Parathyroid hormone-Smad3 axis exerts anti-apoptotic action and augments anabolic action of transforming growth factor beta in osteoblasts.

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Summary

Although several studies indicated that parathyroid hormone (PTH) exerted anabolic action on bone, its precise mechanisms have been unknown. On the other hand, TGF-β, abundantly stored in bone matrix, stimulates bone formation with a local injection in rodents. Although our previous study suggested that Smad3 is an important molecule for the stimulation of bone formation, no reports have been available about the effects of PTH on Smad3. In this present study, we examined the effects of PTH on Smad3 and these physiological significance in mouse osteoblastic cells.

PTH promoted the expression of Smad3 mRNA within 10 minutes and the protein level in a dose-dependent manner in MC3T3-E1 and rat osteoblastic UMR106 cells. PKA activator as well as PKC activators increased Smad3 protein level, and both PKA and PKC inhibitors antagonized PTH-induced Smad3, indicating that PTH promotes the production of Smad3 through both PKA and PKC pathways. Next, we examined anti-apoptotic effects of PTH and Smad3 in these cells, employing Trypan blue, Tunel and Hoechst stainings. Pretreatment with PTH or overexpression of Smad3 decreased the number of apoptotic cells induced by dexamethasone and etoposide. Moreover, a dominant negative mutant, Smad3ΔC, abrogated PTH-induced anti-apoptotic effects. On the other hand, PTH augmented TGF-β-induced transcriptional activity. Furthermore, PTH enhanced TGF-β-induced production of type I collagen, while it did not affect TGF-β-reduced proliferation in MC3T3-E1 cells. These observations implicated that PTH amplified the anabolic effects of TGF-β by accelerating the transcriptional activity of Smad3. In conclusion, we first demonstrated that PTH-Smad3 axis exerts anti-apoptotic effects in osteoblasts and reinforces the anabolic action by TGF-β in osteoblasts. Hence, PTH-Smad3 axis might be involved in the bone anabolic action of PTH.
Introduction

The bone is a highly specialized and dynamic organ with the continuous regeneration, called remodeling. Bone remodeling consists of two different events, resorption by osteoclasts and formation by osteoblasts. For an increase in bone mineral density, a positive balance, in which the formation is prior to the resorption, should be gained. The ability to gain the positive balance might be a therapeutic strategy for osteoporosis.

Although several agents are capable of decreasing bone resorption and halting further bone loss in osteopenic states, the ideal drug would be an anabolic agent that increases bone mass by stimulating bone formation. It has been well established that daily injections of low dose of parathyroid hormone (PTH), a main regulatory hormone in calcium and bone metabolism, increase bone mass in animals and humans (1-8). However, the mechanisms by which PTH possesses bone anabolic action in vivo have not been known enough.

On the other hand, transforming growth factor beta (TGF-β) is most abundant in bone matrix, compared with other tissues (9). TGF- β is stored in an inactive form, released from the bone matrix and activated in the bone microenvironment (10). It is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (11). TGF- β modulates the proliferation, differentiation and production of bone matrix proteins of osteoblasts (10). Several reports demonstrated that TGF- β induced bone formation, when it was locally administered into bone tissues in rat (12-15). The Smad family proteins are critical components of the TGF- β signaling pathways (16,17) and TGF- β regulates the
transcriptional response of the target genes through the two receptor-regulated Smads, Smad2 and Smad3(16,17). Receptor-mediated phosphorylation of Smad2 or Smad3 induces their association with the common partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes (16,17). We recently reported that Smad3 promotes the production of type I collagen, alkaline phosphatase activity and mineralization in mouse osteoblastic MC3T3-E1 cells (18,19). Moreover, the mice with the target disruption of Smad3 exhibited the osteopenia caused by the decreased bone formation (20). Based on these evidence we have proposed that Smad3 is a molecule of promoting bone formation.

Although several studies indicated that PTH increased TGF-β expression and secretion in osteoblasts (21,22), no papers have been available which reported the effects of PTH on Smad3 in osteoblasts. Hence, in our present study, we examined the effects of PTH on the expression and the transcriptional activity of Smad3, and also these physiological significance in osteoblasts.
Experimental Procedures

Materials

MC3T3-E1 and UMR106 cells were kindly provided by Dr. H. Kodama (Ohu Dental College, Japan) and Dr. T. J. Martin (Melbourne, Australia), respectively. Human recombinant TGF-β, human PTH-(1-34), cycloheximide, actinomycin D and, phorbol 12-myristate 13-acetate (PMA), forskolin, N6, O2'-dibutyryl adenosine 3’, 5’-cyclic monophosphosphate (db-cAMPS), PTH-(3-34) amino peptide, staurosporin, H7 and H89 were purchased from SIGMA (St. Louis, MO, USA), and Sp-diastereoisomer of adenosine cyclic 3’, 5’-phosphorothioate (Sp-cAMPS) from Biolog Life Science Institute (Bremen, Germany). Anti-Smad3, Smad2, Smad4, and anti-phosphorylated Smad3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals used were of analytical grade.

Cell Culture

MC3T3-E1 and UMR106 cells were cultured in α-MEM (containing 50 mg/ml ascorbic acid) and DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (GIBCO BRL, Rockville, MD, USA), respectively. The medium was changed twice a week.

