

Parathyroid Hormone Stimulation and PKA Signaling of Latent Transforming Growth Factor- β Binding Protein-1 (LTBP-1) mRNA Expression in Osteoblastic Cells

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Abstract Parathyroid hormone (PTH) regulates bone remodeling and calcium homeostasis by acting on osteoblasts. Recently, the gene expression profile changes in the rat PTH (1–34, 10^{-8} M)-treated rat osteoblastic osteosarcoma cell line, UMR 106-01, using DNA microarray analysis showed that mRNA for LTBP-1, a latent transforming growth factor (TGF- β)-binding protein is stimulated by PTH. Latent TGF- β binding proteins (LTBPs) are required for the proper folding and secretion of TGF- β , thus modifying the activity of TGF- β , which is a local factor necessary for bone remodeling. We show here by real time RT-PCR that PTH-stimulated LTBP-1 mRNA expression in rat and mouse preosteoblastic cells. PTH also stimulated LTBP-1 mRNA expression in all stages of rat primary osteoblastic cells but extended expression was found in differentiating osteoblasts. PTH also stimulated TGF- β 1 mRNA expression in rat primary osteoblastic cells, indicating a link between systemic and local factors for intracellular signaling in osteoblasts. An additive effect on LTBP-1 mRNA expression was found when UMR 106-01 cells were treated with PTH and TGF- β 1 together. We further examined the signaling pathways responsible for PTH-stimulated LTBP-1 and TGF- β 1 mRNA expression in UMR 106-01 cells. The PTH stimulation of LTBP-1 and TGF- β 1 mRNA expression was dependent on the PKA and the MAPK (MEK and p38 MAPK) pathways, respectively in these cells, suggesting that PTH mediates its effects on osteoblasts by several intracellular signaling pathways. Overall, we demonstrate here that PTH stimulates LTBP-1 mRNA expression in osteoblastic cells and this is PKA-dependent. This event may be important for PTH action via TGF- β in bone remodeling. *J. Cell. Biochem.* 95: 1002–1011, 2005. © 2005 Wiley-Liss, Inc.

Key words: PTH; TGF- β ; LTBP-1; osteoblast; PKA signaling

Abbreviations used: PTH, parathyroid hormone; LTBP-1, latent transforming growth factor-beta binding protein-1; TGF- β , transforming growth factor-beta; ECM, extracellular matrix; SLC, small latent TGF- β complex; LLC, large latent TGF- β complex; LAP, latency associated protein; FBS, fetal bovine serum; RT-PCR; reverse transcriptase polymerase chain reaction; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C.

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Parathyroid hormone (PTH) is one of the major calcitropic hormones affecting serum calcium levels and bone remodeling [Tam et al., 1982; Dobnig and Turner, 1997; Swarthout et al., 2002]. PTH acts by binding to the PTH1R, a G-protein-coupled receptor on osteoblasts, resulting in functional changes in the actions of both osteoblasts [Bellows et al., 1990] and osteoclasts [Kanzawa et al., 2000; Qin et al., 2004]. The molecular mechanisms regulating the activities of both cells by PTH are still not completely known.

There is growing evidence that growth factors and cytokines released from bone matrix play important roles in the coupling of bone resorption to bone formation and in repair processes such as fracture healing. Bone extracellular matrix (ECM) is the major storage site in the body for transforming growth factor-beta (TGF- β), which is a multipotent cytokine [Seyedin et al., 1985; Hauschka et al., 1986]. TGF- β is

synthesized as a homodimeric pro-protein and the dimeric pro-peptide is cleaved intracellularly from the growth factor [Taipale et al., 1994]. The TGF- β propeptide binds to TGF- β , and the proteins are secreted as a complex [Annes et al., 2003]. In this small latent complex (SLC), TGF- β cannot bind to its surface receptors. Therefore, the propeptide is called the latency associated protein (LAP). The SLC is secreted by bone cells [Bonewald et al., 1991], chondrocytes [Pedrozo et al., 1999], kidney cells [Marra et al., 1996], and prostate cells [Dallas et al., 2005]. The dissociation or activation of TGF- β from LAP is a critical regulatory event as all TGF- β is secreted in a latent form. The LAP dimer is usually disulfide bonded to a second gene product, latent TGF- β binding protein (LTBP), and the trimolecular aggregate is called the large latent complex (LLC) [Rifkin, 2005].

