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Analysis of Regulator of G-Protein Signaling-2 (RGS-2) Expression and Function in Osteoblastic Cells

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Abstract Regulator of G-protein signaling-2 (RGS-2) belongs to a novel family of GTPase-activating proteins that rapidly turn-off G-protein coupled receptor signaling. RGS proteins contain a characteristic RGS domain by which they interact with the α-subunit of G-proteins and drive them into their inactive GDP-bound forms. Previously, we have reported that RGS-2 mRNA is rapidly and transiently increased by PTH in rat bone and in osteoblast cultures in vitro. In this study, we further explored the molecular basis for the regulation of RGS-2 by cloning and functionally characterizing the RGS-2 gene promoter. We cloned 2.3- and 2.8-kb fragments of the 5′-flanking regions of the rat and mouse RGS-2 genes, respectively, and generated a stable clone of UMR106 osteoblastic cells containing the rat RGS-2 promoter driving the β-gal reporter gene (p2.3RGS-2-β-gal). Treatment of the stable clone with PTH resulted in a maximal 2.2- to 3.6-fold increase in promoter activity at 8 h, reminiscent of the early response observed with endogenous RGS-2 mRNA regulation. Further, PTH (1–38), (1–31), PTHrP (1–34), and forskolin, which elevate cAMP levels, stimulated the promoter, while PTH (3–34) and (7–34), which do not readily stimulate cAMP accumulation, and PMA that directly activates protein kinase C, had no effect on promoter activity. Taken together, these results implicate the involvement of the $G\alpha_s$-adenylate cyclase-protein kinase A pathway in stimulating RGS-2 expression. Maintenance of a hyperphosphorylated state via the inhibition of type 2A protein phosphatases by okadaic acid, resulted in a strong dose-dependent increase in transcriptional activity of the RGS-2 promoter as well as that of the endogenous RGS-2 gene. Furthermore, overexpression of the osteoblast-specific transcription factor Runx2 also led to a stimulation of RGS-2 promoter activity. Functional analysis using RGS-2 overexpression suggests the potential negative regulatory effects of RGS-2 on PTH- and forskolin-induced cAMP production in osteoblastic cells. In summary, our data suggest that PTH treatment results in a direct transcriptional stimulation of RGS-2 that in turn may play a role in modulating the duration/intensity of PTH receptor signaling. J. Cell. Biochem. 85: 837–850, 2002.

Key words: PTH; RGS-2; rat RGS-2 promoter; osteoblasts; Runx2; okadaic acid

Regulator of G-protein signaling (RGS) proteins belong to a family of GTPase activating proteins (GAPs) that act as negative regulators of G-protein coupled receptor (GPCR)-mediated signaling in organisms ranging from yeast and nematodes to mammals [Roush, 1996]. They interact with the α-subunit of G-proteins and accelerate the rate of GTP hydrolysis by Gα, thereby driving them into their inactive GDP-bound form [Berman et al., 1996; Watson et al., 1996; Popov et al., 1997]. The first RGS protein to be discovered was a yeast protein called Sst2p that is involved in the desensitization of mating pheromone response. Loss of function mutations in the *Sst2* gene led to supersensitivity to mating pheromone, resulting in continued growth arrest [Chan and Otte, 1982a,b; Dohlman and Thorner, 1997; Dohlman et al., 1998]. In *C. elegans*, an RGS protein called egl-10 has been shown to modulate locomotion and egg-laying behaviors, by inhibiting the effects of a Gα protein [Lochrie et al., 1991; Koelle and Horvitz, 1996]. In mammals, about 25 members of the RGS protein family have been discovered.

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They are expressed in a wide range of tissues where they have been shown to dampen GPCR signaling involved in regulating a number of physiological processes. RGS proteins have a characteristic 120-amino acid "core domain" called the RGS domain that is responsible for mediating interaction with the Gα subunit. To date, there is increasing evidence for the interaction of various RGS proteins with different subtypes of Gα-, $G\alpha_i$-, $G\alpha_q$-, $G\alpha_s$-, and $G\alpha_{12/13}$), and for the role of RGS proteins in regulating cell proliferation, differentiation, motility, vesicular trafficking, and other important processes. In addition, many of the RGS proteins contain additional domains that enable them to interact with other signaling networks besides regulating G-protein-linked processes [reviewed in De Vries and Farquhar, 1999; De Vries et al., 2000].

Although rapid progress has been made in our understanding of the mechanism of action of RGS proteins, there is limited evidence as to how RGS proteins themselves are regulated. Some of the suggested modes of regulation are known to occur at the levels of transcription, post-translational modification, and intracellular localization, among others [De Vries et al., 2000]. Transcriptional upregulation of RGS proteins has been shown to occur in response to pheromones (yeast *Sst2* gene) [Dietzel and Kurjan, 1987; Dohlman et al., 1995], B-cell mitogens [Hong et al., 1993], p53 [Buckbinder et al., 1997], forskolin [Pepperl et al., 1998], amphetamine [Burchett et al., 1998], haloperidol [Ingi et al., 1998], and a number of other agents. However, there are only a few examples for the regulation of RGS proteins upon agonist stimulation of GPCRs in a negative feedback loop scenario. These include glucose-dependent insulinotropic peptide (GIP)-stimulated induction of RGS-2 mRNA in pancreatic βTC3 cells [Tseng and Zhang, 1998], platelet-activating factor (PAF)-stimulated expression of RGS1 in B lymphocytes [Druey et al., 1996], and protein kinase C-dependent muscarinic receptor stimulation of RGS-2 mRNA in human neuroblastoma cells [Song et al., 1999].

We reported recently that parathyroid hormone (PTH) treatment results in a rapid and transient upregulation of RGS-2 mRNA levels in rat metaphyseal and diaphyseal bone [Miles et al., 2000]. In order to understand the molecular mechanism underlying this increase in RGS-2 mRNA levels, we have cloned and analyzed the promoters of the rat and mouse RGS-2 genes. Based on functional studies of the rat RGS-2 promoter, we show that treatment with PTH and other cAMP-elevating agents, maintenance of a hyperphosphorylated state via the inhibition of type 2A protein phosphatases (PP2A), as well as overexpression of the osteoblast-specific transcription factor Runx2, result in a direct transcriptional stimulation of RGS-2 expression in rat osteoblastic cells. Furthermore, we have shown that overexpression of RGS-2 results in a significant decrease in PTH- and forskolin-induced cAMP accumulation in rat osteoblastic cells. These results suggest a potential role for RGS-2 in regulating PTH signaling in osteoblastic cells.

