Mode of Growth Hormone Action in Osteoblasts*

Received for publication, June 26, 2007, and in revised form, August 9, 2007Published, JBC Papers in Press, August 13, 2007, DOI 10.1074/jbc.M705219200

Douglas J. DiGirolamo‡, Aditi Mukherjee§, Keertik Fulzele‡, Yujun Gan, Xuemei Cao, Stuart J. Frank, and Thomas L. Clemens*1

From the Departments of *Pathology and §Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019 and ‡Veteran’s Affairs Medical Center, Birmingham, Alabama 35233

Growth hormone (GH) affects bone size and mass in part through stimulating insulin-like growth factor type 1 (IGF-1) production in liver and bone. Whether GH acts independent of IGF-1 in bone remains unclear. To define the mode of GH action in bone, we have used a Cre/loxp system in which the type 1 IGF-1 receptor (Igf1r) has been disrupted specifically in osteoblasts in vitro and in vivo. Calvarial osteoblasts from mice homozygous for the floxed IGF-1R allele (IGF-1Rflox/floxflox) were infected with adenoviral vectors expressing Cre. Disruption of IGF-1R mRNA (>90%) was accompanied by near elimination of IGF-1R protein but retention of GHR protein. GH-induced STAT5 activation was consistently greater in osteoblasts with an intact IGF-1R. Osteoblasts lacking IGF-1R retained GH-induced ERK and Akt phosphorylation and GH-stimulated IGF-1 and IGFBP-3 mRNA expression. GH-induced osteoblast proliferation was abolished by Cre-mediated disruption of the IGF-1R or co-incubation of cells with an IGF-1-neutralizing antibody. By contrast, GH inhibited apoptosis in osteoblasts lacking the IGF-1R. To examine the effects of GH on osteoblasts in vivo, mice wild type for the IGF-1R treated with GH subcutaneously for 7 days showed a doubling in the number of osteoblasts lining trabecular bone, whereas osteoblast numbers in similarly treated mice lacking the IGF-1R in osteoblasts were not significantly affected. These results indicate that although direct IGF-1R-independent actions of GH on osteoblast apoptosis can be demonstrated in vitro, IGF-1R is required for anabolic effects of GH in osteoblasts in vivo.

The process of osteogenesis and remodeling of the skeleton is orchestrated by a constellation of local growth factors, cytokines, and systemic hormones (1, 2), of which growth hormone (GH)3 and insulin-like growth factor type 1 (IGF-1) are key components. GH belongs to a family of cytokine peptides (3) and is produced and stored by somatotroph cells within the anterior pituitary. GH actions are mediated by binding to the transmembrane GHR, thereby triggering increased association with and activation of Janus kinases (JAKs) (4–6) to activate signal transducers and activators of transcription (STATs) (7–15), phosphatidylinositol 3-kinase/Akt (16, 17), and ERK (18–20). Growth hormone exerts many, but not all (21), of its effects by stimulation of IGF-1 from liver and peripheral tissues. IGF-1 is a small polypeptide with homology to pro-insulin that is produced by a number of cell types. IGF-1 signals via the type 1 IGF-1 receptor (IGF-1R), engaging ERK and phosphatidylinositol 3-kinase pathways through Src homology 2 domain-containing proteins and insulin receptor substrates-1 and 2 (22, 23). The effects of IGF-1 on bone have been well documented. IGF-1 has been shown to induce proliferation of MC3T3 osteoblast-like cells (24) and is an important survival factor for many mammalian cell types, including osteoblasts. IGF-1 production increases during the initial phases of fetal rat calvarial osteoblast differentiation in vitro and then declines with the appearance of a differentiated phenotype (25). This is followed by a second wave of IGF-1 production that occurs with matrix synthesis and mineralization that may account for the ability of IGF-1 to augment synthesis of type I collagen and inhibit collagen degradation in differentiated fetal rat osteoblasts (26). In mice, targeted overexpression of IGF-1 accelerates new bone formation and increases the rate at which matrix is mineralized (27), whereas osteoblast-specific disruption of the IGF-1R causes markedly impaired mineral apposition rate and increased mineralization lag time (28).

In contrast to the large volume of literature on IGF-1 actions in osteoblasts, little is known regarding direct GH effects in this cell type. High affinity GH receptors are found on rat osteoblast-like UMR106 osteosarcoma cells (29) and human (30) and mouse (31–33) primary cultured osteoblasts. GH can induce osteoblast proliferation in cell culture (30), but whether this action requires IGF-1 production remains uncertain. Genetic mouse models globally deficient in IGF-1 or GH provide circumstantial evidence for independent functions of GH and IGF-1 on osteoblast-mediated bone formation. For example, Mohan et al. (34) reported that mice globally deficient in either IGF-1 or GH (lit/lit) had markedly impaired postnatal bone mineral density but that the defect was more severe in IGF-1 nulls. However, severe disturbances of overall growth of these extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription.