A major mechanism for storage of secreted latent TGF- β in bone matrix is via its association with the latent TGF- β binding protein-1 (LTBP-1) [Taipale et al., 1994; Dallas et al., 1995; Dallas et al., 2000]. LTBP is a member of the LTBP/fibrillin protein family, which comprises fibrillin-1, fibrillin-2 and fibrillin-3, and LTBP-1, LTBP-2, LTBP-3, and LTBP-4 [Ramirez and Pereira, 1999; Oklu and Hesketh, 2000]. LTBPs are required for the proper folding and secretion of TGF- β , thus modifying the activity of TGF- β [Miyazono et al., 1991]. In human and rats, *LTBP-1* appears as two mRNA species, which encode for two different NH₂-terminal variants, the longer LTBP-1L having a 346 amino acid extension not present in the shorter LTBP-1S isoform [Kanzaki et al., 1990; Saharinen et al., 1999]. Both isoforms possess their own, independent promoter regions, capable of regulating the tissue type specific expression of *LTBP-1* isoforms [Koski et al., 1999]. The LLC-containing the LTBP-1L is found in ECM [Kanzaki et al., 1990]; whereas the LLC-containing the LTBP-1S is found in platelets [Wakefield et al., 1988]. This ECM-bound TGF- β stored in a latent form can be released and activated by resorbing osteoclasts [Oreffo et al., 1989; Oursler, 1994]. Once released from the matrix and activated, TGF- β can influence inhibition of osteoclast activity, osteoblast proliferation, and stimulation of production of bone ECM proteins [Hughes et al., 1996; Roberts, 1998; Bonewald, 1999]. TGF- β has therefore been implicated as a coupling factor that coordinates the processes of bone resorption and subsequent bone formation.

The rat osteoblastic cell line, UMR 106-01 is a useful model system for studying the effects of PTH on osteoblastic cells in vitro. Recently, the gene expression profile changes in these cells treated with rat PTH (1–34, 10⁻⁸M) using DNA microarray analysis have been published [Qin et al., 2003]. LTBP-1 expression was stimulated by PTH in these cells. Since PTH stimulates LTBP-1 mRNA expression and that controls the activity of TGF- β , LTBP-1 seems to be a mediator in controlling PTH action on osteoblasts via TGF- β . In this study, we show PTH stimulation of LTBP-1 mRNA expression in the mouse and rat osteoblastic cell lines and in proliferating, differentiating, and mineralizing rat primary osteoblasts. We have also identified the signaling pathways used by PTH in stimulation of LTBP-1 mRNA and TGF- β 1 mRNA expression in rat osteoblastic cells.

MATERIALS AND METHODS

Materials

Rat PTH (1–34) and human TGF- β 1 were purchased from Sigma, St. Louis, MO and Promega, Madison, WI, respectively. Synthetic oligonucleotides were synthesized by Invitrogen, Carlsbad, CA. Tissue culture medium and reagents were also obtained from Invitrogen. The MEK1/2, p38 MAPK, JNK, PKA, and PKC inhibitors were purchased from Calbiochem, San Diego, CA. All other chemicals were obtained from Sigma.

METHODS

Cell Culture

The rat osteoblastic cells (UMR 106–01) and the mouse preosteoblastic cells (MC3T3) were maintained in monolayer in Eagle's minimal essential medium (with Earle's salts; EMEM) supplemented with nonessential amino acids, 25 mM HEPES (pH 7.3), 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Rat Primary Osteoblastic Cells

Rat primary osteoblasts were isolated by the method of Shalhoub et al. [1992]. Osteoblasts were derived from postnatal day 1 rat calvariae by sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A, 0.25% trypsin. Cells from digests one and two were discarded.

Cells from the third digest were plated at 6.4×10^3 cells/cm² and grown in minimal essential medium (MEM) supplemented with 10% FBS. After reaching confluence (day 7), the medium was switched to BGJ_b with 10% FBS containing 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate to allow for initiation of differentiation and mineralization. Medium changes were performed every 2 days. The determination of proliferating, differentiating, and mineralizing stages of osteoblasts has been established by demonstration of alkaline phosphatase activity, osteocalcin production, alizarin red staining, and a sensitive adenyl cyclase response to PTH [Shalhoub et al., 1992; Winchester et al., 1999].

Total RNA Isolation and Real Time Reverse Transcriptase-PCR

The rat osteoblastic cells and the mouse preosteoblastic cells were treated with either rat PTH (1–34, 10^{-8} M) or human TGF-β1 (1 ng/ml) or both together for different time periods. To determine de novo protein synthesis, cells were pretreated with cycloheximide (30 µg/ml) for 1 h before PTH treatment. To determine the signaling pathways, cells were pretreated with DMSO, PD98059, SB203580, SP600125, H89, or GF109203X for 20 min before PTH treatment. Cells were rinsed once with 10 ml of cold (4°C) PBS, pH 7.4, and harvested. Total RNA was isolated using the QIAGEN RNeasy Mini kit. Reverse transcription was carried out using TaqMan reverse transcription reagents (Roche Applied Science, Indianapolis, IN). PCRs were performed using a real time PCR DNA Opticon Engine (MJ Research, Inc., Watertown, MA) according to the manufacturer's instructions, which allow real time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. Each analysis was performed three to four times with independent sets of cells. The data are represented as mean ± SEM. Statistical analysis was performed by Student's *t*-test. Primers for rat LTBP-1, TGF-β1, MMP-13, and β-actin were designed using Primer Express software (PerkinElmer Life Sciences). The sequences of the above primers were as follows: *LTBP-1*: Forward, 5'-CGTGGCTGGAATGGACAATG, Reverse, 5'-TGGTCTGGTGTGGGGCTGTA; *TGF-β1*: Forward, 5'-TTAGGAAGGACCTGGGTTGGA, Reverse, 5'-ACTGTGTGTCCAGGCTCCA-