Construct and Transient Transfection

Myc-tagged Smad2 and Smad3, and flag-tagged Smad4 were prepared, as previously described (23). Smad3 DNA was derived from rat. A mutant form of myc-tagged Smad3
(Smad3ΔC), in which the MH2 domain corresponding to amino acid residues 278-425 was removed, was kindly provided by Dr. Y. Chen. Myc-Smad3, Myc-Smad3ΔC and empty vector (pcDNA3.1+) (each 3µg) were transfected to MC3T3-E1 and UMR106 cells with lipofectamine (GIBCO BRL). Six hr later, the cells were fed with fresh medium containing 10% FBS. Forty-eight hr later, the transfected cells were used for the experiments. To rule out the possibility of clonal variation, we characterized at least three independent clones for each transfection. Empty vector -transfected cells were used as the control.

**Luciferase assay**

Cells were seeded at a density of 2x10^5 per 6-well plate. Twenty-four hr later, cells were transfected with 3 µg of the reporter plasmid (p3TP-Lux) and the pCH110 plasmid expressing β-galactosidase (1µg), using lipofectamine (GIBCO BRL). Fifteen hr later, the medium was changed to the fresh one containing 4% FBS, and the cells were incubated for an additional 9 hr. Thereafter, cells were cultured for 24 hr in the presence or absence of 5.0 ng/ml TGF-β in the medium containing 0.2% FBS. Cells were lysed, and the luciferase activity was measured and normalized to the relative β-galactosidase activity as described (18).

**Protein extraction and Western analysis**

Cells were lysed with radioimmunoprecipitation buffer containing 0.5mM PMSF, complete protease inhibitor mixture, 1% Triton X-100, and 1mM sodium...
orthovanadate. Cell lysates were centrifuged at 12000 x 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, Ill, USA). Twenty µg of proteins were denatured in SDS sample buffer and separated on 10% polyacrylamide-SDS gels. Proteins were transferred in 25 mM Tris, 192mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with TBS [20mM Tris-HCl (pH7.5) and 137mM 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).

For co-immunoprecipitation experiments, cells were lysed with a buffer containing 1% Triton X-100, 1% deoxycholate, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1.5 mM MgCl₂, 2 mM EGTA, plus a protease inhibitor mixture for 30 min at 4 °C, and insoluble materials were separated by centrifugation at 4 °C for 30 min at 14,000 X g. The supernatant containing 1 mg of protein was clarified and incubated with anti-flag antibody (SIGMA) on a rocking platform at 4 °C overnight. The immune complexes were collected with Protein G Plus/Protein A Agarose beads (CALBIOCHEM-NOVABIOCHEM CORPORATION, San Diego, CA, USA) for 30 min at 4 °C. The beads were washed three times with the lysis buffer, resuspended in 2 X sample buffer, and boiled for 5 min. Immunoprecipitated proteins were then analyzed by SDS-PAGE and subjected to Western blot analysis employing anti-myc antibody (SIGMA) as described above.

Subcellular Fractionation

Cultures were trypsinized, and the cells were washed with PBS and collected by centrifugation (24). Cells were gently resuspended in 2ml of buffer containing 5 mM
KCl, 1 mM MgCl₂, 20 mM HEPES, 10 mM EDTA, 0.5 mM PMSF, 0.5% aprotinin and 0.5% leupeptin; allowed to swell for 10 min; processed by 20 strokes in a Dounce tissue homogenizer; and centrifuged at 2000 X g for 10 min. After decanting the supernatant, the pellet was resuspended in 1 ml of radioimmunoprecipitation buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% deoxycholate, 0.1% SDS, 0.5% aprotinin, and 0.5 mM PMSF], briefly sonicated and nuclear pellets were obtained by centrifugation at 15000 X g for 20 min at 4 °C; resuspended in 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT; again Dounce-homogenized. After a 20-min centrifugation at 15000 X g, supernatants were dialyzed for 5 hr against 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Protein quantitation was performed with BCA protein assay reagent (PIERCE, Rockford, Ill, USA). Western blot analysis was performed as described above.

**RNA extraction and Northern analysis**

Total RNA was prepared from cells using the acid guanidinium-thiocyanate-phenol-chloroform extraction method. Twenty µg of total RNA was denatured, run on a 1% agarose gel containing 2% formaldehyde, then transferred to a nitrocellulose membrane and fixed with ultraviolet light (FUNA-UV-LINKER, FUNAKOSHI, Tokyo, Japan). The membrane was hybridized to a 32P (Amersham Pharmacia Biotech)-labeled DNA probe overnight at 42°C. The hybridization probe was the 2.8-kb fragment of the α1 gene of type I procollagen (a gift from Dr. T. Kimura, Osaka University, Japan). After hybridization, the filter was washed twice with 2x standard saline citrate (SSC).
containing 0.5% SDS and subsequently washed twice with 0.1x SSC containing 0.5% SDS at 58 °C for 1 hr. The filter was exposed to x-ray film, using intensifying screen at −80 °C. All values were normalized for RNA loading by probing blots with human β-actin cDNA (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

**Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Reverse transcription of 5 µg of cultured cell total RNA was carried out for 50 min at 42°C and then 15 min at 70°C, using Super Script™ First-Strand Synthesis System for RT-PCR (GIBCO BRL), which contained RT buffer, oligo (dT)12-18, 5x First-Strand Solution, 10 mM of dNTP, 0.1M of DTT, Super Script II (RT-enzyme), and RNaseH (Rnase inhibitor). PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 µl reaction mixture for PCR (as supplied by TaKaRa, Otsu, Japan), supplemented with 2.5 units of TaKaRa Taq™, 1.5 mM of each dNTP (TaKaRa) and PCR buffer (10x), which contained 100mM of Tris-HCl (pH 8.3), 500 mM of KCl and 15 mM of MgCl₂. Twenty-five ng of each primer and 1 µl of template (from a 50 µl RT reaction) were used. Thermal cycling conditions and primer sequences are described below. 1) initial denaturation at 96°C for 2 min; 2) cycling for cDNA-specific number of cycles; 96°C for 1 min, cDNA-specific annealing temperature for 2 min, and 72°C for 2 min; and 3) final extension at 72°C for 5min. Primer sequences, annealing temperature, and cycle numbers were as follows. Smad3 were 5’-GAGTAGAGACGCCAGTTCTACC-3’ and 5’-
GGTTTGGAGAACCTGCGTCCAT-3’ (62°C; 25 cycles) (25), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 5’-ATCCCATCACCACATCTTCCAGGAG-3’ and 5’-CCTGCTTCACCACCTTCTTGATG-3’ (58°C; 22 cycles). For semi-quantitative RT-PCR, the number of cycles was chosen so that amplification remained well within the linear range, as assessed by densitometry (NIH Image J, version 1.08i, public domain program). An equal volume from each PCR was analyzed by 6% non-denaturing polyacrylamide gel electrophoresis and ethidium bromide stained PCR products were evaluated. Marker gene expression was normalized to GAPDH expression in each sample.

Determination of osteoblast apoptosis.

Trypan blue staining (GIBCO BRL; 0.1% final concentration) was used for routine quantification of apoptosis (26). In brief, a drop of the cell suspension was mixed with a drop of the trypan blue solution. The ratio of each volume was 1:1. The total numbers of viable and unviable cells were calculated under the light microscopy (27). Apoptosis cleaves cellular DNA into histone-associated fragments. As the more specific staining to detect apoptosis, transferase-mediated nick end labeling (TUNEL) staining and HOECHST staining were employed. In brief, cells were cultured on round cover glasses (Fisher Scientific, Pittsburgh, PA) set in 6-well plates and the glasses attached by cells were rinsed with ice-cold PBS two times, followed by fixation with 4% neutral formaldehyde. Then, these fixed cell layers on the glasses were stained. TUNEL reaction was performed using Apoptosis in situ Detection kit (Wako), following the
standard protocol, as described (28). HOECHST staining was performed in order to view the pyknotic fragmented nuclei typical of apoptotic cells, using HOECHST 33258 (SIGMA). In each experiment, the percentages of numbers of apoptotic cells per total ones in randomly selected fields were calculated using hemocytometer. Each experiment was performed at least three times.

\[^{3}H\]thymidine incorporation (TdR)-assay

MC3T3-E1 cells were seeded at 2X10^4 cells/well in 24 well plates. These cells were maintained in \(\alpha\)-MEM with 10% FBS. After 48 hr culture, cells were labeled with 0.5 \(\mu\)Ci/ml \([^3H]\) thymidine (Amersham Pharmacia Biotech) for 4 hr. The incubation was terminated by removal of the medium, washed with PBS twice and followed by the addition of 5% trichloroacetic acid (TCA) on ice in 10 min. After removal of the TCA, the residue was dissolved in 20 mM NaOH at 37 °C, and scintillation cocktail was added. Each sample was counted in a liquid scintillation counter.

Statistics

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using an unpaired t-test or ANOVA.
Results

**PTH stimulated the expression of Smad3 mRNA and protein.**

First, we examined the effects of PTH on the expressions of Smad3 mRNA and protein. In MC3T3-E1 cells, PTH-(1-34) stimulated the expression of Smad3 mRNA in a dose-dependent manner (Figure 1A). PTH-(1-34) also stimulated the expression of Smad3 protein in both MC3T3-E1 and UMR106 cells (Figure 1B and 1C). Next, we performed the time course experiments. As shown in Figure 2A, PTH-(1-34) promoted the expression of Smad3 mRNA within 10 minutes in MC3T3-E1 cells. The expression of Smad3 protein was enhanced by PTH-(1-34) within 1 hour in MC3T3-E1 and UMR106 cells (Figure 2B and 2C). These effects of PTH-(1-34) were sustained at least for 24 hours. Moreover, PTH-(1-34) increased the level of phosphorylated Smad3 within 6 hours (Figure 2D). Since the upregulation of Smad3 by PTH-(1-34) was initiated in early times, PTH might stimulate the expression of Smad3 without protein synthesis de novo. We therefore employed cycloheximide, a protein synthesis inhibitor, and actinomycin D, a transcription inhibitor. Although 10µM cycloheximide did not affect PTH-induced expression of Smad3 mRNA, PTH-induced Smad3 protein was reduced with 10µM actinomycin D (Figure 3). These findings suggested that PTH promoted the expression of Smad3 at the transcriptional level independently of protein synthesis de novo in osteoblasts.