MATERIALS AND METHODS

Materials

Synthetic human PTH (1–38), PTH (1–31), and PTHrP (1–34), as well as bovine PTH (3–34) and (7–34) were obtained from Bachem (Torrance, CA), and were resuspended in a vehicle containing 0.001 N HCl, 150 mM NaCl, and 1 mg/ml BSA. Forskolin, 1,9-dideoxy forskolin, and isobutyl methyl xanthine (IBMX) were purchased from Sigma (St. Louis, MO) and were solubilized in dimethyl sulfoxide (DMSO). Okadaic acid was also obtained from Sigma and was first dissolved in 100% ethanol, and further diluted in cell culture medium (containing 0.1% fetal bovine serum (FBS), obtained from Hyclone Laboratories, Logan, UT). DMEM, Ham's F-12, and DMEM/Ham's F-12 (3:1) media, as well as L-glutamine, Hepes buffer and Geneticin (G418) were obtained from GIBCO-BRL Life Technologies, Inc. (Gaithersburg, MD). The control vector pEF/myc/cyto (Invitrogen, Carlsbad, CA) and the Cbfa1(Runx2) expression construct pEF-Cbfa1 were described previously. The rat RGS-2 expression construct (pEF-RGS-2) was constructed by inserting the RGS-2 coding sequence into the PstI and XhoI sites of pEF/myc/cyto. RGS-2 is expressed from this plasmid as a fusion protein containing a myc epitope tag at the C-terminal end.

Cloning of the Rat and Mouse RGS-2 Gene Promoters

The rat and mouse RGS-2 promoters were cloned using the GenomeWalker kit (Clontech, Palo Alto, CA). The procedure involves PCR-based "walking" of genomic DNA adjacent to

known sequences. Gene-specific primers were designed corresponding to the rat and mouse RGS-2 coding sequences available in the GenBank database (accession numbers: AF279918 (rat RGS-2) and U67187 (mouse RGS-2)) [Chen et al., 1997]. Primers rRGS-2R (5′-ATT TTC TCC CGC TTC TCC TCG ACC TTG-3′) and rRGS-2nest (5′-GGC ACT TTG CAT TCT CAG ACA CC-3′) were designed based on the rat sequence, and primers rRGS-2R (same as above) and mRGS-2nest (5′-TGG CAC TTT GCA TTC TCA GAC TCC-3′) were used to amplify the mouse sequence. Two steps of PCR amplification were performed using one of the above primers, in combination with a primer specific to the GenomeWalker adaptor (adaptor primer AP1 or AP2). Fragments of length ~2.3 and ~2.8 kb, respectively, were amplified from the rat and mouse libraries. The PCR products were subcloned using standard procedures into the pCR2.1TOPO vector (Invitrogen) and sequenced (Lilly DNA Technology Group, Indianapolis, IN). All of the amplified fragments from the rat and mouse genomes contained overlapping proximal regions that were identical in sequence, confirming them to be genuine sequences from the 5′-flanking region of the RGS-2 gene. The sequences were confirmed by comparison to one another and with the published human promoter sequence [Siderovski et al., 1994]. All of the mouse RGS-2 promoter clones that we sequenced lacked two base pairs (+1 ggccgcgcgg...) in the 5′-untranslated region of the gene, when compared to the published mouse sequence (GenBank accession # U67187) [Chen et al., 1997]. This could be due to strain variations (DNA polymorphism) between the ICR Swiss mouse genomic libraries used in the GenomeWalker protocol and the strain of mouse from which the published clone was generated.

Construction of Rat RGS-2 Promoter-β-Gal Construct

The pCR2.1TOPO clone containing the 2.3-kb rat RGS-2 promoter fragment was digested with SmaI (present in the genome walker adaptor) and SacII (present in the proximal region of the promoter). The SmaI–SacII fragment was then blunted with Klenow polymerase and cloned into the SmaI site of pβ-gal-Basic (Clontech) to generate the p2.3RGS-2-β-gal promoter-reporter construct that contains the (−2303 to −1) region of the rat RGS-2 promoter. The integrity and orientation of the insert was verified by restriction mapping and DNA sequencing.

Sequence Analysis

The GCG Wisconsin Package (Genetics Computer Group, Madison, WI) was used for sequence comparison and to analyze the sequence for the presence of consensus transcription factor binding sites.

Cell Culture and DNA Transfection

UMR106 rat osteoblastic cells were grown in DMEM/Ham's F-12 (3:1) supplemented with 10% FBS, 50 mM Hepes, and 2 mM L-glutamine. ROS17/2.8 rat osteoblastic cells and COS1 monkey kidney cells were cultured as described previously [Thirunavukkarasu et al., 2000a].

For Runx2 transactivation studies, 1×10^5 cells were plated per well in six-well plates and incubated overnight. The next morning, cells were transfected with 1 μg each of the reporter plasmid (p2.3RGS-2-β-gal, or the control reporter vector pβ-gal-Basic) and the effector plasmid [pEF-Cbfa1 (Runx2) or the control expression vector pEF/myc/cyto] using Fugene6™ transfection reagent (Roche, Indianapolis, IN). β-gal assay was performed 48 h after transfection, as described previously [Thirunavukkarasu et al., 2000a]. In our hands, the Fugene6 reagent consistently yields 85–90% transfection efficiency. We chose not to include an additional reporter construct to monitor transfection efficiency in our experiments, in order to avoid possible squelching of factors that could arise when cotransfecting multiple plasmids [Farr and Roman, 1992; Selvamurugan et al., 1998]. Furthermore, the luciferase reporter vector was not used to normalize for transfection efficiency because of our recent finding that cryptic enhancer elements in the promoterless luciferase reporter vector pGL3-Basic could mediate transactivation by Runx2, leading to spurious background luciferase expression [Thirunavukkarasu et al., 2000b]. However, in all of our transient transfection experiments, we did use as a positive control (and to verify transfection efficiency), separate plates that were transfected with a β-gal expression plasmid (pβ-gal-Promoter, Clontech), that has the β-gal reporter coding sequence under the control of the SV40 early promoter. β-gal expression was quantified either by histochemical staining or by β-gal enzyme assay. The transfection efficiency was 85–90% and comparable across plates. Addi-

tionally, the experiments were repeated 2–3 times using multiple clones of the same construct and different preparations of the plasmids. The results obtained under these conditions were similar or identical in nature.

Generation of stable clones of UMR106 cells containing the 2.3-kb rat RGS-2 gene promoter—UMR106 rat osteoblastic cells were transfected with a 5:1 molar ratio mixture (total 6.5 μg DNA) of p2.3RGS-2-β-gal and pCDNA3.1 (Invitrogen) that contains the neomycin resistance gene, using Fugene6 transfection reagent. Forty-eight hours after transfection in T25 flasks (Corning Incorporated, Corning, NY) cells were re-seeded (1:10) into T75 flasks and selected in media containing 1 mg/ml G418 for 10 days. Regular media changes were made at 3–4 day intervals. On the tenth day, randomly selected G418 resistant colonies (containing more than 50 cells) were picked and expanded in fresh media containing 1 mg/ml G418. Many G418-resistant β-gal-positive clones showed a PTH-dependent increase in β-gal activity (1.3- to 3.6-fold). A representative clone was used for further studies.

Assays of transient and stable-transfected cells. Treatment of the stable clone with PTH and other agents—cells were plated at 5×10^4 cells per well in 96-well plates and grown in media containing 10% FBS for 4 h to allow cells to attach. The medium was then removed, wells were washed with PBS, medium containing 0.1% serum was added, and then incubated for an additional 16 h. Treatments with various agents (diluted in 0.1% serum medium) were done in quadruplicate wells and allowed to proceed for 8, 24, or 48 h. The cells were then lysed and β-gal activity was measured.

