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Bone Morphogenetic Proteins Induce Gremlin, a Protein That Limits Their Activity in Osteoblasts*

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ABSTRACT

Bone morphogenetic proteins (BMP) induce the differentiation of cells of the osteoblastic lineage and enhance the function of the osteoblast. Growth factor activity is regulated by binding proteins, and we previously showed that BMPs induce noggin, a glycoprotein that binds and blocks BMP action. Recently, additional BMP antagonists, such as gremlin, have been described, but there is no information about their expression or function in osteoblasts. We tested for the expression of gremlin and studied its induction by BMPs in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells). BMP-2 caused a time- and dose-dependent increase in gremlin messenger RNA and polypeptide levels, as determined by Northern and Western blot analyses. The effects of BMP-2 on gremlin transcripts were independent of new protein synthesis. BMP-2 increased the rate of gremlin transcription as determined by nuclear run-on assays. Fibroblast growth factor-2 and platelet-derived growth factor BB also induced gremlin, but other hormones and growth factors had no effect. Gremlin prevented the stimulatory effects of BMP-2 on DNA, collagen, noncollagen protein synthesis, and alkaline phosphatase activity in Ob cells. In conclusion, BMPs induce gremlin transcription in Ob cells, a mechanism that probably limits BMP action in osteoblasts. (*Endocrinology* **141:** 4558–4563, 2000)

BONE MORPHOGENETIC proteins (BMPs) were originally identified as proteins that induce bone formation at extraskeletal sites (1, 2). There are over 20 known BMPs, and, excluding BMP-1, they are all structurally related and belong to the transforming growth factor-β (TGFβ) superfamily of peptides (2). Cells of the osteoblastic lineage express primarily BMP-2, -4, and -6 (3–5). BMPs play a central role in the differentiation of mesenchymal cells into cells of the osteoblastic lineage and enhance the differentiated function of the osteoblast (6–8). In bone tissue, BMPs play an autocrine role in skeletal function, and deficiency of selected BMPs, such as BMP-7, leads to abnormalities in skeletal patterning (9).

BMPs, like other growth factors, are regulated at the level of expression and activity, and the effects of BMPs can be modulated by a group of polypeptides that antagonize BMP action (5, 10). BMP antagonists prevent BMP signaling by binding BMPs with high affinity, therefore precluding their binding to specific cell surface receptors (11–15). Polypeptides antagonizing BMP action include noggin, chordin, and the Dan/cerberus family of genes, which is comprised of the head inducer cerberus, the tumor suppresser Dan, gremlin and its rat homologue drm, the protein related to Dan and cerberus, and Dte (11, 12, 14–20). Noggin, but not chordin, was recently shown to be expressed by skeletal cells, and studies in noggin null mice demonstrate that it is essential for proper skeletal development (8, 21, 22). Noggin null mice have excessive cartilage and fail to initiate joint formation, probably due to excessive BMP action. Noggin blocks the effect of BMPs on osteoblastogenesis and osteoblastic function, and although unstimulated osteoblasts express modest levels of noggin, they express noggin messenger RNA (mRNA) and protein after BMP exposure (8, 21). This would suggest that induction of BMP antagonists may be a protective mechanism to prevent excessive exposure of skeletal cells to BMPs. However, except for noggin and chordin, there is no information about the expression and function of BMP antagonists in osteoblasts.

Gremlin was cloned from a *Xenopus* ovary library as a novel gene with axial patterning activities. The product of the gene is a secreted polypeptide with a M_r of 28 kDa, and, like noggin, it prevents BMP receptor binding and activities in a variety of assays, but not the activity of other members of the TGFβ family (14). There is no information about the expression or activity of gremlin in osteoblasts. In the present study we examined the expression of gremlin in cultures of osteoblast-enriched cells from 22-day-old fetal rat calvariae (Ob cells) and determined its regulation by BMPs and other growth regulators. We also examined whether gremlin modified the actions of BMPs in cultured osteoblasts.