* This work was supported by a Veterans Affairs Merit Review grant and National Institutes of Health Grant R01 AR052746. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement is for identification purposes only and should not be regarded as an endorsement of the product named. The abbreviation used is: GH, growth hormone; IGF-1, insulin-like growth factor type 1; IGF-1R, IGF receptor type 1; Cre, Cre recombinase; AdGFP, adenovirus-expressing green fluorescent protein; AdCre, adenovirus-expressing Cre; αMEM, α-minimal essential medium; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription.
mice and problems with reproductive hormone status confound interpretation of these previous models.

Because of the intimate relationship between GH and IGF-1, it has been difficult to precisely define discrete actions of each of these factors. Here we used a Cre-loxP method to selectively disrupt the IGF-1R in osteoblasts. Results from this model suggest that although IGF-1R-independent actions of GH to inhibit osteoblast apoptosis can be demonstrated in vitro, IGF-1R is required for normal GH action in osteoblasts in vivo, presumably through its ability to stimulate the production of IGF-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium, α-minimal essential medium (αMEM), was obtained from Cellgro-Mediatech (Herndon, VA), and fetal bovine serum (FBS) was from Invitrogen. Bovine GH was obtained from the National Hormone and Peptide Program-Monsanto (Torrance, CA) and stored in 200× aliquots for single use. Human IGF-1 was obtained from GroPep (Theberton, SA, Australia) and stored in 1000× aliquots for single use. Platelet-derived growth factor BB (PDGF-BB) was obtained from Sigma and stored in 430× aliquots for single use. Antibodies used for immunoblotting included anti-IGF-1R β subunit (C-20), anti-BAX (N-20), anti-β-actin (C4) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-phospho-STAT5 (Tyr-694), anti-STAT5, anti-phospho-Akt (Ser-473), anti-Akt, anti-phospho-ERK (Thr-202/Tyr-204), and anti-ERK from Cell Signaling Technology (Danvers, MA). Anti-GHR (AL47) was a generous gift from Dr. Stuart Frank. IGF-1-neutralizing antibody (Clone 126002) was obtained from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated rabbit and mouse secondary antibodies were obtained from Pierce Biotechnology. Polyvinylidene difluoride membrane, Laemmli sample buffer, and other electrophoresis supplies were from Bio-Rad. Assay kits for flow cytometry analysis of cell proliferation and apoptosis were purchased from BD Biosciences. All other reagents not specified here were purchased from Sigma.

**Osteoblast Isolation and Culture**—Osteoblasts were isolated from calvaria of newborn Igf1r<sup>floxflox</sup> mice or ROSA26 reporter mice by serial digestion in 1.8 mg/ml collagenase type I (Worthington, Lakewood, NJ) solution. Calvaria were digested in 10 ml of digestion solution for 15 min at 37 °C with constant agitation. The digestion solution was collected, and digestion was carried out for 15 min at 37 °C in a humidified incubator supplied with 5% CO<sub>2</sub>. For in vitro deletion of the IGF-1R, osteoblasts containing floxed IGF-1R alleles were cultured to be 70% confluent and then, in the absence of serum, were infected with adenovirus-encoding Cre recombinase (AdCre) (Vector Biolabs, Philadelphia, PA) at a titer of 100 multiplicity of infection. Infection with 100 multiplicity of infection of adenovirus-encoding green fluorescent protein (AdGFP) (Vector Biolabs) was used as control. After 1 h, culture medium containing 10% FBS was added, and the cells were allowed to recover for the next 48 h. Greater than 90% IGF-1R deletion was confirmed for every infection by quantitative real-time PCR.

**Animal Studies**—Female control (Igf1r<sup>flox/flox</sup>) and ΔIgf1r mice (Igf1r<sup>flox/flox Cre<sup>+</sup></sup>) 3 weeks of age were treated subcutaneously with GH (3 μg/g body weight/day) or vehicle (0.01% NaHCO<sub>3</sub> in phosphate-buffered saline (PBS)) for 7 consecutive days. During this period, all mice were also injected intraperitoneally with 100 μl of a 1% calcein solution (to assess dynamic histomorphometric parameters) on a split dose schedule, leaving 3 days between the first and second dose and 2 days following the second dose before sacrifice. Distal femora were fixed in 100% ethanol, embedded, and sectioned. Five serial sections were stained using the Masson-Goldner trichrome technique, and five more serial sections were left unstained for fluorescent microscopy. Static and dynamic parameters of bone structure and formation were measured using the OsteoMeasure<sup>TM</sup> system (OsteoMetrics). Serum was also collected upon sacrifice of the animals, and serum IGF-1 measurements were performed at MERCORE Laboratory (Bangor, ME).

**LacZ Staining**—Paraformaldehyde-fixed samples of primary osteoblasts from ROSA26 reporter mice were washed three times for 5 min in lacZ wash buffer (2 mm MgCl<sub>2</sub>, 0.01% sodium deoxycholate, 0.02% Nonidet-P40, 5 mm EGTA in PBS). Staining was carried out in lacZ staining buffer (1 mg/ml X-gal, 5 mm potassium ferrocyanide, and 5 mm potassium ferricyanide in lacZ wash buffer) at 37 °C for 4 h to overnight, with shaking and protection from light. After staining, samples were rinsed with PBS, post-fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 m sodium cacodylate buffer, pH 7.3, for 10 min, rinsed twice with PBS, and then twice with 70% ethanol prior to storage in 70% ethanol at 4 °C.