Analysis of cAMP Production

Approximately 90% confluent UMR106 cells (that were serum-starved overnight) were transfected with 20 μg of either the control vector pEF/myc/cyto or pEF-RGS-2 using Fugene6 reagent. Five and a half hours after transfection, the cells were trypsinized and 5×10^4 cells were plated per well in 96-well plates in complete medium (containing 10% FBS) and grown for 4 h. The medium was then replaced with 0.1% serum-containing medium, and cells were incubated for a further 36 h. Treatments were done with either vehicle or the indicated amounts of PTH or forskolin, along with 200 μM IBMX for a period of 1 h. The cells were then lysed and cAMP measurements were performed using a chemiluminescent immunoassay (Tropix, Inc., Bedford, MA).

mRNA Isolation and Northern Blot Analysis

For the analysis of RGS-2 mRNA expression, UMR106 or ROS17/2.8 osteoblastic cells were plated in T150 flasks, allowed to grow to 70–80% confluence, serum-starved overnight (in medium containing 0.1% serum) and then treated with PTH or okadaic acid for the indicated times. Cells from two flasks were pooled, total RNA was isolated using Ultraspec II^{TM} reagent (Biotecx, Houston, TX), and polyA$^+$ RNA was purified using Oligotex resin (Qiagen, Santa Clarita, CA). Two micrograms of polyA$^+$ RNA was used for Northern blot analysis. Radiolabeled rat RGS-2 and GAPDH probes were generated using the Random Primer DNA labeling kit (GIBCO-BRL) with corresponding cDNA templates [Miles et al., 2000]. Prehybridization, hybridization, and washing were carried out as described previously [Miles et al., 2000].

RESULTS

Cloning of the Rat and Mouse RGS-2 Gene Promoters and Analysis of Transcription Factor Binding Sites in the Promoter

We cloned and sequenced 2.3- and 2.8-kb fragments of the 5′-flanking regions of the rat (−2303 to +25) and mouse RGS-2 (−2810 to +31) genes, as described under Materials and Methods. Bioinformatic analysis of the sequences demonstrated the presence of binding sites for many transcription factors that are known to mediate both basal transcription and response to a diverse array of signals. The most notable among these include a noncanonical TATA box (TTAAAA), a CCAAT box, putative Sp1-binding elements, and three OSE2 elements that serve as consensus binding sites for the osteoblast-specific transcription factor, Runx2, an essential factor for osteoblast differentiation and function (Fig. 1) [Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Ducy et al., 1999].

Comparison of the rat and mouse RGS-2 promoter sequences showed them to have 65% identity (Fig. 1). However, the two sequences bear only 45–50% identity to the previously published human RGS-2 promoter sequence [Siderovski et al., 1994]. This is in contrast to

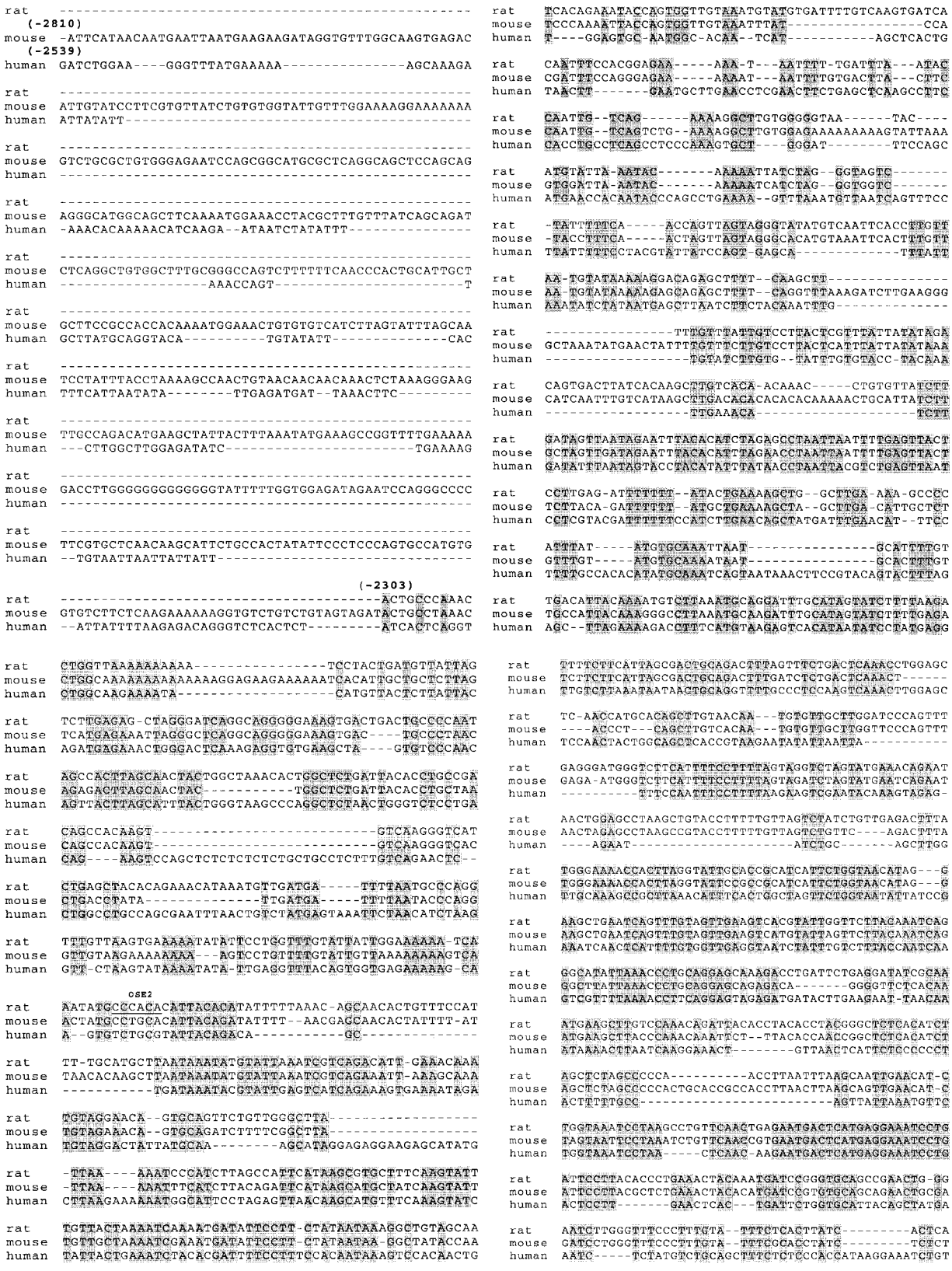

Fig. 1. Cloning of the rat and mouse RGS-2 gene promoters. Sequences of the newly cloned rat (− 2303 to + 25) and mouse (− 2810 to + 31) RGS-2 gene promoters are shown in alignment with the previously published human RGS-2 promoter sequence [Siderovski et al., 1994]. Sequence of the rat RGS-2 gene promoter. Nucleotides that are conserved in all three sequences are shaded. The transcription start site (+ 1), the translation start site (ATG), the TATA box, the CCAAT box, and some of the other key promoter elements are highlighted. Sequence alignments were performed using the GCG program.

