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Role of Smad3, Acting Independently of Transforming Growth Factor-β, in the Early Induction of Wnt-β-Catenin Signaling by Parathyroid Hormone in Mouse Osteoblastic Cells

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ABSTRACT

Parathyroid hormone (PTH) exerts an anabolic action on bone but the mechanisms are incompletely understood. We showed previously that PTH interacts with the canonical Wnt-β-catenin signaling pathway via the transforming growth factor (TGF)-β signaling molecule, Smad3, to modulate osteoblast differentiation and apoptosis. Here, we examined which actions of Smad3 are TGF-β-independent in stimulating the osteoblast phenotype and PTH-induced Wnt-β-catenin signaling. For this, the TGF-β receptor type 1 [activin receptor-like kinase (ALK5)] inhibitor (SB431542), and a Smad3 mutant in which the site normally phosphorylated by ALK5 is mutated from SSVS to AAVA, was used. PTH induced total β-catenin and reduced phosphorylated β-catenin levels at 1, 6, and 24 h in mouse osteoblastic MC3T3-E1 cells. Transient transfection of Smad3AAVA inhibited the PTH induction of total β-catenin and reduction of phosphorylated β-catenin levels at 6 and 24 h, but not at 1 h, indicating that the early effects occur independently of TGF-β receptor signaling. On the other hand, MC3T3-E1 cell clones in which Smad3AAVA was stably expressed demonstrated elevated β-catenin levels, although alkaline phosphatase (ALP) activity and mineralization were unaltered. In contrast, MC3T3-E1 cell clones in which wild-type Smad3 was stably expressed exhibited increased ALP activity and mineralization that were decreased by the ALK5 inhibitor, SB431542, although the β-catenin levels induced in these cells were not modulated. In conclusion, the present study indicates that PTH induces osteoblast β-catenin levels via Smad3 independently of, and dependently on, TGF-β in the early and later induction phases, respectively. J. Cell. Biochem. 108: 285–294, 2009.

KEY WORDS: Smad3; TRANSFORMING GROWTH FACTOR-β; SIGNALING; WNTS; PARATHYROID HORMONE; OSTEOBLASTS

Parathyroid hormone (PTH) is a potent bone-forming agent that, when administered intermittently, increases bone mineral density and reduces fracture risk in osteoporotic patients, although its continuous administration or an excess of endogenous hormone, such as in primary hyperparathyroidism, causes osteopenia [Dempster et al., 1993; Rosen and Bilezikian, 2001]. The anabolic action of PTH is exerted partly through local growth factors and their transcriptional regulators as well as an anti-apoptotic action in osteoblasts [Jilka et al., 1999; Bellido et al., 2003]. PTH stimulates the expression of insulin-like growth factors and their binding proteins [Linkhart and Mohan, 1989; Kaji et al., 1997] and transforming growth factor (TGF)-β [Pfeilschifter et al., 1995; Janssens et al., 2005] in osteoblasts. PTH exerts its effects on genes important for bone formation through transcriptional regulators

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such as activator protein-1 family members [Kano et al., 1994], runt-related transcriptional factor 2 (Runx2) [Selvamurugan et al., 2000] and others [Swarthout et al., 2002]. However, additional signaling pathways are also important and we recently showed that PTH affects bone formation through the TGF-β signaling molecule and transcription factor, Smad3, and Wnt-β-catenin signaling, in osteoblasts [Sowa et al., 2003; Tobimatsu et al., 2006].

TGF-β binds to its type II membrane receptor promoting dimerization with the type I receptor stimulating its Ser/Thr kinase activity causing phosphorylation of the receptor-activated Smads, Smad2 and Smad3, on two serines at their far COOH-termini. The activated Smads associate with the common Smad, Smad4, and translocate into the nucleus where they modulate transcription of specific genes [Massague and Chen, 2000; Feng and Derynck, 2005; Massague et al., 2005]. We recently showed that upregulation of Smad3 expression, by transfection in mouse osteoblastic MC3T3-E1 cells, promotes markers of osteoblast differentiation, such as production of type I collagen, alkaline phosphatase (ALP) activity, and mineralization [Sowa et al., 2002a,b]. However, similar studies in mouse mesenchymal ST-2 cells suggested that Smad3 inhibits commitment to the osteoblast lineage [Kaji et al., 2006]. In vivo, mice with targeted disruption of Smad3 exhibit decreased bone formation and osteopenia [Borton et al., 2001]. Overall these findings suggest that the Smad3 molecule is a promoter of bone formation by acting after commitment of cells to the osteoblast lineage. Although overexpression of Smad3 stimulates ALP activity and mineralization in osteoblasts, these differentiation markers are inhibited by TGF-β [Sowa et al., 2002a,b]. We demonstrated that PTH induced Smad3 levels within 1 h in osteoblastic cells, although the induction of phosphorylated Smad3 levels by PTH took 6 h [Sowa et al., 2003], suggesting that the early induction of Smad3 by PTH is independent of the phosphorylation of Smad3 by TGF-β.

The secreted Wnt glycoproteins are critical regulators of mesenchymal stem cell commitment to the osteogenic and chondrogenic lineages, skeletal development and bone formation [Moon et al., 2002; Kolpakova and Olsen, 2005; Kulkarni et al., 2005; Bodine and Komm, 2006; Krishnan et al., 2006]. Wnt proteins initiate a canonical (β-catenin regulated) signaling cascade by binding to seven-transmembrane receptors of the frizzled family together with coreceptors, members of the low-density lipoprotein receptor-related protein (LRP) family, LRP5 and LRP6. This induces phosphorylation of the cytosolic disheveled protein, that inhibits the phosphorylation of β-catenin by glycogen synthase kinase 3 (GSK3) [Behrens et al., 1996]. The unphosphorylated β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it binds high mobility group box transcription factors of the lymphoid enhancer-binding factor (Lef)/T cell factor (Tcf) family to regulate skeletal cell gene transcription [Behrens et al., 1996]. We recently reported that PTH induced the levels of β-catenin within 1 h independently of endogenous TGF-β [Tobimatsu et al., 2006]. However, this was at a time when PTH had already stimulated Smad3 expression and therefore Smad3 itself was directly implicated in the increased levels of β-catenin.

