Continuous Activation of Gαq in Osteoblasts Results in Osteopenia through Impaired Osteoblast Differentiation*

Received for publication, December 29, 2006, and in revised form, September 5, 2007 Published, JBC Papers in Press, September 5, 2007, DOI 10.1074/jbc.M611902200

Naoshi Ogata‡§, Hiroshi Kawaguchi‡∥, Ung-il Chung¶, Sanford I. Roth‡, and Gino V. Segre‡‡

From the ‡Endocrine Unit and ¶Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114 and §Sensory & Motor System Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113-8655, Japan

We explored the role of Gαq-mediated signaling on skeletal homeostasis by selectively expressing a constitutively active Gαq (mutation of Q209L) in osteoblasts. Continuous signaling via Gαq in mouse osteoblastic MC3T3-E1 cells impaired differentiation. Mice that expressed the constitutively active Gαq transgene in cells of the osteoblastic lineage exhibited severe osteopenia in cortical and trabecular bones. Osteoblast number, bone volume, and trabecular thickness were reduced in transgenic mice, but the osteoclasts were unaffected. Osteoblasts from transgenic mice showed impaired differentiation and matrix formation. In the presence of a protein kinase C inhibitor GF109203X, this impairment was not seen, indicating media-

We first studied the effect of constitutively active Gαq signaling on osteoblast differentiation by overexpressing the epitope-tagged (HA), constitutively active mutant (Q209L) of the Gαq subunit (HA-CA-Gαq) in vitro in osteoblastic cells. Next, to study the role of Gαq signaling in vivo, we used mice that expressed a transgene encoding HA-CA-Gαq under the control of 2.3-kb type I collagen α1 chain (Col.1) promoter (10), thus limiting expression nearly exclusively to osteoblasts. This study demonstrated that Gαq-mediated activation of PKC in osteoblasts plays a crucial role in bone formation by inhibiting the differenti-

We explored the role of Gαq-mediated signaling on skeletal homeostasis by selectively expressing a constitutively active Gαq (mutation of Q209L) in osteoblasts. Continuous signaling via Gαq in mouse osteoblastic MC3T3-E1 cells impaired differentiation. Mice that expressed the constitutively active Gαq transgene in cells of the osteoblastic lineage exhibited severe osteopenia in cortical and trabecular bones. Osteoblast number, bone volume, and trabecular thickness were reduced in transgenic mice, but the osteoclasts were unaffected. Osteoblasts from transgenic mice showed impaired differentiation and matrix formation. In the presence of a protein kinase C inhibitor GF109203X, this impairment was not seen, indicating media-

Continuous Activation of Gαq in Osteoblasts Results in Osteopenia through Impaired Osteoblast Differentiation*

Received for publication, December 29, 2006, and in revised form, September 5, 2007 Published, JBC Papers in Press, September 5, 2007, DOI 10.1074/jbc.M611902200

Naoshi Ogata‡§, Hiroshi Kawaguchi‡∥, Ung-il Chung¶, Sanford I. Roth‡, and Gino V. Segre‡‡

From the ‡Endocrine Unit and ¶Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114 and §Sensory & Motor System Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113-8655, Japan

We explored the role of Gαq-mediated signaling on skeletal homeostasis by selectively expressing a constitutively active Gαq (mutation of Q209L) in osteoblasts. Continuous signaling via Gαq in mouse osteoblastic MC3T3-E1 cells impaired differentiation. Mice that expressed the constitutively active Gαq transgene in cells of the osteoblastic lineage exhibited severe osteopenia in cortical and trabecular bones. Osteoblast number, bone volume, and trabecular thickness were reduced in transgenic mice, but the osteoclasts were unaffected. Osteoblasts from transgenic mice showed impaired differentiation and matrix formation. In the presence of a protein kinase C inhibitor GF109203X, this impairment was not seen, indicating media-

We first studied the effect of constitutively active Gαq signaling on osteoblast differentiation by overexpressing the epitope-tagged (HA), constitutively active mutant (Q209L) of the Gαq subunit (HA-CA-Gαq) in vitro in osteoblastic cells. Next, to study the role of Gαq signaling in vivo, we used mice that expressed a transgene encoding HA-CA-Gαq under the control of 2.3-kb type I collagen α1 chain (Col.1) promoter (10), thus limiting expression nearly exclusively to osteoblasts. This study demonstrated that Gαq-mediated activation of PKC in osteoblasts plays a crucial role in bone formation by inhibiting the differenti-

We explored the role of Gαq-mediated signaling on skeletal homeostasis by selectively expressing a constitutively active Gαq (mutation of Q209L) in osteoblasts. Continuous signaling via Gαq in mouse osteoblastic MC3T3-E1 cells impaired differentiation. Mice that expressed the constitutively active Gαq transgene in cells of the osteoblastic lineage exhibited severe osteopenia in cortical and trabecular bones. Osteoblast number, bone volume, and trabecular thickness were reduced in transgenic mice, but the osteoclasts were unaffected. Osteoblasts from transgenic mice showed impaired differentiation and matrix formation. In the presence of a protein kinase C inhibitor GF109203X, this impairment was not seen, indicating media-