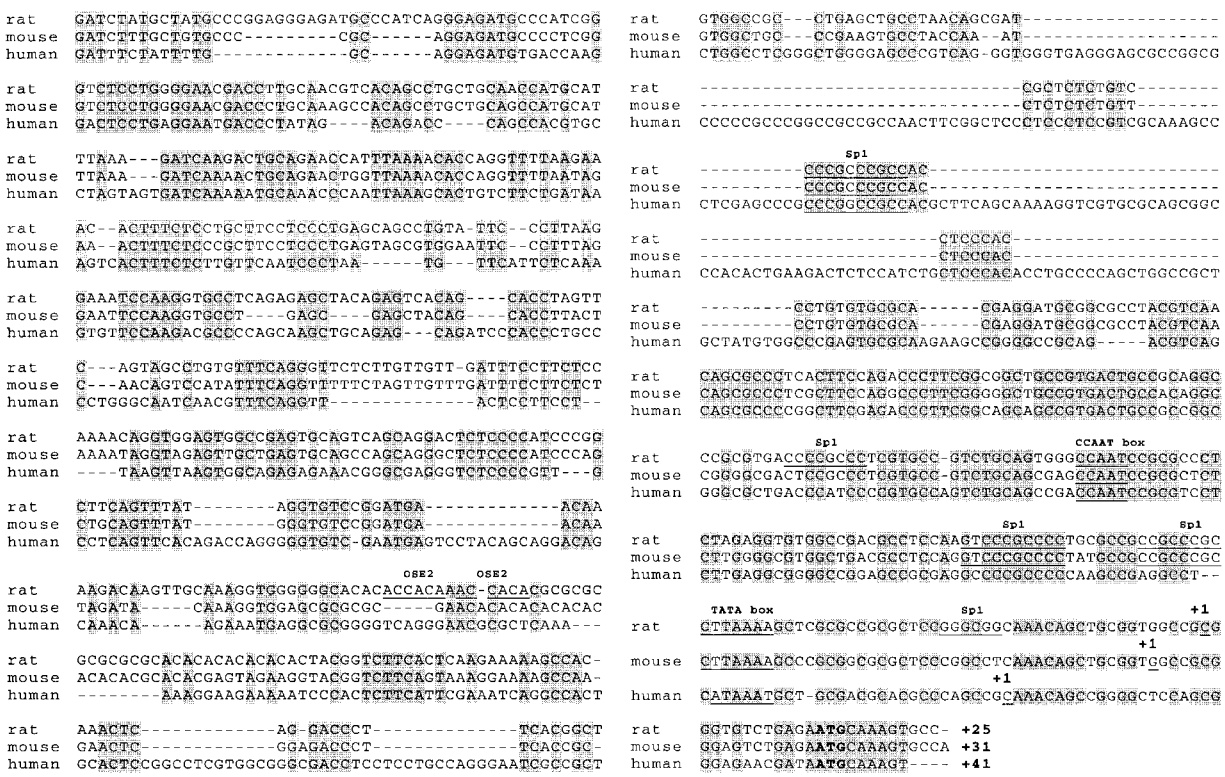

Fig. 1. (*Continued*)

the very high level of conservation in the coding sequences of RGS-2 in all three species (95–97% identity at the amino acid level) [Miles et al., 2000].

Transcriptional Activity of the RGS-2 Promoter

To explore the regulation of the RGS-2 gene, in the rest of this article, we have focused our studies to the functional characterization of the rat RGS-2 promoter. In order to analyze the basal transcriptional activity directed by the 2.3-kb promoter region, we cloned it upstream of the β-gal reporter gene (p2.3RGS-2-β-gal) and performed transient transfection assays in the monkey kidney cell line COS1 [Gulzman, 1981], and in the rat osteoblastic cell lines ROS17/2.8 and UMR106. The β-gal activity in cell extracts was measured 48 h after transfection. As shown in Figure 2, the 2.3-kb promoter directed a high level of expression of the reporter gene in all three cell lines, reminiscent of the widespread expression of RGS-2 (mRNA) in various tissues, including kidney and bone [Miles et al., 2000].

Effect of PTH (1–38) on RGS-2 Expression in Osteoblastic Cells

We have reported earlier that PTH treatment results in a rapid and transient increase in RGS-2 mRNA levels in rat metaphyseal and diaphyseal osteoblasts both in vitro and in vivo, and also in ROS17/2.8 osteoblastic cells. Consistent with these observations, PTH treatment of UMR106 cells also resulted in a rapid and transient increase in RGS-2 mRNA levels at 1 h after treatment, and were back to control levels, thereafter (Fig. 3A). In order to analyze whether PTH has a direct transcriptional effect on RGS-2 expression, we generated a stable line of UMR106 cells containing p2.3RGS-2-β-gal. UMR106 cells were chosen for stable transfection, because of their characteristic clonal stability and their responsiveness to transfection. We screened a total of 40 randomly picked clones for β-gal expression and PTH responsiveness, and identified clones that showed a PTH-dependent increase in β-gal activity, ranging from 1.3- to 3.6-fold. As depicted in Figure 3B, treatment of a representative clone with PTH

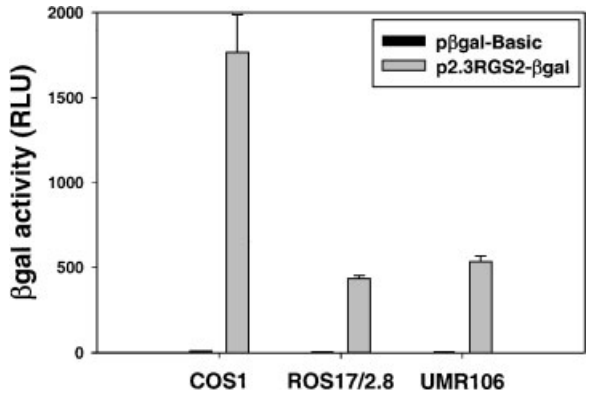

Fig. 2. Basal transcriptional activity directed by the 2.3-kb rat RGS-2 promoter in nonosteoblastic (COS1) and osteoblastic (ROS17/2.8 and UMR106) cell lines. Transient transfection experiments were performed using pβ-gal-Basic or p2.3RGS-2-β-gal in the three cell lines, and the β-gal activity was measured in cell extracts 48 h after transfection. Two independent transfection experiments were performed in triplicate and the mean ± standard error of β-gal activity from a representative experiment is shown.

(1–38) resulted in a 2.2-fold increase in promoter activity at 8 h, reminiscent of the early response observed in the regulation of endogenous RGS-2 mRNA. For this clone, the PTH-induced increase in promoter activity in different experiments ranged from 2.2- to 3.6-fold. Dose–response analysis with PTH (1–38) showed a dose-dependent increase in promoter activity (Fig. 3C). The stimulatory effects of PTH on RGS-2 promoter activity were confirmed in ROS17/2.8 cells that were transiently transfected with p2.3RGS-2-β-gal (data not shown), indicating that the promoter contains *cis*-elements needed for PTH responsiveness.