AAT; *MMP-13*: Forward, 5'-GCCCTATCCCTTGATGCCATT, Reverse, 5'-ACAGTTCAGGCTCAACCTG; *β-actin*: Forward, 5'-TCCTGAGCGCAAGTACTCTGTG, Reverse, 5'-CGGACTCATCGTACTCCTGCTT.

RESULTS

PTH Stimulates LTBP-1 mRNA Expression

To study the effect of PTH on expression of LTBP-1 in the rat osteoblastic osteosarcoma line UMR 106-01, cells were treated with rat PTH (1–34) either for different time periods with 10^{-8} M concentration (Fig. 1A) or at different concentrations for 4 and 24 h (Fig. 1B). Total cellular RNAs were purified and analyzed by real time RT-PCR using specific primers for rat LTBP-1 and β-actins. As shown in Figure 1A, LTBP-1 mRNA expression was maximally stimulated (20-fold) by 10^{-8} M PTH at 4 h in UMR 106-01 cells and was still significant at 24 h. A wide range of PTH concentrations from 10^{-9} to 10^{-7} M stimulated LTBP-1 mRNA expression at 4 h in these cells but with 10^{-7} and 10^{-8} M PTH concentration, the fold stimulation of LTBP-1 mRNA expression was maintained out to 24 h (Fig. 1B).

PTH Stimulates LTBP-1 mRNA Expression in Mouse Preosteoblastic and Rat Primary Osteoblastic Cells

We next determined PTH-stimulated LTBP-1 mRNA expression in other osteoblastic cells. The MC3T3 mouse preosteoblastic cells were treated with control or rat PTH (1–34, 10^{-8} M)-containing media for 1, 4, 12, and 24 h. Total RNA was isolated and examined for LTBP-1 mRNA expression by real time RT-PCR analysis. PTH significantly stimulated LTBP-1 mRNA expression at 4 h in these cells (Fig. 2A) but the fold stimulation was less than that seen in UMR 106-01 cells (Fig. 1A). To determine expression of PTH-regulated LTBP-1 in rat primary osteoblastic cells, proliferating, differentiating, and mineralizing cells were treated with either control or rat PTH (1–34, 10^{-8} M)-containing media for different times. Total RNA was isolated and subjected to examination for LTBP-1 mRNA expression by real time RT-PCR analysis. Our results (Fig. 2B) show that LTBP-1 mRNA expression was stimulated by PTH in proliferating (1 h) and mineralizing (4 h) osteoblasts. In differentiating osteoblasts, LTBP-1 mRNA expression was