**PTH promoted Smad3 expression via PKA and PKC pathways.**
PTH binds to PTH/PTHrP receptor, which is a G-protein coupled 7th transmembrane-type receptor. The PTH signals are known to be transduced through PKA and PKC pathways (1). We therefore investigated whether PTH would upregulate the Smad3 expression through PKA and/or PKC pathway(s). As shown in Figure 4, activators for PKA pathways, db-cAMP and Sp-cAMPS as well as forskolin, and an activator for PKC pathway, PMA, promoted the expression of Smad3 mRNA and protein in MC3T3-E1 cells (A and B) and UMR106 cells (C). In addition, a specific inhibitor of PKA pathway, H89, as well as the inhibitors of PKC pathways, staurosporin and H7, antagonized PTH-induced expression of Smad3 mRNA and protein in MC3T3-E1 cells (Figure 5A and 5B) and UMR106 cells (Figure 5C). These findings indicated that PTH stimulates the expression of Smad3 mRNA and protein through both PKA and PKC pathways in osteoblasts.

**Smad3 is an essential molecule for PTH-induced anti-apoptotic effects in osteoblasts.**

PTH exerts anti-apoptotic signals in osteoblasts (29). Recent study revealed that the mice with the targeted disruption of Smad3 exhibited osteopenia caused by decreased bone formation including the promoted apoptosis of osteoblasts and osteocytes (20). These evidence suggested that Smad3, as well as PTH, possesses anti-apoptotic effects in osteoblasts. We therefore examined the effects of PTH and Smad3 on dexamethsone- or etoposide-induced apoptosis in MC3T3-E1 and UMR106 cells. We employed Trypan blue staining, Tunel staining and Hoechst staining methods to detect the apoptotic cells. Trypan blue stain method is generally used to distinguish the viable cells
from unviable ones. As shown in Figure 6, dexamethasone and etoposide induced the number of unviable cells in MC3T3-E1 (A and B) and UMR106 cells (C and D). Treatment with PTH-(1-34) as well as Smad3 overexpression antagonized dexamethasone- and etoposide-induced cell death. The truncated Smad3 mutant, Smad3ΔC, lacks its MH2 region in C-terminus and possesses dominant negative effects on endogenous Smad3 activity, as previously described (18). Smad3 inactivation with Smad3ΔC antagonized the PTH-induced anti-apoptotic effects on the cells treated with dexamethasone and etoposide, although Smad3ΔC expression itself did not affect them (Figure 6A and 6C). These findings suggested that PTH and the overexpression of Smad3 antagonized dexamethasone- and etoposide-induced apoptosis, and that Smad3 was indispensable in the PTH-induced anti-apoptotic signals in osteoblasts. The cell death detected in Trypan Blue assay might include the death by the mechanisms other than apoptosis. Tunel stain method is more specific for the detection of apoptotic cells, because cells with Tunel-positive nucleus means ones with DNA fragmentations. In both empty vector- and Smad3ΔC-transfected UMR106 cells, dexamethasone increased the numbers of Tunel-positive cells (Figure 7A and 7C). PTH-(1-34) did not affect dexamethasone-induced Tunel-positive cell number in Smad3ΔC-transfected cells, although PTH antagonized it in empty vector-transfected cells (Figure 7A and C). Hoechst staining method is generally recommended to detect apoptotic nucleus morphologically. As shown in Figure 7B, the nucleus of the empty vector-transfected cells treated with dexamethasone exhibited the dot-spot appearance, indicating that the cells fell in apoptosis. As seen in Figure 7D, dexamethasone increased the number of
apoptotic cells transfected with empty vector and Smad3ΔC. However, PTH did not affect the number of apoptotic cells transfected with Smad3ΔC, although PTH antagonized it in empty vector-transfected cells (Figure 7D). These findings indicated that Smad3 is an essential molecule for PTH-induced anti-apoptotic action in osteoblasts.

**PTH augmented Smad3-induced transcriptional activity.**

Our previous study revealed that Smad3 promoted the expression of type I procollagen, resulting in acceleration of mineralization in MC3T3-E1 cells (19). We therefore examined the effects of PTH on Smad3-induced transcriptional activity with luciferase assay using 3TP-Lux containing the promoter of plasminogen inhibitor 1 with a Smad3-specific responsive element. TGF-β increased the transcriptional activity in the absence of PTH-(1-34) in MC3T3-E1 cells (Figure 8A). PTH-(1-34) significantly augmented TGF-β-induced transcriptional activity, although PTH-(1-34) alone did not affect them (Figure 8A). These results suggested that PTH-(1-34) augmented TGF-β-induced transcriptional activity of Smad3. Although PTH increased the expression of TGF-β in MC3T3-E1 and UMR-106 cells (Figure 8B), PTH alone did not affect the transcriptional activity in MC3T3-E1 cells, and TGF-β pretreatment as well as PTH pretreatment alone did not affect the transcriptional activity (data not shown). Moreover, PTH pretreatment did not affect TGF-β-induced phosphorylation of Smad3 (data not shown). These findings suggest that PTH augmented TGF-β-induced transcriptional activity in a manner independent of PTH-induced expression of TGF-β.
and Smad3. In TGF-β signaling, receptor-mediated phosphorylation of Smad2 or Smad3 induces their association with the common partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes (16,17). We examined the effects of PTH on the association of Smad2 and Smad3 with Smad4. As shown in Figure 8D and E, when myc-Smad2 or myc-Smad3 was co-transfected with flag-Smad4 in MC3T3-E1 and UMR-106 cells, Smad2 or Smad3 was coimmunoprecipitated with Smad4 with TGF-β treatment. However, PTH-(1-34) did not affect the association of Smad2 or Smad3 with Smad4 in TGF-β signaling (Figure 8D (for Smad2) and E (for Smad3)). Moreover, PTH did not affect the expression of Smad2 and Smad4 in MC3T3-E1 and UMR-106 cells (Figure 8C). These findings suggest that PTH specifically increased the expression of Smad3, and that PTH-augmented transcriptional activity by TGF-β are independent of Smad2 and Smad4. Next, we examined the nuclear translocation of Smad3 by using Smad3-transfected MC3T3-E1 cells. As shown in Figure 8F, PTH-(1-34) did not affect TGF-β-induced translocation of Smad3 into nucleus. These results suggested that PTH stimulated the transcriptional activity of Smad3, after Smad3 was translocated into nucleus.