We first studied the effect of constitutively active Gαq signaling on osteoblast differentiation by overexpressing the epitope-tagged (HA), constitutively active mutant (Q209L) of the Gαq subunit (HA-CA-Gαq) in vitro in osteoblastic cells. Next, to study the role of Gαq signaling in vivo, we used mice that expressed a transgene encoding HA-CA-Gαq under the control of 2.3-kb type I collagen α1 chain (Col.1) promoter (10), thus limiting expression nearly exclusively to osteoblasts. This study demonstrated that Gαq-mediated activation of PKC in osteoblasts plays a crucial role in bone formation by inhibiting the differenti-

Experimental Procedures

Cell Culture—Mouse osteoblastic MC3T3-E1 cells were cultured in α-minimal essential medium (αMEM), 10% FBS, and 1% penicillin/streptomycin (Invitrogen). In experiments

Continuous Activation of Gαq in Osteoblasts Results in Osteopenia through Impaired Osteoblast Differentiation*

Received for publication, December 29, 2006, and in revised form, September 5, 2007 Published, JBC Papers in Press, September 5, 2007, DOI 10.1074/jbc.M611902200

Naoshi Ogata‡§, Hiroshi Kawaguchi‡∥, Ung-il Chung¶, Sanford I. Roth‡, and Gino V. Segre‡‡

From the ‡Endocrine Unit and ¶Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114 and §Sensory & Motor System Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113-8655, Japan

We explored the role of Gαq-mediated signaling on skeletal homeostasis by selectively expressing a constitutively active Gαq (mutation of Q209L) in osteoblasts. Continuous signaling via Gαq in mouse osteoblastic MC3T3-E1 cells impaired differentiation. Mice that expressed the constitutively active Gαq transgene in cells of the osteoblastic lineage exhibited severe osteopenia in cortical and trabecular bones. Osteoblast number, bone volume, and trabecular thickness were reduced in transgenic mice, but the osteoclasts were unaffected. Osteoblasts from transgenic mice showed impaired differentiation and matrix formation. In the presence of a protein kinase C inhibitor GF109203X, this impairment was not seen, indicating media-

We first studied the effect of constitutively active Gαq signaling on osteoblast differentiation by overexpressing the epitope-tagged (HA), constitutively active mutant (Q209L) of the Gαq subunit (HA-CA-Gαq) in vitro in osteoblastic cells. Next, to study the role of Gαq signaling in vivo, we used mice that expressed a transgene encoding HA-CA-Gαq under the control of 2.3-kb type I collagen α1 chain (Col.1) promoter (10), thus limiting expression nearly exclusively to osteoblasts. This study demonstrated that Gαq-mediated activation of PKC in osteoblasts plays a crucial role in bone formation by inhibiting the differenti-

Experimental Procedures

Cell Culture—Mouse osteoblastic MC3T3-E1 cells were cultured in α-minimal essential medium (αMEM), 10% FBS, and 1% penicillin/streptomycin (Invitrogen). In experiments
Inhibitory Effect of $\alpha_q$ Signal on Osteoblast Differentiation

assessing differentiation, medium was supplemented with $\beta$-glycerophosphate and ascorbic acid (Sigma). Fresh medium was added twice per week.

Stable Transfection—MC3T3-E1 cells were plated at a density of $3 \times 10^4$ cells/cm$^2$. After 24 h, these cells were transfected with the HA-tagged, constitutively active $\alpha_q$ (MC3T3-E1-$\alpha_q$) cloned into pcDNA3.1 (Invitrogen) or with the empty vector (MC3T3-E1-EV) using calcium phosphate precipitation. Replacing amino acids 125–130 in $\alpha_q$ with part of the HA epitope does not interfere with its function (9). After 24 h, and every 48 h thereafter, the medium was replaced with fresh medium containing 200 $\mu$g/ml of hygromycin. By limiting dilution, more than 200 clones of MC3T3-E1-$\alpha_q$ and more than 50 clones of MC3T3-E1-EV were established; clones were selected for further study based on measurement of total inositol phosphates as described previously (11).

Alkaline Phosphatase (ALP) Activity—MC3T3-E1-EV and MC3T3-E1-$\alpha_q$ clones were plated at a density of 2 $\times$ 10$^4$ cells/cm$^2$ and cultured for periods ranging from 3 to 21 days. At various intervals, the ALP activity in cell lysates was assessed in assay buffer (50 mM Tris-HCl (pH 7.6), and 0.1% Triton X-100) containing 1.5 $\mu$M 2-amino-2-methyl-1-propanol for 30 min at 37 °C using p-nitrophenyl phosphate as a substrate. The release of p-nitrophenol was monitored by measuring absorbance at 405 nm.

Generation of Transgenic (TG) Mice—A DNA fragment containing the 2.3-kb osteoblast-specific promoter region of the mouse Col.1 was inserted into KpnI/XbaI site of the intermediate vector to generate the final expression construct. The ends of this fragment were made blunt with Klenow polymerase and ligated to a blunt-ended BamHI site in the pcDNAI plasmid that contained a 1.1-kb cDNA fragment that encoded the entire coding sequence for HA epitope-tagged, constitutively active $\alpha_q$ protein $\alpha$ subunit (HA-$\alpha_q$) (9). Restriction endonuclease digestions and nucleotide sequence analysis confirmed the correct orientation of the construct. The fragment from the final construct, including the 2.3-kb Col1 promoter and HA-$\alpha_q$, was purified according to standard techniques and injected into the pronuclei of fertilized eggs from FVB/N mice by the Massachusetts General Hospital Transgenic Facility (Boston). Founder mice of the FVB/N strain were mated with WT mice to establish individual transgenic lines. The cDNA encoding for mouse HA-$\alpha_q$ (Q209L) was provided by E. J. Neer (Brigham and Women’s Hospital and Harvard Medical School, Boston). The 2.3-kb osteoblast-specific promoter region for the mouse Col1 was provided by B. de Crombrugghe (The University of Texas, Houston). All mice were maintained according to the institutional animal care committee.