Analysis of the Putative Signaling Pathways Involved in RGS-2 Promoter Regulation

Our previous studies [Miles et al., 2000] have suggested the involvement of the cAMP/protein kinase A (PKA) pathway in the regulation of RGS-2 mRNA expression by PTH in osteoblastic cells. The binding of PTH to its G-protein coupled PTH receptor (PTH1R in bone) results in the activation of the adenylate cyclase and phospholipase C pathways, and the subsequent activation of protein kinase A and protein kinase C (PKC) [Civitelli et al., 1988, 1989; Juppner et al., 1991; Abou-Samra et al., 1992; Bringhurst et al., 1993; Guo et al., 1995; Lee et al., 1995; Segre et al., 1995; Behar et al., 1996; Huang et al., 1996; Usdin et al., 1996a,b]. To confirm the putative signaling pathways involved in regulating RGS-2 promoter activity, and to analyze whether the promoter contains elements needed for regulation via the cAMP/PKA pathway, we tested the effects of various signal-selective analogs of PTH. Only those analogs of PTH that readily stimulate cAMP production, PTH (1–38) and PTH (1–31) [Fujimori et al., 1991, 1992; Jouishomme et al., 1992,

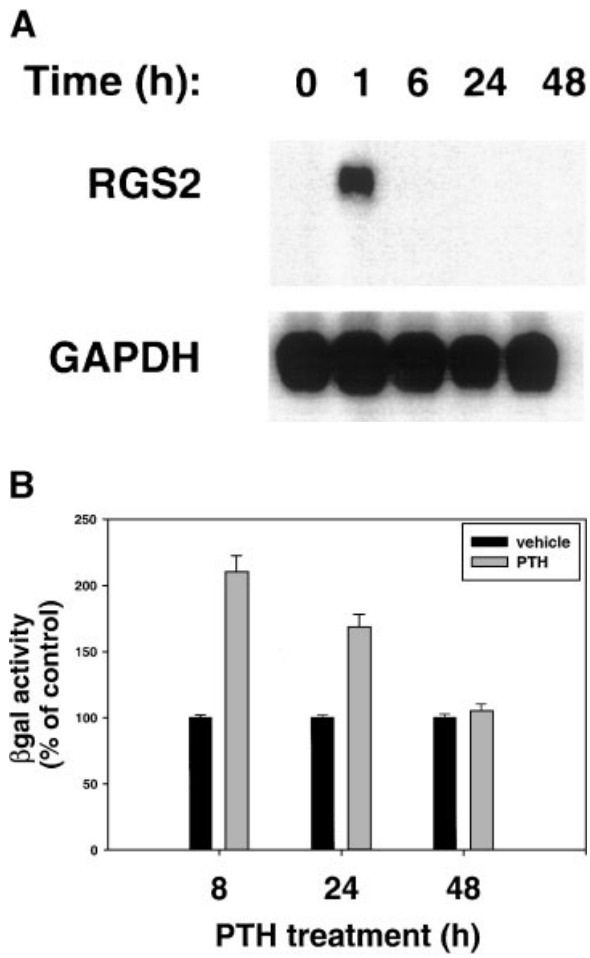

Fig. 3. Effect of PTH (1–38) on RGS-2 expression in osteoblastic cells. **A**: PTH stimulation of RGS-2 mRNA levels in UMR106 cells. Cells were treated with PTH (1–38) (5×10^{-8} M) for the indicated lengths of time. Northern blot analysis was performed using 2 μg of polyA$^+$ RNA and probed with rat RGS-2 and GAPDH-specific probes. **B**: PTH stimulation of RGS-2 promoter activity in a stable clone of UMR106 cells containing the 2.3-kb rat RGS-2 promoter. Cells were treated with PTH (1–38) (5×10^{-7} M) or vehicle for a period of 8, 24, or 48 h. The β-gal activity in cell extracts is shown as a percent of activity in control (vehicle-treated) cells. **C**: Dose-dependent stimulation of RGS-2 promoter activity by PTH in the UMR106 stable clone. Cells were treated with vehicle or a range of concentrations of PTH for a period of 8 h and the β-gal activity measured in cell extracts is shown as a percent of that in control (vehicle-treated cells).

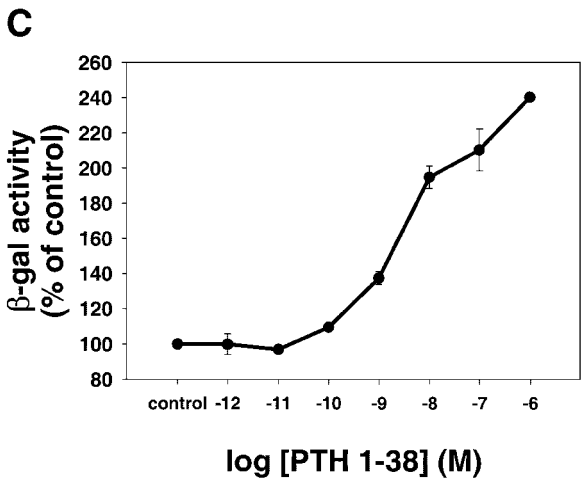

Fig. 3. (*Continued*)

1994; Rixon et al., 1994], and PTHrP (1–34) that also stimulates the cAMP pathway [Juppner et al., 1988, 1991] induced RGS-2 promoter activity, whereas PTH (3–34) and PTH (7–34) that do not readily stimulate cAMP production had no effect on the promoter (Fig. 4A). To further confirm the involvement of the cAMP/PKA pathway in RGS-2 promoter stimulation, we tested the effects of forskolin, a direct activator of adenylate cyclase activity, and IBMX, an inhibitor of cAMP phosphodiesterases. As shown in Figure 4B, treatment with forskolin resulted in a strong dose-dependent increase in RGS-2 promoter activity. Under the same conditions, dideoxy forskolin (dd forskolin), an inactive analog of forskolin, and the vehicle DMSO had no effect on promoter activity. Consistent with the stimulatory effects of forskolin, treatment with IBMX that results in an increase in cAMP accumulation, also led to a dose-dependent increase in RGS-2 promoter activity (Fig. 4C). However, phorbol 12-myristate 13-acetate (PMA), an activator of PKC had no effect on promoter activity (data not shown). Taken together, these results implicate the involvement of the cAMP/PKA pathway in the stimulation of RGS-2 promoter activity in osteoblastic cells.