In the present study, the role of TGF-β-independent Smad3 action in the osteoblast phenotype and PTH-induced Wnt-β-catenin has been investigated in mouse osteoblastic MC3T3-E1 cells by using a TGF-β receptor type 1 [activin receptor-like kinase (ALK5)] inhibitor and a dominant-negative Smad3 mutant in which the site normally phosphorylated by ALK5 is mutated from SSVS to AAVA.

MATERIALS AND METHODS

MATERIALS

MC3T3-E1 cells were provided by Dr. H. Kodama (Ohu Dental College, Japan). Human (h)PTH-(1–34), anti-phosphorylated-β-catenin, and anti-β-actin antibodies were from Sigma (St. Louis, MO), and the ALK5 inhibitor, SB431542, was from Tocris Cookson Ltd (Bristol, UK). Anti-β-catenin and anti-phosphorylated Smad2/3 (Ser 423/425: sc-11769) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Smad3 antibodies were from Zymed Laboratories (San Francisco, CA). All other chemicals used were of analytical grade.

CELL CULTURE

Mouse osteoblastic MC3T3-E1 cells were cultured in α-MEM (containing 50 μg/ml ascorbic acid) with 10% FBS and 1% Penicillin–Streptomycin (Gibco BRL, Rockville, MD). The medium was changed twice a week.

CONSTRUCTS AND TRANSIENT AND STABLE TRANSFECTION

The vectors expressing Myc-tagged Smad3 and a mutant form of Myc-tagged Smad3, (Smad3ΔC), in which the MH2 domain corresponding to amino acid residues 278–425 had been removed, were as described previously [Kaji et al., 2001; Sowa et al., 2003; Tobimatsu et al., 2006]. The Myc-tagged Smad3AAVA mutant (wild-type is SSVS) was generated by PCR (Expand High Fidelity kit, Roche) with Myc-Smad3 cDNA (in pCMV-tag3 vector, Stratagene) as template. Forward primer (nucleotides 1013–1037 of Smad3 cDNA): 5′-CAACCTGAAGATCTTCAACAACCA-3′ with a *Bgl*II site (unique in the Smad3 cDNA and absent in the vector) in boldface type; reverse (mutant) primer: 5′-AGCTCTCGAGCTAAGCCACGGCGGCACAGCGGATGCT-3′ with an *Xho*I site in boldface type and the mutation (encoding AAVAX where X is a stop codon) in underlined boldfaced type. PCR conditions were: denaturation; 94°C, 40 s; annealing; 58°C, 40 s; amplification; 72°C, 40 s for 25 cycles, and extension at 72°C for 10 min. The 274-bp PCR product was purified (MinElute PCR Purification kit, Qiagen) and the Myc-Smad3 wild-type construct and the mutant PCR product were digested with *Bgl*II and *Xho*I, gel-purified (MinElute Gel Extraction kit, Qiagen) and ligated together to generate Myc-Smad3AAVA.

Myc-Smad3, Myc-Smad3AAVA, Myc-Smad3ΔC, and empty vector (pcDNA3.1+) (each 3 μg) were transfected into MC3T3-E1 cells with Lipofectamine (Invitrogen). Six hours later, the cells were fed with fresh α-MEM containing 10% FBS. Forty-eight hours later, the transiently transfected cells were used for experiments. For experiments with stably transfected cells, after incubation in α-MEM containing 10% FBS for 48 h, the cells were passaged and clones were selected in α-MEM supplemented with G418 (0.3 mg/ml) (Invitrogen) and 10% FBS for 3 weeks. To rule out the possibility of clonal variation, at least three independent clones for each stable transfection were characterized. Empty vector (V)-transfected cells were used as control.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM PMSF, complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000*g* for 20 min at 4°C, and the supernatants were stored at −80°C. Protein quantitation was performed with BCA protein assay reagent (Pierce, Rockford, IL). Twenty micrograms protein aliquots were denatured in SDS sample buffer and separated on 10% polyacrylamide–SDS gels. Proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with TBS [20 mM Tris–HCl (pH 7.5) and 137 mM NaCl] plus 0.1% Tween-20 containing 3% dried milk powder. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma), and the enhanced chemiluminescence detection system, as recommended by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, England).

Results depicted in each figure are representative of at least three separate cell preparations. Each experiment was repeated three times.

COTRANSFECTION OF MYC-SMAD AND p3TP-LUX CONSTRUCTS AND LUCIFERASE ASSAY

MC3T3-E1 cells were seeded at a density of 2×10^5/6-well plate. Twenty-four hours later, the cells were transfected with 3 μg of the TGF-β-responsive luciferase reporter plasmid (p3TP-Lux) [Kaji et al., 2001], and the β-galactosidase-expressing plasmid, pCH110 (Amersham Pharmacia Biotech) (1 μg) as the transfection efficiency control, using Lipofectamine (Invitrogen). Fifteen hours later, the medium was changed to α-MEM containing 4% FBS, and the cells were incubated for an additional 9 h. Thereafter, the cells were cultured for 24 h in the absence or presence of 5 ng/ml TGF-β in α-MEM containing 0.2% FBS. The cells were lysed, and the luciferase activity was measured and normalized to the relative β-galactosidase activity as described [Kaji et al., 2001].