**Cell Lysis and Immunoblotting Analysis**—For signaling experiments, ΔIGF-1R and control cells were cultured in 10% FBS αMEM to 90% confluence and then serum-starved in 0.1% bovine serum albumin containing αMEM for 24 h to reduce cellular activity to quiescent levels. At the end of the study, the cells were washed twice with ice-cold PBS and resuspended in lysis buffer (50 mm Tris, pH 7.4, 150 mm NaCl, 1 mm MgCl<sub>2</sub>, 1 mm EDTA, 1% Triton X-100, and 10% glycerol). Protease and phosphatase inhibitors (Sigma) were added to the lysis buffer. The cell lysates were homogenized by needle aspiration, and protein concentration was measured by Bradford protein assay (Bio-Rad). For immunoblotting of whole cell lysates, equal amounts of protein (10 or 20 μg/lane) were solubilized in Laemmli sample buffer and loaded onto a mini-SDS-PAGE system. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a Bio-Rad semi-dry transfer system. Protein transfer efficiency was verified using prestained protein markers. Membranes were then blocked with 5% nonfat dry milk for 1 h at room temperature and subsequently incubated overnight at 4 °C with antibodies directed against the protein of interest. Signals were detected using a horseradish peroxidase-conjugated secondary antibody, and bound antibodies were visualized using the Supersignal West Femto Substrate (Pierce). Western blot photographic results were scanned with a Canon flatbed scanner. The relative levels of proteins of interest were then determined by measuring the density of the corresponding bands with AlphaEaseFC<sup>™</sup> soft-
Growth Hormone Action in Osteoblasts

FIGURE 1. Cre-mediated excision of IGFR-1R in primary mouse osteoblasts. Cre excision efficiency was first assessed in the ROSA26 reporter locus. Calvarial osteoblasts were isolated from newborn ROSA26 reporter mice. The cells were infected with AdGFP as control or AdCre for deletion. After 2 days in culture, cells were stained for lacZ (A). Cre excision efficiency was then assessed in the Igfr locus. Calvarial osteoblasts isolated from newborn mice carrying floxed IGFR-1R alleles were infected with AdGFP as control or AdCre for deletion. After 2 days in culture, cells were analyzed for IGFR-1 mRNA and protein expression by real-time PCR (B) and immunoblotting (D), respectively. GHR protein level was also analyzed by immunoblotting (C). Control or ΔIGFR-1R osteoblasts were serum-starved for 24 h before induction of proliferation with PDGF. A portion of cells were lysed 10 min following stimulation, and cell lysates were immunoblotted with anti-phospho-ERK (E). The remaining cells were treated with BrdUrd for the final 12 h before harvesting at 48 h of treatment for BrdUrd and 7-amino-actinomycin D (7-AAD) staining. The -fold increase of BrdUrd incorporation following PDGF treatment is shown here for both control and ΔIGFR-1R osteoblasts (F). Data from three separate experiments are represented as mean ± S.E. *, p < 0.05.

ware (Alpha Innotech). All values were averages of at least three experiments and were normalized to the protein expression of a normalization protein.

Quantitative Real-time PCR—Total RNA was extracted from cells using the TRIzol® method as recommended by the manufacturer (Invitrogen). The RNA concentration was estimated spectrophotometrically and only pure RNA (A260:A280 ratio ≥ 1.8) was used for further analysis. First strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). The cDNA was amplified in the Opticon Continuous Fluorescent Detector (MJ Research, Waltham, MA) using IQ™ SYBR Green supermix (Bio-Rad) and sequence-specific primers. PCR reactions were performed in triplicate for each cDNA, averaged, and normalized to endogenous β-actin reference transcripts. Primer sequences used were as follows: IGFR-1R, F5′-CTGTGTGCTCGCGGTGTGAC-3′, R5′-TGCTTTTCTGCTGGAAGGGGC-3′; IGFR-1, F5′-GCTCTTCAGTTCGTGTTGGAC-3′, R5′-TTGGGCTGTCAGTGTTGGCC-3′; IGFBP3, F5′-ACAGACACCCAGAAGTTCTCCTCTC-3′, R5′-TGGTTTCTGCTTTGGAAAGGCC-3′; β-actin, F5′-ACCTCTCAATGAGCTGTCC-3′, R5′-TGCAATAGTGAATGGC-3′.

