NF-κB Specifically Activates BMP-2 Gene Expression in Growth Plate Chondrocytes in Vivo and in a Chondrocyte Cell Line in Vitro*

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Bone morphogenetic protein-2 (BMP-2) regulates growth plate chondrogenesis during development and postnatal bone growth, but the control mechanisms of BMP-2 expression in growth plate chondrocytes are unknown. Here we have used both in vitro and in vivo approaches to demonstrate that transcription factor, NF-κB, regulates BMP-2 gene expression in chondrocytes. Two putative NF-κB response elements were found in the −2712/+165 region of the BMP-2 gene. Cotransfection of mutant IκBα expression plasmids with BMP-2 promoter-luciferase reporters into TMC-23 chondrocyte cell line suppressed BMP-2 transcription. Mutations in NF-κB response elements in the BMP-2 gene lead to decreases in BMP-2 promoter activity. Electrophoretic mobility shift assay using nuclear extracts from TMC-23 chondrocytic cells revealed that the NF-κB subunits p50 and p65 bound to the NF-κB response elements of the BMP-2 gene. Thus, NF-κB may positively regulate BMP-2 gene transcription. Consistent with these findings, expression of BMP-2 mRNA was significantly reduced in growth plate chondrocytes in NF-κB p50/p52 double KOs, which associated with decreased numbers of 5-bromo-2′-deoxyuridine (BrdUrd)-positive cells in the proliferating zone of growth plate in these mice. Therefore, in postnatal growth plate chondrocytes, expression of BMP-2 is regulated by NF-κB, which may play an important role in chondrogenesis.

In long bones, morphogenesis is followed by an extended period of growth. Longitudinal growth occurs at the growth plate by endochondral ossification, a process in which cartilage is formed and then remodeled into bone (1, 2). The multistep process of chondrogenesis involves: 1) the aggregation of mesenchymal cells into prechondrogenic condensations, 2) differentiation of the condensed prechondrogenic mesenchyme into small immature chondrocytes, and 3) chondrocyte maturation followed by ossification, starting in the center of the element.

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† The abbreviations used are: BMP, bone morphogenetic protein; KO, knock-out; dKO, double KO; dpc, days postcoitum; BrdUrd, 5-bromo-2′-deoxyuridine; NRE, NF-κB response element; PDTC, pyrrolidine dithiocarbamate; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift assay.
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2 This paper is available on line at http://www.jbc.org.
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EXPERIMENTAL PROCEDURES

BMP-2 Promoter-LacZ Transgenic Mice—The BMP-2 promoter fragment (−2712 to +165) linked to AUG-LacZ plasmid (gift of Dr. Eric Olson, University of Texas Southwest Medical Center, Dallas, TX) was removed and purified from vector, and the transgene was injected into eggs from CB6F1 females. After fertilization the eggs were implanted in pseudopregnant CB6F1 females and lines were screened for the presence of the transgene by Southern hybridization. Transgenic lines had 2–20 copies per genome and LacZ staining patterns were independent of copy numbers. Three lines of transgenic mice with the −2712/+165 bp BMP-2 promoter construct were evaluated and all gave similar patterns of expressions at 13.5 to 15.5 days postcoitum (dpc).

LacZ Staining—Male transgenic animals containing the BMP-2 promoter-LacZ transgene were crossed to CB6F1 females and embryos were collected at appropriate times, fixed, and stained overnight as described by Lazik et al. (16). Embryos were embedded in plastic and sectioned at 15 μm and viewed using Zeiss Axioplot microscope under dark-field illumination (16).

Cell Culture and Luciferase Assay—TMC-23 cells were cultured with Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. Cells were plated in 24-well plates at density of 2 × 10^4 cells/well. 24 h later, cells were transfected with BMP-2 promoter constructs (−2712/+165/pGL3 and −150/+165/pGL3) or pNF-κB-luc reporter and β-galactosidase with either empty vector (pCMX) or mutant IκBα expression plasmids (mIκBα-pCMX and mIκBα-pCMX/13) using LipofactAMINE Plus reagents (Invitrogen). 48 h after transfection, cells were lysed with lysis buffer, and luciferase activities were measured using a luciferase assay kit (Promega, Madison, WI). The luciferase activities were normalized by the β-galactosidase activities.

Site-specific mutations were created in the intact promoter by using PCR-directed mutagenesis (Stratagene, La Jolla, CA) on a construct containing a 2.7-kb BMP-2 promoter (−2712/+165) or BMP-2 minimal promoter (−150/+165) upstream of a luciferase reporter gene. The designed mutations were verified by sequencing.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extract was prepared from TMC-23 cells. Double-stranded oligonucleotides corresponding to consensus NF-κB response element were prepared and used in competition assays. NRE-1 and NRE-2 binding sites (Table I) were end-labeled and purified as described previously (17). Five fmol of labeled oligonucleotide was incubated with 10 μg TMC-23 cell nuclear extract. The incubation mix for nuclear extract binding assay consisted of binding buffer and 2 μg/ml poly(dI-dC). Incubation took place at room temperature for 5 min. Supershift experiments were carried out as described above, except that the TMC-23 nuclear extract was preincubated for 10 min at room temperature with antibodies against p50, p52, and p65 in binding buffer prior to their incubation with the labeled oligonucleotide.