ALP ACTIVITY ASSAY AND ALP STAINING

Confluent cells in 24-well plates were rinsed three times with PBS, and 600 μl of distilled water were added to each well. ALP activity was assayed at 37°C, as previously described [Sowa et al., 2002a]. In brief, the assay mixtures contained 0.1 M 2-amino-2-methyl-1-propanol (Sigma), 1 mM $MgCl_2$, 8 mM *p*-nitrophenyl phosphate disodium, and cell homogenates. After 3 min of incubation, the reaction was stopped with 0.1 N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with *p*-nitrophenol (Sigma). Each value was normalized with the value in protein content. ALP staining was performed by a standard protocol. In brief, cultured cells were rinsed in PBS, fixed in 100% methanol, rinsed with PBS, and then overlaid with 0.5 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Invitrogen) plus 0.3 mg/ml nitro blue tetrazolium chloride (Invitrogen) in 0.1 M Tris–HCl, pH 9.5, 0.01 N NaOH, 0.05 M $MgCl_2$, followed by incubation at room temperature for 2 h in the dark.

MINERALIZATION ASSAY

The mineralization of MC3T3-E1 cells was determined in 6-well and 12-well plates using Von Kossa staining and Alizarin Red staining, respectively. After confluent cells were grown in α-MEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 10 mM of β-glycerophosphate for 2 weeks, the cells were fixed with 95% ethanol and stained with $AgNO_3$ by the Von Kossa method to detect phosphate deposits in bone nodules [Sowa et al., 2002a]. At the same time, the other plates were fixed with ice-cold 70% ethanol and stained with Alizarin Red (Sigma) to detect calcification. For quantitation, cells stained with Alizarin Red were destained with ethylpyridinium chloride (Wako Industries, Ltd) and then the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader, as previously described [Sowa et al., 2002a].

STATISTICS

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using ANOVA.

RESULTS

SMAD3AAVA MUTANT

The activated TGF-β receptor type 1 (ALK5) phosphorylates Smad3 on serine residues within the SSXS motif (where X is a nonconserved residue among species) at the far COOH-terminus and activates the signaling molecule. To examine the role of TGF-β receptor-independent Smad3 signaling in osteoblastic cells, we prepared a rat Smad3 mutant in which the SSVS motif was mutated to AAVA. Transient transfection of the Smad3AAVA mutant inhibited TGF-β-induced transcriptional activity in MC3T3-E1 cells (Fig. 1A). Moreover, transfection of the Smad3AAVA mutant antagonized the phosphorylation of endogenous Smad2/3 induced by TGF-β in these cells (Fig. 1B). These data indicate that transient expression of the Smad3AAVA mutant exerts a dominant-negative effect on TGF-β signaling in MC3T3-E1 cells. Therefore, transient overexpression of this mutant is useful to examine the roles of Smad3 that are independent of activation of the TGF-β type I receptor/ALK-5 signal in osteoblastic cells. In contrast, the TGF-β-induced transcriptional activity was unimpaired in MC3T3-E1 cells stably expressing the Smad3AAVA mutant (Fig. 1C) suggesting that under these conditions the mutant was not acting in a dominant-negative manner.

EFFECTS OF SMAD3AAVA MUTANT ON β-CATENIN LEVELS INDUCED BY PTH

We previously demonstrated that PTH increases β-catenin levels in a time-dependent fashion from as early as 1 h up to 48 h in MC3T3-E1 cells [Tobimatsu et al., 2006]. In addition, PTH inhibited phosphorylation of β-catenin on serine residue 45. These effects were mediated in part by Smad3. Here, we examined whether transient transfection of the Smad3AAVA mutant would affect the induction of β-catenin levels and the inhibition of phosphorylation of β-catenin induced by PTH in MC3T3-E1 cells. Transient transfection of the Smad3AAVA mutant abrogated the PTH induction of β-catenin levels at 6 and 24 h, but had no effect at