Effect of Phosphatase Inhibition (Maintenance of a Hyperphosphorylated State) on RGS-2 Promoter Activity

The effects of PKA on gene expression are mediated via the phosphorylation of cAMP-response element binding protein (CREB) and other proteins that in turn bind to response

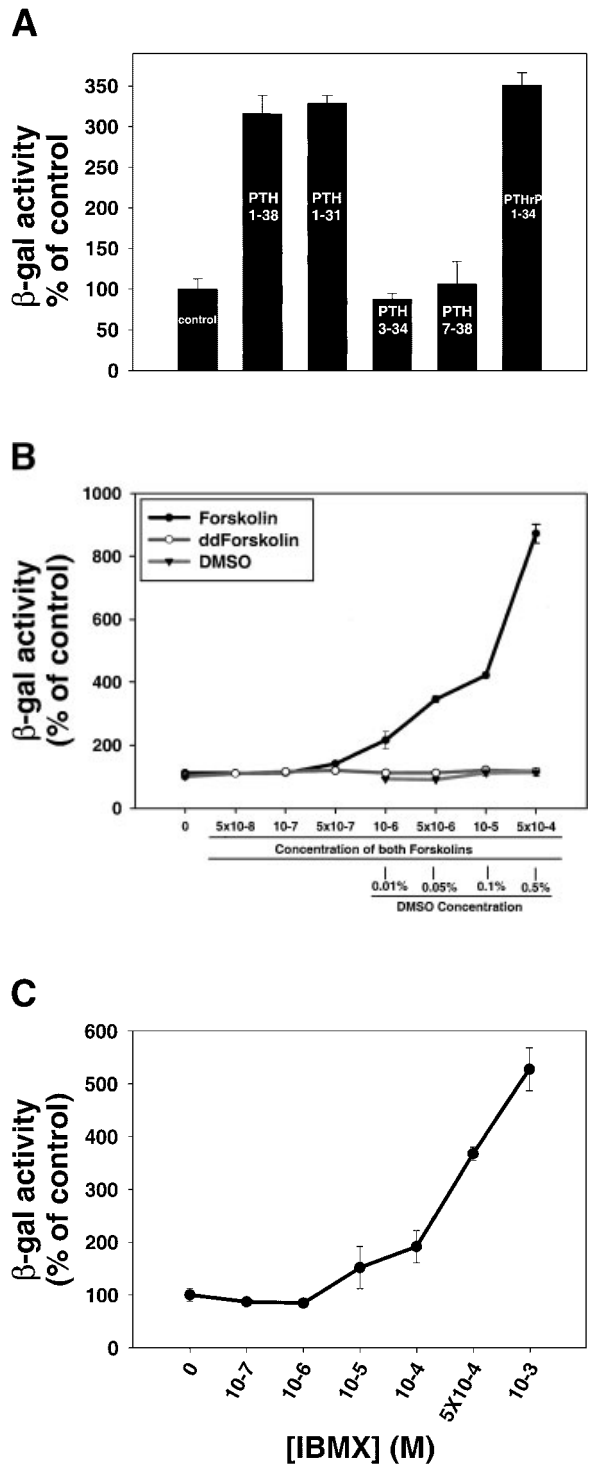

Fig. 4. Stimulation of RGS-2 promoter activity by cAMP-elevating agents. The UMR106 stable clone was treated with various analogs of PTH or with PTHrP (1–34) at a concentration of 10^{-7} M, and the β-gal activity was measured in cell extracts after 8 h of treatment. **B**: Effect of forskolin, dideoxy forskolin (dd forskolin), and vehicle (DMSO) on RGS-2 promoter activity. **C**: Dose-dependent stimulation of the RGS-2 promoter by IBMX. Treatments were done for 8 h, and the data are represented as a percent of activity in vehicle-treated cells.

elements on target gene promoters and activate transcription of those genes. Attenuation of gene transcription occurs via the dephosphorylation of CREB by the PP2A class of protein phosphatases [Wadzinski et al., 1993]. So, we hypothesized that inhibitors of PP2A [Suganuma et al., 1988; Cohen et al., 1990; Goad et al., 1992] that would result in the maintenance of CREB (and possibly other proteins) in the phosphorylated state should lead to an increase in RGS-2 promoter activity. We tested this hypothesis in both ROS17/2.8 and UMR106 cells and obtained similar results except that the magnitude of response was consistently greater in ROS17/2.8 cells. Okadaic acid treatment of p2.3RGS-2-β-gal transfected ROS17/2.8 cells led to a strong dose-dependent increase in RGS-2 promoter activity (Fig. 5). Consistent with this observation, treatment with okadaic acid also resulted in a time-dependent increase in endogenous RGS-2 mRNA levels (Fig. 5 (inset)).

Regulation of RGS-2 Promoter Activity by the Osteoblast-Specific Transcription Factor, Runx2

As shown in Figure 1, analysis of the rat RGS-2 promoter revealed the presence of three perfect OSE2 elements that serve as binding sites for Runx2, an essential factor for osteoblast formation and maturation, and bone formation [Ducy et al., 1997, 1999; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997]. Based on this observation and our recent finding that PTH stimulates the transactivation ability of Runx2 to stimulate new bone formation [Moore et al., 2001], we speculated that Runx2 could regulate RGS-2 promoter activity. To test this hypothesis, we analyzed the effect of Runx2 overexpression on RGS-2 promoter activity. Cotransfection of a Runx2 expression construct along with the p2.3RGS-2-β-gal construct in ROS17/2.8 cells resulted in a 4.0-fold increase in RGS-2 promoter activity, compared to that in empty vector cotransfected cells (Fig. 6). In addition, in COS1 cells that do not express detectable levels of endogenous Runx2 [Kurokawa et al., 1996], overexpression of exogenous Runx2 resulted in a 4.8-fold increase in promoter activity (Fig. 6). This level of induction is substantial considering the fact that the basal transcriptional activity directed by the 2.3-kb promoter is high in both cell lines (Fig. 2). As a control, Runx2 did not significantly transactivate the promoterless reporter construct pβ-gal-Basic [Thirunavukkarasu et al., 2000a]. These results suggest that the RGS-2 promoter could be a potential target of Runx2 action.

In order to determine the mechanism by which Runx2 stimulates RGS-2 promoter activity, we generated sequential 5′ deletions in the

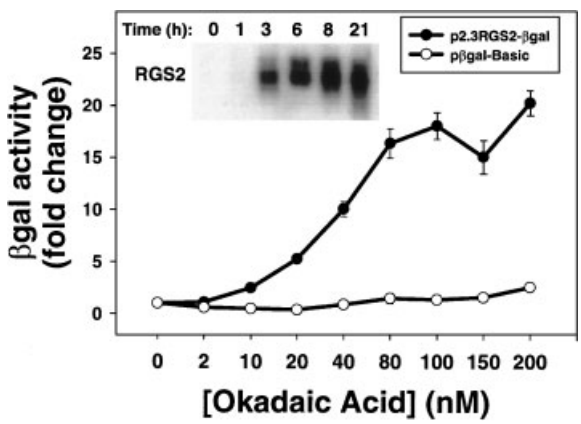

Fig. 5. Effect of phosphatase inhibitors on RGS-2 promoter activity. Dose-dependent stimulation of RGS-2 promoter activity by okadaic acid in ROS17/2.8 cells. Cells transfected with p2.3RGS-2-β-gal were treated with increasing doses of okadaic acid and the β-gal activity was measured in cell extracts after 8 h of treatment. Treatments in promoterless reporter vector (pβ-gal-Basic)-transfected cells served as a control. Inset: Northern blot analysis of mRNA from ROS17/2.8 cells that were treated with 20 nM okadaic acid for the indicated periods of time, showing a time-dependent increase in RGS-2 mRNA levels.