Materials and Methods

Culture technique

The culture method used was described in detail previously (23). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area or by CO_2 asphyxiation. This project was approved by the institutional animal care and use committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II, Worthington Biochemical Corp., Freehold, NJ). Cell populations harvested from the third to fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics (23). Ob cells were plated at a density of 8,000–12,000 cells/cm^2, and cultured in a humidified 5% CO_2 incubator

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at 37 C until reaching confluence (~50,000 cells/cm^2). For the nuclear run-on experiment, first passage cultures were used. Ob cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY), supplemented with nonessential amino acids and 10% FBS (Summit Biotechnologies, Fort Collins, CO). Immortalized osteoblastic mouse MC3T3-E1 cells were plated at a density of 14,000 cells/cm^2 in αMEM (Life Technologies, Inc.) containing 20 mM HEPES and 10% FBS and were grown under the same conditions as Ob cells to confluence (~100,000 cells/cm^2) (24). Cells were exposed to serum-free medium for 20–24 h and then exposed to test or control medium in the absence of serum for 2–24 h. Recombinant human BMP-2 (a gift from Genetics Institute, Cambridge, MA), TGFβ1 (a gift from Genentech, Inc., South San Francisco, CA), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor BB (PDGF-BB), insulin-like growth factor I (IGF-I; Austral, San Ramon, CA), and gremlin (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) were added directly to the medium. Recombinant human BMP-4 and BMP-6 were dissolved in 0.1% trifluoroacetic acid and diluted 1:8,000 and 1:4,000 in culture medium. PTH-(1–34) (Bachem, Torrance, CA) was dissolved in 0.05 N HCl containing 4 mg/ml BSA and diluted 1:10,000 or greater in culture medium. Porcine insulin (Sigma, St. Louis, MO) was dissolved in 0.001 N HCl and diluted 1:1,000 in DMEM, recombinant human GH (a gift from P. A. Kelly, Paris, France) was dissolved in distilled water, and T_3 (Sigma) was added directly to the culture medium. 1,25-Dihydroxyvitamin D_3 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), cortisol, and cycloheximide (both from Sigma) were dissolved in ethanol and diluted 1:1,000 in DMEM. Control cultures contained equal amounts of solvent. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70 C. For the nuclear run-on assay, nuclei were isolated by Dounce homogenization (Kontes Co., Vineland, NJ). For gremlin protein analysis, the medium was collected in the presence of 0.1% polyoxyethylene sorbitan monolaurate (Tween-20, Pierce Chemical Co., Rockford, IL). For DNA and protein synthesis and determination of alkaline phosphatase activity (APA), the cell layer was extracted and stored at −70 C.

Northern blot analysis

Total cellular RNA was isolated using the RNeasy kit according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, confirming equal RNA loading of the various experimental samples. The RNA was blotted onto GeneScreen Plus charged nylon (DuPont/NEN, Wilmington, DE), and the uniformity of transfer was confirmed by revisualization of ethidium bromide-stained ribosomal RNA. A 0.6-kb *Xho*I/*Not*I human gremlin complementary DNA (cDNA; Regeneron Pharmaceuticals, Inc.) was purified by agarose gel electrophoresis and labeled with [α-^{32}P]deoxy-CTP and [α-^{32}P]deoxy-ATP (50 μCi each at a specific activity of 3000 Ci/mmol; DuPont/NEN) using the random hexanucleotide primed second strand synthesis method (25). Hybridizations were carried out at 42 C for 16–72 h, followed by two posthybridization washes at room temperature for 15 min in 1 × saline sodium citrate (SSC) and a third wash performed at 65 C for 30 min in 1 × SSC. The blots were stripped and rehybridized with an α-^{32}P-labeled 752-bp *Bam*HI/*Sph*I restriction fragment of the murine 18S ribosomal RNA cDNA (American Type Culture Collection, Manassas, VA) under conditions previously described (8). The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY), employing Cronex Lightning Plus (DuPont/NEN) or Biomax MS (Eastman Kodak Co.) intensifying screens. Relative hybridization levels were determined by densitometry, and levels of gremlin expression were normalized to the levels of 18S hybridization. The Northern analyses shown are representative of three or more cultures.

Nuclear run-on assay

To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% IGEPAL-650 (Sigma) (26). Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM each of ATP, CTP, and GTP; 150 U RNasin (Promega Corp., Madison, WI); and 250 μCi [α-^{32}P]UTP (800 Ci/mM; DuPont/NEN) (26). RNA was isolated by treatment with DNase I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized gremlin cDNA was immobilized onto GeneScreen Plus by slot blotting according to the manufacturer's directions (DuPont). The plasmid vector pGL2-Basic (Promega Corp.) was used as a control for nonspecific hybridization, and 18S ribosomal RNA cDNA was used to estimate uniformity of counts applied to the membrane. Equal counts per min of [α-^{32}P]RNA from each sample were hybridized to cDNAs at 42 C for 72 h and washed in 1 × SSC at 65 C for 60 min. Hybridized cDNAs were visualized by autoradiography.