**PTH augmented anabolic but not catabolic effects of TGF-β in osteoblasts.**

TGF-β stimulates type I collagen expression and inhibits proliferation of MC3T3-E1 cells, as previously described (30). As shown in Figure 9A and B, PTH-(1-34) accelerated TGF-β-induced expression of type I procollagen mRNA and type I collagen protein in MC3T3-E1 cells. These findings suggested that PTH-(1-34) augmented TGF-
β-induced expression and synthesis of type I collagen. On the other hand, PTH-(1-34) did not affect TGF-β-reduced [³H] thymidine incorporation in MC3T3-E1 cells (Figure 9C). These results suggested that PTH-(1-34) augmented anabolic but not catabolic effects of TGF-β in osteoblasts.
Discussion

The present study indicated three important new points in osteoblasts; First, PTH promoted the expression of Smad3, a crucial mediator in TGF-β signaling. Second, Smad3 was involved in the anti-apoptotic effects of PTH. Finally, PTH augmented bone anabolic actions of TGF-β.

In this study, PTH promoted the expression of Smad3 in osteoblasts. Smad3 is a critical component of the TGF-β signaling pathways and receptor-mediated phosphorylation of Smad2 or Smad3 induces their association with the common partner Smad4, followed by translocation into the nucleus where these complexes activate transcription of specific genes (16,17). Thus, Smad3 is considered to play as a signal transduction molecule and a transcriptional regulator in cytoplasm and nucleus, respectively. Previous studies indicated that PTH stimulates TGF-β expression in osteoblasts (22). In the present study, PTH stimulated Smad3 expression within only one hour and its effects were independent of the protein synthesis de novo. These findings indicated that Smad3 expression induced by PTH was not through TGF-β. Smad3 might be a transcriptional regulator for bone formation partly in a manner independent of TGF-β in osteoblasts, as previously reported (18, 19). It has been reported that PTH affects the transcriptional factors through the activator protein-1 (AP-1), the cAMP responsive element binding protein (CREB), and the Runt related transcriptional factor 2 (Runx2) (31). As for the AP-1 family, c-fos, fra-1 and ΔfosB play important roles in bone formation in the previous studies (32-34), and PTH stimulated the expression of c-fos, c-jun, fra-1, fra-2 and fosB (35-39). Moreover, the
phophorylation of CREB in response to PTH treatment was required for PTH-stimulated expression of c-fos (35,39). Runx2 has been characterized as an osteoblast specific transcriptional activator (40-42) and it induced promoter activity in the osteocalcin, osteopontin, type I collagen and collagenase-3 promoters (40,43-48). In UMR106-01 cells, rat osteoblastic cell line, PTH lead to activation of Runx2 (49), and the physical interaction between AP-1 and Runx2 was required for the PTH-stimulated transcriptional activity of the collagenase-3 promoter in MC3T3-E1 cells (50). These evidence suggest that those transcriptional regulators are important in PTH actions in bone. Our previous study suggested that Smad3 is a crucial molecule in bone formation (18, 19). The present findings, therefore, suggested that Smad3 functions, as a transcriptional regulator at the down stream of PTH signaling.

It was reported that several nuclear receptors of the steroid hormones were interacted with Smad3; Smad3 potentiated ligand-induced transactivation of vitamin D receptor (VDR) as a coactivator of this receptor (51), and glucocorticoid receptor inhibited transcriptional activation of Smad3 (52). Smad3 was an androgen receptor co-regulator in prostate cancer cells, while the androgen receptor repressed TGF-β signaling through interaction with Smad3 (53,54). Estrogen receptor (ER)-mediated transcriptional activation enhanced by TGF-β signaling was through Smad3 (55). Although these findings suggested that Smad3 is related to the action of steroid hormones, no reports have been available about the significance of these interactions in osteoblasts. Moreover, whether the peptide hormone would regulate the expression and the transcriptional activity of Smad3 has not been known. Our present report was the first that calcitropic
hormone affected the expression and transcriptional activity of Smad3.