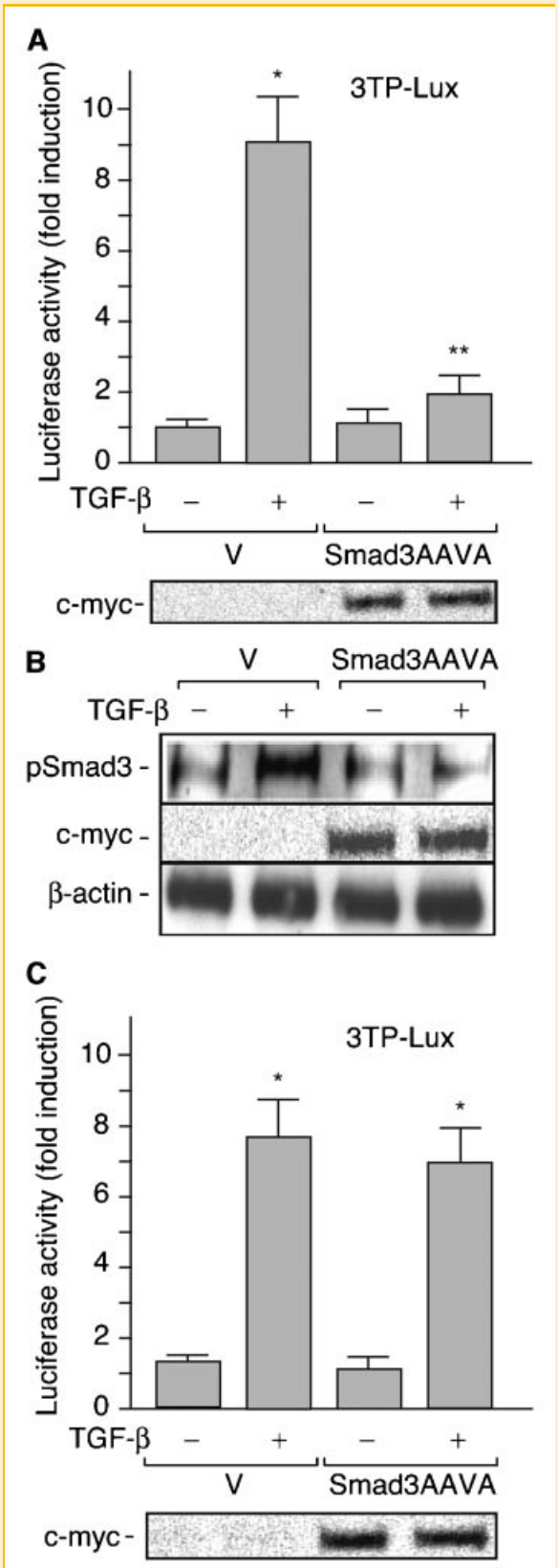

Fig. 1. The Smad3AAVA mutant has dominant-negative activity on TGF-β-induced effects when transiently transfected in osteoblastic cells. A: MC3T3-E1 cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux), the β-galactosidase-expressing pCH110 plasmid (1 μg) with or without Smad3AAVA mutant (1 μg) per well in 6-well plates. Twenty-four hours later, cells were treated without (–) and with (+) 5 ng/ml TGF-β for 24 h. The cells were then harvested and relative luciferase activity was measured. Values are mean ± SEM. $^{*}P < 0.01$, compared with control group; $^{**}P < 0.01$, compared with TGF-β (+) Smad3AAVA mutant (–) group. Lysates were analyzed by Western blot to monitor expression of the transfected Smad3AAVA. B: Confluent MC3T3-E1 cells were cultured without (–) or with (+) 5 ng/ml TGF-β for 1 h. Protein extraction of the cells and western blot analysis were performed as described in Materials and Methods Section. C: Stably empty vector (V) or Smad3AAVA mutant-transfected MC3T3-E1 cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux) and the β-galactosidase-expressing pCH110 plasmid (1 μg) per well in 6-well plates. Twenty-four hours later, cells were treated without (–) and with (+) 5 ng/ml TGF-β for 24 h. The cells were then harvested and relative luciferase activity was measured. Values are mean ± SEM. $^{*}P < 0.01$, compared with control group. Lysates were analyzed by Western blot to monitor expression of the transfected SmadAAVA.

1 h (Fig. 2A) and 3 h (data not shown). Moreover, while overexpression of the Smad3AAVA mutant antagonized the inhibition of phosphorylated β-catenin levels by PTH at 24 h, it had no effect on this PTH action at 1 h (Fig. 2B). These data indicate that the early effects of PTH (at 1 h) in inducing β-catenin levels and inhibiting phosphorylation of β-catenin are independent of TGF-β receptor signaling.

ELEVATION OF β-CATENIN LEVEL BY THE SMAD3AAVA MUTANT

We previously demonstrated that MC3T3-E1 cell clones stably expressing wild-type Smad3 have higher levels of β-catenin (as assessed by immunoblot) than empty vector-transfected cells [Tobimatsu et al., 2006]. To assess the contribution that Smad3 regulated by TGF-β might play in this situation, we prepared MC3T3-E1 clones stably expressing the Smad3AAVA mutant. In contrast to the transiently transfected cells in which the Smad3AAVA mutant exerts a dominant-negative effect on TGF-β signaling, the stably transfected cells retain this signaling pathway (Fig. 1A vs. C). In the stably expressing Smad3AAVA clones, the β-catenin levels were elevated relative to those in vector alone expressing clones (Fig. 3A). This indicates that Smad3-induced effects on β-catenin are, at least in part, independent of endogenous TGF-β signaling. Moreover, MC3T3-E1 cells stably expressing a different Smad3 mutant, SmadΔC, in which the COOH-terminal 148 amino acids (encompassing the MH2 domain) are deleted, only expressed the lower amounts of β-catenin seen in vector-alone expressing cells (Fig. 3B). These baseline levels of β-catenin can be contrasted with the elevated levels of both the Smad3 wild-type and Smad3AAVA mutant cells (Fig. 3B). Therefore, Smad3 sequence within amino acids 278–421 appears to be important in mediating the upregulation of β-catenin.

We previously showed that stable expression of wild-type Smad3 induced ALP activity and mineralization in MC3T3-E1 cells, although TGF-β inhibited these parameters [Sowa et al., 2002a,b]. Therefore, in the present study we examined ALP activity