Western blot analysis

To examine changes in gremlin protein levels, aliquots of the culture medium representing one culture well were precipitated with 5% trichloroacetic acid and mixed with Laemmli sample buffer to give a final concentration of 2% SDS. To test for the presence of oligosaccharides, the precipitated samples were resuspended in sodium phosphate buffer and exposed to a mixture of deglycosylating enzymes before electrophoresis, in accordance with the manufacturer's instructions (glycoprotein deglycosylation kit, Calbiochem, La Jolla, CA). Supernatant from COS-7 cells overexpressing human gremlin was used as a standard. Samples were fractionated by PAGE on a 15% denaturing gel in the presence of reducing agents (27). Proteins were transferred to Immobilon P membranes (Millipore Corp., Bedford, MA), blocked with 2% BSA, and exposed to 20 ng/ml of a rat monoclonal antibody raised against human gremlin (Regeneron Pharmaceuticals, Inc.) in 1% BSA overnight. Blots were exposed to a goat antirat IgG antiserum conjugated to horseradish peroxidase and developed with a horseradish peroxidase chemiluminescent detection reagent. Western blots are representative of three or more cultures.

DNA, collagen, and noncollagen protein synthesis

DNA synthesis was studied by measuring effects on the incorporation of [*methyl*-^{3}H]thymidine (5 μCi/ml; SA, 78 Ci/mmol; DuPont/NEN) into acid-insoluble extracts during the last 2 h of culture, as previously described (28). Data are expressed as disintegrations per min/0.32-cm^2 culture well. Collagen and noncollagen protein synthesis were determined by measuring the incorporation of [2,3-^{3}H]proline (12.5 μCi/ml; SA, 40 Ci/mmol; DuPont/NEN) into collagenase-digestible protein and noncollagen protein according to the method of Peterkofsky and Diegelmann (28, 29). Data are expressed as disintegrations per min/2-cm^2 culture well.

APA

APA was determined in 0.5% Triton X-100 cell extracts by hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol and was measured by spectroscopy at 410 nm after 30 min of incubation according to the manufacturer's instructions (Sigma). Data are expressed as picomoles of *p*-nitrophenol released per min/μg protein. Total protein content in the extracts was determined by the Coomassie brilliant blue G-250 dye-binding assay of Bradford in accordance with the manufacturer's instructions (Bio-Rad Laboratories, Inc., Richmond, CA).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical differences were determined by ANOVA and *post-hoc* examination by Ryan-Einot-Gabriel-Welch F test, using a Crunch Statistical Package (Crunch Software Corp., Oakland, CA) (30).

Results

Untreated cultures of Ob cells expressed limited levels of gremlin mRNA, as determined by Northern blot analysis. Treatment with BMP-2 caused a time- and dose-dependent induction of gremlin transcripts of 4.1 kb. BMP-2 at 1 nM (not shown) and 3.3 nM increased gremlin mRNA levels from virtually undetectable to clearly detectable levels after 6 and

24 h (Fig. 1). The effect was more pronounced after 24 h and varied somewhat from experiment to experiment, so that BMP-2 at 3.3 nM increased gremlin mRNA by about 4- to 8-fold after 6 h and by about 15-fold after 24 h. BMP-4 caused a similar stimulation of gremlin transcripts, whereas BMP-6 had a more modest effect (not shown). The stimulatory effect of BMP-2 was dose-dependent, and treatment of Ob cells with BMP-2 at 0.3–3.3 nM (10–100 ng/ml) for 6 h increased gremlin mRNA levels (Fig. 2). Western blot analysis of conditioned medium from Ob cells revealed that BMP-2 at 3.3 nM for 24 h, but not for 2 or 6 h, induced a protein reacting with the gremlin antibody and migrating with a M_r of approximately 28 kDa, the known M_r of gremlin (Fig. 3) (15). The protein comigrated with one of the products of COS-7 cells overexpressing human gremlin; however, the conditioned medium of COS-7 and Ob cells contained additional proteins that reacted with the gremlin antibody and could not be characterized. To determine whether some of the proteins in Ob cell-conditioned medium represented different degrees of gremlin glycosylation, medium samples were precipitated and exposed to a mixture of deglycosylating enzymes. The predominant immunoreactive band induced by BMP-2 migrated with a M_r of 28 kDa before and 26 kDa after deglycosylation, confirming prior observations that glycosylation plays a role in the M_r heterogeneity of gremlin (31). BMP-2 increased gremlin polypeptide at 1 and 3.3 nM, and at lower doses it had an inconsistent effect (not shown).