Osteoblast Proliferation Assays—For the Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), the ΔIGFR-1R and control osteoblasts were plated in 96-well plates at low cell density (3 × 10³ cells/well) and cultured in αMEM with 1% FBS for 24 h to arrest the cells in G₀ phase. The cells were restimulated with 40 ng/ml PDGF-BB, 500 ng/ml GH, or 100 ng/ml IGF-1 for 48 h. For IGF-1-neutralizing antibody experiments, the manufacturer-indicated ND50 dose of IGF-1 was co-incubated with GH or IGF-1. CellTiter reagent was then added to each well according to the manufacturer’s instruction, and plate results were read spectrophotometrically 1.5 h later at 490 nm (Thermo Labsystems OpsysMR and Revelation Quicklink software). For the flow cytometric analysis of BrdUrd incorporation, the ΔIGFR-1R and control osteoblasts were plated in 6-well plates at low cell density (9 × 10⁴ cells/well) and cultured in αMEM with 1% FBS for 24 h to arrest the cells in G₀ phase. The cells were then restimulated with 500 ng/ml GH or 100 ng/ml IGF-1 for 48 h. For proliferation analysis of the cells, 10 μM BrdUrd was added to the medium 12 h before harvesting cells. The cells were stained for anti-BrdUrd-APC and 7-amino-actinomycin D (7-AAD) for proliferation and analyzed by FACSCalibur (BD Biosciences). 20,000 events were collected for each sample, and results were analyzed with WinMDI version 2.8.

Osteoblast Apoptosis Assays—Control and ΔIGFR-1R osteoblasts were grown to confluent monolayers in 6-well plates on
coverslips for terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) and cultured in αMEM with 1% FBS containing 500 ng/ml GH for 24 h prior to induction of apoptosis with 8 ng/ml staurosporine for 24 h. For BAX analysis, protein lysates were harvested and subjected to SDS-PAGE as described above. For TUNEL staining, coverslips were fixed and stained according to the manufacturer’s instructions for the In Situ Cell Death Detection kit, Fluorescein (Roche Applied Science). TUNEL-positive cell numbers were determined by direct counting.

Statistical Analysis—All statistical analyses were performed using the Microsoft Excel data analysis program for analysis of variance or Student’s t test analysis with an assigned significance level of 0.05 (α). All experiments were repeated at least three times unless otherwise stated. Values were expressed as mean ± S.E.

RESULTS

Disruption of IGF-1R in Osteoblasts—To establish a system for Cre-mediated gene excision of the IGF-1R in vitro, primary osteoblasts from calvaria of ROSA26 reporter mice were infected with adenoviral Cre (AdCre) or GFP control (AdGFP) vectors and stained by X-gal staining. In this model system, Cre excises a floxed neomycin cassette within the lacZ gene, allowing expression and positive blue staining in cells in which Cre is expressed (35). A 100 multiplicity of infection dose of AdCre resulted in nearly 100% excision as shown by the presence of blue-staining cells (Fig. 1A). We next assessed AdCre-mediated deletion in the Igf1r locus by infecting primary osteoblasts of calvaria from mice carrying the floxed Igf1r allele. Real-time PCR analysis of IGF-1R mRNA expression (Fig. 1B), as well as immunoblotting for IGF-1R (Fig. 1C), indicated >90% removal of IGF-1R at a 100 multiplicity of infection dose of AdCre. The level of GHR as assessed by immunoblotting (Fig. 1D) was not affected by the removal of IGF-1R.

To assess the effect of IGF-1R disruption on the responsivity of osteoblasts to other mitogenic stimuli, calvarial osteoblasts from Igf1r floxed mice were treated with PDGF, a potent mitogen for osteoblasts (36). Immunoblotting for phospho-ERK (Fig. 1E) in extracts of cells treated for 10 min showed equivalent phosphorylation in AdGFP- and AdCre-treated osteoblasts. PDGF induced similar BrdUrd uptake in AdGFP- and AdCre-treated cells (Fig. 1F), indicating that those osteoblasts lacking IGF-1R retained responsiveness to other osteoblastic mitogens.

GHR Signaling in the Absence of IGF-1R—To examine the effect of IGF-1R on GH signaling, calvarial osteoblasts from mice carrying the floxed Igf1r allele were infected with AdGFP (hereafter referred to as control) or AdCre (hereafter referred to as ΔIGF-1R) and treated with GH or IGF-1. Immunoblotting for phospho-STAT5 (Fig. 2A) showed slightly greater activation in control cells compared with ΔIGF-1R cells. Signaling through Akt and ERK (Fig. 2, B and C, respectively) was assessed by immunoblot in the same manner. IGF-1-induced phospho-ERK was reduced to basal levels following removal of the IGF-1R (Fig. 2C, lane 3 versus lane 6) whereas GH-induced ERK activation actually increased modestly in the absence of the IGF-1R (Fig. 2C, lane 2 versus lane 5). Similar results were seen for Akt activation (Fig. 2B). In osteoblasts with an intact IGF-1R, GH induced only modest Akt activation compared with the robust induction seen following IGF-1 treatment. Following removal of IGF-1R, however, IGF-1-induced Akt activation was nearly abolished (Fig. 2B, lane 3 versus lane 6) whereas GH retained levels of Akt activation similar to those seen in the cells containing IGF-1R (Fig. 2B, lane 2 versus lane 5).

IGF-1R Is Not Required for GH-stimulated IGF-1 and IGFBP-3 mRNA Production—We next determined the effects of loss of IGF-1R on the transcription of established GH target genes IGF-1 and IGFBP-3. GH stimulated IGF-1 mRNA by 2-fold over basal levels in control osteoblasts (Fig. 3A). Interestingly, osteoblasts lacking IGF-1R had basal levels of IGF-1 mRNA comparable with those of stimulated control cells, but GH treatment still showed further significant increase in IGF-1 mRNA. Similar results were seen for IGFBP3 mRNA (Fig. 3B).