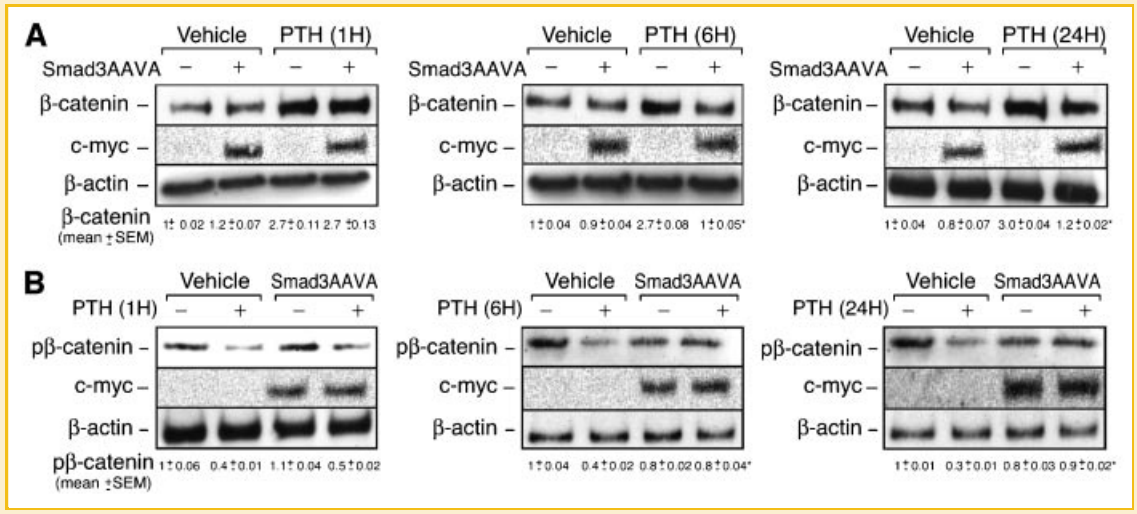

Fig. 2. The SmadAAVA mutant blocks the PTH induction of β-catenin levels at later (6 and 24 h) but not early (1 h) times. A: Transiently transfected empty vector (−) or Smad3AAVA mutant-transfected MC3T3-E1 cells that had been treated (+) or not (−) with 10^{-8} M PTH-(1-34) for 1, 6, or 24 h were analyzed by Western blot with antibodies against β-catenin, β-actin, and c-myc. Densitometric analysis of the β-catenin signal for triplicate experiments (mean ± SEM) is shown. $^{*}P < 0.01$, compared to the PTH-treated Smad3AAVA (−) group. B: Transiently transfected empty vector (−) or Smad3AAVA mutant-transfected MT3T3-E1 cells that had been treated (+) or not (−) with 10^{-8} M PTH-(1-34) for 1, 6, or 24 h were analyzed by Western blot with antibodies against phosphorylated (p) β-catenin, β-actin, and c-myc. Densitometric analysis of the pβ-catenin signal for triplicate experiments (mean ± SEM) is shown. $^{*}P < 0.01$, compared to the PTH-treated Smad3AAVA (−) group.

and mineralization in MC3T3-E1 cell clones stably expressing either Smad3 wild-type or Smad3AAVA mutant. Although stable expression of wild-type Smad3 induced ALP activity in MC3T3-E1 cells, stable expression of the Smad3AAVA mutant did not affect this parameter (Fig. 4A,B). Likewise, while stable expression of wild-type Smad3 induced mineralization in MC3T3-E1 cells, stable expression of the Smad3AAVA mutant did not stimulate either Von Kossa or Alizarin Red staining (Fig. 5A,B). These data show that the induction of ALP activity and mineralization by Smad3 in osteoblastic cells requires the COOH-terminal SSXS phosphorylation motif to be intact.

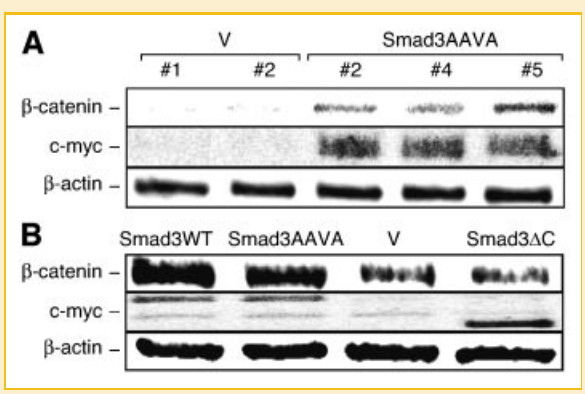

Fig. 3. A: The Smad3AAVA mutant induces β-catenin levels in MC3T3-E1 cells. Lysates of stable empty vector (V: #1, 2) or Smad3AAVA mutant (#2, 4, 5)-transfected MC3T3-E1 cells were analyzed by Western blot as described in Materials and Methods Section. B: MH2 domain is indispensable for induction of β-catenin levels by Smad3. Lysates of stable empty vector (V)-, wild-type (WT) Smad3-, Smad3ΔC mutant-, or Smad3AAVA mutant-transfected MC3T3-E1 cells were analyzed by Western blot as described in Materials and Methods Section.

EFFECT OF AN ALK-5 INHIBITOR

SB431542 specifically inhibits ALK5 (TGF-β receptor type I) and subsequent phosphorylation of Smad2/3, and is therefore useful to assess effects of endogenous TGF-β action [Maeda et al., 2004; Tobimatsu et al., 2006]. We examined the consequences of ALK5 inhibition by SB431542 in MC3T3-E1 cells stably expressing wild-type Smad3. The β-catenin levels were increased in these cells relative to those stably expressing the vector alone and SB431542 did not modulate the β-catenin levels in either the vector alone control or Smad3 overexpressing cells (Fig. 6A). The activity of the inhibitor was demonstrated by the reduction in phosphorylated Smad2/3 (Fig. 6A). The Smad3 overexpressing cells demonstrated a marked increase in ALP activity over control cells and the magnitude of the increase was reduced (although not to control levels) by treatment of the cells with SB431542 (Fig. 6B,C).

Marked mineralization as assessed by Von Kossa and Alizarin Red staining was induced in the Smad3 overexpressing cells and this was completely reduced to control cell levels by SB431542 (Fig. 7A–C).

DISCUSSION

PTH regulates bone metabolism by controlling the expression of many osteoblast genes, with responses being apparent at early and/or later times for particular genes [Ishizuya et al., 1997]. Here, we have shown that PTH induced the levels of β-catenin as well as Smad3 as early as 1 h and at later times in MC3T3-E1 cells. In