Screening for TG Mice by the PCR—To screen for TG animals, we performed PCR using TaqDNA polymerase (Promega Corp., Madison, WI) and 100–200 ng of DNA prepared from mice tails using the DNeasy tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s directions. The PCR was performed for 40 cycles using the primer pairs encompassing nucleotides 397–421 (GAGTCCTCAGACTACGCGGCTTAC) of the HA-$\alpha_q$ cDNA, including an HA tag and nucleotides 579–599 (TATTCCGATGATCCTGTAGTG) of the $\alpha_q$ cDNA with the thermal cycle set at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min.

Transgene mRNA Expression—To investigate tissue-specific expression of the transgene, we reverse-transcribed total cellular RNA from mouse tissues and then performed PCR (RT-PCR). The reverse transcription reaction was performed with Superscript reverse transcriptase (Invitrogen) and oligo(dt) primers using 2 $\mu$g of total cellular RNA prepared with the TRIzol reagent (Invitrogen) according to the manufacturer’s directions. Prior to the PCR, total cellular RNA was treated with DNase (Invitrogen) according to the manufacturer’s directions. The reverse transcription reaction was performed using Superscript reverse transcriptase (Invitrogen) and oligo(dt) primers according to the manufacturer’s directions. PCR was performed for 30 cycles using hstQ DNA polymerase (Promega) and primer pairs encompassing nucleotides 397–421 (GAGTCCTCAGACTACGCGGCTTAC) of the HA-$\alpha_q$ cDNA, including an HA tag and nucleotides 579–599 (TATTCCGATGATCCTGTAGTG) of the $\alpha_q$ cDNA with the thermal cycler set at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min. Control PCRs were performed for 30 cycles using glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5’-CATGTAGGCACTTGCCACAC-3’ and 5’-TGAAGTCGGTGTGAACGGATTTGGC-3’, with the thermal cycler set at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min. PCR products were separated on 1.2% agarose gels and visualized by staining with ethidium bromide.

Serology—Serum was collected at 1 month of age for ionized calcium. Ionized calcium was measured by the 634 Ca$^{2+}$/pH analyzer (Ciba-Corning Diagnostics Corp., Medfield, MA). Intact immunoreactive PTH was measured in duplicate using enzyme-linked immunosorbent assay (Immutopics Inc., San Clemente, CA), which uses two affinity-purified polyclonal antibodies raised to peptides common to rat and mouse PTH.

Analysis of Bone Morphology—Bone radiographs of the excised femora and tibiae from 8-week-old WT and TG mice were taken with a soft x-ray apparatus (type SRO-M50; Sofron, Tokyo, Japan). Three-dimensional computed tomography scans were reconstructed using a composite x-ray analyzing system (NS-ELEX, Tokyo, Japan). Bone mineral density (BMD) was measured by single energy x-ray absorptiometry utilizing a bone mineral analyzer (DCS-600R, Aloka Co., Tokyo, Japan). For histological analyses, mice were killed at birth, 2, 4, and 8 weeks of age, and tibial and calvarial bone sections (5 $\mu$m) were fixed in 4% paraformaldehyde, 0.1 M PBS, decalcified by 10% EDTA, embedded in paraffin, and stained with hematoxylin and eosin. Collagen fiber deposition was assessed under polarized light in tibial and calvarial bone sections (5 $\mu$m) that had been fixed in 4% paraformaldehyde, 0.1 M PBS, decalcified by 10% EDTA, embedded in paraffin, and stained with hematoxylin and eosin. For the assessment of dynamic histomorphometric indices, mice were injected twice with calcein at a dose of 0.16 mg/10 g body weight and analyzed at 4 or 8 weeks of age. The 4-week group received dual injections 6 and 3 days before sacrifice, and the 8-week group received them 10 and 3 days before sacrifice. Long bones were fixed with ethanol, and the undecalced bones were embedded in glycolmethacrylate. Three-$\mu$m longitudinal sections from the proximal tibiae and
20-μm cross-sections from mid-diaphyses of femora were stained with toluidine blue and analyzed using a semi-automated system (Osteoplan II; Zeiss). Parameters for trabecular bone were measured in an area 1.2 mm in length from 0.1 mm below the growth plate at the proximal metaphysis of the tibiae. Parameters for cortical bone were measured at the midshaft of the tibiae.

In Situ Hybridization—In situ hybridizations were performed as described (12) using complementary 35S-labeled riboprobes (complementary RNAs and cRNAs) transcribed from the plasmids encoding human Col.I and mouse osteocalcin.