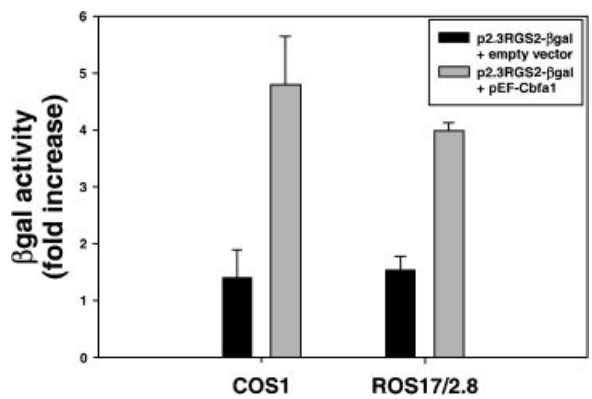

Fig. 6. Regulation of RGS-2 promoter activity by Runx2. To examine the effect of overexpression of Runx2 on RGS-2 promoter activity, COS1 and ROS17/2.8 cells were transiently cotransfected with p2.3RGS-2-β-gal and either the empty vector (pEF/myc/cyto) or the Runx2 expression vector (pEF-Cbfa1). The β-gal activity was measured in cell extracts 48 h after transfection. Two independent transfection experiments were performed in triplicate and the data from a representative experiment is shown.

rat RGS-2 promoter hooked to the β-gal reporter gene. Cotransfection of these promoter constructs along with the Runx2-expression construct (pEF-Cbfa1) showed that promoter fragments that lacked all of the OSE2 elements were still responsive to Runx2 (data not shown), suggesting that Runx2 transactivates the RGS-2 promoter probably via an indirect mechanism or through a novel *cis*-element in the proximal promoter.

Effect of RGS-2 Overexpression on PTH Signaling

Since RGS proteins are known to be involved in downregulating GPCR signaling [De Vries et al., 2000], we analyzed whether the PTH-stimulated increase in RGS-2 expression could in turn contribute to the termination of PTH receptor signaling in a negative feedback loop fashion. UMR106 cells were transfected with either an RGS-2 expression construct or a control construct, and were subsequently treated with vehicle, or the indicated concentrations of PTH (1–38). Measurement of cAMP levels in cell extracts showed that RGS-2 overexpression resulted in a ~55% reduction in the PTH-induced accumulation of cAMP (Fig. 7A), suggesting that RGS-2 could negatively regulate PTH activity mediated via the $G\alpha_s$-adenylate cyclase-protein kinase A pathway. To evaluate whether RGS-2 exerts its effects by accelerating the GTPase activity of $G\alpha_s$ or by inhibiting the activity of adenylate cyclase, the transfected cells were treated with forskolin, a direct stimulator of adenylate cyclase. Interestingly, RGS-2 overexpression also resulted in a ~55% reduction in forskolin-stimulated increase in cAMP levels (Fig. 7B), consistent with recent observations in olfactory epithelium membranes, in which RGS-2 inhibits the activity of adenylate cyclase type III [Sinnarajah et al., 2001].

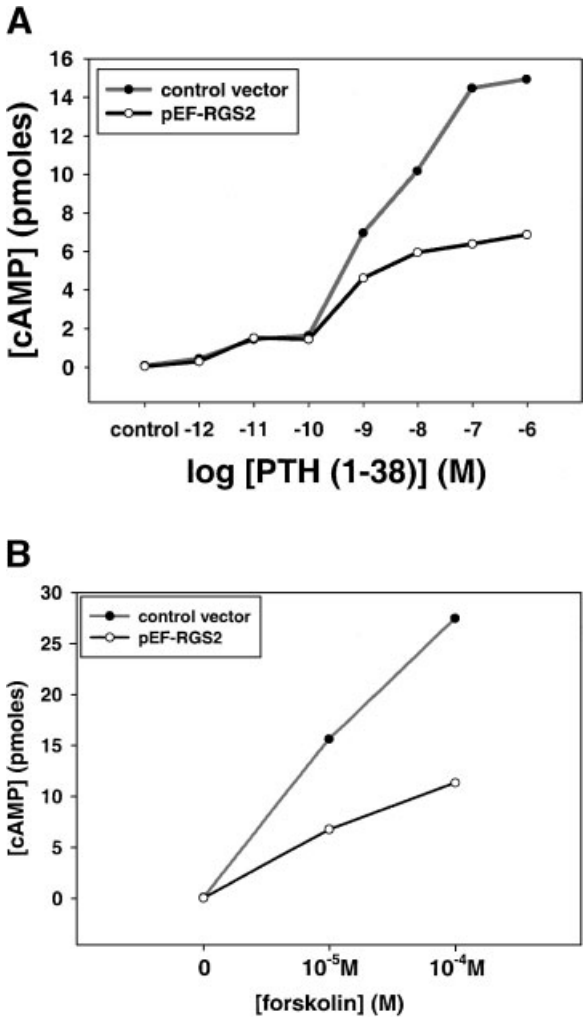

Fig. 7. Effect of RGS-2 overexpression on PTH signaling. To examine the effect of RGS-2 overexpression on PTH and forskolin-induced cAMP production, UMR106 osteoblast-like cells were transiently transfected with either the control vector (pEF/myc/cyto) or the rat RGS-2 expression vector (pEF-RGS-2), as described in the Materials and Methods section. Approximately 48 h after transfection, the cells were treated with the indicated concentrations of PTH (1–38) (**A**) or forskolin (**B**), along with 200 μM IBMX (eight wells per treatment). Cell extracts were prepared 1 h after treatment and the mean values of cAMP measured using a chemiluminescent immunoassay are shown.

DISCUSSION

RGS proteins play a major role in downregulating signaling mediated by GPCRs. They exert their negative regulatory effects by serving as GAPs for Gα subunits or by acting as effector antagonists by physically blocking G-protein-effector interactions [Hepler, 1999; Melliti et al., 2000; Sinnarajah et al., 2001]. The mRNA levels of different RGS proteins are known to be regulated in response to a wide variety of signals [Dietzel and Kurjan, 1987; Hong et al., 1993; Dohlman et al., 1995; Druey et al., 1996; Buckbinder et al., 1997; Burchett et al., 1998; Ingi et al., 1998; Pepperl et al., 1998; Tseng and Zhang, 1998; Song et al., 1999]. The resultant increase in RGS protein levels are predicted to result in a negative feedback loop leading to the termination of signaling [Druey et al., 1996; Tseng and Zhang, 1998; Song et al., 1999]. We reported recently that PTH treatment results in an increase in RGS-2 mRNA

levels in rat bone. However, there is limited data concerning the regulation of RGS-2 and other RGS genes at the promoter level [Buckbinder et al., 1997; Chatterjee et al., 1997]. In the present study, we have cloned and characterized the rat and mouse RGS-2 gene promoters, and provided evidence that PTH, other cAMP-elevating agents, okadaic acid, and the osteoblast-specific transcription factor, Runx2, stimulate RGS-2 gene transcription in rat osteoblastic cells.