TABLE I

| Putative NF-κB binding sites in BMP-2 gene and probes used in EMSA |
|---------------------------------------------------------------|
| Site                        | Sequences (bases)                          |
| NRE-1 (−838/−829)          | GGGRNYYCC                                 |
| NRE-2 (−123/−132)          | GAATTCGCC                                 |
| NRE-2                      | TGGGCTCCC                                 |
| S1P/NRE-1 (−849/−829)      | CCGCCGGCCCAGGGGATCCCC                     |
| NRE-2                     | S1P S1P NRE-1                             |

p50/p52 dKO Mice—Heterozygous NF-κB p50 and p52 knock-out mice (p50/− and p52/−) were obtained from Dr. Ulrich Siebenlist (15). NF-κB p50 and p52 homozygous knock-out mice (p50/− and p52/−) were generated by breeding p50/− and p52/− mice; respectively and NF-κB p50/p52 dKO mice (p50/− and p52/−) were generated by inter-crossing NF-κB p50/− and p52/− mice. The mice were genotyped by PCR as described previously (15).

In Situ Hybridization—Bone samples were fixed overnight in fresh 10% paraformaldehyde in RNase-free phosphate-buffered saline, transferred to ethanol series to 100%, followed by xylene and ethanol, and finally embedded in wax. 10 μm sections were placed on microscope slides, dewaxed, refixed, and utilized for in situ hybridization. A 1.0-kb mouse BMP-2 RNA probe was prepared and hybridized to appropriate sections as described by Wilkinson and Nieto (18) and Shamim et al. (19). To analyze changes in message levels of BMP-2 and BMP-6 in NF-κB p50/p52 dKO mice, in situ hybridization slides were viewed by the Nikon E400 microscope that is linked to a color video monitor, and images were captured. The message levels of BMP-2 and BMP-6 were quantitated using a software Image-Pro Plus, version 3.0 (Media Cybernetics, Silver Spring, MD).

BrdUrd Labeling—BrdUrd labeling was performed using Zymed Laboratories Inc. (South San Francisco, CA) BrdUrd labeling reagent and BrdUrd staining kit according to manufacturer’s protocols (Zymed Laboratories Inc.). Briefly, BrdUrd was injected intraperitoneally into mice 24 h before the animals were sacrificed. Bone tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. 3–4-μm thick sections were cut and placed on polylysine-coated slides. Slides were dried in a 60 °C oven for overnight and deparaffinized in 2 changes of xylene for 5 min each and then rehydrated in a series of graded alcohol. Slides were stained for BrdUrd according to manufacturer’s protocols. Streptavidin-peroxidase was used as a signal generator for the BrdUrd system, and deaminobenzidine in the presence of hydrogen peroxide was used as a chromagen, staining BrdUrd-incorporated nuclei dark brown.