Ex Vivo Cell Cultures—Osteoblastic cells were isolated from calvariae of neonatal (1–2 days old) WT and TG mice as described previously (13). Calvariae were digested at 37 °C for 10 min in an enzyme solution containing 0.1% collagenase and 0.2% dispase in αMEM for five cycles, and cells isolated in the last four digestions were combined as osteoblastic cells. Bone marrow cells were collected from long bones of 8-week-old mice and were plated at a density of 10⁴ cells on a 6-multwell plate. Osteoblastic cells and marrow cells were cultured in αMEM containing 10% FBS, 50 mg/ml ascorbic acid, and 1% penicillin/streptomycin. To assess mineralization, medium was supplemented with 0.1% collagenase and 0.5% Triton X-100. ALP activity in the lysate was measured by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. The protein content was determined using BCA protein assay reagent (Pierce). For measurement of mineralized matrix formation, primary osteoblastic cells were inoculated at a density of 1 × 10⁴ cells/well in a 24-multwell plate, and cultured in the same medium for 3, 6, 9, and 12 days. [3H]Thymidine (1 mCi/ml of medium) was added for the final 3 h of incubation after 24 h of culture, and incorporation was measured. To measure ALP activity, primary osteoblastic cells were inoculated at a density of 2 × 10⁴ cells/well in a 24-multwell plate and cultured in the same medium in the absence or presence of PKC inhibitor, GF109203X (100 ng/ml) (Calbiochem-Novabiochem). After 14 days of culture, the cells were washed with PBS and sonicated in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl₂ and 0.5% Triton X-100. ALP activity in the lysate was measured by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. The protein content was determined using BCA protein assay reagent (Pierce). For measurement of mineralized matrix formation and calcium deposition, primary osteoblastic cells were plated at a density of 2 × 10⁵ cells/cm² and cultured for 28 days. The formation of mineralized matrix nodules was assessed by Alizarin red staining, and the Alizarin red dye was eluted and measured spectrophotometrically to quantify calcium.

Northern Blotting and RT-PCR—For Northern blot analysis, osteoblasts were incubated at a density of 2 × 10⁴ cells per dish in 100-mm dishes and cultured in αMEM containing 10% FBS with 50 mg/ml ascorbic acid. Total RNA was extracted using a TRizol kit (Invitrogen). Ten μg of total RNA was electrophoresed in 1.2% agarose-formaldehyde gels and transferred onto nylon membrane filters (Hybond-N; Amersham Biosciences). cDNAs were labeled with [32P]dCTP by a random primed labeling kit (Amersham Biosciences) according to the manufacturer’s protocol. The membranes were hybridized for 2 h at 68 °C with cDNA probes for mouse Runx2, Col.I, osteocalcin, RANKL, and osteoprotegerin, prepared from the plasmids described previously. The membranes were then washed and analyzed by a Cyclone PhosphorImager (Hewlett-Packard, Palo Alto, CA). Control hybridization with G3PDH verified that equal amounts of RNA had been loaded. Semi-quantitative RT-PCR was performed within the exponential amplification range to assess for expression of the transgene. Total mRNA (1 μg) was reverse-transcribed, and PCR was performed using specific primer pairs for transgene and G3PDH described above.

Statistical Analysis—All data are expressed as means ± S.E. Means of groups were compared by analysis of variance, and significance of differences was determined by post hoc testing using the Bonferroni method.

RESULTS

Constitutive Signaling by Gαq Inhibits Differentiation of MC3T3-E1 Cells—We initially evaluated whether expression of the constitutively active Gαq subunit affected proliferation and/or differentiation of MC3T3-E1 cells. MC3T3-E1 cells were chosen because they are “pre-osteoblasts”; specifically, they differentiate into mature osteoblasts and form mineralized nodules under a defined culture condition.

We have established more than 200 MC3T3-E1 cell clones in total that stably overexpressed Gαq and the range of increases in the protein level was 1.4–5.0-fold. Among them, three clones (Gαq clones -29, -135, and -155) with ~2-fold higher Gαq protein expression were selected, because they also caused higher levels of PKC activity determined by the total inositol phosphate uptake than those of three control clones transfected with the empty vector (EV-1, -2, and -3) (Fig. 1A). Interestingly, most of the clones that expressed more than 3-fold of the Gαq protein neither showed higher uptake of total inositol phosphate nor did they grow well, resulting in abnormal morphological changes of cell shape (data not shown).

Proliferation of the selected three MC3T3-E1-Gαq and the MC3T3-E1-EV cell lines was indistinguishable within each group and between groups, as assessed by counting cells at regular intervals; cell morphology also was not notably different (data not shown). However, the differentiation of the MC3T3-E1-Gαq cells determined by ALP activity was significantly lower during 21 days of culture (Fig. 1B), and a mineralized bone nodule formation determined by Alizarin red staining was also suppressed after 28 days (Fig. 1C), as compared with MC3T3-E1-EV clones. Additionally, mRNA levels of osteoblast differentiation markers Runx2, Col.I, and osteocalcin were lower in MC3T3-E1-Gαq clones than in MC3T3-E1-EV clones (Fig. 1D). Because the differences were more conspicuous at the earlier days than at 14 days, the Gαq-mediated signaling was likely to affect not only matrix formation by mature osteoblasts but also early stages of osteoblast differentiation.