GH Inhibits Osteoblast Apoptosis in Vitro in the Absence of IGF-1R—To determine the effect of GH on osteoblast apoptosis, control and ΔIGF-1R osteoblasts were pretreated with GH or vehicle in medium containing 1% FBS before the induction of apoptosis with staurosporine. Apoptosis was measured by immunoblotting for BAX, a pro-apoptotic protein, and TUNEL staining (Fig. 4, A and B, respectively). In the presence of IGF-1R, GH reduced staurosporine-induced apoptosis as indicated by reduced BAX levels and decreased TUNEL-positive cells. Following removal of IGF-1R, GH still modestly attenuated the apoptotic effects of staurosporine, although the basal apoptotic

FIGURE 2. GHR signaling in osteoblasts lacking the IGF-1R. Control or ΔIGF-1R osteoblasts were grown to confluence and serum-starved for 24 h before stimulation with vehicle (NT), GH, or IGF-1 for 10 min. Cells were lysed and cell lysates immunoblotted with anti-phospho-STAT5 and anti-STAT5 (A), anti-phospho-Akt and anti-Akt (B), or anti-phospho-ERK and anti-ERK (C). Immunoblots were quantified by densitometry and plotted to compare the ratio of phospho- to total protein in control cells (black bars) and ΔIGF-1R cells (striped bars). Data from three separate experiments are represented as mean ± S.E. *p < 0.05. Two-way analysis of variance showed significant differences among treatment groups in control and ΔIGF-1R for both phospho-ERK and phospho-Akt (p < 0.05).
Growth Hormone Action in Osteoblasts

rates were increased in osteoblasts lacking the IGF-1R, likely due to the loss of autocrine IGF-1 survival signals since osteoblasts express both IGF-1 and IGF-1R.

**GH Requires IGF-1R to Induce Osteoblast Proliferation in Vitro**—To assess the effect of IGF-1R on GH-induced osteoblast proliferation, control and ΔIGF-1R osteoblasts were serum-starved for 24 h and then treated with GH or IGF-1. Proliferation was assessed by flow cytometry for BrdUrd incorporation (Fig. 5A) as well as Promega CellTiter 96® AQueous Cell Proliferation Assay (Fig. 5B). In control osteoblasts, GH and IGF-1 induced osteoblast proliferation by both assays, whereas GH failed to increase proliferation in ΔIGF-1R osteoblasts.

This result suggests that GH induces proliferation in osteoblasts primarily by increasing IGF-1 production. To test this idea, wild type osteoblasts treated with GH or IGF-1 following serum starvation were cultured in the presence of an anti-IGF-1-neutralizing antibody. As shown in Fig. 5C, the proliferative effects of GH were ablated in the presence of the IGF-1-neutralizing antibody, demonstrating that GH promotes osteoblast proliferation through the production of IGF-1.

**GH Requires the IGF-1R to Induce Osteoblast Proliferation in Vivo**—In previous studies (28), we found that 3-week-old ΔIgf1r mice had significantly fewer osteoblasts and reduced trabecular bone formation rate compared with controls. To evaluate the effect of GH on osteoblasts in vivo, ΔIgf1r mice and control littermates were treated subcutaneously with 3 μg/g body weight/day GH for 7 days. Serum IGF-1 measurements showed equivalent -fold increases in serum IGF-1 levels in both control and knock-out mice (Fig. 6E). Treatment of control mice with GH (Fig. 6B, arrows, and Fig. 6F) nearly doubled osteoblast number compared with vehicle treatment (Fig. 6, A and F). Osteoblast number (Fig. 6, C and F) and bone formation rate (Fig. 6G) were decreased ~50% in the knock-out animals, in agreement with our previous characterization of these mice. In contrast to control mice, however, GH treatment of mice lacking IGF-1R in osteoblasts had no effect on osteoblast number (Fig. 6, D and F) or bone formation rate (Fig. 6G).

**DISCUSSION**

GH profoundly influences bone growth and mass, but despite decades of work, the cellular targets and mechanisms of action of GH in bone remain unclear. The principal difficulty in studying GH action is the fact that GH stimulates IGF-1 production, making it virtually impossible to distinguish actions due to GH versus those resulting indirectly through IGF-1. A number of previous studies have provided circumstantial evidence that GH directly influences skeletal development and bone formation independent of IGF-1. For example, administration of IGF-1 to GH-deficient rats results in sub-optimal growth (37). Further, mice globally deficient in either IGF-1 or GH (lit/lit) had a markedly impaired postnatal bone mineral density, but that defect was more severe in the IGF-1 nulls (34). Moreover, it has been reported that GH administration significantly increased trabecular bone formation rates in IGF-1 null mice (38). Taken together, these studies suggest that the GH and IGF-1 sig-
signaling pathways serve both independent and overlapping functions in the skeleton. However, severe disturbances in overall growth of these mice and problems with reproductive hormone status limit conclusions about direct effects of GH in bone.

