Nuclear Factor-κB p65 Facilitates Longitudinal Bone Growth by Inducing Growth Plate Chondrocyte Proliferation and Differentiation and by Preventing Apoptosis

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NF-κB is a group of transcription factors involved in cell proliferation, differentiation, and apoptosis. Mice deficient in the NF-κB subunits p50 and p52 have retarded growth, suggesting that NF-κB is involved in bone growth. Yet, it is not clear whether the reduced bone growth of these mice depends on the lack of NF-κB activity in growth plate chondrocytes. Using cultured rat metatarsal bones and isolated growth plate chondrocytes, we studied the effects of two NF-κB inhibitors (pyrrolidine dithiocarbamate (PDTC) or BAY11-7082 (BAY)), p65 short interference RNA (siRNA), and of the overexpression of p65 on BMP-2 expression and activity. To test our hypotheses, we first evaluated the protein expression of p65 (one of the most studied members of the NF-κB family), p50 and p52 in the rat metatarsal and tibial growth plate. Secondly, we cultured whole rat metatarsal bones in the presence of pyrrolidine dithiocarbamate (PDTC) or BAY11-7082 (BAY), two specific NF-κB inhibitors, and studied their effects on cell growth and survival.

Previous evidence indicates that NF-κB exerts a regulatory role in bone growth and development. In chick embryo, overexpression of IκB-α, which prevents NF-κB activation, results in abnormal limb development (7). It has also been shown that mice deficient in both the NF-κB subunits p50 and p52 have retarded growth and shortened long bones, suggesting that NF-κB may be involved in bone formation and growth (8).

Bone morphogenetic proteins (BMPs) play an important role in skeletal development (10–12). At an early stage of bone development, BMP signaling is required for prechondrogenic condensation of mesenchymal cells (13). At a late stage, BMP-2 accelerates longitudinal bone growth by stimulating growth plate chondrocyte proliferation and hypertrophy (14–16). Previous experimental evidence suggests that NF-κB and BMPs may interact in growth plate chondrocytes (17). Based on these findings, we hypothesized that 1) NF-κB promotes longitudinal bone growth by stimulating growth plate chondrogenesis and preventing chondrocyte apoptosis, and 2) the growth-promoting effect of NF-κB is mediated by BMP-2.
effects on metatarsal longitudinal growth. PDTC (18–21) and BAY (22–24) inhibit NF-κB activation by suppressing IκB-ubiquitin ligase activity and IκB kinase activity, respectively. In addition, we analyzed the effects of both NF-κB inhibitors, p65-specific siRNA, and of the overexpression of p65 in cultured growth plate chondrocyte proliferation, differentiation, and apoptosis. Thirdly, we evaluated the effects of the NF-κB inhibitors, p65 siRNA, and overexpressed p65 on the expression of BMP-2 in growth plate chondrocytes. Lastly, we studied cell proliferation, differentiation, and apoptosis in chondrocytes transfected with the p65 plasmid and cultured in the presence of Noggin (a BMP-2 antagonist).

**MATERIALS AND METHODS**

**Organ Culture**—The second, third, and fourth metatarsal bone rudiments were isolated from Sprague-Dawley rat fetuses at 20 days postconception and cultured individually in 24-well plates (25, 26). Each well contained 0.5 ml of minimum essential medium (Invitrogen), supplemented with 0.05 mg/ml ascorbic acid (Sigma), 1 mM sodium glycerophosphate (Sigma), 0.2% bovine serum albumin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Bone rudiments were cultured for 3 days in a humidified incubator with 5% CO₂ in air at 37 °C. The medium was changed on day 2. Metatarsals were cultured for 3 days in serum-free medium in the absence or presence of PDTC (1 and 2 μM, Sigma) or BAY (1–10 μM, EMD Biosciences Inc., San Diego, CA), two specific NF-κB inhibitors. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication [National Institutes of Health] 85-23, revised 1988).

**Measurement of Longitudinal Growth**—The length of each bone rudiment was measured under a dissecting microscope, using an eyepiece micrometer. To calculate the metatarsal growth rate, length measurements were performed at the beginning and at the end of the experiments.

**Quantitative Histology**—At the end of the culture period, metatarsals were fixed in 4% paraformaldehyde overnight. After routine processing, three longitudinal, 5- to 7-μm-thick sections were obtained from each metatarsal bone and stained with toluidine blue. From each of the three sections, we measured the height of the epiphyseal, proliferative zones, and then averaged. For each treatment group, we sampled eight bones and analyzed both growth plates of each of three longitudinal sections per bone. All determinations were made by the same observer blinded to the treatment category.

To assess proliferation in cultured chondrocytes, 2.5 μCi/ml [³H]thymidine (Amersham Biosciences) was added to the culture medium for an additional 3 h at the end of the culture period. Cells were then released by trypsin and stained onto glass fiber filters. Incorporation of [³H]thymidine was measured by liquid scintillation counting.