Generation of Mice Overexpressing Constitutively Active Gαq in Osteoblastic Cells—We then generated mice that express the constitutively active mutant of Gαq in cells of the osteoblast lineage, by controlling expression using the 2.3-kb proximal promoter of Col.I gene (Fig. 2A). Independent transgenic lines were established from two male founders, and indistinguishable results were obtained from analyses of the progeny from both founder lines. Bone-specific expression of the transgene was confirmed by reverse transcription of total RNA, after it had been extracted from numerous tissues, followed by PCR using HA epitope-specific sense and Gαq antisense primers.
A total inositol phosphates and Western blotting using an anti-Gαq antibody in selected stable MC3T3-E1 cell lines that express either constitutively active Gαq (-29, -135, and -155) or empty vector (EV-1, -2, and -3). B, ALP activity of Gαq (-155) and MC3T3-E1-EV (-1) at 7, 14, and 21 days of culture. C, Alizarin red staining of MC3T3-E1-Gαq (-155) and MC3T3-E1-EV (-1) cultured for 28 days. D, Runx2, type I collagen (Col.1), and OCN mRNA levels determined by Northern blotting of MC3T3-E1-Gαq (-155) and MC3T3-E1-EV (-1) cells. Control hybridization with a G3PDH probe verified the amount of RNA loaded. Blots were quantified using densitometry and indicated as ratios normalized to those of respective G3PDH.

At birth, TG mice appeared normal, and their body weights and bone lengths were indistinguishable from WT littermates. Both males and females of the two transgenic lines showed qualitatively similar skeletal changes at all stages of postnatal development; however, the TG mice were smaller and had shorter limbs than WT littermates at 8 weeks and thereafter (Fig. 2B). A PCR product of the appropriate size was detected in bone extracts from TG mice, but none was detected either in extracts from other tissues from TG mice nor in any tissues from the WT littermates (Fig. 2B).

The skeleton of TG mice was fragile. Craniofacial development and tooth eruption were normal in TG mice. Blood-ionized calcium concentration and intact PTH levels were comparable between WT and TG mice at 4 weeks of age (ionized Ca²⁺ (mM): WT mice, 1.26 ± 0.02, n = 10; TG mice, 1.28 ± 0.03, n = 10, p > 0.1 versus WT mice and PTH (pg/ml); WT mice, 35.3 ± 3.3, n = 10; TG mice, 34.2 ± 4.4, n = 10, p > 0.1 versus WT mice).

Inhibitory Effect of Gαq Signal on Osteoblast Differentiation

All data are expressed as means (bars and symbols) ± S.E. (error bars) of three independent experiments. *, p < 0.01 versus MC3T3-E1-EV. Similar results were obtained in other cell lines (Gαq, 29 or 135 versus EV2 or EV3).
Radiographic and Histological Skeletal Analyses—Long bones of WT and TG mice at birth were grossly indistinguishable as were the radiological and histological analyses of the physis, epiphysis, and diaphysis (data not shown). Also, at all ages examined, no difference was apparent in height or morphology of growth plate cartilage. Plain x-ray of long bones showed that TG mice at 8 weeks were proportionally shorter than WT mice and showed severe osteopenia (Fig. 3A). Three-dimensional femoral computed tomography analyses demonstrated thinner cortices and fewer trabeculae in TG mice compared with WT littermates, indicating that both cortical and trabecular bone were affected (Fig. 3A). BMDs of the entire femur and tibia of TG mice were significantly lower than those of WT mice at 4 and 8 weeks of age (Fig. 3B).

By histological examination of the proximal tibiae, osteopenia began to be detected in TG mice at 2 weeks of age, which became evident at 4 weeks with decreases in both cortical thickness and trabecular number, as compared with WT littermates (Fig. 3C). In addition to being thinner, a polarized microscopic image of the TG cortical bone revealed that the extracellular matrix was more disorganized, suggesting a woven bone pattern, than the WT matrix with lamellar distribution. Marrow fibrosis that can sometimes accompany woven bone (14), however, was not detected in the marrow cavity of TG mice. The decreases in cortical and trabecular bone volumes were still evident without widening of osteoid seams at 8 weeks in TG mice, as shown in the toluidine blue staining (Fig. 3C). We further examined the in vivo expressions of Col.1 and osteocalcin by in situ hybridization at 2 weeks of age when osteopenia became detectable. The expressions were decreased in trabecular and cortical bones of TG mice (Fig. 3D).

Bone Histomorphometric Analyses—Bone histomorphometric measurements of trabecular bones from mice of 4 and 8 weeks of age showed that parameters of bone formation, bone volume (BV/TV), trabecular thickness (Tb.Th), osteoblast number (Ob.S/BS), and osteoid surface (OS/BS), were markedly reduced in TG mice at both ages, whereas parameters of bone resorption (number of mature osteoclasts (Oc.N/B.Pm), percentage of bone surface covered by mature osteoclasts (Oc.S/BS), percentage of eroded surface (ES/BS)) were not significantly different (Fig. 4A). Cortical thickness (Ct.Th) at the midshaft of tibiae was also decreased in TG mice at both ages.

Dynamic analyses of bone formation performed by double labeling with calcein at a 3-day interval in 4-week-old mice and a 7-day interval in 8-week-old mice in trabecular bone showed two widely spaced bands of calcein deposition in 4-week-old WT mice (Fig. 4B). In contrast, bone from TG mice showed only a single or two only narrowly separated lines. Trabecular dynamic histomorphometry was performed on longitudinal sections of 4- and 8-week-old bone. Compared with the WT littermates, TG mice had significantly lower bone formation and mineral apposition rates but similar mineralization lag time at both ages (Fig. 4B). No sex differences were apparent for these quantitative analyses. Our attempts at comparison of the apoptosis of osteoblasts and osteocytes in WT and TG mice showed no significant difference (data not shown).