The rat and mouse promoter sequences show a high level of evolutionary conservation (65% identity), but they share only 45–50% identity with the human RGS-2 promoter sequence, with stretches of similarity spread along the length of the promoter. Using promoter-reporter constructs, we have shown that PTH treatment results in a direct transcriptional activation of the rat RGS-2 gene. Based on treatments with signal-selective analogs of PTH, we have implicated the involvement of the cAMP/PKA pathway in mediating PTH stimulation of the RGS-2 promoter. This finding was confirmed by the stimulatory effects observed with forskolin and IBMX that induce cAMP accumulation, and the lack of an effect by direct activators of PKC in osteoblastic cells [Miles et al., 2000] (data not shown). The role for cAMP in stimulating RGS-2 mRNA expression has been demonstrated in PC12 pheochromocytoma cells [Pepperl et al., 1998] and in GIP-treated βTC3 pancreatic cells [Tseng and Zhang, 1998]. In contrast, muscarinic receptor stimulation in human neuroblastoma cells has been shown to increase RGS-2 mRNA levels through a PKC-dependent mechanism [Song et al., 1999].

The rapid increase in RGS-2 mRNA levels observed upon PTH treatment in vivo [Miles et al., 2000] is paralleled by an increase in RGS-2 promoter activity in vitro at an early time point (8 h) and a subsequent decline at later time points (24 and 48 h) (we allow at least an 8 h period in order to facilitate the β-gal protein to be synthesized and available in sufficient amounts). This confirms that the rapid effects of PTH on RGS-2 expression occur, at least in part, through changes in transcriptional initiation. Such a rapid increase in RGS-2 levels would be predicted to be important in modulating the delicate kinetics required for implementing PTH effects. It is conceivable that stabilization of the RGS-2 mRNA upon PTH treatment may also contribute to the increase in steady state mRNA levels, and may in part explain the difference in the magnitude of response observed at the promoter vs. the mRNA. Also, the possibility exists that the 2.3-kb fragment that we have studied may not constitute the entire promoter region that is needed for in vivo function (in the chromosomal context). However, the fact that the proximal promoter region typically contains most of the regulatory elements in a vast majority of genes, combined with our observation that the 2.3-kb fragment contains elements needed for regulation by okadaic acid and Runx2 (in addition to PTH) would suggest the presence of most, if not all, of the elements needed for in vivo regulation.

Consistent with the widespread expression of RGS-2 mRNA [Miles et al., 2000], the 2.3-kb promoter was functional in all the cell lines we have tested (ROS17/2.8 and UMR106 osteoblastic cells, and COS1 kidney cells). In spite of the high basal levels of expression in ROS17/2.8 and COS1 cells, okadaic acid and Runx2 were still able to significantly stimulate the transcriptional activity of the promoter in these two cell lines. The strong dose-dependent stimulatory effects of okadaic acid implicate the involvement of the PP2A class of protein phosphatases in regulating basal and induced levels of RGS-2 expression. Our data suggest the possibility that maintenance of the phosphorylated form of CREB (a substrate of PP2A) could result in enhanced RGS-2 expression. However, we did not find any consensus CREB-binding element in the rat RGS-2 promoter sequence, implying that the effects of CREB on the RGS-2 promoter are probably indirect. In addition, the possibility exists that okadaic acid (and probably the other inhibitors and agonists used in the study) could also stimulate other signaling pathways that directly and/or indirectly affect promoter activity.

Since, we noted the presence of putative Runx2-binding elements (OSE2) in the RGS-2 promoter, and based on the observation that Runx2 is a potential molecular mediator of PTH anabolic actions in bone [Moore et al., 2001], we speculated that Runx2 could mediate, at least in part, the stimulatory effects of PTH on the RGS-2 promoter. Indeed, overexpression of Runx2 resulted in a four-fold increase in RGS-2 promoter activity in ROS17/2.8 cells and a 4.8-fold increase in the nonosteoblastic COS1 cells that

do not express detectable levels of endogenous Runx2 [Kurokawa et al., 1996]. This suggests that the bone-selective upregulation of RGS-2 by PTH may be mediated, at least in part, by the action of Runx2. Sequential 5′-deletion analysis showed that promoter fragments that lacked all of the OSE2 elements were still responsive to Runx2 (data not shown), suggesting that Runx2 transactivates the RGS-2 promoter probably via an indirect mechanism or through a novel *cis*-acting element in the proximal promoter.

The increase in RGS-2 expression upon PTH treatment is thought to be involved in the desensitization of PTH receptor signaling. RGS-2 protein has been shown to interact with and inhibit either $G\alpha_s$- or $G\alpha_q$-mediated signaling in different systems [Heximer et al., 1997, 1999; Tseng and Zhang, 1998]. Since PTH is known to activate multiple signaling pathways that are mediated by the interaction of $G\alpha_s$ and $G\alpha_q$ subunits with the receptor, it is likely that the increase in RGS-2 levels could negatively regulate either or both of the signaling pathways. Our observation that overexpression of RGS-2 leads to a significant decrease in PTH-induced cAMP accumulation, suggests the involvement of RGS-2 in downregulating the $G\alpha_s$-adenylate cyclase pathway. It remains to be determined whether RGS-2 plays any role in downregulating the $G\alpha_q$-phospholipase C pathway. Recent studies demonstrate that RGS-2 can function as an effector antagonist and attenuate the activation of adenylate cyclase and/or phospholipase C [Melliti et al., 2000; Sinnarajah et al., 2001]. Interestingly, we have shown that overexpression of RGS-2 also results in a significant decline in forskolin-induced accumulation of cAMP, which would be consistent with the inhibition of adenylate cyclase activity by RGS-2, as suggested by Sinnarajah et al. [2001].

In order to investigate the role of RGS-2 in vivo, *RGS-2*-deficient mice were generated recently [Oliveira-Dos-Santos et al., 2000]. RGS-2 deficiency resulted in impaired T-cell activation and neuronal function, reduced male aggressive behavior, and increased anxiety, behaviors that can be regulated by multiple GPCRs. It would be interesting to analyze the bone phenotype in these animals, and to examine the regulation of PTH signaling in osteoblasts in these animals.

To summarize, we have cloned and functionally characterized the rat RGS-2 gene promoter, and have shown that PTH treatment results in a direct transcriptional stimulation of the RGS-2 gene. Using functional studies involving overexpression of RGS-2, we have demonstrated an inhibitory effect for RGS-2 on PTH- and forskolin-induced cAMP accumulation in UMR106 cells. This suggests the possibility that the PTH-induced increase in RGS-2 levels could result in a negative feedback inhibition of PTH signaling. The 2.3- and 2.8-kb promoter sequences of the rat and mouse RGS-2 genes that we have reported here would serve as valuable tools for further research on RGS-2 gene regulation, and would also enable us to understand the mechanism of PTH signal termination.

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