In these studies, we used a Cre-loxP technique to selectively disrupt the IGF-1R in osteoblasts, enabling us to examine the actions of GH on this cell type in the absence of confounding effects brought about by IGF-1R signaling. In osteoblasts with an intact IGF-1R, GH effectively coupled to its downstream effector STAT5 and stimulated activation of both ERK and Akt pathways. However, removal of IGF-1R caused a modest but significant attenuation of GH-induced STAT5 activation, suggesting that IGF-1R and GHR might normally interact in cells that express both receptors. Such a collaboration between GHR and IGF-1R appears to occur in 3T3-F442A preadipocyte cells and might account for synergistic activation of c-Fos in response to combined GH and IGF-1 treatment (39). Indeed, it has been proposed that synergy could occur in these cells through physical interaction of GHR and IGF-1R at the level of Janus kinase 2 (JAK2) (40). Whether, or to what extent, such a mechanism exists in osteoblasts is unknown and is currently under further investigation in our laboratory.

Despite the potential for interaction of GH and IGF-1R signaling just described, disruption of IGF-1R in osteoblasts in vitro did not abolish the ability of GH to stimulate its target genes, IGF-1 and IGFBP-3, and block apoptosis. Interestingly, osteoblasts lacking IGF-1R had elevated basal levels of IGF-1 and IGFBP-3 mRNA, possibly due to loss of negative feedback signaling, and increased basal apoptotic frequency. In addition, previous studies by Morales et al. (42) in osteoblast-like UMR 106 cells also showed that GH inhibited apoptosis.

**FIGURE 5.** GH-induced proliferation is ablated upon IGF-1R removal or IGF-1 neutralization. Control or ΔIGF-1R osteoblasts were cultured at low density and starved for 24 h before stimulation with GH or IGF-1 to induce proliferation. In separate experiments proliferation was assessed by BrdUrd incorporation in control cells (black bars) and ΔIGF-1R cells (striped bars) (A) or by CellTiter® in control cells (black bars) and ΔIGF-1R cells (striped bars) (B). Wild type osteoblasts were serum-starved and treated with GH or IGF-1 in the presence of an IGF-1-neutralizing antibody or IgG control before assessment of proliferation with CellTiter® (C). Data from three separate experiments are represented as mean ± S.E., *p < 0.05. Two-way analysis of variance for the neutralizing antibody studies (C) showed significant differences among GH and IGF-1 treatment groups (p < 0.05) in IgG- and anti-IGF-1-neutralizing antibody groups.
By contrast, the growth-promoting actions of GH appear to require IGF-1R signaling since they are eliminated in osteoblasts lacking the IGF-1R. Studies in osteoblasts from human trabecular bone explants and SaOS-2 osteosarcoma cells (33), as well as human bone marrow stromal osteoblast-like cells (43), have shown that GH induces proliferation in all of these cell types. However, the proliferative effects of GH on the SaOS-2 cells are abolished in the presence of an IGF-1-neutralizing antibody (33), in agreement with our findings. Most importantly, our studies show that in vivo administration of GH increased the number of osteoblasts only in mice with an intact IGF-1R. This finding demonstrates that, although GH can prevent osteoblast apoptosis in vitro independent of IGF-1R, the in vivo effects of GH on osteoblast number in vivo are likely to be mediated indirectly via IGF-1 arriving from the circulation or produced locally in bone. We think it unlikely that the anti-apoptotic effect of GH shown in vitro would have a significant impact on osteoblast number in vivo, at least under the short-term treatment protocol used in this study. Analysis of GH effects beyond 1 week in this model would be complicated by the transient nature of the bone phenotype of this mouse; decreased osteoblast numbers seen in ΔIgf1r mice at 3 weeks of age are reversed by 6 weeks of age, likely due to the engagement of compensatory mechanisms (28).

Previous studies by Bikle et al. (38) led to a different conclusion regarding the mode of GH action in the skeleton. These authors reported that GH treatment was able to increase bone formation rates in mice globally deficient in IGF-1. In this regard, significant differences in model systems are relevant and may explain the apparent differences in the conclusions reached by these investigators. Mice globally deficient in IGF-1 exhibit severe disturbances in postnatal growth and sex hormone production. Thus, administration of GH to these animals might have altered bone formation secondary to a correction of growth plate abnormalities or gonadal hormone status. Additionally, because these mice possess intact GHR and IGF-1R, it is possible that these receptors might interact upon GH binding and enable activation of IGF signaling pathways in the absence of IGF-1. Most important is the possibility that GH stimulated production of IGF-2, which could have compensated for the loss of IGF-1. In our model, removal of the IGF-1R eliminates all IGF-ligand signaling and hence the possibility of compensation by IGF-2.