**In Situ Hybridization**—Metatarsals were fixed overnight in 4% paraformaldehyde at 4 °C, then dehydrated in ethanol and embedded in paraffin. Sections (5 μm thick) were hybridized to ³⁵S-labeled Col10a1 antisense Riboprobes. Slides were exposed to photographic emulsion at 4 °C for 4 days, then developed, fixed, and cleared. Sections were counterstained with hematoxylin and viewed using a light microscope. Sections hybridized with a labeled-sense Col10a1 Riboprobe were used as negative controls. The mouse Type X collagen (Col10a1) probe (a gift from Dr. Bjorn Olsen, Harvard Medical School, Boston, MA) was a 650-bp HindIII fragment containing 400 bp of non-collagenous (NC1) domain and 250 bp of 3’-untranslated sequence of the mouse Col10a1 gene in pBluescript (27).

**Immunohistochemistry**—Bone rudiments were fixed overnight in 4% paraformaldehyde and embedded in paraffin. 5- to 7-μm-thick longitudinal sections were obtained and deparaffinized in xylene and rehydrated in graded ethanol. Sections were incubated in 1% H₂O₂ for 10 min followed by three rinses with phosphate-buffered saline. For digestion, 0.1% trypsin for 12 min was used at room temperature followed by a triple wash in phosphate-buffered saline. After preincubation with 1.5% blocking serum for 30 min at room temperature, sections were incubated for 30 min at room temperature with rabbit polyclonal antisera raised against mouse type X collagen peptides, anti-NC2 domain (1:200) (28) or rabbit anti-p65, anti-p50, or clone antisera raised against mouse type X collagen peptides, anti-NC2 domain (1:200) (28) or rabbit anti-p65, anti-p50, or anti-p52 polyclonal antibodies (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was an anti-rabbit antibody conjugated with biotin (ABC staining system, Santa Cruz Biotechnology), applied for 30 min at a dilution of 1:200. This step was followed by an incubation for 30 min with avidin and biotinylated horseradish peroxidase. The sections were then visualized with peroxide substrate for 5 min and mounted with Permound medium. Control bone sections were
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incubated with a nonspecific rabbit IgG instead of the primary antibody.

RT-PCR—At the end of the culture period, total RNA was extracted from the growth plates of fifteen rat metatarsals per group or from cultured chondrocytes using the Qiagen RNeasy Mini kit (Qiagen). Primers specific for rat collagen X (5’ primer, 5’-atatcctggatctggctg-3’; 3’ primer, 3’-agttctgctgtaatgga-3’; product size, 241 bp) (AJ131848); BMP-2 (5’ primer, 5’-ttctggctctagtattctgctc-3’; 3’ primer, 3’-tagttctagtattctgctc-3’; product size, 386 bp) (NM 017178); and NF-κB p65 (Santa Cruz Biotechnology) were used. The housekeeping gene β-actin (5’ primer, 5’-CTGACGACTCTCATGAAGATCC-3’; 3’ primer, 5’-CATAGAGGTCTTTACGGATGTCAC-3’; product size, 330 bp) (NM 031144) was used as a normalization control. The recovered RNA was further processed using a 1st Strand cDNA Synthesis kit for RT-PCR avian myeloblastosis virus (Roche Applied Science) to produce cDNA. 1 μg of total RNA and 1.6 μg of oligo-p(dT)15 primer were incubated for 10 min at 25 °C, followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. The reaction (100-μl total volume) was performed using a PerkinElmer Life Sciences GeneAmp PCR system 9600 in the presence of 20 pmol of primers, 20 nmol of dNTP, 150 nmol of MgCl2, 1×PCR buffer (Expand High Fidelity PCR buffer; Roche Applied Science), and 2.5 units of Expand High Fidelity DNA polymerase (Roche Applied Science). The conditions for amplification were 2 min 30 s at 96 °C, followed by 35 cycles of denaturation for 45 s at 96 °C, annealing for 1 min at 55 °C, elongation for 1 min 30 s at 72 °C, and finally, extension for 10 min at 72 °C. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (1.5 μg/ml).

In Situ Cell Death—At the end of the culture period, metatarsals were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and cut in 5- to 7-μm-thick longitudinal sections. From each bone, three sections parallel to the long axis of the bone were obtained. Apoptotic cells in the growth plate were identified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling, according to the manufacturer’s instructions (TDT-FragEL kit, Oncogene Research Products, Boston, MA) with slight modifications (deparaffinized and rehydrated sections were treated with protease K for 10 min instead of 20 min) (29). A positive control was generated by covering the entire tissue section with 1 μg/μl DNase I in 1×Tris-buffered saline/1 mM MgSO4 for 20 min following protease K treatment, whereas a negative control was generated by substituting dH2O for the TdT in the reaction mixture.