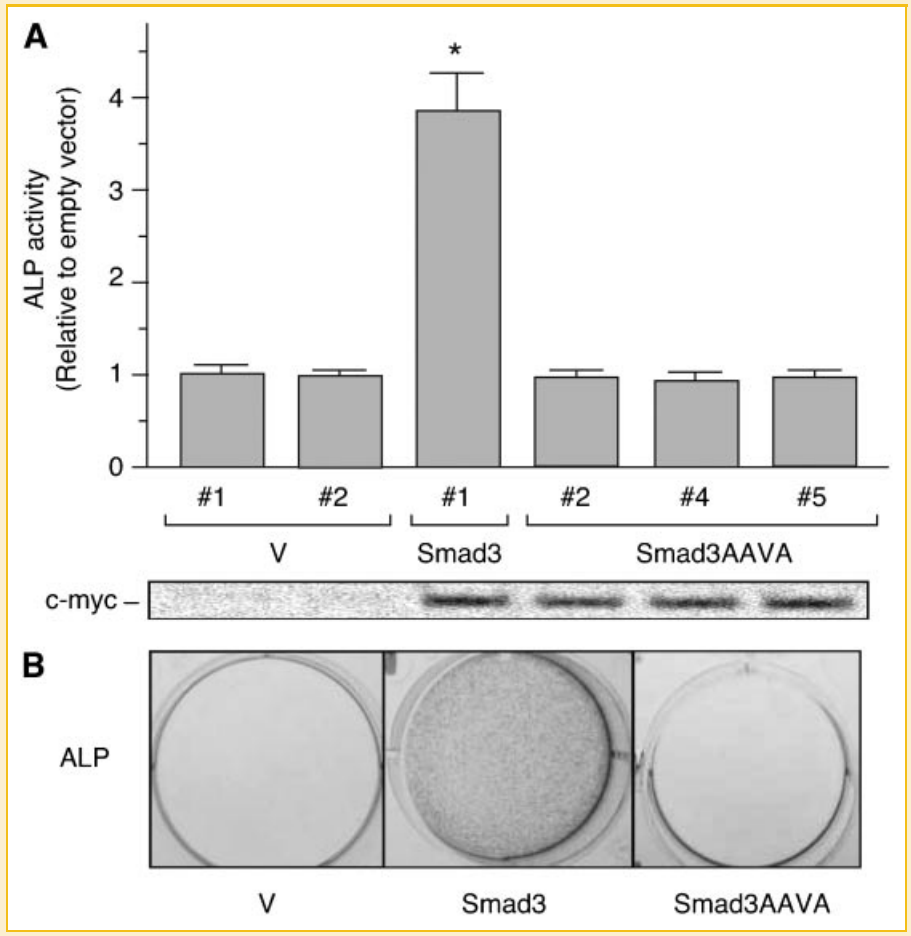

Fig. 4. The Smad3AAVA mutant does not affect ALP activity in MC3T3-E1 cells. A: Effects of Smad3 mutant on ALP activity in MC3T3-E1 cells. Confluent stable empty vector (V: #1, 2), Smad3 or Smad3AAVA mutant (#2, 4, 5)-transfected MC3T3-E1 cells were cultured for 48 h in FBS-free medium. ALP activity was measured, as described in the Materials and Methods Section. Each bar is expressed as the mean ± SEM (a ratio of the empty vector #1 value) of six determinations. *Significantly different ($P < 0.01$) from the value in empty vector-transfected cells. Lysates were analyzed by Western blot to monitor expression of the transfected Smad constructs. B: Effects of Smad3AAVA mutant on ALP staining in MC3T3-E1 cells. Confluent stable empty vector (V)-, Smad3, or Smad3AAVA mutant-transfected MC3T3-E1 cells were cultured for 48 h in FBS-free medium. ALP staining was performed as described in Materials and Methods Section.

previous studies we showed that in contrast PTH induced the levels of TGF-β and phosphorylated Smad3 [Sowa et al., 2003] only at 6 h and later. Moreover, ALK5 (TGF-β receptor type I) inhibition by SB431542, blocked PTH-induced β-catenin levels at later times, although it did not alter the PTH induction of total β-catenin and reduction of phosphorylated β-catenin levels at early times [Tobimatsu et al., 2006]. In addition, the present study using the phosphorylation-defective Smad3AAVA mutant emphasizes the importance of phosphorylation of Smad3 for PTH induction of β-catenin levels at 6 and 24 h, although phosphorylation of Smad3 had not occurred at 1 h and was therefore not a contributor to this response at this time. Taken together, these findings indicate that PTH stimulates stabilization of β-catenin levels via Smad3 independently of and dependently on endogenous TGF-β in the early and later induction phases, respectively.

We demonstrated previously that some of the other effects, besides the early PTH induction of β-catenin mediated by Smad3, such as enhancement of ALP activity and mineralization in MC3T3-E1 cells occur independently of TGF-β [Sowa et al., 2002a,b]. Studies in other systems have provided evidence that Smads may also function in additional ways to their well-appreciated roles as TGF-β signaling molecules [Derynck and Zhang, 2003]. For example, Smad4 binds to the positive regulatory Lef/Tcf binding elements (TBE1) of the c-myc promoter and transactivates the c-myc gene independently of TGF-β signaling [Lim and Hoffman, 2006]. Moreover, Mps1, a protein kinase that plays critical roles in normal mitotic progression and mitotic checkpoint signaling, phosphorylates the carboxyl terminal SSXS motifs of Smad2/3 independently of TGF-β action [Zhu et al., 2007].

In the present study, while the phosphorylation-defective Smad3AAVA mutant induced the early β-catenin response like wild-type Smad3, the mutant Smad3ΔC lacking 148 amino acids from the COOH-terminus that includes the MH2 domain lacked this ability. Hence, this TGF-β-dispensable activity appears to reside within the MH2 domain (that is well-conserved in the receptor-regulated Smads, Smad 2 and Smad3, as well as the inhibitory Smads, Smad6 and Smad7) that is already known to be indispensable for receptor interaction, Smad oligomerization, and