Results

To understand the regulatory mechanism of BMP-2 gene in cartilage and bone tissues, previously we have isolated the BMP-2 gene and characterized the 2.7-kb BMP-2 promoter in vivo (9, 10). To further determine whether this 2.7-kb BMP-2 promoter fragment controls BMP-2 gene expression in cartilage and bone in vivo, we generated transgenic mice which express lacZ reporter gene driven by the 2.7-kb BMP-2 promoter (−2712/+165-LacZ). We analyzed the LacZ expression pattern of these mice and found strong expression of lacZ reporter gene in cartilage perichondrium and proliferating chondrocytes in 15.5 dpc mouse embryos (Fig. 1). As shown in Fig. 1A, a whole mount staining of LacZ showed predominate expression in almost all developing bones, mainly at the perichondrium and periosteum in this 15.5-dpc embryo. In Fig. 1B, an example of LacZ staining is shown, using darkfield microscopy, where the LacZ staining is red, in the perichondrium and...
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Fig. 2. NF-κB is a transcriptional activator for BMP-2 gene transcription. a, a schematic description of the BMP-2 promoter and two potential NF-κB binding sites, NRE-1 and NRE-2. b, transfection of mutant I-κBα expression plasmids reduces BMP-2 promoter (−2712/+165) activity. Wild-type (wt) and mutant BMP-2 promoter (−2712/+165) constructs were co-transfected with empty vector or mutant I-κBα expression plasmids into TMC-23 cells. The luciferase activity was measured 24 h after transfection. *, p < 0.01; #, p < 0.01, unpaired t test, compared with the group in which the wild-type or mutant BMP-2 promoter was co-transfected with empty vector into TMC-23 cells. c, regulation of BMP-2 promoter activity by TNF-α and PDTC. TMC-23 cells were transfected with the BMP-2 promoter (−2712/+165) and treated with different concentrations of TNF-α (50–200 ng/ml) or PDTC (0.25–1 μM). TNF-α significantly stimulated and PDTC inhibited BMP-2 promoter activities in a dose-dependent manner in TMC-23 cells. *, p < 0.05; #, p < 0.05, one-way analysis of variance followed by Dunnett’s test, compared with untreated control. d, expression of mutant I-κBα proteins inhibits the activity of BMP-2 minimal promoter. Wild-type and mutant BMP-2 minimal promoters (−150/+165) were co-transfected with empty vector or mutant I-κBα expression plasmids into TMC-23 cells. Transfection of mutant I-κBα expression plasmids inhibited wild-type BMP-2 minimal promoter but had no effect on mutant BMP-2 minimal promoter. *, p < 0.01, unpaired t test, compared with empty vector control. e, mutations in NRE-1 and NRE-2 sites of BMP-2 promoter. TMC-23 cells were transfected with wild-type or mutant BMP-2 promoter in the presence or absence of mutant I-κBα expression plasmids. Mutations in NRE-1 and NRE-2 caused 50% decrease in promoter activity. Expression of mutant I-κBα proteins inhibited wild-type BMP-2 promoter activity but not mutant BMP-2 promoter. *, p < 0.01, unpaired t test, compared with empty vector control.

Growing chondrocytes of the manubrium of a 15.5-dpc embryo. Fig. 1C showed the in situ hybridization of BMP-2 32P-cRNA probe to a similar embryo in the manubrium and ventricle of the heart. We also detected strong staining of LacZ in the hypertrophic regions of several endochondral bones, indicating that this promoter fragment of the BMP-2 gene can direct BMP-2 expression in this region of the growth plate (data not shown).

To determine whether NF-κB regulates BMP-2 gene transcription in chondrocytes, we analyzed the sequence of the −2712/+165 region of BMP-2 gene. Two putative NF-κB response elements were found and are designated as NRE (NF-κB response element)-1 and NRE-2. The NRE-1 is located in the 5’-flanking region and the NRE-2 is located in Exon 1 of BMP-2 gene (Table I and Fig. 2a). To determine whether these NF-κB response elements are functional in chondrocytes, we co-transfected two different mutant mouse I-κBα expression plasmids with BMP-2 promoter (−2712/+165)-luciferase reporter construct into TMC-23 cells, a clonal chondrocyte cell line (20). In one mutant I-κBα construct, the inducible phosphorylation sites (Ser32 and Ser36) and constitutive phosphorylation site at its carboxyl terminus were substituted with alanine residues (mI-κBα), and the other mutant was generated by substituting three lysine residues at the ubiquitination sites.
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Fig. 3. Binding of NF-κB to NRE-1 and NRE-2 elements in BMP-2 gene. a, TMC-23 cell nuclear extract binds NRE-1 and NRE-2 in BMP-2 promoter. Labeled oligonucleotides of NF-κB consensus element, NRE-1 and NRE-2 elements, were incubated with TMC-23 cell nuclear extract. DNA binding was analyzed by EMSA. The arrow indicates the complex of interest. b, p50 and p65 bind NRE-1 site in BMP-2 promoter. Supershift of EMSA was performed using antisera against p50, p52, and p65. The arrow indicates the complex of lower mobility observed after incubation with the anti-p50 and anti-p65 antibodies.

(I-κBΔUb). These mutant I-κBα proteins retain their ability to bind with NF-κB proteins and cannot be degraded properly and therefore block NF-κB activity in vitro and in vivo (13). Transfection of mutant I-κBα expression plasmids inhibited BMP-2 promoter activity ~50% (Fig. 2b), indicating that NF-κB response elements in BMP-2 gene serve as positive regulatory elements for BMP-2 gene transcription. To confirm that these mutant I-κBα expression plasmids do inhibit NF-κB activity in chondrocytes, we co-transfected them with pNF-κB-luc reporter construct (Promega), which contains six copies of NF-κB consensus response elements, into TMC-23 cells. Transfection of mutant I-κBα expression plasmids significantly inhibited luciferase activity of the NF-κB reporter construct (data not shown).

To confirm that NF-κB transcription factors are transcriptional activators for BMP-2 gene, we transfected BMP-2 promoter luciferase construct into TMC-23 cells and treated cells with NF-κB inducer TNF-α and NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). TNF-α induces phosphorylation of I-κBα and causes dissociation of I-κBα/NF-κB complex, resulting in nuclear translocation of NF-κB. Treatment with TNF-α (50–200 ng/ml) for 24 h caused a dose-dependent increase in BMP-2 promoter activity in TMC-23 cells (Fig. 2c). In contrast, treatment with PDTC (0.25–1 μM) for 24 h significantly inhibited BMP-2 promoter activity in a dose-dependent manner in the same cells (Fig. 2c).