Apoptosis was quantitated by determining the apoptotic index (calculated as the number of apoptotic cells per grid divided by the total number of cells per grid). The grid consisted of a portion of the growth plate analyzed through a 40× objective and generally contained an average of 50 cells. In each growth plate, the apoptotic index was calculated separately in three distinct grid locations of the epiphyseal, the proliferative, and the hypertrophic zones, and then averaged. For each treatment group, we sampled five bones and analyzed both growth plates of each of three longitudinal sections per bone. All determinations were made by the same observer blinded to the treatment category.

Chondrocyte Culture—The cartilaginous regions of the metatarsal rudiments were dissected, rinsed in phosphate-buffered saline, and then incubated in 0.2% trypsin for 1 h and 0.2% collagenase for 3 h. Cell suspension was aspirated repeatedly and filtered through a 70-μm cell strainer, rinsed first in phosphate-buffered saline then in serum-free DMEM, and counted. Chondrocytes were seeded in 100-mm dishes at a density of 5 × 104/cm2 in DMEM with 100 units/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10% fetal bovine serum. The culture medium was changed on day 2. Cells at day 4 were washed with serum-free medium and treated with graded concentrations of PDTC or BAY. To confirm the chondrogenic phenotype, we studied the expression of type I, type II, and type X collagen (by immunocytochemistry and Western blot, data not shown) in a subset of cells isolated from the cartilaginous portion of the metatarsal bone. Cells were cultured only if at least 95% of the cells studied were type II and type X collagen-positive and type I collagen-negative.

Western Blot—Whole cell lysates were solubilized with 1% SDS sample buffer and electrophoresed on a 4–15% SDS-PAGE gel (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane and were probed with the following primary antibodies: rabbit polyclonal antibodies against p65, Bcl-2 and Bax (Santa Cruz Biotechnology), and goat polyclonal antibody against BMP-2 (Santa Cruz Biotechnology). The blots were developed using a horseradish-peroxidase-conjugated polyclonal goat-anti-rabbit IgG or donkey-anti-goat IgG antibody and enhanced chemiluminescence system (Amersham Biosciences). The intensity of the bands on Western blots was analyzed by ImageJ (software from NCBI). The protein size was confirmed by molecular weight standards (Invitrogen).

p65 siRNA and p65 Plasmid Transfection—Chondrocytes were transfected with siRNA targeted for NF-κB p65 (Santa Cruz Biotechnology). An siRNA consisting of a scrambled sequence was similarly transfected as control. siRNA was introduced to cells using Lipofectamine 2000 (Invitrogen), according to the procedure recommended by the manufacturer. One day before transfection, cells were plated in 500 μl of growth medium without antibiotics such that they were 30–50% confluent at the time of transfection. The transfected cells were cultured in DMEM containing 10% fetal calf serum for 72 h after transfection. In another series of experiments, chondrocytes were transfected with the expression plasmid for the NF-κB component p65-pcDNA3 (kindly provided by Dr. Tom Maniatis, Harvard University, Cambridge, MA). The empty parental pcDNA3 vector DNA was similarly transfected as control. The expression vector was introduced to cells using Lipofectamine PLUS (Invitrogen), according to the procedure recommended by the manufacturer. The transfected cells were cultured in DMEM containing 10% fetal calf serum for 48 h after transfection, with or without PDTC (1 and 2 μM), or Noggin (3 μg/ml).

To determine transfection efficiency, we transfected chondrocytes with fluorescein-conjugated control siRNA in two
separate experiments. Transfection efficiency was determined by using a fluorescence microscope, with chondrocytes exhibiting both the green (due to fluorescein-conjugated control siRNA) and blue (due to 4′,6-diamidino-2-phenylindole, a specific nuclear staining) colors being considered positive (transfected) cells (supplemental Fig. 1s). We calculated the number of positive cells divided by the total number of cells per grid, and analyzed 5 grids per sample (total of 10 grids) The grid circumscribed a portion of the cell sample analyzed through a 10× objective and generally contained an average of 40 cells. Transfection efficiency was 45.0 ± 2.4% (range of 30.8–51.4%).

Caspase 3 Assay—Cytosolic caspase 3 activity was determined in the chondrocyte culture medium containing 50 mM Tris-HCl buffer (pH 7.0), 0.5 mM Na-EDTA, 20% glycerol, 500 μg of cytosolic protein, and 75 μM of a synthetic fluorogenic substrate containing the recognition sequence for caspase 3 (Ac-DEVD-aminomethyl coumarin, Upstate Biotechnology). The caspase activity was measured spectrofluorometrically at 460 nm using 380 nm excitation wavelength at 37 °C for 300 s. The relative level of caspase 3 activity was expressed as nanomoles per mg of protein per hour. Results were expressed as percentage of control.

