0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 5
Printed in U.S.A.

Parathyroid Hormone Increases mac25/Insulin-Like Growth Factor-Binding Protein-Related Protein-1 Expression in Cultured Osteoblasts*

RENATA C. PEREIRA† AND ERNESTO CANALIS

Departments of Research and Medicine, Saint Francis Hospital and Medical Center (R.C.P., E.C.), Hartford, Connecticut 06105; University of Connecticut School of Medicine (E.C.), Farmington, Connecticut 06030; and Universidade de Sao Paulo (R.C.P.), Sao Paulo, Brazil

ABSTRACT

PTH induces the synthesis of insulin-like growth factor I (IGF-I) and regulates the expression of IGF-binding proteins (IGFBP) in osteoblast cultures. IGFBP-related protein-1 (IGFBP-RP-1), the product of the mac25 gene, binds IGF-I, IGF-II, and insulin. We tested the actions of PTH on the expression of mac25/IGFBP-RP-1 in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells). PTH at 0.1–10 nM for 6–48 h increased mac25/IGFBP-RP-1 messenger RNA (mRNA) levels in Ob cells, an effect not altered by cycloheximide. PGE_2 increased mac25/IGFBP-RP-1 mRNA levels, but indomethacin did not modify basal or PTH-stimulated mac25/IGFBP-RP-1 expression. The decay of mac25/IGFBP-RP-1 mRNA in transcriptionally arrested Ob cells was not modified by PTH, and PTH increased the rate of IGFBP-RP-1 transcription. GH, insulin, bone morphogenetic protein-2, fibroblast growth factor-2, platelet-derived growth factor BB, IGF-I, and IGF-II did not modify mac25/IGFBP-RP-1 expression, whereas transforming growth factor-β1 was modestly stimulatory. In conclusion, PTH stimulates mac25/IGFBP-RP-1 transcription in osteoblasts, an effect that could be relevant to the actions of PTH in bone. (*Endocrinology* **140:** 1998–2003, 1999)

INSULIN-LIKE growth factor I (IGF-I) is an important local regulator of bone formation, and its activity can be modified at the level of synthesis, receptor binding, and IGF-binding proteins (IGFBP) (1–3). Recent studies have shown that in addition to the six characterized IGFBPs, the protein encoded by the mac25 gene shares biochemical and functional properties with the IGFBPs and has been termed IGFBP-7 or IGFBP-related protein-1 (IGFBP-RP-1) (2, 4). The protein encoded by mac25 has a molecular mass of 31,000 and has 40–45% similarity and 20–25% amino acid sequence identity with IGFBPs (2, 4–7). It is also identical to tumor adhesion factor and prostacyclin-stimulating factor (8–10). Although the protein encoded by mac25 binds IGF-I and IGF-II, it has higher affinity for insulin and inhibits insulin-stimulated phosphorylation of its receptor, suggesting that the product of mac25 acts primarily as an insulin-binding protein (11). The product of mac25 stimulates cell growth in BALB/c3T3 cells, enhancing the mitogenic effects of IGF-I, whereas in selected osteosarcoma cells it inhibits cell growth, suggesting additional and diverse biological functions (8, 12). The mac25 gene is expressed by a variety of normal and malignant cells of epithelial and mesenchymal origin, although its expression by skeletal cells has not been reported (4–6, 10). Mac25 expression is increased after exposure to retinoic acid and glucocorticoids, but it is not known whether polypeptide hormones and growth factors regulate its expression (6) (Pereira, R. C., *et al.*, unpublished observations).

PTH has complex effects on bone formation and resorption (13). PTH has mitogenic effects on cells of the osteoblastic lineage, and when osteoblasts are continuously exposed to PTH, it inhibits collagen synthesis (13, 14). However, intermittent exposure of bone cells to PTH results in anabolic effects *in vitro* and *in vivo* (14, 15). PTH is a major inducer of IGF-I, and selected stimulatory effects in bone seem to be mediated by IGF-I (14, 16). Skeletal cells express the six classic IGFBPs, and the actions of PTH in bone could be modified by effects on IGFBP expression (17–19). For example, through its ability to stimulate cAMP, PTH is likely to induce the expression of IGFBP-5, a binding protein that can stimulate bone cell growth (18, 20). In view of the known actions of PTH on the IGF-IGFBP axis and on the possible relationship between mac25 gene expression and cell regulation, we postulated mac25/IGFBP-RP-1 gene regulation by PTH in osteoblasts.

The present studies were undertaken to examine the effects of PTH on mac25 or IGFBP-RP-1 gene expression and to compare them to the effects of other polypeptide hormones and growth factors in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells).