It is important to emphasize that the conclusions from our studies are relevant to mature osteoblasts and do not rule out possible direct effects of GH that might occur on different populations of skeletal cells or in other GH-responsive tissues. In this regard, Wang et al. (44) compared long bone growth in mice with targeted deletions of either IGF-1 or GHR. Mice lacking IGF-1 with elevated GH had a reduction in chondrocyte hypertrophy whereas in mice lacking GHR both chondrocyte generation and hypertrophy were compromised, producing a compound deficit in long bone growth. Such observations support dual roles for GH in promoting longitudinal bone growth: an IGF-1-independent role in growth plate chondrocyte generation and an IGF-1-dependent role in promoting chondrocyte hypertrophy. Furthermore, in skeletal muscle, loss of STAT5 (STAT5MKO)
causes marked sarcopenia in association with dramatic reductions in muscle IGF-1 production (45). However, recent work by Sotiropoulos et al. (21) showed that GH signaling profoundly influenced muscle mass, predominantly by promoting fusion of myoblasts with nascent myotubes. In vitro assays demonstrated this GH effect was exerted in an IGF-1-independent fashion, albeit without direct demonstration by blockade of IGF-1 signaling. These data appear to provide evidence that the extent to which GH and IGF-1 act independently, in tandem, or even synergistically likely depends on the tissue and context.

Our studies in mice reported herein have implications for the use of GH in humans. Postmenopausal women treated with recombinant human GH for 36 months showed a 5% increase in total body and 14% increase in lumbar spine bone mineral content after 4 years (46). IGF-1 levels increased from ~150 μg/liter to ~350 μg/liter in patients treated with GH. Further, Szulc et al. (47) have shown a positive correlation between serum IGF-1 levels and bone mineral density in a cohort of 721 men age 19–85. Such studies in humans suggest that GH increases bone mineral density primarily by elevating IGF-1. This notion is further supported by a case study of a patient with homozygous, partial deletion of the IGF-1 gene (48). Despite abnormally high circulating GH levels, this patient suffered severe osteopenia of the lumbar spine.

In summary, we have established a model system to explore the interactions between GH and IGF-1 signaling in osteoblasts. Using this system, we demonstrate that GH exerts antiapoptotic actions through IGF-1-independent mechanisms but cannot acutely increase osteoblast numbers in vivo in the absence of an intact IGF-1R, at least under the experimental conditions used here. Thus, we conclude that the main anabolic activity of GH is exerted through increasing the production of IGF-1 and the subsequent action of this growth factor on osteoblast proliferation.

Acknowledgment—We thank the MERCORE laboratory for serum IGF-1 measurements.