Several studies indicated that PTH exerted skeletal anabolic effects. In animals and humans, intermittent treatment with PTH resulted in increased bone mineral density, while continuous exposure to high concentration of PTH in vivo lead to progressive bone loss and osteopenia (1-8). On the other hand, PTH possesses bi-directional actions in vitro, bone anabolic and catabolic ones. PTH increased the expression of insulin-like growth factor I (IGF-I) (56,57) and osteocalcin (58), while it decreased the expression of type I collagen (59,60), alkaline phosphatase (ALP) (61,62), osteonectin (63), osteopontin (64), DNA synthesis (65). The different effects of PTH were also reported among cells from different species. For example, PTH stimulated proliferation of primary osteoblastic cells isolated from human trabeculae (66) and chick calvariae (67), although PTH inhibited proliferation of UMR106 cells (68,69). Thus, the actions of PTH on osteoblasts are still controversy.

The anabolic mechanism in vivo may be partially explained by the growth factor genes expressed in activated osteoblasts following exposure to PTH. PTH is known to stimulate the expression of IGF-I, IGF-II, IGF-binding proteins, and TGF-β in osteoblasts (21,70,71). IGF-I is known to stimulate bone cell replication and matrix synthesis (72,73), and mice with a bone-specific mutation in IGF-I receptor exhibited a striking decrease in bone volume (74). Moreover, a neutralizing antibody against IGF-I blocked the stimulatory effect on ALP activity and the expression of osteocalcin induced by the intermittent exposure to PTH in osteoblast cells from rat calvariae (75). These lines of evidence suggested that the anabolic action of PTH in bone is partly mediated
by IGF-I (21,70,76,77). Furthermore, IGF-I binding proteins also might be related to anabolic action of PTH-IGF-I signals (78). On the other hand, the anabolic action of PTH has been partly explained by the evidence that PTH exerts anti-apoptotic effects on osteoblasts (29). The mice with the targeted disruption of Smad3 exhibited osteopenia caused by decreased bone formation including the promoted apoptosis of osteoblasts and osteocytes (20). These evidence raised the possibility that Smad3 possesses anti-apoptotic effects in osteoblasts. In the present study, Smad3 inhibited apoptosis and inactivation of Smad3 by dominant negative mutant attenuated PTH-induced anti-apoptotic effects in mouse and rat osteoblastic cells. These findings indicated that Smad3 was required for PTH-induced anti-apoptotic effects on osteoblasts. Apoptosis plays a critical role during embryonic limb development, skeletal maturation, bone turnover, fracture healing and bone degeneration. The balance of osteoblast proliferation, differentiation, and apoptosis determines the size of the osteoblast population at any given time (79). Previous studies revealed that glucocorticoid as well as estrogen withdrawal promoted apoptosis in osteoblasts (80-82). It was also suggested that the anti-apoptotic effects of PTH are mediated by signals transduced through Gs pathway (83). However, it is still unclear what signal transduction pathways are used in apoptosis of osteoblasts and what steps in apoptosis are targeted by PTH in its rescue effects.

Since PTH augmented TGF-β-induced transcriptional activity in these cells and our previous reports indicated that TGF-β as well as Smad3 inhibited osteoblast proliferation, it is possible that PTH induces some signaling leading to the enhancement
of Smad3 signaling-mediated reduction in proliferation. Borton et al. reported that TGF-β did not affect osteoblast proliferation in Smad3-deficient osteoblasts (20). Therefore, it seemed improbable that TGF-β inhibited osteoblast proliferation through the signal other than Smad3. Surprisingly, although PTH augmented TGF-β-induced type I collagen expression, anabolic effects of TGF-β on osteoblasts, PTH did not affect the TGF-β-reduced proliferation, catabolic effects in the present study. These data suggested that the regulation of Smad3 by PTH specifically contributed to anabolic action of TGF-β in osteoblasts.

Since PTH increased Smad3 expression and its phosphorylation level in the present study, it might be somewhat puzzling that PTH itself did not affect the transcriptional activity. The reason is exactly unknown. However, the existence of sufficient endogenous TGF-β stimulation might be necessary to induce transcriptional activity of 3TP-Lux. Moreover, it seems that PTH-induced Smad3 levels are not sufficient for the activation of the reporter gene or that an additional factor or mechanism is needed for this activity. Previous studies indicated that TGF-β as well as Smad3 increased the expression of type I collagen in MC3T3-E1 cells (18, 19, 30). However, PTH itself did not increase the expression of type I collagen (data not shown), which was compatible with the previous reports (59, 60), although PTH increased the expression of Smad3 in the present study. Since PTH also regulates the expression of numerous genes which inhibit the expression of type I collagen, PTH-induced proteins might inhibit the expression of type I collagen against the effects of Smad3 or TGF-β. Alternatively, it appears that PTH-mediated induction of Smad3 alone is not sufficient for gene
activation.

In the present study, although PTH stimulates the expression of TGF-β, PTH did not affect TGF-β-induced phosphorylation of Smad3, association of Smad2/3 and Smad4, the nuclear translocation of Smad3 in MC3T3-E1 cells, suggesting that the augmentation of PTH on transcriptional activity of Smad3 was through the events in nucleus. The previous evidence suggested that PTH affected osteoblasts through some transcriptional regulators, such as AP-1 family, CREB and Runx2. It was also reported that Smad3 interacted with c-Jun, CREB and Runx2 (84-86). We therefore speculated that those transcriptional regulators induced by PTH affected the transcriptional activity of Smad3 by the interaction. Further study is necessary to clarify these issues.