To determine whether the effects of BMP-2 on gremlin mRNA expression were dependent on protein synthesis, Ob cells were exposed to BMP-2 in the presence and absence of the protein synthesis inhibitor cycloheximide at 3.6 μM, a dose known to block protein synthesis in Ob cell cultures (32). Cycloheximide caused a small increase in gremlin mRNA levels, suggesting transcript superinduction due to mRNA stabilization and did not modify the effect of BMP-2 on gremlin mRNA, indicating the lack of protein synthesis dependency (Fig. 4). BMP-2 increased gremlin transcripts by (mean ± SEM; n = 3) 7.7 ± 0.5-fold in the absence of cycloheximide and by 10.1 ± 4.1-fold in the presence of cycloheximide. To determine whether BMP-2 modified gremlin gene transcription, nuclear run-on assays were performed twice on nuclei from Ob cells. The assay demonstrated that BMP-2 for 6 h induced the rate of gremlin transcription by about 2-fold (Fig. 5).

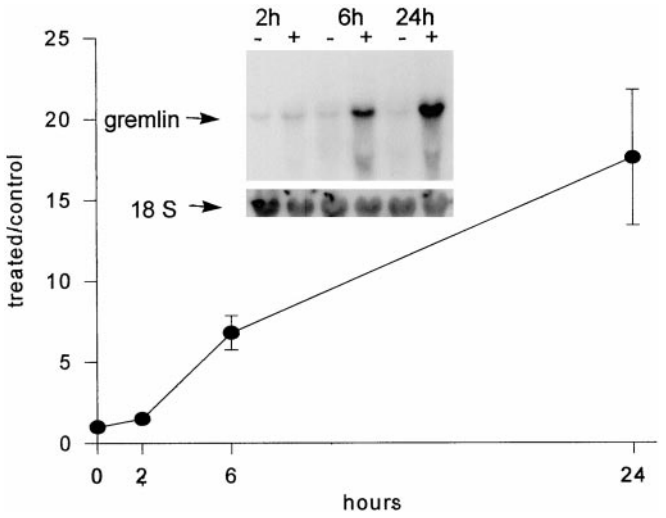

FIG. 1. Effect of BMP-2 at 3.3 nM on gremlin mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control or BMP-2-treated cultures was subjected to Northern blot analysis and hybridized with an α-^{32}P-labeled gremlin cDNA. The blot was stripped and rehybridized with labeled 18S cDNA. The effect of BMP-2 is expressed, relative to untreated control cultures, following gremlin mRNA visualization by autoradiography, quantitation by densitometry, and correction by the level of 18S hybridization. Symbols represent the mean ± SEM for three cultures. *Inset*, A representative Northern blot.