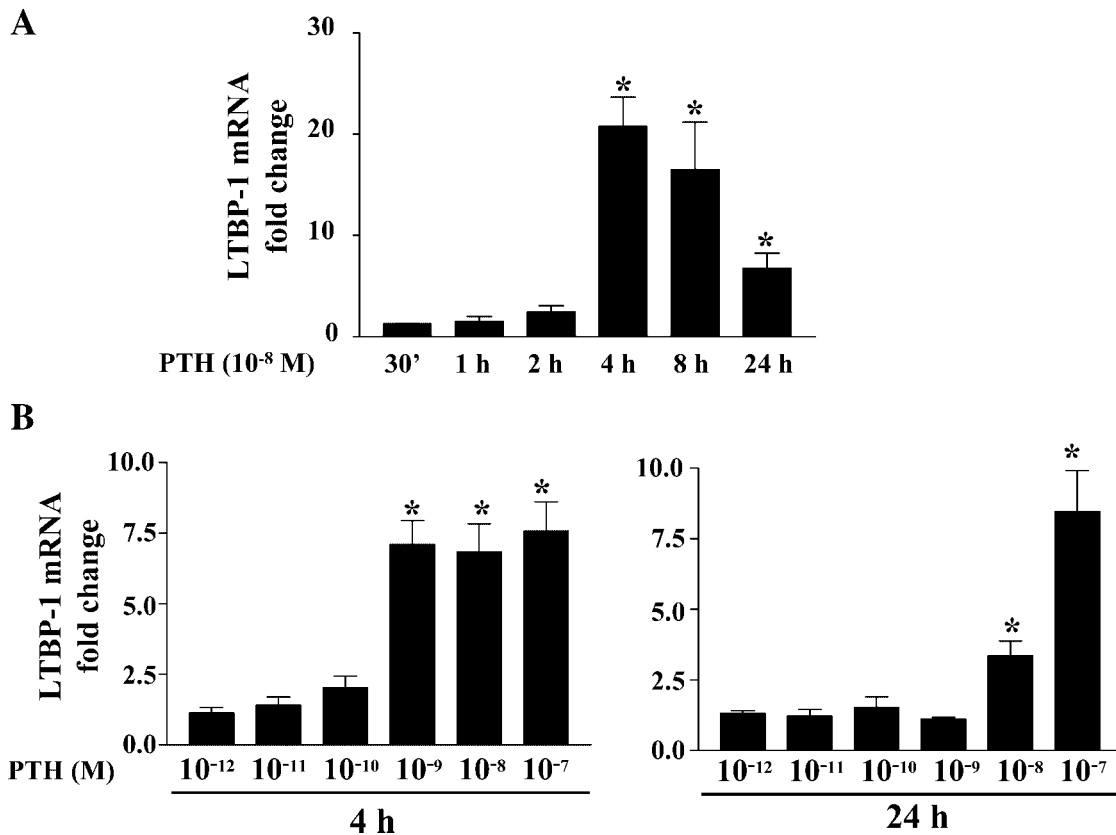


Fig. 1. Effect of PTH on expression of *LTBP-1* mRNA levels in the rat osteoblastic cell line, UMR 106-01. **A:** Time course of the PTH stimulation of *LTBP-1*. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10⁻⁸M) for different times as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. **B:** Concentration-dependence of the PTH stimulation of *LTBP-1*

mRNA. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing PTH at different concentrations as indicated for 4 or 24 h. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

found at 1 h and its level persisted up to 4 h with PTH-treatment.

PTH Stimulation of *LTBP-1* mRNA Expression Is a Primary Effect

To determine whether the PTH-mediated increase in *LTBP-1* mRNA is a primary response, UMR 106-01 cells were treated with control medium or medium containing rat PTH (1–34, 10⁻⁸M) for 4 h in the presence or absence of 30 μ g/ml cycloheximide added 1 h before treatment. Total RNA was subjected to real time RT-PCR analysis using specific primers for rat *LTBP-1* and β -actin. As shown in Figure 3A, cycloheximide did not inhibit PTH induction of *LTBP-1* mRNA expression, indicating that the PTH stimulation of *LTBP-1* expression is a primary effect and de novo protein synthesis is not required for this purpose. In fact, cyclohex-

imide increased the PTH-response, indicating the inhibition of de novo synthesis of repressor proteins for this effect. As a positive control for cycloheximide treatment, we analyzed mRNA expression of MMP-13 (matrix metalloproteinase-13; collagenase-3) in UMR 106-01 cells (Fig. 3B). We have previously shown that the PTH stimulation of MMP-13 expression is a secondary effect in these cells [Scott et al., 1992].

PTH Stimulates TGF- β 1 mRNA Expression and Both PTH and TGF- β 1 Have an Additive Effect on *LTBP-1* mRNA Expression

Since *LTBPs* are required for the proper folding and secretion of TGF- β [Miyazono et al., 1991], the increased *LTBP-1* expression caused by PTH may be correlated with increased expression of TGF- β . Hence, we determined if