It has been widely believed that both PTH and TGF-β possess the divergent and complex effects on osteoblasts, bone-anabolic and catabolic effects. Our previous and present study suggested that Smad3 was an important molecule in anti-apoptotic action of PTH and anabolic action of TGF-β. Smad3 seemed to be an important molecule that activates the bone anabolic signals in the intracellular pathways of PTH and TGF-β.

Mammalians have got the complex network systems in order to maintain homeostasis under variable circumstances in development. The systems consist of systemic and local regulators, such as systemic hormones, local growth factors, and cytokines. Those factors exert the positive or negative effects in the appropriate balance on the tissues for homeostasis, and the endocrine disease is caused by the disorder of homeostasis. Bone is a dynamic connective tissue, undergoing remodeling, a continual process of resorption and renewal. In the remodeling, osteoblasts, which contribute to the renewal process, are
regulated by several hormones and growth factors. These systemic and local factors cross-talk with each other, resulting in the regulation of the number and function of osteoblasts. We speculate that Smad3 is one of key molecules that arrange the signals from systemic and local factors and convert them into anabolic signals in osteoblasts, because our previous study suggested that Smad3 signal might exert anabolic actions of TGF-β in osteoblasts (18,19), and Smad3 leads to PTH-induced anti-apoptotic effects. Furthermore, PTH augmented anabolic effects of Smad3 on osteoblasts in the present study.

In conclusion, we first demonstrated that PTH stimulated the expression of Smad3 in osteoblasts. The pathway, “PTH-Smad3 axis”, was essential in PTH-induced anti-apoptotic effects and reinforced the anabolic effects of Smad3. Hence, PTH-Smad3 axis might be involved in the bone anabolic action of PTH, and nominated for the therapeutic strategy, targeting bone degenerated disease, including osteoporosis.
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Footnotes

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Abbreviations

TGF-β, transforming growth factor beta; PKA/C, protein kinase A/C; TdR, [³H] thymidine incorporation.
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Figure legends

Figure 1. PTH stimulates the expression of Smad3 in a dose-dependent manner.

A. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were treated with the indicated concentrations of $10^{-8}$ M PTH-(1-34) for 1 hr. Then, RNA extraction and semi-quantitative RT-PCR assay were performed, as described in Experimental Procedures. B-C. Confluent MC3T3-E1 cells (B) or UMR106 cells (C) were cultured in serum-free α-MEM and DMEM, respectively, for 12 hr, cells were treated with the indicated concentrations of PTH-(1-34) for 6 hr. Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures.

Figure 2. PTH stimulates the expression of Smad3 in the early times.

A. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were treated with $10^{-8}$ M PTH-(1-34) for the indicated time. Then, RNA extraction and semi-quantitative RT-PCR assay were performed, as described in Experimental Procedures. B-C. After confluent MC3T3-E1 cells (B) or UMR106 cells (C) were cultured in serum-free α-MEM or DMEM, respectively, for 12 hr, cells were treated with $10^{-8}$ M PTH-(1-34) for the indicated time. Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures. D. The membrane used in B was re-hybridized by anti-phosphorylated Smad3 antibody.
Figure 3. PTH stimulates the expression of Smad3 at the transcriptional level.

A. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were pretreated with 10μM cycloheximide (CHX) for 6 hr and treated with 10^{-8} M PTH-(1-34) for 1 hr. Then, RNA extraction and semiquantitative RT-PCR analysis were performed, as described in Experimental Procedures. B. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were pretreated with 10μM actinomycin D (Act. D) for 6 hr and treated with 10^{-8} M PTH-(1-34) for 6 hr. Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures.

Figure 4. PKA and PKC agonists stimulate the expression of Smad3.

A. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were treated with 10^{-5}M Phorbol 12-myristate 13-acetate (PMA), 10^{-5}M forskolin (fors), 10^{-4}M N^{6}, O^{2'持有的 dinucleoside 3', 5'-cyclic monophosphate (db-cAMP), and 10^{-5}M Sp-diastereoisomer of adenosine cyclic 3’, 5’-phosphorothioate (Sp-cAMPS) for 1 hr. Then, RNA extraction and semi-quantitative RT-PCR assay were performed, as described in Experimental Procedures. B-C. Confluent MC3T3-E1 cells (B) or UMR106 cells (C) were cultured in serum-free α-MEM or DMEM respectively for 12 hr, cells were treated with the PKA or PKC agonists for 6 hr (B and C). Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures.
Figure 5. Both PKA and PKC inhibitors antagonize PTH-induced expression of Smad3.

A. After confluent MC3T3-E1 cells were cultured in serum-free α-MEM for 12 hr, cells were pretreated with a PKA inhibitor, 10^{-8} M H89 or PKC inhibitors, 10nM staurosporin or 50μM H7 for 6 hr and treated with 10^{-8} M PTH-(1-34) for 1 hr. Then, RNA extraction and semiquantitative RT-PCR analysis were performed, as described in Experimental Procedures. B-C. After confluent MC3T3-E1 cells (B) and UMR106 cells (C) were cultured in serum-free α-MEM or DMEM, respectively, for 12 hr, cells were pretreated with PKA- or PKC- inhibitors for 6 hr and treated with 10^{-8} M PTH-(1-34) for 6 hr. Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures.

Figure 6. The effects of PTH and Smad3 on the cell viability in osteoblasts.