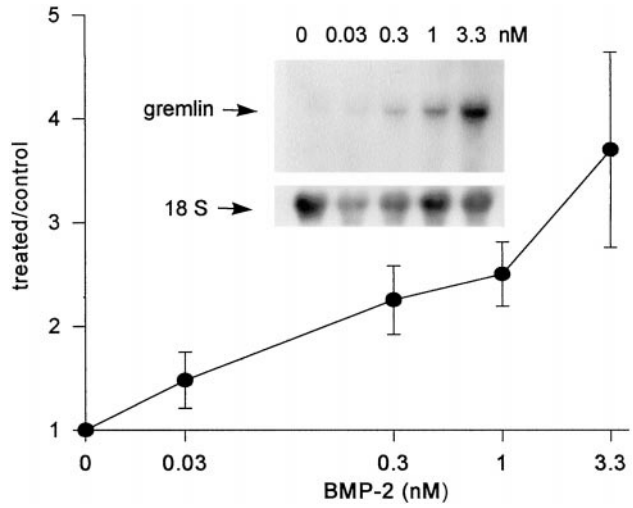

FIG. 2. Effect of BMP-2 at 0.03–3.3 nM on gremlin mRNA expression in cultures of Ob cells treated for 6 h. Total RNA from control (0) or treated cultures was subjected to Northern blot analysis and hybridized with an α-^{32}P-labeled gremlin cDNA. The blot was stripped and rehybridized with labeled 18S cDNA. The effect of BMP-2 is expressed, relative to untreated control cultures, after gremlin mRNA visualization by autoradiography, quantitation by densitometry, and correction by the level of 18S hybridization. Symbols represent the mean ± SEM for four cultures. *Inset*, A representative Northern blot.

Similar to the effect of BMP-2, FGF-2 at 1.7 nM and PDGF BB at 3.3 nM induced gremlin mRNA levels in Ob cells (Fig. 6). The degree of the stimulatory effect in the experiment shown in Fig. 6 was difficult to determine accurately because gremlin mRNA levels were below the level of detection in control cultures. After 6 h, FGF-2 and PDGF BB increased gremlin mRNA, after correction for 18S hybridization, by 3- to 4-fold. In contrast, TGFβ1 at 1.2 nM and IGF-I at 100 nM had no effect (not shown). Therefore, the induction of gremlin appeared to be selective to BMPs, FGF-2, and PDGF-BB. Treatment with hormones, such as GH at 50 μM, insulin at 100 nM, T_3 at 10 nM, cortisol at 1 μM, PTH at 10 nM, and 1,25-dihydroxyvitamin D_3 at 100 nM, for 2, 6, and 24 h did not modify gremlin expression in Ob cells (not shown). Similar to its effects in Ob cells, BMP-2 also increased gremlin mRNA levels in the mouse MC3T3 osteoblastic cell line (Fig. 7). The effect was noted after 2 h, was maximal after 6 h, and was sustained for 24 h.

To confirm that gremlin modified cellular functions regulated by BMPs, the effects of BMP-2 at 3.3 nM on DNA, collagen, and noncollagen protein synthesis and on APA in Ob cells were examined in the presence and absence of gremlin at 30 nM. After 24 h, BMP-2 at 3.3 nM stimulated the

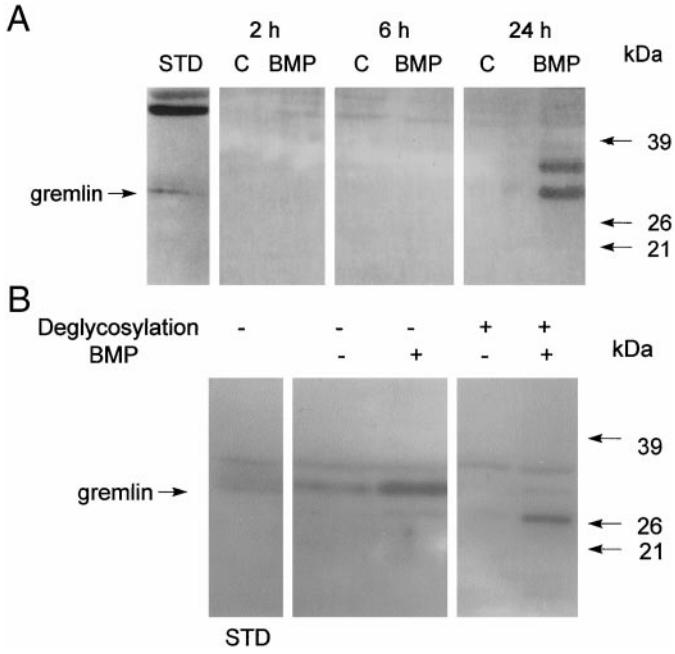

FIG. 3. Effect of BMP-2 on gremlin polypeptide levels in cultures of Ob cells. In A, cells were treated with BMP-2 at 3.3 nM for 2, 6, and 24 h, and a 750-μl aliquot of conditioned medium, representing a culture well, from control (−) and BMP-2-treated cultures (+) was trichloroacetic acid precipitated and subjected to Western immunoblot analysis. In B, cells were treated with BMP-2 at 1 nM for 24 h, and aliquots were precipitated and processed in the absence (−) or presence (+) of deglycosylating agents. Supernatant from cultured COS-7 cells overexpressing human gremlin was used as a standard (STD). Gremlin was detected using an antigremlin antibody and a chemiluminescence detection system. Migration of M_r markers in kilodaltons is indicated on the *right*.