Ex Vivo Analyses of Primary Osteoblasts—To further examine the cellular mechanism underlying the abnormalities in bone formation in TG mice, osteoblasts were isolated from neonatal calvariae of TG and WT mice, grown in culture and studied after two passages. First, expression of the transgene was detected only in osteoblasts from TG lines, not in cells from WT littermates, by RT-
PCR using transgene-specific primers (Fig. 5A). Proliferation rates of osteoblasts from TG and WT littermates were similar at 3, 6, 9, and 12 days in culture, when pulse-labeled with [3H]thymidine, confirming that the impaired bone formation in TG mice is not because of diminished proliferation (Fig. 5B).

Next, we assessed osteoblast maturation. Both ALP activity and osteocalcin expression were lower in TG osteoblasts than in WT (Fig. 5, C and D). Alizarin red staining and extracellular matrix-bound calcium levels were also severely decreased, confirming a decrease of matrix formation, in the TG osteoblast culture (Fig. 5E). Conversely, there was a slight suppression of ALP and Alizarin red staining in cultures of bone marrow cells from 8-week-old TG mice, indicating that marrow cells were less affected by Gαq activation than osteoblasts (Fig. 5F).

Inhibitory Effect of Gαq Signal on Osteoblast Differentiation

FIGURE 4. Histomorphometric analyses of trabecular and cortical bones. A, static parameters of trabecular bones from 0.1 to 1.2 mm below the growth plate of the proximal tibiae metaphysis were compared between 4- and 8-week-old WT and TG mice. BV/TV, trabecular bone volume as a percentage of total volume; Tb.Th, trabecular thickness; Ob.S/BS, percentage of the bone surface covered by cuboidal osteoblasts; OS/BS, percentage of the bone surface covered with osteoid; Oc.N/B.Pm, number of mature osteoclasts in 10 cm of the bone perimeter; Oc.S/BS, percentage of bone surface covered by mature osteoclasts; Es/BS, percentage of eroded surface. Cortical thickness (Ct. Th) was also compared at the midshaft of tibiae. B, dynamic parameters of bone formation in trabecular bone. Tibial mineralization fronts of 4-week-old WT and TG mice were imaged by fluorescent microscopy, after labeling with calcine at days 3 and 6 (scale bar, 50 μm), and longitudinal sections from proximal parts of tibiae of WT and TG mice at 4 and 8 weeks of age were used for measurement of mineral apposition rate (MAR), bone formation rate (BFR), and mineralization lag time (MLT). Data are expressed as means (bars) ± S.E. (error bars) for five mice/group/time. *, p < 0.01 versus WT mice.

FIGURE 5. Ex vivo analyses of cultured osteoblasts or bone marrow cells. A, RT-PCR for the transgene using total RNA extracted from primary cultured osteoblasts from neonatal calvariae of WT or TG mice. B, time course of [3H]thymidine incorporation into calvarial osteoblasts. C, time course of ALP activity of calvarial osteoblasts. D, Northern blotting for OCN expression in calvarial osteoblasts. E, Alizarin red staining of calvarial osteoblasts cultured for 28 days (top) and quantification of matrix-bound calcium (bottom). F, ALP and Alizarin red stainings of bone marrow cells from 8-week-old WT or TG mice at 14 and 28 days of culture, respectively. G, Northern blotting for RANKL and osteoprotegerin (OPG) expressions in calvarial osteoblasts cultured with or without 1,25(OH)2D3 (1 nM) (VD) or PTH (10 nM) for 7 days. Control hybridization with a G3PDH probe verified the amount of RNA loaded. Blots were quantified using densitometry, and relative RANKL/osteoprotegerin ratio was calculated after the density of each blot was normalized to that of the respective G3PDH. H, ALP activity of calvarial osteoblasts in the presence and absence of GF109203X (100 nM) at 14 days of culture. Data are expressed as means (bars and symbols) ± S.E. (error bars) of three independent experiments; each experimental condition was done in triplicate. *, p < 0.01 versus WT osteoblasts; #, p < 0.05 versus WT osteoblasts without GF109203X; ##, p < 0.01 versus TG osteoblasts without GF109203X.
inhibition of ALP activity by Goq overexpression was not seen (Fig. 5H), indicating that the impaired osteoblast differentiation by Goq is at least partly mediated by the PKC pathway.

DISCUSSION

We have shown that mice with osteoblast-specific expression of a constitutively active Goq transgene exhibited proportionate dwarfism that became apparent by the 2nd postnatal week, and it progressed at least until 8 weeks after birth. There was no unusual gestational or perinatal lethality, nor did the mice manifest an atypical life span. The phenotype was characterized by deficient intramembranous and endochondral bone, despite apparently normal growth-plate chondrogenesis, osteopenia, and skeletal fragility, manifested by nontraumatic long bone fractures. Histology revealed reduced thickness of cortical and trabecular bones, with a decrease in lamellar bone and an increase in immature bone. The rate of bone formation was decreased because of an inhibition of osteoblast differentiation, without an alteration in osteoblast proliferation. The decrease of mineralized matrix in the cultures of osteoblasts with higher Goq expression (Fig. 1C and Fig. 5E) represents the cumulative consequence of impairment in osteoblast differentiation and matrix production rate during 28 days of cultures. However, the absence of widened osteoid seams in vivo indicates the lack of defect in the mineralization process. The fact that inhibitory effects of the Goq overexpression on osteoblastic markers were more conspicuous at earlier days (Fig. 1D) also supports the suppression in the process of osteoblast differentiation rather than the later stages. In addition, treatment of TG osteoblasts ex vivo with the PKC inhibitor, GF109203X, confirmed and extended these observations by showing that the impaired differentiation in TG osteoblasts was because of the continuous activation of PKC. Therefore, PKC activation by Goq may impair osteoblast differentiation, causing the reduced bone growth and bone formation.