Materials and Methods

Culture technique

The culture method used was described in detail previously (21). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area. This project was approved by the institutional animal care and use committee of Saint Francis Hospital and Medical Center (Hartford, CT). Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II, Worthington Biochemical Corp., Freehold, NJ). Cell

Received August 31, 1998.
Address all correspondence and requests for reprints to: Ernesto Canalis, M.D., Department of Research, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, Connecticut 06105-1299. E-mail: ecanalis@stfranciscare.org.
* This work was supported by NIDDK Grant DK-42424.
† Supported by a scholarship from CAPES, Universidade de Sao Paulo (Sao Paulo, Brazil).

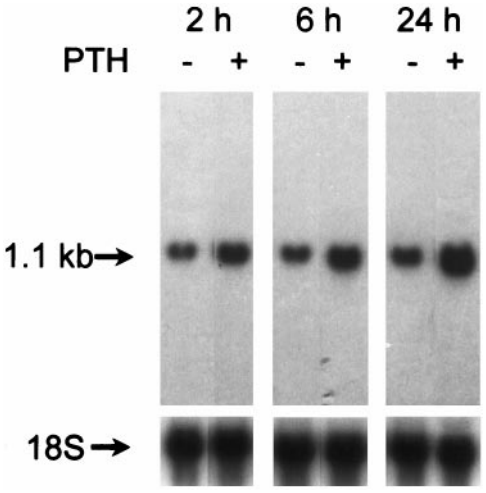

FIG. 1. Effect of PTH at 10 nM on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (−) or PTH-treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics (21). Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and cultured in a humidified 5% CO_2 incubator at 37 C until reaching confluence (~50,000 cells/cm^2). Cells were cultured in DMEM supplemented with nonessential amino acids and 10% FBS (both from Summit Biotechnology, Fort Collins, CO). Ob cells were grown to confluence, transferred to serum-free medium for 20–24 h, and exposed to test or control medium in the absence of serum for 2–48 h, as indicated in the text and legends. Cultures treated for 48 h were switched to fresh control and test solutions after 24 h. For nuclear run-on assays, Ob cells were grown to subconfluence, trypsinized, replated, and grown to confluence, at which time they were serum deprived and exposed to test or control solutions for 2–6 h. Rat PTH-(1–34) (Bachem, Torrance, CA) was dissolved in 0.05 N HCl containing 4 mg/ml BSA and diluted 1:10,000 or more in culture medium. Porcine insulin (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.001 N HCl and diluted 1:1,000 in DMEM, and recombinant human GH (a gift from P. A. Kelly, Paris, France) was dissolved in distilled water and added to DMEM. Recombinant human transforming growth factor-β1 (TGFβ1) (a gift from Genentech, Inc., South San Francisco, CA), bone morphogenetic protein-2 (BMP-2; a gift from Genetics Institute, Cambridge, MA), fibroblast growth factor-2, and platelet-derived growth factor BB (both from Austral, San Ramon, CA) were added directly to the medium. Recombinant human IGF-I (Austral) was dissolved in 20 mM sodium citrate and diluted 1:1,000 in DMEM, and IGF-II (a gift from Eli Lilly & Co. Research Laboratories, Indianapolis, IN) was dissolved in 0.1 M acetic acid and diluted 1:100 in DMEM. Cycloheximide, PGE_2, and indomethacin (all from Sigma Chemical Co.) were dissolved in ethanol and diluted 1:1,000 or more in DMEM, and 5,6-dichlorobenzimidazole riboside (DRB; Sigma Chemical Co.) was dissolved in absolute ethanol and diluted 1:200 in DMEM. Control and experimental cultures were exposed to equal amounts of solvent. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70 C. For nuclear run-on assays, nuclei were isolated by Dounce homogenization (Kontes Co., Vineland, NJ).

Northern blot analysis

Total cellular RNA was isolated using an RNeasy kit following manufacturer's instructions (Qiagen, Chatsworth, CA). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel following denaturation. The gel was stained with ethidium bromide to