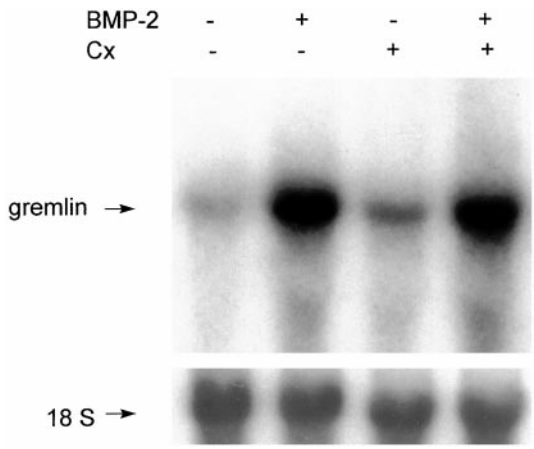

FIG. 4. Effect of BMP-2 (1 nM) in the presence or absence of cycloheximide (Cx) at 3.6 μM on gremlin mRNA expression in cultures of Ob cells treated for 6 h. Total RNA from control or BMP-2 treated cultures was subjected to Northern blot analysis and hybridized with an α-^{32}P-labeled gremlin cDNA. The blot was stripped and rehybridized with labeled 18S cDNA. Gremlin mRNA was visualized by autoradiography and is shown in the *upper panel*, and 18S ribosomal RNA is shown *below*.

incorporation of [^{3}H]thymidine into DNA and of [^{3}H]proline into collagen and noncollagen protein and increased APA (Fig. 8). Gremlin at 30 nM decreased basal DNA synthesis and

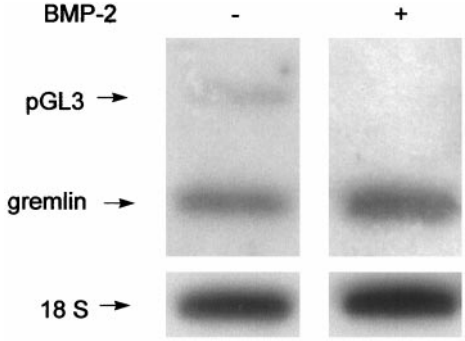

FIG. 5. Effect of BMP-2 (1 nM) on gremlin transcription rates in cultures of Ob cells treated for 6 h. Nascent transcripts from control (−) or BMP-2-treated (+) cultures were labeled *in vitro* with [α-^{32}P]UTP, and the labeled RNA was hybridized to immobilized gremlin cDNA. 18S cDNA was used to demonstrate loading, and pGL2-Basic vector DNA was used as a control for nonspecific hybridization.

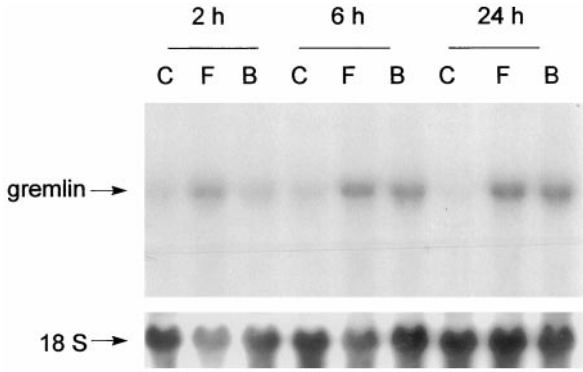

FIG. 6. Effect of FGF-2 (F) at 1.7 nM and PDGF-BB (B) at 3.3 nM on gremlin mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (C) or treated cultures was subjected to Northern blot analysis and hybridized with an α-^{32}P-labeled gremlin cDNA. The blot was stripped and rehybridized with labeled 18S cDNA. Gremlin mRNA was visualized by autoradiography and is shown in the *upper panels*, and 18S ribosomal RNA is shown in the *lower panel*.

prevented the effect of BMP-2 at 3.3 nM on DNA, collagen and noncollagen protein synthesis, and APA in Ob cells.