Empty vector-, Smad3ΔC-, or Smad3-transfected MC3T3-E1 cells (A, B) and UMR106 cells (C, D) were treated with 10^{-7} M dexamethasone (Dex) or 5x10^{-5} M etoposide (Etop) for 6 hr with or without pretreatment with 10^{-8} M PTH-(1-34) for 1 hr in serum-free α-MEM or DMEM. Apoptotic cells were enumerated by trypan blue staining, as described in Experimental Procedures. Bars represent mean ± SEM of ratio of trypan blue positive cell-numbers in untreated empty vector-transfected cells (control group). * p<0.01 from PTH-untreated group. ** p<0.01 from corresponding vector-transfected group.
Figure 7. Smad3 is involved in PTH-induced anti-apoptotic action in osteoblasts.

Empty vector- or Smad3ΔC-transfected UMR106 cells were treated with 10^{-7} M dexamethasone (Dex) for 6 hr with or without pretreatment with 10^{-8} M PTH-(1-34) for 1 hr in serum-free DMEM. A-B. Apoptotic cells were detected with Tunel staining (A) or Hoechst stain (B). C-D. The numbers of apoptotic cells, the tunnel positive cells (C) and cells whose nuclease show dot-spot appearance (D), were calculated. Bars represent mean ±SEM of ratio of the values in control group. * p<0.01 from PTH-untreated group.

Figure 8. PTH stimulates the transcriptional activity of Smad3.

A. MC3T3-E1 cells were transfected with 3 μg of the reporter plasmid (p3TP-Lux), the pCH110 plasmid expressing β-galactosidase (1μg) per well in 6-well plate. Forty-eight hr later, cells were treated with 10^{-8} M PTH for 12 hr, and fed with fresh α-MEM with or without 5ng/ml TGF-β. Then, 24 hr later, cells were harvested and relative luciferase activity was measured. Values of relative luciferase activity represent the mean ± SEM. * p<0.01 from TGF-β-untreated group, ** P<0.01 from PTH-untreated group. B, C. After confluent MC3T3-E1 cells were cultured in serum-free medium for 12hr, cells were treated with 10^{-8} M PTH-(1-34) for the indicated times. Then, protein extraction and Western blot analysis were performed, as described in Experimental Procedures. D, E. Myc-tagged Smad2 (D) or Smad3 (E) was co-transfected into MC3T3-E1 cells with flag-tagged Smad4. Cells were treated with 5 ng/ml TGF-β for 1 hr after pretreatment with 10-8 PTH-(1-34) for 12 hr.
extracts were immunoprecipitated (IP) with anti-flag, followed by immunoblotting (IB) with anti-myc, as described in Experimental Procedures. F. Smad3-transfected MC3T3-E1 cells were treated with $10^{-8}$ M PTH for 12 hr, and fed with fresh $\alpha$-MEM with or without 5ng/ml TGF-$\beta$. One hr later, subcellular fractionations and Western blot analysis were performed, as described in Experimental Procedures.

Figure 9. PTH augments TGF-$\beta$-induced expression of type I collagen but not affects TGF-$\beta$-reduced proliferation in osteoblasts.

MC3T3-E1 cells were treated with $10^{-8}$ M PTH-(1-34) for 24 hr, and fed with fresh $\alpha$-MEM with or without 5ng/ml TGF-$\beta$. Then, 24 hr later, RNA extraction and Northern blot analysis (A), protein extraction and Western blot analysis (B), and $[^3]$H thymidine incorporation (TdtR)-assay (C) were performed, as described in Experimental Procedures.
Figure 2

A

Time (min) 0 5 10 15 30 60 360

Smad3 mRNA

GAPDH

B

Time (hr) 0 1 3 6 12 24

Smad3

β-actin

C

Time (hr) 0 1 3 6

Smad3

β-actin

D

Time (hr) 0 1 3 6 12 24

P-Smad3

β-actin
Figure 3

A

|       | CHX |
|-------|-----|
| PTH   | -   | +   |

Smad3 mRNA

GAPDH

B

|       | Act. D |
|-------|--------|
| PTH   | +      | -   | +   |

Smad3

β-actin
Figure 5

A

|       | PTH | +  | Staurosporin | H7   | H89 |
|-------|-----|----|--------------|------|-----|
| Smad3 mRNA | -   | +  | -            | +    | -   |
| GAPDH  | -   | +  | -            | +    | -   |

B

|       | PTH | +  | Staurosporin | H7   | H89 |
|-------|-----|----|--------------|------|-----|
| Smad3 | -   | +  | -            | +    | -   |
| β-actin| -   | +  | -            | +    | -   |

C

|       | PTH | +  | Staurosporin | H7   | H89 |
|-------|-----|----|--------------|------|-----|
| Smad3 | -   | +  | -            | +    | -   |
| β-actin| -   | +  | -            | +    | -   |
Figure 9

A

PTH - + - +

TGF-β

COLI mRNA

β-actin

B

PTH - + - +

TGF-β

COLI

β-actin

C

\[ \Delta \text{dR(CPM)} \]

vehicle  TGF-β

*  *
Parathyroid hormone-Smad3 axis exerts anti-apoptotic action and augments anabolic action of transforming growth factor beta in osteoblasts
Hidekai Sowa, Hiroshi Kaji, Mey Fway Iu, Tatsuo Tsukamoto, Toshitsugu Sugimoto and Kazuo Chihara

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