Electrophoretic Mobility Shift Assay—Nuclear protein extract was prepared from cultured chondrocytes. Briefly, chondrocytes incubated without or with PDTC or BAY for up to 24 h, or transfected with the expression plasmid for the NF-κB component p65-pcDNA3 for 48 h, were washed and scraped in phosphate-buffered saline, resuspended in buffer (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol) and rotated at 4 °C for 40 min. After centrifugation, the supernatant containing nuclear proteins was collected, analyzed by Bradford, and stored at −80 °C.

NF-κB binding activity was studied by using double-stranded oligonucleotides (5′-AGTGAGGCGCCCAAGGCG-3′, corresponding to the consensus NF-κB binding site, Promega, Madison, WI). The oligonucleotide probe was prepared by phosphorylation with T4 polycombine kinase (Promega) in the presence of [γ-32P]ATP (Amersham Biosciences), followed by inactivation of the kinase by adding 1 μl of 0.5 M EDTA. Nuclear proteins (10 μg) were preincubated for 10 min in NF-κB binding buffer (Promega). Radioactively labeled oligonucleotide was added, and the mixture was incubated for 30 min at room temperature. The complexes were then subjected to 6% nondenaturing acrylamide gel, electrophoresed, and analyzed by autoradiography. To assess the specificity of the NF-κB DNA binding, competition experiments were performed by using excess (10×) of unlabeled NF-κB oligonucleotides and nonspecific competitor DNA sequence (SP1).

Expression of p65 in Growth Plate Chondrocytes—We first evaluated p65 expression in the fetal (20 days post coitus) rat metatarsal growth plate by immunohistochemistry. Whole metatarsal bones were isolated from rat fetuses (20 days post coitus) and immediately processed for immunohistochemistry. Panel A is the negative control metatarsal section treated with nonspecific rabbit IgG. Panel B shows immunolocalization of p65 using a rabbit anti-p65 polyclonal antibody (1:100). Brown staining (arrow) indicates p65 protein expression. Panel C shows a representative Western blot of the p65 protein expression in control or p65-transfected cultured chondrocytes isolated from metatarsal growth plates. EZ, epiphyseal zone; PZ, proliferative zone; and HZ, hypertrophic zone.

Statistics—All data are expressed as the mean ± S.E. Statistical significance was determined by t test or by analysis of variance.

RESULTS

Expression of p65 in Growth Plate Chondrocytes—We first evaluated p65 expression in the fetal (20 days post coitus) rat metatarsal growth plate by immunohistochemistry. Whole metatarsal bones were isolated from rat fetuses (20 days post coitus) and immediately processed for immunohistochemistry. Panel A shows a representative Western blot of the p65 protein expression in control or p65-transfected cultured chondrocytes isolated from metatarsal growth plates. EZ, epiphyseal zone; PZ, proliferative zone; and HZ, hypertrophic zone.

Effects of PDTC and BAY on Longitudinal Bone Growth and Growth Plate Chondrogenesis—During the 3 days of the culture period, 2 μM PDTC induced a significant suppression of the metatarsal longitudinal growth (n = 33–35/group, Fig. 2). Consistent with this finding, 10 μM BAY11-7082 induced a similar suppression of metatarsal longitudinal growth (n = 22/group, Fig. 2). Because the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis, we evaluated the effects of PDTC and BAY on chondrocyte hypertrophy/differentiation and chondrocyte proliferation. To assess chondrocyte hypertrophy, we examined the bone rudiments histologically. After 3 days in culture, treatment with 2 μM PDTC or 10 μM BAY reduced the height of the growth plate hypertrophic zone (n = 6–9/group, Table 1, representative sections of control, PDTC-treated, and BAY-treated bones, supplemental Fig. 4s). Treatment with 2 μM PDTC or 10 μM BAY

FIGURE 1. Expression of NF-κB-p65 in the fetal (20 days post coitus) rat metatarsal growth plate and cultured growth plate chondrocytes.

A

B

C

Control

p65-transfected
chondrocytes

EZ

PZ

HZ

65 KDa

80 °C.

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Effects of PDTC and BAY on collagen X expression in the metatarsal growth plate. Collagen X protein and mRNA expression were detected in the growth plate of control (A and B), 2 μM PDTC-treated (C and D), and 10 μM BAY-treated (E and F) metatarsal bones by immunohistochemistry (protein expression) and in situ hybridization (mRNA expression). Upper panels show immunolocalization of collagen X protein using a rabbit polyclonal antibody directed against mouse collagen X, at a dilution of 1:200. Lower panels show in situ hybridization for collagen X mRNA. Brown staining (arrow) indicates collagen X protein expression, whereas black punctuate staining (arrow) indicates collagen X mRNA expression. PZ, proliferative zone; HZ, hypertrophic zone; and OC, ossification center.