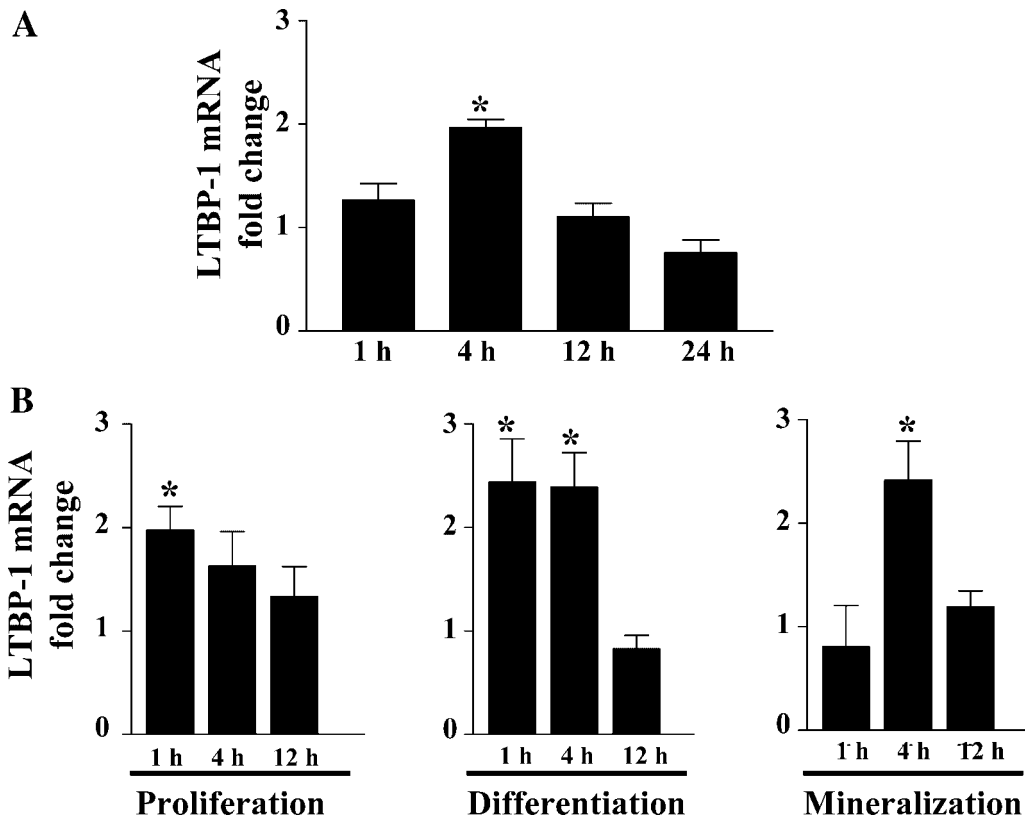


Fig. 2. Effect of PTH on expression of *LTBP-1* mRNA levels in mouse preosteoblastic cells and in rat primary osteoblastic cells. **A:** MC3T3 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for different time periods as indicated. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. **B:** Osteoblasts derived from postnatal day 1, rat calvariae were grown in 6-well plates in MEM, 10% FBS to confluence (day 7), after which the cells were switched to differentiation and mineralizing medium (BGJb, 10% FBS, 50 μ g/

ml ascorbic acid, and 10 mM β -glycerophosphate). Proliferating, differentiating, and mineralizing osteoblasts were treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 1, 4, and 12 h at days 7, 14, and 21 of culture. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

PTH stimulated TGF- β 1 mRNA expression in rat primary osteoblastic cells treated with rat PTH (1–34, 10^{-8} M) for different times during the three stages of differentiation. The results (Fig. 4) indicate that PTH stimulated TGF- β 1 mRNA expression in differentiating and mineralizing osteoblasts at 4 h treatment indicating that osteoblasts respond to PTH and synthesize TGF- β 1 only at these stages. Since PTH stimulates mRNA expression of both *LTBP-1* and TGF- β 1 mRNAs, we wanted to determine whether there is a synergistic effect with combined treatment with PTH and TGF- β 1 on *LTBP-1* expression in rat osteoblastic cells. UMR 106-01 cells were treated with human TGF- β 1 (1 ng/ml), rat PTH (1–34, 10^{-8} M) or both together at different time periods. Total RNA was isolated and subjected to real time RT-PCR analysis.

TGF- β 1 and PTH stimulated *LTBP-1* mRNA expression to 3.5 ± 1.1 -fold and 11.7 ± 2.1 -fold, respectively at 4 h and to 1.4 ± 0.2 -fold and 7.3 ± 2.4 -fold, respectively at 24 h in UMR 106-01 cells. When cells were treated with TGF- β 1 and PTH together, an additive effect was observed at both 4 h (17.1 ± 1.6 -fold) and 24 h (9.5 ± 2.7 -fold) in these cells (Fig. 5).

PTH Stimulation of *LTBP-1* mRNA Expression Is Dependent on the PKA Signaling Pathway

To identify the signaling pathways in PTH-stimulated *LTBP-1* mRNA expression, we used MAPK, PKA, and PKC pathway inhibitors. UMR 106-01 cells were pretreated with DMSO, PD98059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNKII inhibitor), H89 (PKA inhibitor), or GF109203X (PKC

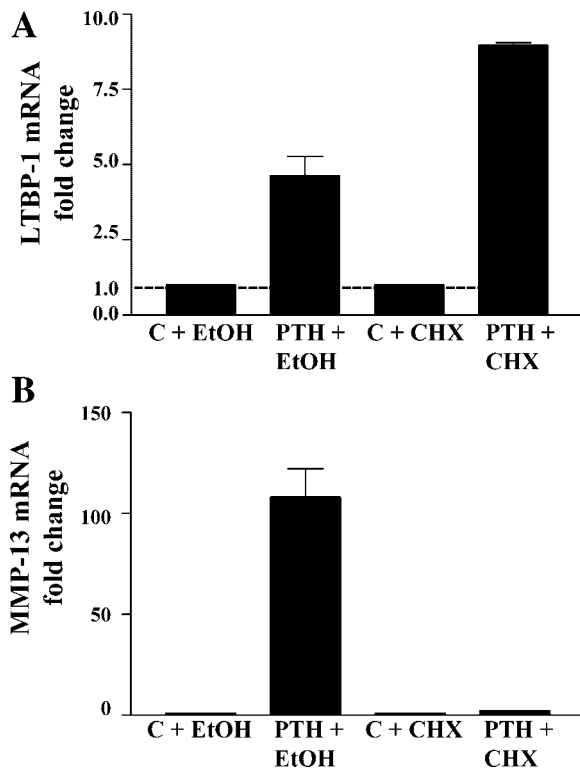


Fig. 3. PTH-stimulated *LTBP-1* mRNA expression is a primary effect. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 4 h in the presence or absence of 30 μ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. **B:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 24 h in the presence or absence of 30 μ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat *MMP-13* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls.