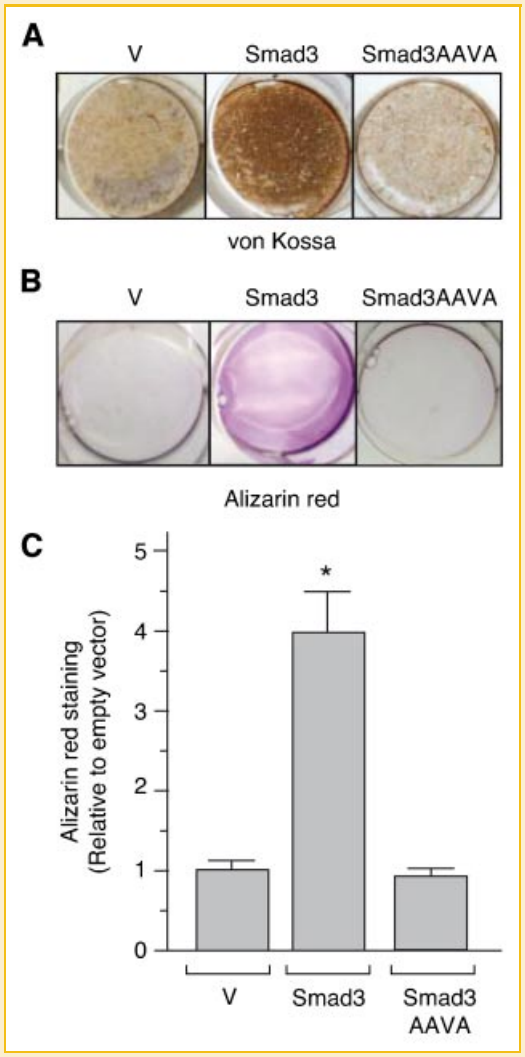

Fig. 5. The Smad3AAVA mutant does not affect mineralization in MC3T3-E1 cells. A: Confluent cells were cultured in the presence of β-glycerophosphate for 2 weeks. The mineralized matrix was stained by the Von Kossa method. B: Confluent cells were cultured in the presence of β-glycerophosphate for 2 weeks. The mineralized matrix was stained with Alizarin Red. C: Quantitation of mineralization using Alizarin Red staining, as described in the Materials and Methods Section. Data were expressed as a ratio of the empty vector value. *Significantly different ($P < 0.01$) from the empty vector group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

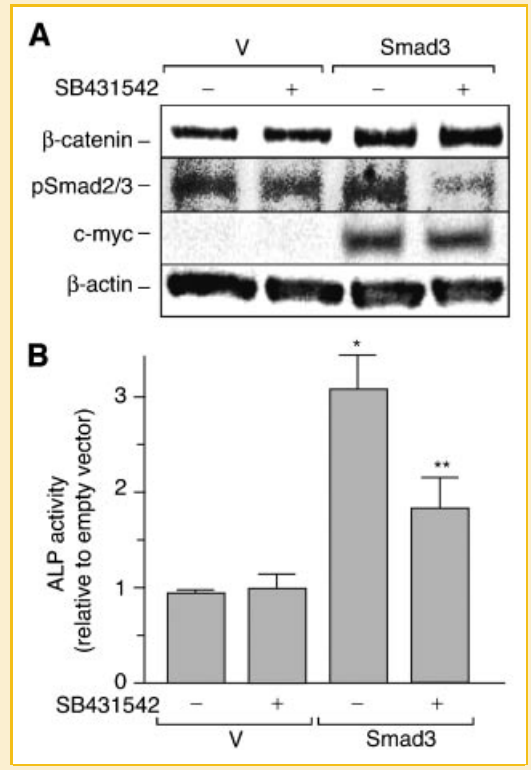

Fig. 6. Effects of ALK-5 inhibition on β-catenin levels and ALP activity induced by Smad3 in MC3T3-E1 cells. A: Confluent stable empty vector (V)- and Smad3-transfected MC3T3-E1 cells were cultured with or without 3 μM SB431542 for 24 h. Total cell lysates were analyzed by Western blot as described in Materials and Methods Section. B: Confluent stable empty vector (V)- and Smad3-transfected MC3T3-E1 cells were cultured with or without 3 μM SB431542 for 24 h. ALP activity was measured, as described in Materials and Methods Section. Data were expressed as the mean ± SEM (a ratio of the empty vector value) of six determinations. *Significantly different ($P < 0.01$) from the value in empty vector-transfected cells. **Significantly different ($P < 0.01$) from the value in Smad3-transfected cells.

transcriptional activation [Derynck and Zhang, 2003; Janssens et al., 2005]. Further studies will be needed to identify the precise region required for the activities described here.

We demonstrated previously that Smad3 itself inhibited cell proliferation and enhanced the expression of bone matrix proteins similar to TGF-β in MC3T3-E1 cells [Sowa et al., 2002a,b]. On the other hand, Smad3 enhanced, and TGF-β inhibited, ALP activity and mineralization of MC3T3-E1 cells. The effects of TGF-β might be accomplished by downregulation of Smad3 expression and/or inhibition of ALP activity and mineralization of osteoblasts via a different mechanism than the Smad3 pathway. Support for effects operating via TGF-β is provided by our previous study indicating that TGF-β-activated ERK1/2 and JNK cascades negatively regulated Smad3 transcriptional activity as well as ALP activity and mineralization induced by Smad3 in mouse osteoblastic cells [Sowa et al., 2002b]. JNK as well as c-Jun and JunB have been shown to repress Smad3-mediated transcriptional activity in other cell systems [Verrecchia et al., 2001; Derynck and Zhang, 2003]. Alternatively, Smad3 might have specific roles independent of TGF-β actions in osteoblasts analogous to what occurs in other cell types. For example, in a study of the role of Smad3 in Angiotensin (Ang) II-induced fibrosis in vascular smooth muscle cells it was demonstrated that Ang II-induced Smad signaling was independent of TGF-β at early times (30 min) (and dependent on ERK1/2), but TGF-β-dependent at later times (24 h) [Wang et al., 2008]. In the present study, the Smad3AAVA mutant (unlike wild-type Smad3) did not stimulate ALP activity and mineralization in MC3T3-E1 cells. Moreover, an inhibition of ALK-5 by SB 431542 antagonized the stimulation of ALP activity (partially) and mineralization induced by wild-type Smad3 in these cells. These findings indicate that enhancement of ALP activity and mineralization by Smad3 is dependent, in part, on TGF-β in osteoblasts.