TABLE 2
Effects of PDTC and BAY on metatarsal [3H]thymidine incorporation and in situ cell death

| Treatment (n = 27) | Labeling index | Apoptotic index |
|--------------------|----------------|-----------------|
|                    | Control 100.0 ± 2.3 | 4.3 ± 2.3 | EZ a 2.4 ± 0.2 | PZ a 2.1 ± 0.1 | HZ b 4.3 ± 0.3 |
| PDTC 2.3 ± 5.6  | 4.7 ± 3.4 | 6.2 ± 0.2 | 5.8 ± 0.3 | 7.3 ± 0.4 |
| BAY 4.9 ± 1.8 | 5.7 ± 4.2 | 6.6 ± 0.3 | 5.5 ± 0.2 | 7.1 ± 0.3 |

a PZ, proliferative zone; EZ, epiphyseal zone; and HZ, hypertrophic zone. b p < 0.01 versus control.

developed significantly decreased [3H]thymidine incorporation into the bone rudiments at the end of the culture period. Both 2 μM PDTC and 10 μM BAY significantly decreased [3H]thymidine incorporation into the growth plate epiphyseal and proliferative zones (Labeling index, Table 2; representative sections of control, PDTC-treated, and BAY-treated bones (supplemental Fig. 6s)). Consistent with these findings, treatment with PDTC (2 μM) or BAY (10 μM) significantly reduced the height of the growth plate proliferative zone (n = 6–9 bones/group, Table 1). To investigate the mechanisms underlying the reduced chondrocyte proliferation, we examined the expression of two cell cycle regulatory proteins, p27 (a cyclin-dependent kinase inhibitor) and cyclin D1 (a cyclin that controls progression through the G1 phase of the cell cycle). Western blot of lysates from chondrocytes isolated from the metatarsal growth plates and transfected with p65 siRNA revealed increased expression of p27 and decreased expression of cyclin D1 (supplemental Fig. 7s).

In light of the regulatory role of NF-κB on apoptosis in other cell types, we evaluated the effects of both NF-κB inhibitors on growth plate chondrocyte apoptosis by in situ cell death. 2 μM PDTC and 10 μM BAY significantly induced apoptosis in the metatarsal growth plate (Apoptotic index, Table 2; representative sections of control, PDTC- and BAY-treated bones (supplemental Fig. 8s)).

Effects of PDTC and BAY on NF-κB-DNA Binding in Chondrocytes—To determine whether PDTC and BAY specifically inhibited NF-κB activation, we studied the binding of NF-κB to DNA by performing EMSA. Chondrocytes isolated from metatarsal growth plates were cultured up to 6 h without or with PDTC or BAY, and nuclear extracts were then obtained.
Effects of p65 siRNA on NF-κB p65 Expression in Chondrocytes—To evaluate the effects of the selective inhibition of NF-κB p65 expression in growth plate chondrocytes, we transfected cultured chondrocytes with p65 siRNA. p65 siRNA reduced both mRNA and protein p65 expression (assessed by RT-PCR and Western blot respectively (Fig. 4, C and D)). When assessed by EMSA, p65 siRNA-transfected chondrocytes exhibited reduced NF-κB-DNA binding when compared with chondrocytes transfected with a control siRNA (Fig. 4E), suggesting that NF-κB p65 is the predominant component of the NF-κB-DNA complex in chondrocytes.

Effects of PDTC, BAY, and p65 siRNA on Growth Plate Chondrocyte Proliferation and Differentiation—In cultured chondrocytes isolated from the metatarsal growth plates, 2 μM PDTC and 3 μM BAY (the lowest concentrations inhibiting NF-κB activation) suppressed chondrocyte proliferation (assessed by total [3H]thymidine incorporation (Table 3)) and differentiation (assessed by RT-PCR analysis of collagen X mRNA expression; representative blots (Fig. 5, A and B), PDTC and BAY, respectively (supplemental Table 1)). Similarly, p65 siRNA-transfected chondrocytes exhibited reduced chondrocyte proliferation (Table 3) and differentiation (representative blot (Fig. 5C and supplemental Table 1)) when compared with chondrocytes transfected with control siRNA.

Effects of PDTC, BAY, and p65 siRNA on Growth Plate Chondrocyte Apoptosis—To determine the molecular mechanisms underlying growth plate chondrocyte apoptosis, we studied the effects of PDTC, BAY, and p65 siRNA on caspase 3 activity and Bcl-2/bax protein expression. In cultured chondrocytes, PDTC, BAY, and p65 siRNA significantly induced caspase 3 activity (Table 4) and decreased the ratio of bcl-2/bax protein expression (Table 4) (representative blots (Fig. 5, D–F, and Table 4).