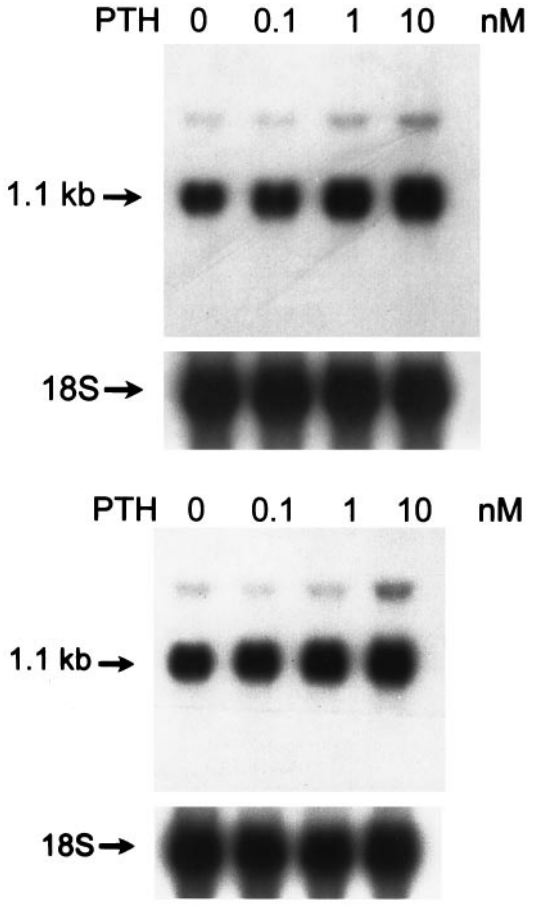

FIG. 2. Effect of PTH at 0.1–10 nM on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 6 (a) or 24 h (b). Total RNA from control or PTH-treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

visualize RNA standards and ribosomal RNA, documenting equal RNA loading of the various experimental samples. The RNA was then blotted onto GeneScreen Plus charged nylon (DuPont, Wilmington, DE), and the uniformity of transfer was documented by revisualization of ribosomal RNA. A 1.1-kb *Eco*RI/*Xho*I restriction fragment of the mouse mac25/IGFBP-RP-1 complementary DNA (cDNA; provided by M. Kato, Ibaraki, Japan) was purified by agarose gel electrophoresis (12). Mac25/IGFBP-RP-1 cDNA was labeled with [α-^{32}P]deoxy (d)-CTP and [α-^{32}P]dATP (50 μCi each at a SA of 3,000 Ci/mmol; DuPont) using the random hexanucleotide primed second strand synthesis method (22). Hybridizations were carried out at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 0.2 × SSC (saline-sodium citrate) for 30 min. The blots were stripped and rehybridized with a 752-bp *Bam*HI/*Sph*I restriction fragment of the murine 18S cDNA (American Type Culture Collection, Manassas, VA) at 42 C for 16–72 h, and posthybridization washes were performed at 65 C in 0.1 × SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY), employing Cronex Lightning Plus intensifying screens (DuPont). Relative hybridization levels were determined by densitometry. The Northern analyses shown are representative of three or more cultures.

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% Nonidet P-40. Nascent transcripts were labeled by incubation of nuclei in a

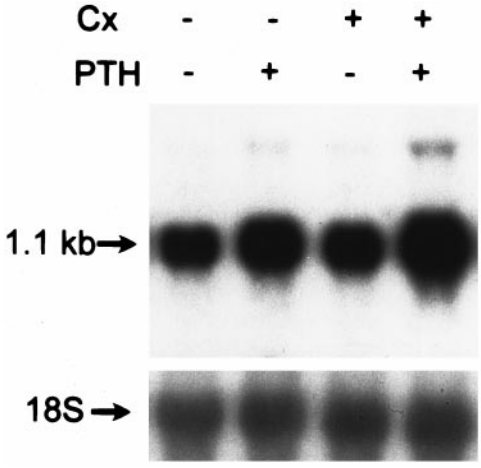

FIG. 3. Effect of PTH in the presence or absence of cycloheximide (Cx) at 3.6 μM on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (−) or treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

reaction buffer containing 500 μM each of adenosine, cytidine, and guanosine triphosphates; 150 U RNasin (Promega Corp., Madison, WI); and 250 μCi [α-^{32}P]UTP (3000 Ci/mM; DuPont) (23). RNA was isolated by treatment with deoxyribonuclease I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid pBluescript SK$^+$ DNA containing about 1 μg of the mac25/IGFBP-RP-1 cDNA used for Northern blotting was immobilized onto GeneScreen Plus by slot blotting according to the manufacturer's directions (DuPont). The plasmid vector pGL3-Basic (Promega Corp.) was used as a control for nonspecific hybridization, and 18S cDNA was used to estimate loading of the radiolabeled RNA. Equal counts per min of [^{32}P]RNA from each sample were hybridized to cDNAs at 42 C for 72 h and washed in 1 × SSC at 65 C for 20 min. Hybridized cDNAs were visualized by autoradiography. The nuclear run-on assay was performed twice.

Statistical methods

Values are expressed as the mean ± SEM. Statistical differences were determined by ANOVA, and *post-hoc* examination was performed using the Bonferroni method (24). Slopes to determine messenger RNA (mRNA) decay were analyzed by the method of Sokal and Rohlf (25).