inhibitor) for 20 min, then treated with or without rat PTH (1–34, 10^{-8} M) for 4 h. Total RNA was isolated and real time RT-PCR was performed. The PTH-stimulated *LTBP-1* mRNA expression was not significantly decreased by MAPK and PKC inhibitors, suggesting that the MEK, p38 MAPK, JNK, and PKC signaling pathways are not involved in PTH stimulation of *LTBP-1* mRNA expression in UMR 106-01 cells. The PKA inhibitor, H89 inhibited PTH-stimulated *LTBP-1* mRNA expression in these cells (Fig. 6). The effective concentrations and specificity of these inhibitors have been previously determined [Selvamurugan et al., 2002; Selvamurugan et al., 2004].

PTH Stimulation of TGF- β 1 mRNA Expression Is a Secondary Effect and Is Dependent on the MAPK Signaling Pathway

Since PTH stimulated TGF- β 1 mRNA expression in rat osteoblastic cells (Fig. 4), we wanted to determine whether this stimulation is a primary effect and if it requires the PKA signaling pathway as we found for PTH stimulation of *LTBP-1* mRNA expression in UMR 106-01 cells (Figs. 3A and 6). UMR 106-01 cells were treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 4 h in the presence or absence of 30 μ g/ml cycloheximide added 1 h before treatment. Total RNA was subjected to real time RT-PCR analysis using specific primers for rat TGF- β 1 and β -actin. As shown in Figure 7A, cycloheximide inhibited PTH induction of TGF- β 1 mRNA expression, indicating that PTH stimulation of TGF- β 1 expression is a secondary effect and de novo protein synthesis is required for this purpose. To identify the signaling pathways in PTH-stimulated TGF- β 1 mRNA expression, we used MAPK, PKA, and PKC pathway inhibitors. Similar to Figure 6, UMR 106-01 cells were pretreated with DMSO, PD98059, SB203580, SP600125, H89, or GF109203X for 20 min, then treated with or without rat PTH (1–34, 10^{-8} M) for 4 h. Total RNA was isolated and real time RT-PCR was performed. The PTH stimulation of TGF- β 1 mRNA expression was not significantly decreased by JNK, PKA, and PKC inhibitors; whereas MEK and p38 MAPK inhibitors inhibited PTH-stimulated TGF- β 1 mRNA expression in rat osteoblastic cells (Fig. 7).

DISCUSSION

The gene expression profile changes in UMR 106-01 cells treated with rat PTH (1–34, 10^{-8} M) using DNA microarray analysis showed that *LTBP-1* is one of the genes stimulated by this hormone [Qin et al., 2003]. We report here that PTH stimulates *LTBP-1* mRNA expression in rat osteoblastic and mouse preosteoblastic cells (Figs. 1 and 2). *LTBP-1* is required for the proper folding and secretion of TGF- β [Miyazono et al., 1991], which is a local factor produced by both osteoblasts and osteoclasts [Pfeilschifter and Mundy, 1987; Bonewald and Dallas, 1994]. Bone ECM is the major storage site in the body for TGF- β [Seyedin et al., 1985; Hauschka et al., 1986]. This ECM-bound TGF- β ,