Discussion

BMPs play an essential role in bone remodeling and in maintaining the structural integrity of the skeletal system due to their ability to induce bone formation and osteoblast differentiation. Gremlin was recently shown to bind BMPs and, like noggin, to diminish their biological effects. In the present studies, we demonstrate that in unstimulated rat osteoblasts, gremlin is expressed at low levels, but its expression is up-regulated by BMPs. The stimulatory effect of BMP-2 on gremlin was time and dose dependent and was associated with an increase in gremlin polypeptide levels. BMP-2 acted by transcriptional mechanisms, and the effect was also observed in MC3T3 cells, a murine osteoblastic line. However, the effects of BMP-2 on the rate of gremlin transcription were of lesser magnitude than those on gremlin mRNA levels, indicating the possibility of a posttranscrip-

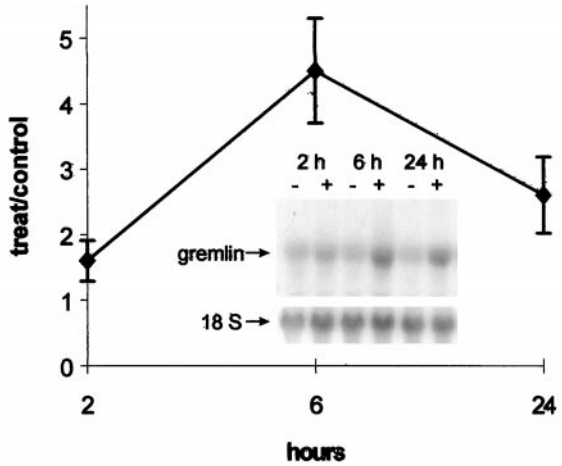

FIG. 7. Effect of BMP-2 at 3.3 nM on gremlin mRNA expression in cultures of MC3T3 cells treated for 2, 6, or 24 h. Total RNA from control or BMP-2-treated cultures was subjected to Northern blot analysis and hybridized with an α-^{32}P-labeled gremlin cDNA. The blot was stripped and rehybridized with labeled 18S cDNA. The effect of BMP-2 is expressed, relative to untreated control cultures, following gremlin mRNA visualization by autoradiography, quantitation by densitometry, and correction by the level of 18S hybridization. Symbols represent the mean ± SEM for four cultures. *Inset*, A representative Northern blot.

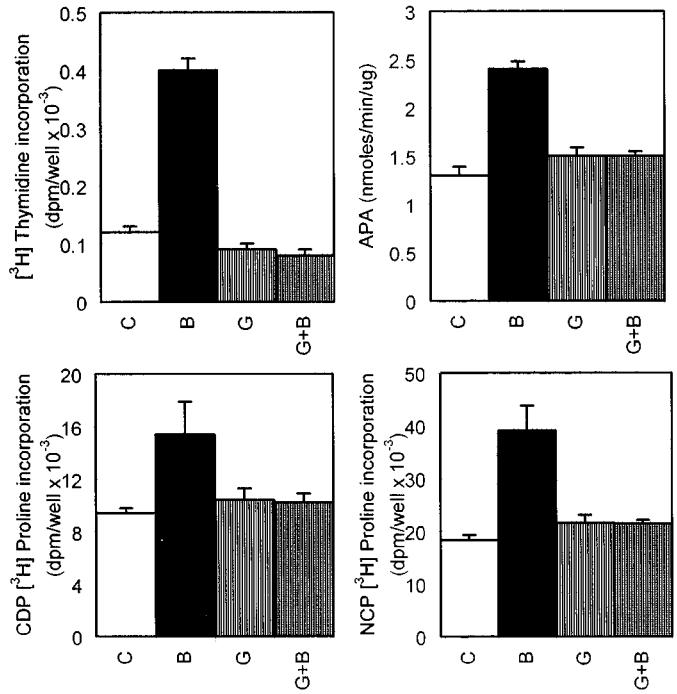

FIG. 8. Effect of BMP-2 at 3.3 nM (B), in the presence and absence of gremlin at 30 nM (G) on [^{3}H]thymidine incorporation into DNA, APA, and [^{3}H]proline incorporation into collagen (CDP) and noncollagen protein (NCP) in Ob cells treated for 24 h. Values represent the mean ± SEM for five or six cultures. For all parameters studied, the effect of BMP-2 was significantly different from the control ($P < 0.05$), and the effect of gremlin and BMP-2 was significantly different from that of BMP-2 alone ($P < 0.05$). The effect of gremlin alone on DNA labeling, but not on APA or CDP and NCP labeling, was significantly different from the control ($P < 0.05$).

tional level of regulation. This was not explored due to the virtually undetectable levels of gremlin mRNA in control cultures. Thus, it was not possible to establish decay rates of gremlin mRNA in control cultures after transcriptional arrest.