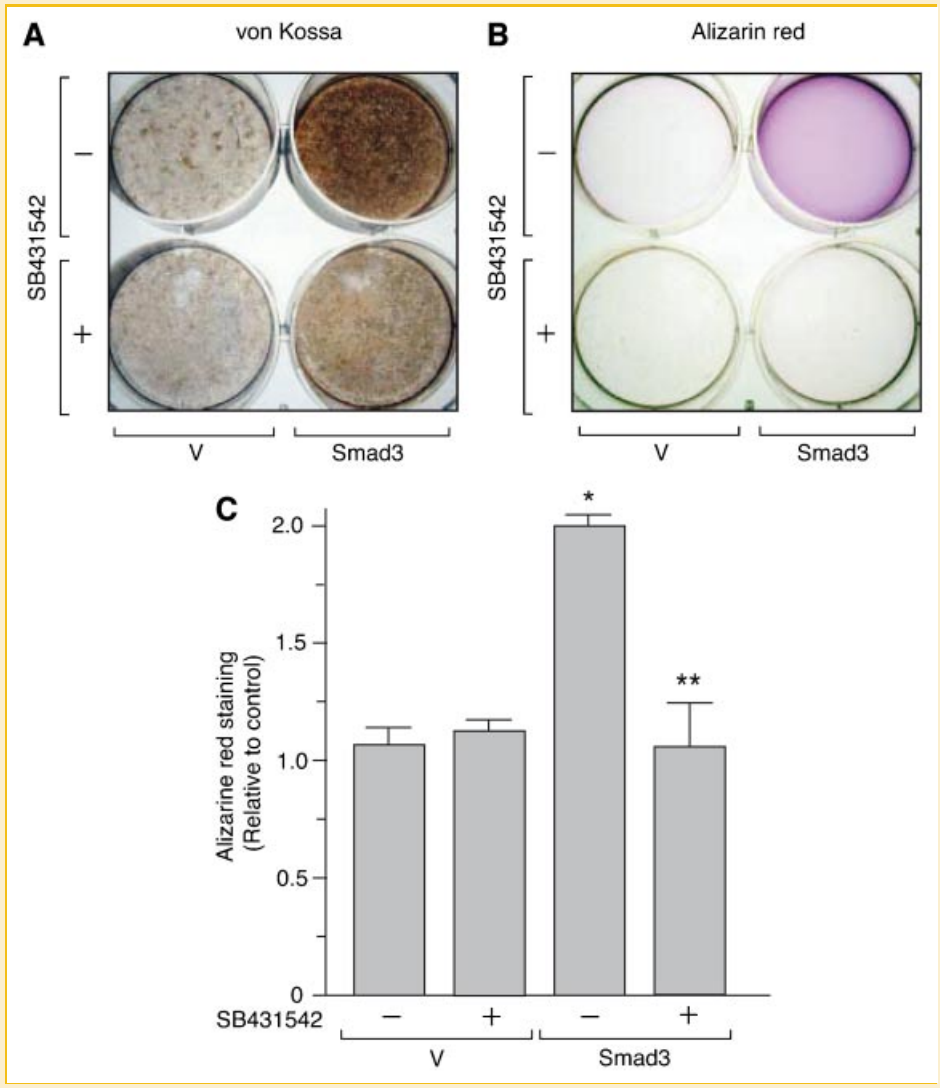

Fig. 7. Effects of ALK-5 inhibition on Smad3-induced mineralization in MC3T3-E1 cells. A: Confluent stable empty vector (V)- and Smad3-transfected MC3T3-E1 cells were cultured in the presence of β-glycerophosphate with or without 3 μM SB431542 for 2 weeks. The mineralized matrix was stained by the Von Kossa method. B: Confluent stable empty vector (V)- and Smad3-transfected MC3T3-E1 cells were cultured in the presence of β-glycerophosphate with or without 3 μM SB431542 for 2 weeks. The mineralized matrix was stained with Alizarin Red. C: Quantitation of mineralization using Alizarin Red staining, as described in the Materials and Methods Section. Data were expressed as a ratio of the empty vector value. *Significantly different ($P < 0.01$) from the value in empty vector-transfected cells. **Significantly different ($P < 0.01$) from the value in Smad3-transfected cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Here, we show that MC3T3-E1 cells stably expressing the Smad3AAVA mutant have elevated β-catenin levels in contrast to those transiently overexpressing the same mutant. Hence, under transient transfection conditions, the Smad3AAVA mutant behaves in a dominant-negative manner similar to the Smad3ΔC mutant as we showed previously [Sowa et al., 2003; Tobimatsu et al., 2006]. Moreover, MC3T3-E1 cells stably expressing the SmadΔC mutant did not exhibit the altered proliferation, bone matrix protein expression, ALP activity, and mineralization induced by stable expression of wild-type Smad3 [Sowa et al., 2002a] similar to the findings for the Smad3AAVA mutant described here. The present study has focused upon the role of COOH-terminal phosphorylation of Smad3 and has not directly examined the consequences of phosphorylation of Smad2. However, under conditions in which the Smad3AAVA mutant behaves in a dominant-negative fashion, any TGF-β-induced transcriptional activity of Smad2 would be compromised also. Under conditions in which the Smad3AAVA does not act in a dominant-negative manner the role of specific Smad2 phosphorylation remains to be explored.

The precise mechanisms of the bone anabolic actions of PTH are unclear at present. Although the anabolic effect of transient PTH administration has been suggested to occur via osteoblast differentiation rather than an increase in progenitor cell population [Wang et al., 2005], TGF-β generally inhibits differentiation, ALP activity, and mineralization in osteoblasts [Sowa et al., 2002a,b, 2004; Janssens et al., 2005]. Moreover, endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells [Maeda et al., 2004].

In conclusion, the present study indicates that PTH induces osteoblast β-catenin levels via Smad3 independently of and dependently on TGF-β in the early and later induction phases, respectively. Therefore, the early induction of Smad3 and β-catenin by PTH might constitute a bone anabolic effect occurring before TGF-β exerts its suppressive effect on osteoblast differentiation. These mechanisms may partly explain how intermittent and continuous exposure of the osteoblast to PTH has bone anabolic and catabolic actions, respectively.

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