Results

Northern blot analysis of total RNA extracted from confluent cultures of Ob cells revealed a predominant mac25/IGFBP-RP-1 transcript of 1.1 kb, although a secondary transcript of larger size was detected in some experiments (Fig. 1). Control mac25/IGFBP-RP-1 mRNA levels varied slightly, and densitometric analysis revealed a 10–20% variation in transcript levels over a 24-h period. Continuous treatment of Ob cells with PTH caused a time- and dose-dependent increase in mac25/IGFBP-RP-1 steady state mRNA levels. The effect was first observed after 2 h of exposure to PTH at 10 nM, and it was maximal and of comparable magnitude after 6, 24 (Fig. 1), and 48 h (not shown). PTH at 0.1, 1, and 10 nM after 6 h, increased mac25/IGFBP-RP-1 mRNA levels by (mean ± SEM; n = 3–6) 1.2 ± 0.1-fold, 1.7 ± 0.4-fold (both $P >$ 0.05 *vs.* control), and 2.8 ± 0.3-fold ($P < 0.05$ *vs.* control and PTH at 0.1 and 1 nM), respectively. PTH at 0.1, 1, and 10 nM after 24 h increased mac25/IGFBP-RP-1 mRNA by 1.3 ± 0.1-fold, 1.4 ± 0.2-fold (both $P > 0.05$ *vs.* control), and 2.2 ± 0.3-fold ($P < 0.05$ *vs.* control and PTH at 0.1 and 1 nM; Fig. 2), respectively. To determine whether the effects observed on mac25/IGFBP-RP-1 mRNA levels were dependent on protein synthesis, serum-deprived confluent cultures of Ob cells were treated with PTH in the presence or absence of cycloheximide at 3.6 μM. In earlier experiments, cycloheximide at a dose of 2 μM or higher was found to inhibit protein synthesis in Ob cell cultures by 80–85% (26). Northern blot analysis revealed that treatment with cycloheximide for 24 h did not alter mac25/IGFBP-RP-1 mRNA levels and did not prevent the stimulatory effect of PTH (Fig. 3). PGE_2 also increased mac25/IGFBP-RP-1 mRNA levels in Ob cells. The effect was noted after 2 h and was sustained for 24 h (Fig. 4a), when PGE_2 at 100 nM and 1 μM increased mac25/IGFBP-RP-1 levels by (mean ± SEM; n = 3) 1.9 ± 0.1- and 2.2 ±

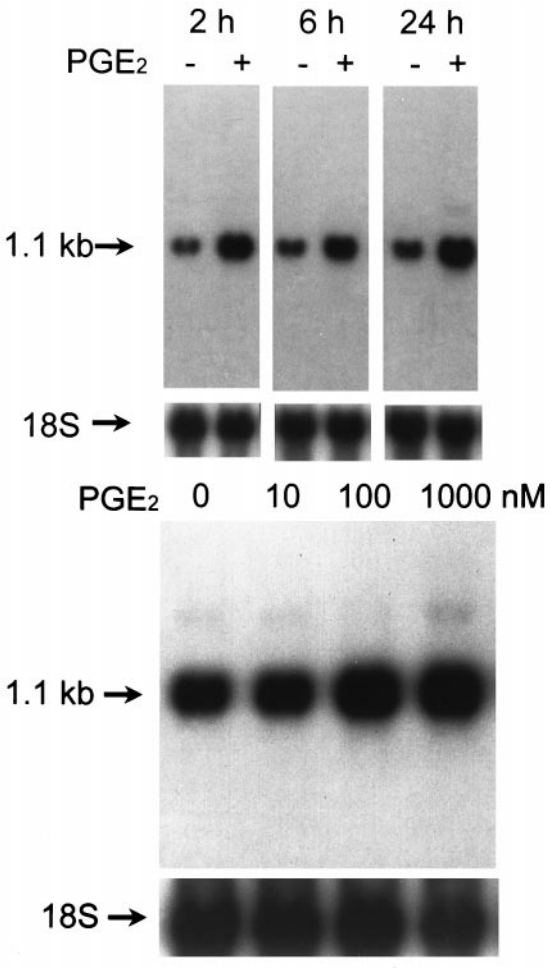

FIG. 4. Effect of PGE_2 on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 2–24 h. In a, cells were exposed to control medium (−) or PGE_2 at 1 μM (+) for 2, 6, or 24 h. In b, cells were exposed to PGE_2 at 10–1000 nM for 24 h. Total RNA from control or PGE_2-treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