Overexpression of p65 on Growth Plate Chondrocyte Proliferation, Differentiation, and Apoptosis—To further elucidate the role of NF-κB-p65 in the growth plate, we transiently transfected cultured growth plate chondrocytes with a NF-κB-p65 plasmid. Overexpression of p65 in chondrocytes increased NF-κB-DNA binding activity when compared with cells trans-

![FIGURE 4. Effects of PDTC, BAY, and p65 siRNA on NF-κB-DNA binding activity, and effects of p65 siRNA on NF-κB p65 expression.](image)

![FIGURE 5. Effect of PDTC, BAY, and p65 siRNA on collagen X mRNA and Bcl-2/Bax protein expression.](image)

### TABLE 3

| Control | PDTC (2 μM) | BAY (3 μM) | p65 siRNA | p65 siRNA + BMP-2 | p65 plasmid | p65 plasmid + PDTC | p65 plasmid + noggin |
|---------|-------------|------------|-----------|------------------|-------------|-------------------|---------------------|
| % of control | 75.2 ± 3.8 | 191.1 ± 23.6 | 103.7 ± 10.7 | 97.4 ± 5.8 |

[^p]: p < 0.01 versus control.
[^a]: p < 0.05 versus p65 siRNA.
[^b]: p < 0.05 versus p65 plasmid.
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TABLE 4
Effects of PDTC, BAY, p65 siRNA, and p65 plasmid on caspase activity, and Bcl-2/Bax expression in cultured chondrocytes

|                  | Control | PDTC     | BAY     | p65 siRNA | p65 siRNA + BMP-2 | p65 plasmid | p65 plasmid + PDTC | p65 plasmid + noggin |
|------------------|---------|----------|---------|-----------|-------------------|-------------|--------------------|----------------------|
| Caspase 3 activity | 100.0 ± 16.7 | 219.8 ± 25.2 | 286.9 ± 46.3 | 244.3 ± 18.4 | 92.8 ± 2.0 | 61.8 ± 7.2 | 77.5 ± 29.4 | 119.0 ± 11.0 |
| Bcl-2/Bax        | 100.0 ± 15.9 | 32.9 ± 7.6 | 20.7 ± 5.0 | 43.9 ± 3.0 | 75.4 ± 4.6 | 561 ± 64.7 | 964 ± 9.3 | 94.9 ± 38.7 |

* p < 0.05 versus control.
* p < 0.05 versus p65 siRNA.
* p < 0.05 versus PDTC.
* p < 0.05 versus p65 plasmid.

FIGURE 6. Effects of the overexpression of NF-κB-p65 plasmid on NF-κB-DNA binding activity, collagen X mRNA expression, and Bcl-2/Bax protein expression. A, chondrocytes were transfected with either an empty vector (control) or a NF-κB-p65 plasmid. After transfection, cells were cultured for 48 h with or without PDTC (1 μM). A labeled oligonucleotide of NF-κB consensus element was incubated with the growth plate chondrocyte nuclear extract. DNA binding was analyzed by EMSA. The arrow indicates the NF-κB-DNA complex. Representative results of three experiments are depicted. For specificity, NF-κB-DNA binding was competed out with a NF-κB cold probe and with the SP-1 cold probe. B, total RNA was extracted from chondrocytes transfected with an empty vector (control) or NF-κB-p65 plasmid, cultured in the presence or absence of 1 μM PDTC, and then reverse-transcribed to cDNA. Collagen X mRNA expression was detected by RT-PCR. The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. Representative results of three independent experiments are presented. C, chondrocytes were transfected with either an empty vector (control) or a plasmid containing NF-κB-p65. After transfection, cells were cultured for 48 h with or without PDTC (2 μM) and subsequently harvested, lysed, electrophoresed, and immunoblotted for Bcl-2, Bax, and the loading control, β-actin. A representative blot from three independent experiments is presented.

FIGURE 7. Effects of PDTC, BAY, and p65 siRNA on BMP-2 expression. A–C, cultured chondrocytes were treated without or with PDTC (1 and 2 μM) (A), BAY (1 and 3 μM) (B) for 24 h, or transfected with p65 siRNA (C) for 72 h. Total RNA was then extracted and then reverse-transcribed to cDNA. BMP-2 mRNA expression was detected by RT-PCR. The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. Representative results of three independent experiments are presented. D–F, chondrocytes cultured in the absence or presence of PDTC (1 and 2 μM) (D), BAY11-7082 (1 and 3 μM) (E), or transfected with p65 siRNA (F) were harvested, lysed, electrophoresed, and immunoblotted for BMP-2 and the loading control, β-actin. A representative blot from three independent experiments is presented.

BMP-2 mRNA (representative gels (Fig. 7, A–C, and supplemental Table 2s)) and protein (representative blots (Fig. 7, D–F, and supplemental Table 2s)). Conversely, overexpression of p65 markedly increased BMP-2 mRNA and protein expression (Fig. 8, A and B, and supplemental Table 2), with the addition of 1 μM PDTC reversing both effects.