Most previous data suggested that regulation of osteoblast differentiation by GPCR was mainly mediated via cAMP/PKA signaling (15), and little attention has been given to how Goq-mediated signaling affects bone formation (16, 17). In vitro, continued treatment of calvariae or osteoblastic cells with PTH has been shown to inhibit type I collagen expression and to increase osteocalcin expression largely via cAMP-dependent pathways (18, 19). Our data, showing suppression of osteoblast differentiation by continuous activation of Goq-undetectable expression of osteocalcin and impaired mineralization, may be the first to highlight a crucial inhibitory signaling role(s) for Goq/PKC in bone formation.

Cortical bone of TG mice was composed mainly of immature bone just like the woven bone in which the extracellular matrix ran in irregular directions and instead of the mature lamellar bone seen in the WT bone. Typically, woven bone is formed in pathological conditions like osteosarcoma and fracture callus, in which the bone turnover rate is accelerated (20). The present histomorphometric analysis of the TG bone, however, showed that bone formation was decreased and bone resorption was unaffected (Fig. 4). In addition, although Goq signaling is known to be involved in the regulation of RANKL and osteoproterogerin (21), none of the expression levels or the ratio was changed in cultured TG osteoblasts (Fig. 5G). A recent work by Komori and co-workers (22) showed that transgenic mice that overexpressed Runx2 in osteoblasts exhibited woven bone, which was caused by the maturational suppression of osteoblasts at a late stage. Their work supports our finding that inhibition of osteoblast differentiation could lead to woven bone formation, although the impairment stage of differentiation was somewhat earlier in our TG mice than the Runx2 transgenic mice. Further studies will elucidate the mechanism underlying the organization of extracellular matrix regulated by osteoblast differentiation.

To date, no human diseases have been attributed to mutations in Goq. The lack of syndromes because of loss-of-function mutations are readily understood because of the redundant functionality of Goq and Go11, making it necessary to have appropriate, simultaneous mutations in both genes. The absence of a syndrome because of a dominant-acting mutation is less apparent. If widespread in many tissues, perhaps it is lethal, as the cardiac overexpression of Goq leads to hypertrophy and dilated cardiomyopathy with a short life span (23–25). Wettschureck et al. (26) recently reported that mice with parathyroid-specific double knock-out of Goq and Go11, exhibited a phenotype resembling germ line knock-out of the extracellular Ca2+-sensing receptor: severe hypercalcemia, hyperparathyroidism, hypocalciuria, retarded growth, and early postnatal death. Similarly, by using cre/lox technology, we are now generating double knock-out mice with osteoblast-specific ablation of Goq and Go11, to learn more about the loss-of-function of Goq/11 in osteoblasts.

Classical teaching was that there was a linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth and that limb-shortening defects were because of defects in the growth plate (27–30). Studies from Karsenty and co-workers (31) have challenged this paradigm. They first showed that ganciclovir ablation of osteoblasts in mice expressing the herpes simplex virus thymidine kinase gene under control of the osteocalcin promoter showed dwarfism with severely reduced longitudinal bone growth (31). Then they showed that overexpression of the Runx2 DNA binding domain, without the transcriptional activating domain, resulted in shortening of long bones and impaired bone formation (32). In addition, Pantschenko et al. (33) reported that overexpression of Bcl-2, an important regulator of apoptosis, in osteoblasts also resulted in shortening of long bones and an inhibition of bone loss with age. As with our mice, neither of these models had apparent chondrocyte defects in the growth plate. Although we cannot, of course, exclude expression of transgene at the growth plates at levels we could not detect, longitudinal bone growth might be determined by the deposition of bone matrix and/or bone formation, in addition to the chondrocyte proliferation and hypertrophy in the growth plates.

In conclusion, the present study demonstrated that Goq-mediated activation of PKC suppressed osteoblast differentiation, but not proliferation, in vivo and in vitro. Together, the effects on these phenomena led to decreased bone formation and pointed to a crucial, novel role for Goq/PKC signaling in osteoblast biology. We predict that a human syndrome(s) in which
Inhibitory Effect of $G_{\alpha_q}$ Signal on Osteoblast Differentiation

bone formation is impaired, but as yet not reported, will be due to dominant-acting mutation(s) of $G_{\alpha_q}$. Because PTH signals through $G_{\alpha_q}$/PKC as well as $G_{\alpha_q}$/PKA (34), further work to sort out the PTH signaling pathways in bone cells may reveal a potential pharmacological target for treatment of osteoporosis and other bone-loss states.

Acknowledgment—We thank the hard tissue research team at Kureha Chemical Co., Ltd. for technical assistance.