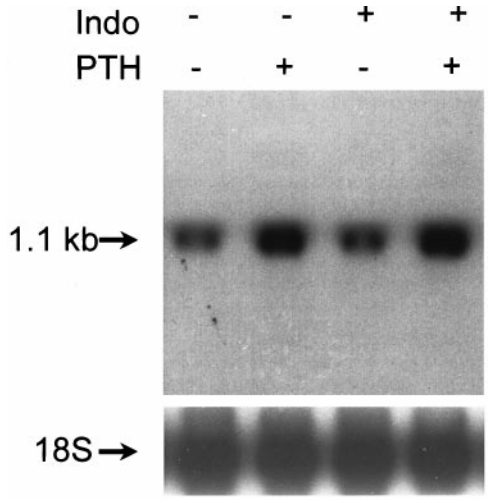

FIG. 5. Effect of PTH in the presence or absence of indomethacin (Indo) at 10 μM on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (−) or treated (+) cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

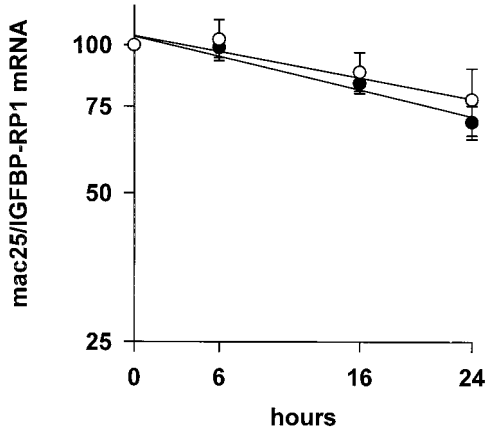

FIG. 6. Effect of PTH at 10 nM on mac25/IGFBP-RP-1 mRNA decay in transcriptionally blocked Ob cells. Cultures were exposed to DMEM (●) or were treated with PTH (○) 2 h before and 6, 16, or 24 h after the addition of DRB. RNA was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA, visualized by autoradiography, and quantitated by densitometry. Ethidium bromide staining of ribosomal RNA was used to check uniform loading of the gels and transfer. Values are the mean ± SEM for three cultures. Values were obtained by densitometric scanning and are presented as a percentage of the mac25/IGFBP-RP-1 mRNA levels relative to the time of DRB addition. Slopes were analyzed by the method of Sokal and Rohlf and were not statistically different.

0.1-fold, respectively (Fig. 4b). However, indomethacin at 10 μM, a dose known to inhibit PG synthesis in skeletal and nonskeletal cells, did not modify basal or PTH-induced mac25/IGFBP-RP-1 expression (Fig. 5) (27, 28).

To examine whether the effect of PTH on mac25/IGFBP-RP-1 mRNA levels was due to changes in transcript stability, confluent cultures of Ob cells were exposed to DMEM or PTH for 2 h, and then treated with the RNA polymerase II

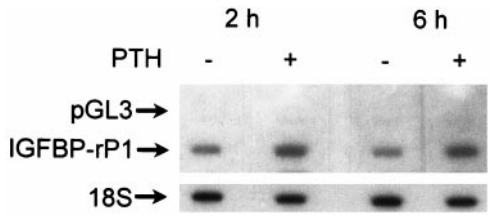

FIG. 7. Effect of PTH at 10 nM on mac25/IGFBP-RP-1 transcription rates in cultures of Ob cells treated for 2 and 6 h. Nascent transcripts from control (−) or PTH-treated (+) cultures were labeled *in vitro* with [α-^{32}P]UTP, and the labeled RNA was hybridized to immobilized cDNA for mac25/IGFBP-RP-1. Murine 18S cDNA was used to demonstrate loading, and pGL3-Basic vector DNA was used as a control for nonspecific hybridization.

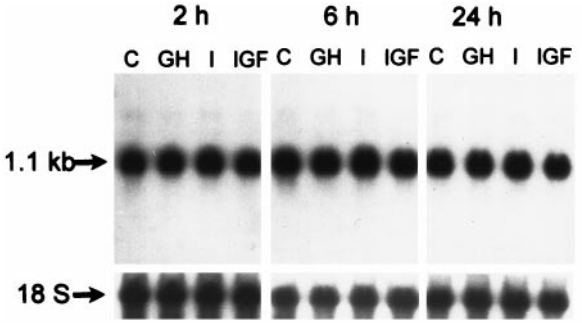

FIG. 8. Effect of GH at 1 μg/ml (50 μM), insulin at 100 nM, and IGF-I at 100 nM on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (C), GH-treated, insulin-treated (I), or IGF-I-treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

inhibitor DRB in the absence or presence of PTH at 10 nM for 6, 16, or 24 h (29). About 75% of Ob cells are viable in the presence of DRB for 24 h, but cell viability is impaired after exposure to DRB for longer periods of time, as determined by trypan blue exclusion (Canalis, E., unpublished observations). After 24 h of DRB exposure, a 35% decay in mac25/IGFBP-RP-1 mRNA was detected, and the change was not different in control and PTH-treated samples (Fig. 6). The half-life of mac25/IGFBP-RP-1 mRNA in transcriptionally arrested Ob cells was estimated to be more than 36 h by extrapolation of the values obtained during the first 24 h. To confirm whether PTH modified the transcription of the mac25/IGFBP-RP-1 gene, a nuclear run-on assay was performed on nuclei from Ob cells treated for 2 and 6 h. This assay demonstrated that PTH increased the rate of mac25/IGFBP-RP-1 transcription by 2- to 3-fold after 2 and 6 h (Fig. 7).