To determine whether the effects of NF-κB-p65 on chondrocyte function (proliferation, differentiation, and apoptosis) are mediated by BMP-2, we cultured chondrocytes in the presence of Noggin, a BMP antagonist. The addition of Noggin to the culture medium of chondrocytes transfected with the NF-κB-p65 plasmid reversed the p65-mediated increase in chondrocyte proliferation (assessed by total [3H]thymidine incorporation, Table 3) and differentiation (assessed by RT-PCR analysis of collagen X mRNA expression; representative blot (Fig. 8C and supplemental Table 1)). Furthermore, Noggin neutralized the p65-mediated anti-apoptotic effect in transfected chondrocytes, as assessed by caspase 3 activity (Table 4) and bcl-2/bax ratio of protein expression (Table 4) (representative blot (Fig. 8D)).

To confirm the specific role of BMP-2 in the NF-κB-65 effects on chondrocytes, recombinant human BMP-2 was added in the medium of cultured chondrocytes transfected

Functional Interaction between NF-κB p65 and BMP-2—To determine whether NF-κB p65 regulates BMP-2 mRNA and protein expression in growth plate chondrocytes, we performed RT-PCR and Western blot in cultured chondrocytes. 2 μM PDTC, 3 μM BAY, and p65 siRNA reduced the expression of
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FIGURE 8. Functional interaction between NF-κB-p65 and BMP-2. A, total RNA was extracted from chondrocytes transfected with NF-κB-p65 plasmid (control chondrocytes were transfected with an empty vector), cultured in the presence or absence of PDTC (1 μM), and then reverse-transcribed to cDNA. BMP-2 mRNA expression was detected by RT-PCR. The housekeeping gene β-actin was used as normalization control. PCR products were separated by electrophoresis in a 2% agarose gel with ethidium bromide. Representative results of three independent experiments are presented. B, chondrocytes transfected with NF-κB-p65 plasmid and cultured in the presence or absence of PDTC (1 μM) were harvested, lysed, electrophoresed, and immunoblotted for BMP-2 and the loading control, β-actin. A representative blot from three independent experiments is presented. C, chondrocytes were transfected with either the empty vector (control) or a plasmid containing NF-κB-p65. After transfection, cells were cultured for 48 h without or with Noggin (3 μg/ml). Total RNA was extracted from chondrocytes and then reverse-transcribed to cDNA. Collagen X mRNA expression was detected by RT-PCR. D, chondrocytes transfected with NF-κB-p65 plasmid and cultured in the absence or presence of 3 μg/ml Noggin were harvested, lysed, electrophoresed, and immunoblotted for Bcl-2, Bax and the loading control, β-actin. A representative blot from three independent experiments is presented. E, total RNA was extracted from chondrocytes transfected with p65 siRNA and cultured in the presence or absence of recombinant human BMP-2 (100 ng/ml). RNA was reverse-transcribed to cDNA. Collagen X mRNA expression was detected by RT-PCR. F, chondrocytes transfected with p65 siRNA and cultured in the absence or presence of recombinant human BMP-2 (100 ng/ml) were harvested, lysed, electrophoresed, and immunoblotted for Bcl-2, Bax and the loading control, β-actin.

with p65 siRNA. Recombinant human BMP-2 significantly neutralized the p65 siRNA-mediated inhibition on total [3H]thymidine incorporation (Table 3) and collagen X mRNA expression (representative blot (Fig. 8E and supplemental Table 1)). Recombinant human BMP-2 also reversed the proapoptotic effect of p65 siRNA, as analyzed by caspase 3 activity assay (Table 4) and bcl-2/bax ratio of protein expression (Table 4) (representative blot (Fig. 8F).

DISCUSSION

In mammals, the rate of longitudinal bone growth depends primarily on the rate of growth plate chondrogenesis. Chondrocyte proliferation and hypertrophy/differentiation are the primary determinants of the formation of new cartilage in the growth plate, chondrogenesis (30). After the terminally differentiated chondrocytes die by apoptosis at the metaphyseal junction, the growth plate is invaded from the metaphysis by blood vessels and bone cell precursors, which remodel the cartilage into bone tissue (31). The net result of these two well-coordinated processes is long bone elongation. Growth plate chondrogenesis is regulated by a network of endocrine and paracrine factors, which modulate the chondrocyte function via several intracellular transcription factors.

The five genes of the NF-κB family (NF-κB1, NF-κB2, RelA, c-Rel, and RelB) encode seven proteins: p65, RelB, c-Rel, p105, p100, p50, and p52 (1). These proteins exist in unstimulated cells as homo- or heterodimers bound to the IκB family proteins. Once IκBs undergo stimulus-coupled phosphorylation, ubiquitination, and proteasome-mediated degradation, the NF-κB heterodimer rapidly translocates into the nucleus, where it modulates the expression of a number of target genes.

Previous evidence has suggested that NF-κB may be implicated in the regulation of longitudinal bone growth. Mice deficient in both the p50 and p52 subunits of NF-κB fail to generate mature osteoclasts. As a result, they develop severe osteopetrosis (8). In addition, these mice show reduced growth and shortened long bones. On the other hand, their impaired longitudinal growth may be secondary to the lack of osteoclasts and not necessarily reflect the lack of activity of NF-κB in growth plate chondrocytes. We have previously demonstrated that the inhibition of the proteasomal function in growth plate chondrocytes results in decreased NF-κB activation and reduced chondrogenesis (32). Yet, these findings provide only indirect evidence of NF-κB regulating growth plate function.