REFERENCES

1. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) Annu. Rev. Biochem. 60, 653–688
2. Spiegel, A. M. (1997) Horm. Res. 47, 89–96
3. Spiegel, A. M., Weinstein, L. S., and Shenker, A. (1992) J. Clin. Investig. 92, 1119–1125
4. Weinstein, L. S., Shenker, A., Gejman, P. V., Merino, M. J., Friedman, E., and Spiegel, A. M. (1991) N. Engl. J. Med. 325, 1688–1695
5. Weinstein, L. S., Yu, S., Warner, D. R., and Liu, J. (2001) Endocr. Rev. 22, 675–705
6. Patten, J. L., Johns, D. R., Valle, D., Eil, C., Gruppuso, P. A., Steele, G., Smallwood, P. M., and Levine, M. A. (1990) N. Engl. J. Med. 322, 1412–1419
7. Sakamoto, A., Chen, M., Nakamura, T., Xie, T., Karsenty, G., and Weinstein, L. S. (2005) J. Biol. Chem. 280, 21369–21375
8. Quarles, L. D., and Siddhanti, S. R. (1996) J. Bone Miner. Res. 11, 1375–1383
9. Wu, D. Q., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) J. Biol. Chem. 267, 1811–1812
10. Rossert, J., Eberspaecher, H., and Crombrugghe, B. D. (1995) J. Cell Biol. 129, 1421–1432
11. Iida-Klein, A., Guo, J., Xie, L. Y., Juppner, H., Potts, J. T., Kronenberg, H. M., Bringhurst, F. R., Abou-Samra, A. B., and Segre, G. V. (1995) J. Biol. Chem. 270, 8458–8465
12. Calvi, L. M., Sims, N. A., Hunzelman, J. L., Knight, M. C., Giovanni, A., Saxton, J. M., Kronenberg, H. M., Barom, R., and Schipani, E. (2001) J. Clin. Investig. 107, 277–286
13. Ogata, N., Chikazu, D., Kubota, N., Terauchi, Y., Tobe, K., Azuma, Y., Ohta, T., Kadowaki, T., Nakamura, K., and Kawaguchi, H. (2000) J. Clin. Investig. 105, 935–943
14. Weinstein, L. S. in Principles of Bone Biology (Bilezikian, J., Raisz, L., and Rodan, G., eds), 2nd Ed., pp. 877–888, Academic Press, San Diego
15. Bowler, W. B., Gallagher, J. A., and Bilbe, G. (1998) Front. Biosci. 3, 769–780
16. Swarthout, J. T., Doggett, T. A., Lemker, J. L., and Partridge, N. C. (2001) J. Biol. Chem. 276, 7586–7592
17. Kim, H. J., Kim, J. H., Bae, S. C., Choi, J. Y., Kim, H. J., and Ryoo, H. M. (2003) J. Biol. Chem. 278, 319–326
18. Partridge, N. C., Dickson, C. A., Kopp, K. F., Teitelbaum, S. L., Crouch, E. C., and Kahn, C. (1989) Mol. Endocrinol. 3, 232–239
19. Swarthout, J. T., D’Alonzo, R. C., Selvamurugan, N., and Partridge, N. C. (2002) Gene (Amst.) 282, 1–17
20. Monier-Faugere, M. C. (1998) in Metabolic Bone Disease (Avioli, L. V., and Krane, S. M., eds) 3rd Ed., pp. 230–240, Academic Press, San Diego
21. Kondo, H., Guo, J., and Bringhurst, F. R. (2002) J. Bone Miner. Res. 17, 1667–1679
22. Liu, W., Toyosawa, S., Furutchi, T., Kanatani, N., Yoshida, C., Liu, Y., Himeno, M., Narai, S., Yamaguchi, A., and Komori, T. (2001) J. Cell Biol. 155, 157–166
23. Offermanns, S., Toombs, C. F., Hu, Y. H., and Simon, M. I. (1997) Nature 389, 183–186
24. Mende, U., Kagen, A., Cohen, A., Aramburu, J., Schoen, F. J., and Neer, E. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13893–13898
25. Wettchvereck, N., Rutten, H., Zywietz, A., Gehring, D., Wilke, T. M., Chen, J., Chien, K. R., and Offermanns, S. (2001) Nat. Med. 7, 1236–1240
26. Wettchvereck, N., Lee, E., Libutti, S. K., Offermanns, S., Robey, P. G., and Spiegel, A. M. (2007) Mol. Endocrinol. 21, 274–280
27. Breur, G. J., VanEnkevort, B. A., Farumn, C. E., and Wilsman, N. J. (1991) J. Orthop. Res. 9, 348–359
28. Breur, G. J., Turgai, J., Vanenkevort, B. A., Farumn, C. E., and Wilsman, N. J. (1994) Anat. Rec. 239, 255–268
29. Kember, N. F. (1978) Cell Tissue Kinet. 11, 477–485
30. Kember, N. F. (1979) J. Theor. Biol. 78, 365–374
31. Corral, D. A., Amlling, M., Priemel, M., Loyer, E., Fuchs, S., Ducy, P., Baron, R., and Karsenty, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13835–13840
32. Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinerio, G., Geoffroy, V., Amlling, M., and Karsenty, G. (1999) Genes Dev. 13, 1025–1036
33. Pantschenko, A. G., Wang, W., Nahounou, M., McCarthy, M. B., Stover, M. L., Lichtler, A. C., Clark, S. H., and Gronowicz, G. A. (2005) J. Bone Miner. Res. 20, 1414–1429
34. Lanks, B., and Kovacs, C. S. (1998) Recent Prog. Horm. Res. 53, 283–301