The induction of mac25/IGFBP-RP-1 was virtually selective to PTH. Other polypeptide hormones and growth factors, such as GH, insulin, and IGF-I, tested at doses known to stimulate aspects of bone formation for 2–24 h (n = 4–5), did not modify the expression of mac25/IGFBP-RP-1 mRNA in Ob cells (Fig. 8) (1, 16, 30). TGFβ1 at 1.2 nM for 24 h increased mac25/IGFBP-RP-1 transcripts by (mean ± SEM; n = 5) 1.6 ± 0.1-fold, whereas BMP-2, fibroblast growth factor-2, platelet-derived growth factor BB, and IGF-II, tested

at concentrations known to increase bone cell replication or alter the differentiated function of the osteoblast, for 2–24 h (n = 3), did not modify mac25/IGFBP-RP-1 mRNA expression (Fig. 9) (1, 26, 31–33).

Discussion

Recent studies have shown that PTH has significant effects on the IGF-IGFBP axis in osteoblasts, but it has not been reported to alter the expression of IGFBP-RPs (14, 16, 18). The present investigation was undertaken to determine whether PTH regulates mac25/IGFBP-RP-1 in osteoblasts. We demonstrated that PTH causes a time- and dose-dependent increase in mac25/IGFBP-RP-1 mRNA levels in Ob cells, and that the effect does not require *de novo* protein synthesis. Experiments in transcriptionally blocked Ob cells, using the RNA polymerase II inhibitor DRB, revealed that PTH did not modify mac25/IGFBP-RP-1 mRNA stability (29). This, in conjunction with an increase in the rate of transcription, indicates that PTH stimulates mac25/IGFBP-RP-1 expression at the transcriptional, but not at the posttranscriptional, level.

In our study, the effects of PTH on mac25/IGFBP-RP-1 synthesis were observed at doses that modify other parameters of metabolic function in Ob cells, suggesting that the stimulation of IGFBP-RP-1 synthesis by PTH is physiologically relevant. IGF-I and IGF-II are abundant in skeletal tissue, and studies in mice with targeted IGF-I and IGF-II gene disruption and studies of transgenic mice overexpressing IGF-I and IGF-II have documented their relevance to skeletal growth (34–36). As PTH induces IGF-I and IGFBP-RP-1, and this protein has the potential to bind IGFs, its induction by PTH could be a mechanism to regulate the actions of IGFs in bone. However, IGFBP-RP-1 binds insulin with higher affinity than IGFs, suggesting that its increased expression is more likely to modify insulin actions (11).

The stimulatory effect observed on mac25 gene expression was fairly selective to PTH, and it was not detected in the presence of polypeptide hormones or growth factors, with the exception of TGFβ1. Although PTH is known to increase TGFβ levels in intact bones in culture, this is probably secondary to increased activation and release of the growth factor from the matrix and not to a change in the synthesis of TGFβ1 by the osteoblast (37). This would indicate that the effect of PTH on mac25/IGFBP-RP-1 in osteoblasts is independent of its actions on TGFβ.

Mac25/IGFBP-RP-1 seems to play a role in the differentiation of certain cells, and it has been found to have stimulatory and inhibitory effects on cell growth (7, 8, 12). It could, therefore, play a role mediating the modest mitogenic actions of PTH and TGFβ on cells of the osteoblastic lineage (14, 38). Although PTH inhibits the differentiation of chondrocytes and bone collagen synthesis in cells continuously exposed to the hormone, it stimulates IGF-I production by osteoblasts, and as a consequence it has the potential to increase the differentiated function of this cell (1, 14, 15, 39, 40). Therefore, the induction of mac25/IGFBP-RP-1 is compatible with the effects of PTH in bone. Similarly, TGFβ1 enhances the differentiated function of the osteoblast, an effect compatible with the previously described effects of mac25 in other cells (38). It is possible that mac25/IGFBP-RP-1 simply binds IGF-I, IGF-II, and insulin in osteoblasts, and its increase by PTH may be a local mechanism to reduce the amount of biologically active IGFs in the bone microenvironment. PTH and insulin have opposing effects on signal transduction pathways in osteoblastic cells, and an increase in a binding protein that prevents insulin effects on osteoblasts may be an additional mechanism to limit the actions of insulin in bones exposed to PTH (41, 42). However, it is important to note that our studies do not address the function of IGFBP-RP-1 in osteoblasts, and additional work is needed to define the exact function of this protein in skeletal tissue.