FGF-2 and PDGF-BB induced gremlin, but other hormones and growth factors known to regulate bone cell function failed to up-regulate gremlin expression. Although PDGF-BB and FGF-2 have important mitogenic activity for skeletal cells, they do not acutely modify the differentiated function of osteoblasts like BMPs (33, 34). It is possible that during periods of mitogenesis, there is a need to prevent the effects of BMPs on cell differentiation, and gremlin induction could play such a role. It is important to note that in contrast to their actions on gremlin induction, neither FGF-2 nor PDGF-BB induces noggin transcripts in osteoblasts, whereas BMPs induce both noggin and gremlin (8).

In the present studies we confirm that BMP-2 has modest mitogenic properties for Ob cells, stimulates collagen and noncollagen protein synthesis, and increases APA in osteoblasts (8). Gremlin, like noggin, decreased the biological effect of BMP-2 on osteoblastic function, indicating that it is capable of attenuating the effects of BMPs in osteoblasts, as previously shown in stromal cells (14). Our studies also indicate that gremlin, like noggin, can antagonize selected actions of endogenous BMPs, such as DNA labeling and collagenase expression (35). These observations in conjunction with an induction of gremlin and noggin after exposure to BMPs indicate a possible role for these two factors in limiting the actions of BMPs in cells of the osteoblastic lineage. It is intriguing that the expression of noggin and gremlin in unstimulated cells is limited. This would suggest that they act as BMP-inducible binding proteins to prevent overexposure of osteoblasts to the morphogenetic proteins. There have been no reports of direct cellular actions of gremlin or noggin or of the existence of specific receptors for either peptide, suggesting that their primary function is to act as BMP-binding proteins. As such, they could prevent the effects of BMP, prolong their half-lives, and store or transport BMPs to other target cells in the bone microenvironment. Our studies also confirm recent observations demonstrating posttranslational modifications of gremlin in the form of glycosylation (31).

The induction of noggin and gremlin by BMPs should not be surprising, as they may regulate the amount of effective or biologically available BMPs in a manner analogous as that described for IGFs and IGF-binding proteins (IGFBP) (36). Frequently, agents that induce IGF I expression also enhance the synthesis of selected IGFBPs by the osteoblast. This would suggest not only a coordinated synthesis among binding proteins and IGF-I, but also a possible role for the binding proteins in modifying the activity of IGF I in bone (37, 38). In addition, IGF I itself induces the transcription of IGFBP-5 in osteoblasts, so that the induction of a binding protein by a growth factor is not unique to BMPs (39).

Binding proteins for other members of the TGFβ family of peptides also have been reported. Follistatin binds and regulates the activity of activin in skeletal and nonskeletal cells, although there are no reports of follistatin induction by activin in osteoblasts (40, 41). The induction of gremlin and noggin by BMPs may be a necessary protective mechanism to avoid excessive exposure of skeletal cells to BMPs in the

bone healing and remodeling processes. In fact, inappropriate expression of BMP-4 is associated with fibrodysplasia ossificans progressiva, and transgenic mice overexpressing TGFβ2 in the bone microenvironment develop osteopenia, suggesting that excessive cellular exposure to growth factors may be detrimental (42, 43). Recently, we demonstrated the suppression of BMP-4 by BMPs, suggesting additional negative feedback loops to regulate BMP levels and action in the skeletal system (5). These observations, in conjunction with the induction of gremlin after exposure to BMPs, indicate a complex interaction between BMPs and their antagonists to control BMP activity in the bone microenvironment.

In conclusion, the present studies demonstrate that BMPs cause an induction of gremlin mRNA and protein levels in skeletal cells by transcriptional mechanisms, and gremlin prevents the effects of BMPs in osteoblasts. The induction of gremlin by BMPs could be an additional mechanism to limit the effects of BMP in bone.

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