In the present study, we have shown for the first time that NF-κB p65 subunit is expressed throughout the growth plate, predominantly in the epiphyseal and hypertrophic zones. The addition of two specific inhibitors of NF-κB activation (PDTC and BAY) in the culture medium caused a significant suppression of metatarsal longitudinal growth. Moreover, PDTC and BAY treatment reduced metatarsal growth plate chondrocyte proliferation (both in the epiphyseal and proliferative zones) and differentiation/hypertrophy, and induced in situ cell death in all the three zones of the growth plate. The ubiquitous localization of p65 supports the finding of a uniformly suppressed chondrogenesis throughout the growth plate. The increased cell death in the growth plate was confirmed in cultured chondrocytes treated with PDTC and BAY or transfected with a short interfering RNA specifically silencing p65 expression. Both NF-κB inhibitors and p65 siRNA reduced the expression of Bcl-2 (an anti-apoptotic molecule) and induced the expression of Bax and the activity of caspase 3 (two pro-apoptotic molecules). All these findings indicate that NF-κB p65 expressed in growth plate chondrocytes facilitates cell proliferation and differentiation/hypertrophy and prevents apoptosis. In support of a facilitatory role of NF-κB p65 in growth plate chondrogenesis, in our study the overexpression of NF-κB-p65 in chondrocytes induced chondrocyte proliferation and differentiation and prevented apoptosis.

When compared with NF-κB p65, NF-κB p50- and p52-reduced expression in the growth plate would suggest a lesser role in growth plate chondrogenesis for each of these two subunits. In addition, the fact that the double knockout mice deficient in both p50 and p52 exhibit skeletal defects not seen with either the p50 or the p52 single knockout mice indicates a redundant activity of these two subunits in the skeletal system (33, 34).

With respect to the intracellular signaling pathways activating NF-κB p65 in chondrocytes, in ATDC5 cells Nkx3.2 (an
intracellular differentiation factor) mediates the constitutive activation of p65, which in turn maintains chondrocyte viability (35). In articular chondrocytes, it has been shown that cytokine-dependent up-regulation of protein kinase Cζ leads to proteoglycan degradation by inducing the activation of NF-κB (36). However, nothing is known on the upstream signals modulating NF-κB activity in growth plate chondrocytes.

BMPs are essential for skeletal development and growth. Early in embryogenesis, BMP-2 is localized in the posterior limb bud where it participates in the pattern formation of the extremities. In addition, BMPs play a critical role in facilitating the commitment of the condensing mesenchyme undergoing chondrogenesis (14). At a late stage of development, BMPs have been shown to regulate growth plate chondrogenesis during longitudinal bone growth (15, 16). In whole metatarsal bones in culture, we have previously demonstrated that BMP-2 stimulates longitudinal bone growth and growth plate chondrogenesis (15). The overexpression of a constitutively active BMP receptor in chondrocytes caused acceleration of their differentiation toward hypertrophic chondrocytes (16).

It has recently been suggested that NF-κB may regulate BMP-2 expression in chondrocytes. Two NF-κB response elements have been found in the promoter region of the BMP-2 gene, with the nuclear extracts from a chondrocytic cell line revealing that the NF-κB subunits p65 and p50 can bind to these response elements (17). In ATDC5 cells, gene silencing of NF-κB p65 significantly inhibits BMP-2 expression (37). However, these findings do not demonstrate that the functional interaction between NF-κB and BMP-2 modulates growth plate chondrogenesis. In NF-κB p50/p52 double knockout mice, the expression of the BMP-2 mRNA in the growth plate is significantly decreased. However, the complex phenotype of these mice (they exhibit severe osteopetrosis due to the absence of osteoclast development) renders difficult the determination whether the reduced BMP-2 expression directly depends on the lack of NF-κB activity in growth plate chondrocytes. Our findings of NF-κB inhibitors suppressing and overexpressed p65-increasing BMP-2 expression support the functional interaction of NF-κB and BMP-2 specifically in the growth plate. Such interaction is further confirmed by Noggin, a BMP-2 antagonist, neutralizing the effects of overexpressed p65 on growth plate chondrocyte proliferation, differentiation, and apoptosis.

In conclusion, our findings show for the first time that NF-κB-p65 expressed in growth plate chondrocytes facilitates longitudinal bone growth by inducing chondrocyte proliferation and differentiation and by preventing apoptosis. The p65-dependent induction of BMP-2 expression and the effects of Noggin in p65-transfected chondrocytes indicate that the NF-κB-p65-promoting effects on growth plate chondrogenesis are mediated, at least in part, by BMP-2.

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