In conclusion, the present studies demonstrate that PTH stimulates IGFBP-RP-1 transcripts in skeletal cells through mechanisms that involve increased transcription. The increased level of IGFBP-RP-1 in the bone microenvironment may bind IGFs and insulin and be relevant to the actions of PTH on bone formation.

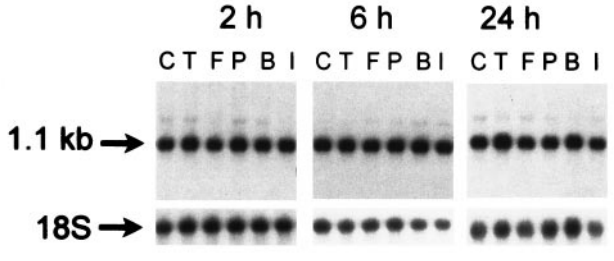

FIG. 9. Effects of growth factors on mac25/IGFBP-RP-1 mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Cells were exposed to control medium (C), TGFβ1 (T) at 1.2 nM, fibroblast growth factor 2 (F) at 1.7 nM, platelet-derived growth factor BB (P) at 3.3 nM, BMP-2 (B) at 1 nM, or IGF-II (I) at 100 nM. Total RNA from control or treated cultures was subjected to Northern blot analysis and hybridized with α-^{32}P-labeled mac25/IGFBP-RP-1 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-RP-1 mRNA was visualized by autoradiography and is shown in the *upper panel*; 18S mRNA is shown *below*.

Acknowledgments

The authors thank Dr. M. Kato for the murine mac25/IGFBP-RP-1 cDNA clone; Dr. P. A. Kelly, Eli Lilly & Co. Research Laboratories, Genentech, Inc., and Genetics Institute for the gifts of GH, IGF-II, TGFβ1, and BMP-2, respectively; Ms. Cathy Boucher, Ms. Deena Durant, and Ms. Kris Sasala for technical assistance; and Ms. Charlene Gobeli for secretarial help.

References

1. **Hock JM, Centrella M, Canalis E** 1988 Insulin-like growth factor I (IGF-I) has independent effects on bone matrix formation and cell replication. Endocrinology 122:254–260
2. **Rechler MM** 1993 Insulin-like growth factor binding proteins. Vitam Horm 47:1–114
3. **Canalis E, Pash J, Varghese S** 1993 Skeletal growth factors. Crit Rev Eukaryot Gene Expr 3:155–166
4. **Oh Y, Nagalla SR, Yamanaka Y, Kim H-S, Wilson E, Rosenfeld RG** 1996 Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. J Biol Chem 271:30322–30325
5. **Murphy M, Pykett MJ, Harnish P, Zang KD, George DL** 1993 Identification and characterization of genes differentially expressed in meningiomas. Cell Growth Differ 4:715–722
6. **Swisshelm K, Ryan K, Tsuchiya K, Sager R** 1995 Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mam-