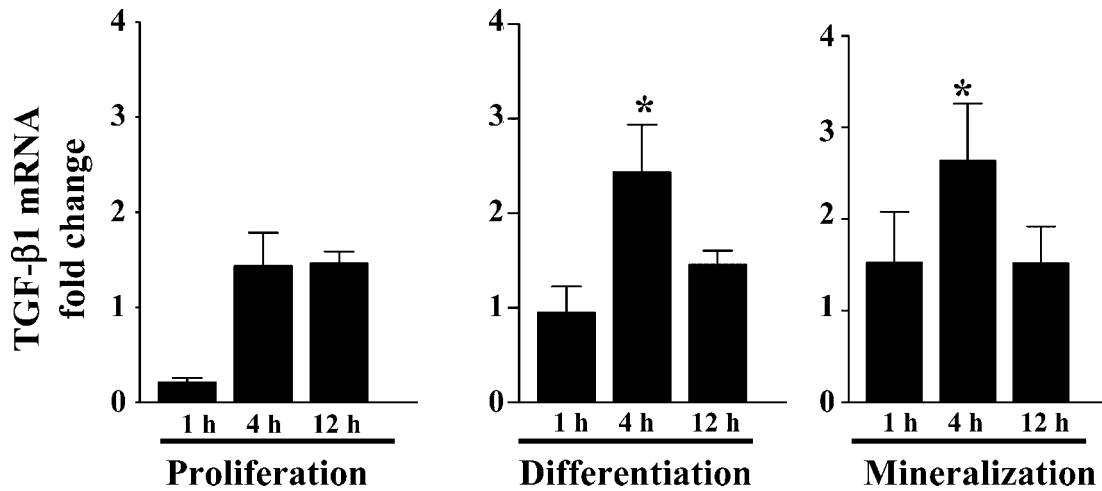


Fig. 4. Effect of PTH on expression of *TGF-β1* mRNA levels in rat primary osteoblastic cells. Proliferating, differentiating, and mineralizing rat primary osteoblasts were treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 1, 4, and 12 h at days 7, 14, and 21 of culture. Total RNA was isolated

and subjected to real time RT-PCR using specific primers for rat *TGF-β1* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

which is predominantly the *TGF-β1* isoform, is stored in a latent form and can be released and activated by resorbing osteoclasts [Oreffo et al., 1989; Oursler, 1994]. Several mechanisms for the activation of latent *TGF-β* complexes have been well documented [Munger et al., 1997; Koli et al., 2001].

TGF-β can influence many of the steps in the bone remodeling pathway. It can both inhibit [Hughes et al., 1996] and stimulate osteoclast

activity [Horwood et al., 1999; Sells Galvin et al., 1999] depending on conditions. *TGF-β* inhibits osteoclast activity, both by stimulating osteoclasts to undergo apoptosis and by inhibiting formation of osteoclasts from their precursors. *TGF-β* is also a powerful chemoattractant and mitogen for osteoblast precursors [Bonewald, 1999]. The effect of *TGF-β* on mature osteoblasts is then to inhibit proliferation and stimulate production of bone ECM proteins, including

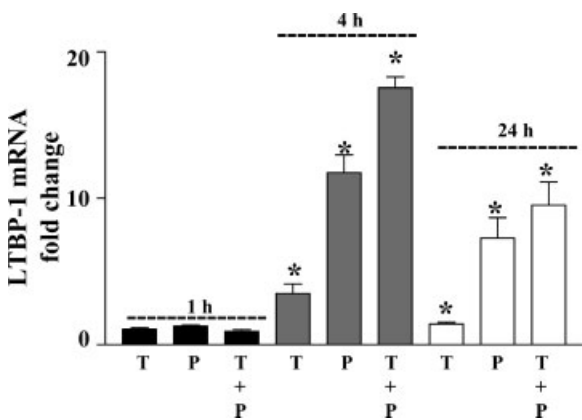


Fig. 5. Effect of PTH and *TGF-β1* on expression of *LTBP-1* mRNA levels in rat osteoblastic cells. UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) (P), human *TGF-β1* (1 ng/ml) (T), or both together for 1, 4, and 24 h. Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

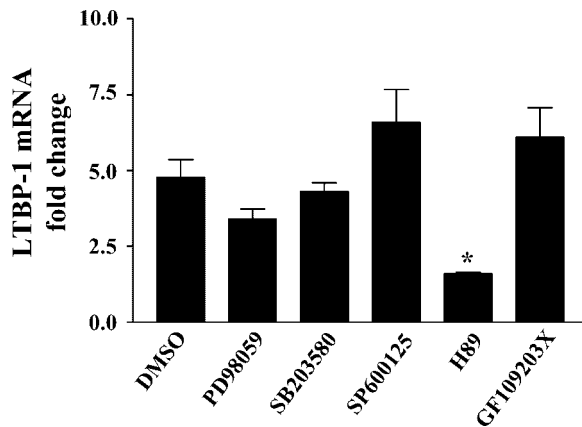


Fig. 6. PTH-stimulated *LTBP-1* mRNA expression depends on the PKA signaling pathway. UMR 106-01 cells were serum-starved for 24 h and then treated with control or rat PTH (1–34, 10^{-8} M)-containing medium for 4 h in the presence or absence of inhibitors PD98059, SB203580, SP600125, H89, and GF109203X (added 20 min before PTH). Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat *LTBP-1* and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

