate of specific deacetylases (22). Interestingly, we found that the acetylation of endogenous Smad2 was enhanced when cells were exposed to the deacetylase inhibitor TSA, indicating that the acetylation of Lys19 in Smad2 could be a dynamic process. Further studies will be necessary to determine which deacetylases remove the acetyl group from Smad2 in vivo.

Acetylation is not the only post-translational modification that targets lysine residues. It is well established that polyubiquitination of lysine residues targets proteins for proteasome-mediated degradation. Acetylation has been shown to stabilize certain proteins, including Smad7, by preventing ubiquitination of the acetylated lysine residues (18, 19). However, mutation of Lys19 did not affect the steady-state levels of Smad2(FL) or Smad2(ΔE3), nor did we detect stabilization of any of the Smad2 isoforms in response to p300 expression. Taken together, these results indicate that acetylation does not affect the degradation of Smad2.

Expression of p300 enhanced the transcriptional activity of Smad2(ΔE3) in a manner dependent on Lys19. Acetylation has been shown to regulate the DNA binding activities of certain transcription factors (39–44). Smad2(ΔE3), unlike Smad2(FL), is able to bind DNA through its DNA binding domain, located in the MH1 domain. Thus, one possibility was that acetylation of Lys19 in Smad2(ΔE3) affects its DNA binding activity. Indeed, expression of p300 enhanced the DNA binding activity of Smad2(ΔE3) in vitro. The K19R mutant of Smad2(ΔE3) bound DNA poorly, and its DNA binding activity was insensitive to p300 expression, suggesting that acetylation of Lys19 promotes the DNA binding of Smad2(ΔE3). This possibility was supported by our observation that expression of p300 enhanced the recruitment of Smad2(ΔE3) to the promoters of target genes in vivo. In contrast, the K19R mutant of Smad2(ΔE3) failed to interact with target promoters in vivo and was insensitive to p300 expression.

Taken together, our results suggest that acetylation of Lys19 in Smad2(ΔE3) promotes its DNA binding activity, both in vitro and in vivo. As a result, acetylation of Smad2(ΔE3) enhances its transcriptional activity. The sequence that confers DNA binding activity to Smad2(ΔE3) is localized to its MH1 domain and insertion of exon 3 in Smad2(FL) prevents DNA binding. It has been suggested that the DNA binding activity of the MH1 domain is blocked by interactions with the MH2 domain of the same molecule. Receptor-mediated phosphorylation of the C terminus of Smad2(ΔE3), and Smad3 is thought to prevent this intramolecular interaction, thereby making the MH1 domain accessible for DNA binding. One interpretation of our results is that acetylation of Lys19 affects the structure of the MH1 domain of Smad2(ΔE3), making it more accessible for interactions with DNA. This hypothesis was supported by our observation that purified GST-Smad2(ΔE3) containing a mutation that mimics acetylation (K19Q) was able to bind DNA, whereas the corresponding wild-type protein did not. Thus, our results strongly suggest that coactivator-mediated acetylation of the MH1 domain in Smad2(ΔE3) enhances its DNA binding activity. In fact, the K19R mutant of Smad2(ΔE3) failed to associate with target promoters in vivo, suggesting that acetylation of Lys19 could play an important role in DNA binding in vivo. Our results also suggest that the acetylation of Smad2(ΔE3) could promote its interaction with DNA in the absence of receptor-mediated phosphorylation, e.g. following dephosphorylation of the transcription factor in the nucleus (45). However, the physiological relevance of this possibility will require further investigation. It has been suggested that structural alterations in response to acetylation of transcription factors could affect their DNA binding activity, both positively and negatively (39–44). Further studies are necessary to determine the exact mechanisms involved in the enhanced DNA binding activity of Smad2(ΔE3) in response to the acetylation of Lys19. Detailed structural studies of acetylated Smad2(ΔE3) may prove especially helpful in these efforts.

We found that also Smad2(FL) was acetylated by p300, CBP and P/CAF in transfected cells and the acetylation of the two isoforms of Smad2 was more pronounced than the acetylation of Smad3. Smad2(FL) is the dominating isoform in most cell lines, suggesting that the acetylated endogenous Smad2 that we detected in our experiments corresponds to Smad2(FL). However, this hypothesis will have to be confirmed following isoform-specific knock-down experiments. We were unable to detect any difference in transcriptional activity between wild-type Smad2(FL) and the corresponding K19R mutant on the ARE-Luc promoter-reporter gene in the presence of FAST. However, when Smad2(FL) was fused to the DNA binding domain of Gal4 and cotransfected with a promoter-reporter gene containing five Gal4 binding sites, the transcriptional activity of the K19R mutant was considerably lower than that of the wild-type protein (data not shown), suggesting that acetylation of Lys19 could play a role in regulating the transcriptional
activity of the full-length form of Smad2. A more detailed analysis of the functional consequence(s) of acetylation of Smad2(FL) is therefore warranted.

We found that the acetylation of Smad3 was significantly lower that that of Smad2(FL) and Smad2(ΔE3). However, we found that mutation of Lys$^{19}$ in Smad3 resulted in a significant decrease in p300-mediated acetylation, suggesting that Lys$^{19}$ is acetylated also in Smad3. This notion was supported by our observation that Smad3 was recognized by the AcK19 antibody in response to p300 expression. We also found that p300 enhanced the DNA binding activity of Smad3 in a manner dependent on Lys$^{19}$, suggesting that acetylation of Lys$^{19}$ enhances its DNA binding activity. A recent study suggested that Smad3 is acetylated on Lys$^{378}$ in response to p300 expression. We also found that p300 acetylated Smad3. This notion was supported by our analysis of the functional consequence(s) of acetylation of Smad3, an essential target genes downstream of TGFβ receptors (48–52).

Inoue et al. (46) found that the receptor-mediated phosphorylation of the K378R mutant of Smad3 was decreased, which could potentially attenuate its nuclear translocation and affect its interaction with coactivators, including p300, as well as its transcriptional activity. Even so, Smad3 appears to be acetylated on multiple lysine residues in both its MH1 and MH2 domain. Further studies are needed before all these sites and their functional roles have been elucidated.

The two isoforms of Smad2 are coexpressed throughout mouse development, but Smad2(FL) is the dominant isoform in most adult tissues. It has been demonstrated that expression of Smad2(ΔE3), but not Smad2(FL), in Smad2-deficient mice results in viable animals (25). These results suggest that Smad2(ΔE3), but not Smad2(FL), has the ability to activate all essential target genes downstream of TGFβ during development. Our data indicate that acetylation of Lys$^{19}$ in Smad2(ΔE3) is important for its transcriptional activity in vivo. It will, therefore, be very important to analyze the acetylation of Smad2(ΔE3) during development. Hopefully, the acetylation-specific Smad2 antibody described in the current study will be helpful in such efforts. It will also be important to determine if the acetylation of Smad2(ΔE3) is important during embryonal development.

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