mary epithelial cells and induced expression with retinoic acid. Proc Natl Acad Sci USA 92:4472–4476
7. **Akaogi K, Okabe Y, Sato J, Nagashima Y, Yasumitsu H, Sugahara K, Miyazaki K** 1996 Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. Proc Natl Acad Sci USA 93:8384–8389
8. **Akaogi K, Sao J, Okabe Y, Sakamoto Y, Yasumitsu H, Miyazaki K** 1996 Synergistic growth stimulation of mouse fibroblasts by tumor-derived adhesion factor with insulin-like growth factors and insulin. Cell Growth Differ 7:1671–1677
9. **Wilson EM, Oh Y, Rosenfeld RG** 1997 Generation and characterization of an IGFBP-7 antibody: identification of 31 kD IGFBP-7 in human biological fluids and Hs578T human breast cancer conditioned media. J Clin Endocrinol Metab 82:1301–1303
10. **Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H** 1994 Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 303:591–598
11. **Yamanaka Y, Wilson EM, Rosenfeld RG, Oh Y** 1997 Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. J Biol Chem 272:30729–30734
12. **Kato MV, Sato H, Tsukada T, Ikawa Y, Aizawa S, Nagayoshi M** 1996 A follistatin-like gene, mac25, may act as a growth suppressor of osteosarcoma cells. Oncogene 12:1361–1364
13. **Dietrich JW, Canalis EM, Maina DM, Raisz LG** 1976 Hormonal control of bone collagen synthesis in vitro: effects of parathyroid hormone and calcitonin. Endocrinology 98:943–949
14. **Canalis E, Centrella M, Burch W, McCarthy T** 1989 Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. J Clin Invest 83:60–65
15. **Canalis E, Hock JM, Raisz LG** 1994 Parathyroid hormone: anabolic and catabolic effects on bone and interactions with growth factors. In: Bilezikian JP, Marcus R, Levine MA (eds) The Parathyroids. Raven Press, New York, pp 65–82
16. **McCarthy TL, Centrella M, Canalis E** 1989 Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. Endocrinology 124:1247–1253
17. **Hassager C, Fitzpatrick LA, Spencer EM, Riggs BL, Conover CA** 1992 Basal and regulated secretion of insulin-like growth factor binding proteins in osteoblast-like cells is cell line specific. J Clin Endocrinol Metab 75:228–233
18. **McCarthy TL, Casinghino S, Canalis E, Centrella M** 1994 Complex pattern of insulin-like growth factor binding protein expression in primary rat osteoblast enriched cultures: regulation by prostaglandin E2, growth hormone, and the insulin-like growth factors. J Cell Physiol 160:163–175
19. **Okazaki R, Riggs BL, Conover CA** 1994 Glucocorticoid regulation of insulin-like growth factor binding protein expression in normal human osteoblast-like cells. Endocrinology 134:126–132
20. **Andress DL, Birnbaum RS** 1992 Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467–22472
21. **McCarthy TL, Centrella M, Canalis E** 1988 Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res 3:401–408
22. **Feinberg A, Vogelstein B** 1984 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267
23. **Greenberg ME, Ziff EB** 1984 Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature 311:433–438
24. **Wall FJ** 1986 Statistical Data Analysis Handbook, ed 1. McGraw-Hill, New York
25. **Sokal RR, Rohlf FJ** 1981 Biometry, ed 2. Freeman, San Francisco
26. **Centrella M, McCarthy TL, Canalis E** 1991 Glucocorticoid regulation of transforming growth factor β, (TGF β) activity and binding in osteoblast-enriched cultures from fetal rat bone. Mol Cell Biol 11:4490–4496
27. **Levine L, Hinkle PM, Voelkel EF, Tashjian Jr AH** 1972 Prostaglandin production by mouse fibrosarcoma cells in culture: inhibition by indomethacin, and aspirin. Biochem Biophys Res Commun 47:888–896
28. **Pilbeam CC, Fall PM, Alander CB, Raisz LG** 1997 Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. J Bone Miner Res 12:1198–1203
29. **Zandomeni R, Bunick D, Ackerman S, Mittleman B, Weinmann R** 1983 Mechanism of action of DRB. Effect on specific in vitro initiation of transcription. J Mol Biol 167:561–574
30. **Canalis EM, Dietrich JW, Maina DM, Raisz LG** 1977 Hormonal control of bone collagen synthesis in vitro. Effects of insulin and glucagon. Endocrinology 100:668–675
31. **Varghese S, Canalis E** 1997 Regulation of collagenase-3 by bone morphogenetic protein-2 in bone cell cultures. Endocrinology 138:1035–1040
32. **McCarthy TL, Centrella M, Canalis E** 1989 Effects of fibroblast growth factors on deoxyribonucleic acid and collagen synthesis in rat parietal bone cells. Endocrinology 126:2118–2126
33. **Hock JM, Canalis E** 1994 Platelet-derived growth factor enhances bone cell replication but not differentiated function of osteoblasts. Endocrinology 134:1423–1428
34. **DeChiara TM, Efstratiadis A, Robertson EJ** 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 345:78–80
35. **Ward A, Bates P, Fisher R, Richardson L, Graham CF** 1994 Disproportionate growth in mice with Igf-2 transgenes. Proc Natl Acad Sci USA 91:10365–10369
36. **Baker J, Liu J, Robertson EJ, Efstratiadis A** 1993 Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73–82
37. **Pfeilschifter J, Mundy GR** 1987 Modulation of type B transforming growth factor activity in bone cultures by osteotropic hormones. Proc Natl Acad Sci USA 84:2024–2028
38. **Centrella M, McCarthy TL, Canalis E** 1987 Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. J Biol Chem 262:2869–2874
39. **Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ** 1996 Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273:613–622
40. **Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra A-B, Juppner H, Segre GV, Kronenberg HM** 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273:663–666
41. **Iida-Klein A, Varlotta V, Hahn TJ** 1989 Protein kinase C activity in UMR-106–01 cells: effects of parathyroid hormone and insulin. J Bone Miner Res 4:764–774
42. **Iida-Klein A, Hahn TJ** 1991 Insulin acutely suppresses parathyroid hormone second messenger generation in UMR-106–01 osteoblast-like cells: differential effects on phospholipase C and adenylate cyclase activation. Endocrinology 129:1016–1024