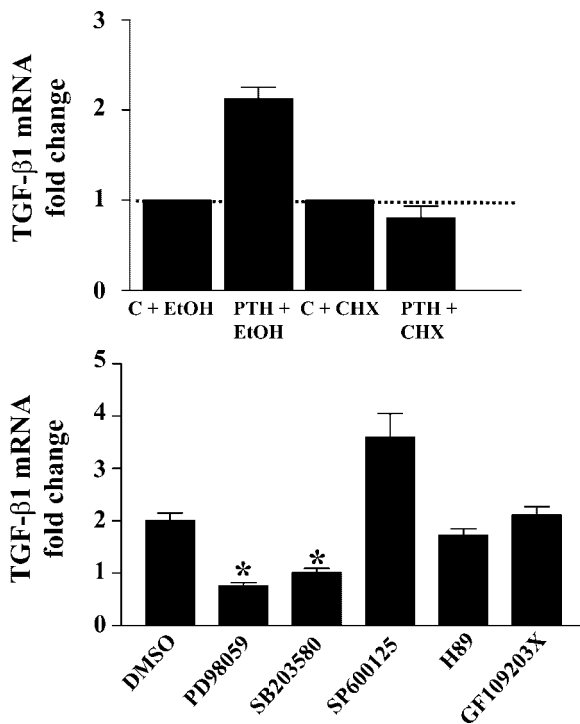


Fig. 7. PTH-stimulated TGF- β 1 mRNA expression requires de novo protein synthesis and depends on the MEK and p38 MAPK signaling pathways. **A:** UMR 106-01 cells were serum-starved for 24 h and treated with control medium or medium containing rat PTH (1–34, 10^{-8} M) for 4 h in the presence or absence of 30 μ g/ml cycloheximide (CHX) added 1 h before PTH treatment, and total RNA was subjected to real time RT-PCR using specific primers for rat TGF- β 1 and β -actin. **B:** UMR 106-01 cells were serum-starved for 24 h and then treated with control or rat PTH (1–34, 10^{-8} M)-containing medium for 4 h in the presence or absence of inhibitors PD98059, SB203580, SP600125, H89, and GF109203X (added 20 min before PTH). Total RNA was isolated and subjected to real time RT-PCR using specific primers for rat TGF- β 1 and β -actin. The relative levels of mRNAs were normalized to β -actin, and the PTH-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control.

type I collagen, fibronectin, and osteocalcin [Roberts, 1998]. TGF- β has therefore been implicated as a coupling factor that coordinates the processes of bone resorption and subsequent bone formation. A functional role for LTBP in regulating the local activity of TGF- β has emerged using antibodies to LTBP-1 [Miyazono et al., 1991]. TGF- β 1 also induces expression of its own mRNA as well as expression of LTBP-1 [Dallas et al., 1994; Roberts, 1998]. This is consistent with our results that TGF- β 1 stimulates LTBP-1 mRNA expression in rat primary osteoblastic cells (Fig. 4). We report here that PTH stimulated expression of both LTBP-1 and TGF- β 1 in rat osteoblastic cells and this effect may be required to maintain the level of LTBP

and its bound latent TGF- β in bone matrix. It is most likely that the PTH stimulation of LTBP-1 expression in osteoblasts has an effect on osteoclasts via TGF- β . It is possible that LTBP-1 may play an important role to link signaling between the systemic (PTH) and local (TGF- β) factors. This may be one of the PTH regulatory mechanisms that is necessary for maintaining the balance between osteoblastic and osteoclastic activity.

The PTH effect on LTBP-1 expression is a primary effect thus, not requiring de novo protein synthesis (Fig. 3A). This result supports the fact that, by association of TGF- β with the ECM, it is stored in a readily mobilized form, which could allow extracellular signaling to proceed rapidly in the absence of new protein synthesis. This event is particularly important in situations such as tissue repair following injury. PTH and TGF- β 1 stimulated LTBP-1 mRNA expression (Fig. 5) and both together had an additive effect on LTBP-1 mRNA expression, indicating that PTH and TGF- β may have separate intracellular components to activate LTBP-1 gene expression. It is well documented that PTH mediates its effects by the PTH1R, and TGF- β mediates its effects by TGF- β type II and type I receptors [Attisano and Wrana, 1998; Massague and Wotton, 2000; Swarthout et al., 2002; Derynck and Zhang, 2003; Qin et al., 2004]. Even though PTH activates both PKA and PKC signaling pathways [Swarthout et al., 2002; Qin et al., 2004], we identified that the PKA signaling pathway is responsible for PTH-stimulated LTBP-1 mRNA expression in UMR 106-01 cells (Fig. 6). The requirement of the PKA signaling pathway for PTH stimulation of LTBP-1 mRNA expression (Fig. 6) and the requirement of de novo protein synthesis and the MEK and p38 MAPK signaling pathways for PTH-stimulated TGF- β 1 mRNA expression (Fig. 7) suggest that PTH mediates its effects on osteoblasts by several intracellular signaling components in rat osteoblastic cells. In summary, we provide evidence for PTH stimulated and PKA-dependent LTBP-1 mRNA expression in osteoblastic cells, which appears to be important for PTH regulation of the TGF- β system to mediate bone remodeling activities.

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