REFERENCES

1. Karsenty, G. (2000) Matrix Biol. 19, 85–89
2. Mundy, G. R., Boyce, B., Hughes, D., Wright, K., Bonevald, L., Dallas, S., Harris, S., Ghosh-Choudhury, N., Chen, D., and Dunstan, C. (1995) Bone 17, 715-755
3. Miller, W. L., and Eberhardt, N. L. (1983) Endocr. Rev. 4, 97–130
4. Argentisger, L. S., Campbell, G. S., Yang, X., Wtith unn, B. A., Silvennoinen, O., Ihle, J. N., and Carter-Su, C. (1993) Cell 74, 237–244
5. Foster, C. M., Schauer, J. A., Rosza, F. W., Wang, X. Y., Lewis, S. D., Renken, D. A., Natale, J. E., Schwartz, J., and Carter-Su, C. (1988) Biochemistry 27, 326–334
6. Silva, C. M., Day, R. N., Weber, M. J., and Thorner, M. O. (1993) Endocrinology 133, 2307–2312
7. Campbell, G. S., Meyer, D. J., Raz, R., Levy, D. E., Schwartz, J., and Carter-Su, C. (1995) J. Biol. Chem. 270, 3974–3979
8. Sotiropoulos, A., Moutoussamy, S., Binart, N., Kelly, P. A., and Finidori, J. (1995) FEBS Lett. 369, 169–172
9. Meyer, D. J., Campbell, G. S., Cochran, B. H., Argentisger, L. S., Larner, A. C., Finbloom, D. S., Carter-Su, C., and Schwartz, J. (1994) J. Biol. Chem. 269, 4701–4704
10. Choi, H. K., and Waxman, D. J. (2000) Growth Horm. IGF Res. 10, Suppl. B, S1–S8
11. Waxman, D. J. (2000) Novartis. Found. Symp. 227, 61–74
12. Waxman, D. J., Ram, P. A., Park, S. H., and Choi, H. K. (1995) J. Biol. Chem. 270, 13262–13270
13. Bergad, P. L., Shih, H. M., Towle, H. C., Schwarzenberg, S. J., and Berry, S. A. (1995) J. Biol. Chem. 270, 24903–24910
14. Delenque-Touchard, N., Park, S. H., and Waxman, D. J. (2000) J. Biol. Chem. 275, 34173–34182
15. Lahuna, O., Rastegar, M., Maiter, D., Thissen, J. P., Lemaigre, F. P., and Rousseau, G. G. (2000) Mol. Endocrinol. 14, 285–294
16. Costoya, J. A., Finidori, J., Moutoussamy, S., Sears, R., Devesa, J., and Arce, V. M. (1999) Endocrinology 140, 5937–5943
17. Liang, L., Jiang, J., and Frank, S. J. (2000) Endocrinology 141, 3328–3336
18. Campbell, G. S., Pang, L., Miyasaka, T., Saltiel, A. R., and Carter-Su, C. (1992) J. Biol. Chem. 267, 6074–6080
19. Moller, C., Hansson, A., Enberg, B., Lobe, P. E., and Norstedt, G. (1992) J. Biol. Chem. 267, 23403–23408
20. Winston, L. A., and Bertics, P. J. (1992) J. Biol. Chem. 267, 4747–4751
21. Sotiropoulos, A., Ohanna, M., Kedzia, C., Menon, R. K., Kopchick, J. J., Kelly, P. A., and Pende, M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 7315–7320
22. Dietrich, P., Dragatis, I., Xuan, S., Zeitlin, S., and Estratiadis, A. (2000) Mamm. Genome 11, 196–205
23. LeRoith, D. (2000) Endocrinology 141, 1287–1288
24. Merriman, H. L., La Tour, D., Linkhart, T. A., Mohan, S., Baylink, D. J., and Strong, D. D. (1990) Calcif. Tissue Int. 46, 258–262
25. Birnbaum, R. S., Bowsher, R. R., and Wieren, K. M. (1995) J. Endocrinol. 144, 251–259
26. Rydziel, S., Delany, A. M., and Canalis, E. (1997) J. Cell. Biochem. 67, 176–183
27. Zhao, G., Monier-Faugere, M. C., Langub, M. C., Geng, Z., Nakayama, T., Pike, I. W., Chernausek, S. D., Rosen, C. I., Donahue, L. R., Malhue, H. H., Fagin, A. J., and Clemens, T. L. (2000) Endocrinology 141, 2674–2682
28. Zhang, M., Xuan, S., Bouxsein, M. L., von Stechow, D., Akeno, N., Faugere, M. C., Malhue, H., Zhao, G., Rosen, C. J., Estratiadis, A., and Clemens, T. L. (2002) J. Biol. Chem. 277, 44005–44012
29. Barnard, R., Ng, K. W., Martin, T. J., and Waters, M. J. (1991) Endocrinol. 128, 1459–1464
30. Nilsson, A., Swolin, D., Enerback, S., and Ohsorn, C. (1995) J. Clin. Endocrinol. Metab. 80, 3483–3488
31. Slootweg, M. C., Salles, J. P., Olszewski, P., de Vries, C. P., Engelbregt, M. J., and Netelenbos, J. C. (1996) J. Endocrinol. 150, 465–472
32. Slootweg, M. C., van Buil-Offers, S. C., Herrmann-Erlee, M. P., van der Meer, J. M., and Duursma, S. A. (1988) J. Endocrinol. 116, R11–R13
33. Scheven, B. A., Hamilton, N. J., Fakkeldij, T. M., and Duursma, S. A. (1991) J. Endocrinol. Growth Regul. 1, 160–167
34. Cohen, N., Richman, C., Guo, R., Amaar, Y., Donahue, L. R., Wegedral, J., and Baylink, D. J. (2003) Endocrinology 144, 929–936
35. Soriano, P. (1999) Nat. Genet. 21, 70–71
36. Hock, J. M., and Canalis, E. (1994) Endocrinology 134, 1423–1428
37. Fielder, P. J., Mortensen, D. L., Mallet, P., Carlsson, B., Baxter, R. C., and Clark, R. G. (1996) Endocrinology 137, 1913–1920
38. Bikle, D., Majumdar, S., Laib, A., Powell-Braxton, L., Rosen, C., Beamer, W., Nauman, E., Leary, C., and Halloran, B. (2001) J. Bone Miner. Res. 16, 2320–2329
39. Ashcom, G., Gurland, G., and Schwartz, J. (1992) Endocrinology 131, 1915–1921
40. Huang, Y., Kim, S. O., Yang, N., Jiang, J., and Frank, S. J. (2004) Mol. Endocrinol. 18, 1471–1485
Growth Hormone Action in Osteoblasts

41. Morales, O., Samuelsson, M. K., Lindgren, U., and Haldosen, L. A. (2004) Endocrinology 145, 87–94
42. Dumon, S., Santos, S. C., Bierre-Grockiego, F., Gouilleux-Gruart, V., Co-cault, L., Boucheron, C., Mollat, P., Gisselbrecht, S., and Gouilleux, F. (1999) Oncogene 18, 4191–4199
43. Kassem, M., Mosekilde, L., and Eriksen, E. F. (1994) Growth Regul. 4, 131–135
44. Wang, J., Zhou, J., Cheng, C. M., Kopchick, J. J., and Bondy, C. A. (2004) J. Endocrinol. 180, 247–255
45. Klover, P., and Hennighausen, L. (2007) Endocrinology 148, 1489–1497
46. Landin-Wilhelmsen, K., Nilsson, A., Bosaeus, I., and Bengtsson, B. A. (2003) J. Bone Miner. Res. 18, 393–405
47. Szulc, P., Joly-Pharaboz, M. O., Marchand, F., and Delmas, P. D. (2004) Calcif. Tissue Int. 74, 322–329
48. Woods, K. A., Camacho-Hubner, C., Savage, M. O., and Clark, A. I. (1996) N. Engl. J. Med. 335, 1363–1367