To determine the function of NRE-1 in BMP-2 gene transcription, we mutated 3 nucleotides in this response element (Table I) and found that BMP-2 promoter activity was decreased over 30% when NRE-1 site is mutated (Fig. 2b). Co-transfection of mutant I-κBα expression plasmids with the 2.7-kb BMP-2 promoter, in which three nucleotides at NRE-1 site were mutated, caused reduction in BMP-2 promoter activity in TMC-23 cells (Fig. 2b), suggesting that NRE-2 site located at Exon 1 may also serve as a positive regulatory element. We then examined the effects of mutant I-κBα on BMP-2 minimal promoter (~150/ +165). The luciferase activity of BMP-2 minimal promoter is about 30% lower than that of larger BMP-2 promoter (~2712/+165) in TMC-23 cells (data not shown). Transfection of mutant I-κBα expression plasmids with BMP-2 minimal promoter (~150/+165) suppressed the activity of BMP-2 minimal promoter in TMC-23 cells (Fig. 2d). Mutation of NF-κB response element NRE-2 in this region completely abolished the inhibitory effect of mutant I-κBα (Fig. 2d). When both NRE-1 and NRE-2 sites of BMP-2 promoter (~2712/+165) were mutated, the promoter activity was reduced about 50%. Mutant I-κBα had no significant effects on this mutant BMP-2 promoter (Fig. 2c), Taken together, these results suggest that both NRE-1 and NRE-2 sites in BMP-2 gene are functional and NF-κB transcription factors positively regulate BMP-2 gene transcription.

To examine the binding of NF-κB to its response element in BMP-2 gene, we performed EMSA. The nuclear proteins were extracted from TMC-23 cell nuclear preparations. Labeled oligonucleotides containing NRE-1 and NRE-2 sequences of the BMP-2 gene (Table I) were incubated with TMC-23 cell nuclear extract, leading to the formation of a protein-DNA complex that migrated at the same location as that formed upon incubation of TMC-23 cell nuclear extract with labeled oligonucleotides containing NF-κB consensus sequence (Table I and Fig. 3a). Addition of different amounts of unlabeled oligonucleotides containing NF-κB consensus element inhibited formation of this protein-DNA complex in a dose-dependent manner (Fig. 2a). Addition of unlabeled oligonucleotides containing Sp1 element had no significant effects on NF-κB binding to NRE-1 response element (data not shown). To show that NF-κB was part of this protein-DNA complex, supershift experiments were performed using antibodies against different NF-κB subunits, p50, p52, and p65. The incubation of TMC-23 nuclear extract with antibodies against p50 and p65 prior to the addition of labeled oligonucleotides led to the formation of a slower mobility complex. This complex was specific, as it was not observed when using an antibody against p52 (Fig. 3b).

To determine whether NF-κB regulates BMP-2 gene expression in chondrocytes in vivo, we examined BMP-2 mRNA expression in 2-week-old NF-κB knock-out (KO) mice and their...
wild-type littermates by in situ hybridization (n = 3 in each group and three non-consecutive sections per mouse were analyzed). In wild-type mice, BMP-2 mRNA was predominantly expressed in the lower portion of proliferating zone, the junction between the proliferating and hypertrophic zone and the upper layer of hypertrophic zone of growth plate. BMP-2 was also highly expressed in articular chondrocytes (Fig. 4a, upper panel). In NF-κB p50 KO or p52 KO mice, no significant changes in BMP-2 mRNA expression were observed (data not shown). In contrast, in NF-κB p50/p52 dKO mice, BMP-2 mRNA expression was significantly reduced over 80% in growth plate chondrocytes (Fig. 4a, lower panel). The effect of NF-κB on BMP-2 expression seems specific, since expression of BMP-6 mRNA in growth plate chondrocytes was not significantly changed in p50/p52 dKO mice (Fig. 4b). It has been reported that BMP-2 stimulates chondrocyte proliferation (7, 8). To investigate whether decreased BMP-2 expression in NF-κB p50/p52 dKO mice causes reduction in chondrocyte proliferation, we monitored BrdUrd incorporation in growth plate chondrocytes in these mice. The numbers of BrdUrd-positive cells were decreased over 65% in proliferating chondrocytes in dKO mice (Fig. 5a and b). These results suggest that reduced BMP-2 expression in growth plate chondrocytes in dKO mice has important biological impacts on growth plate chondrogenesis.

**DISCUSSION**

The major finding of this study was that NF-κB selectively modulates BMP-2 mRNA expression in growth plate chondrocytes in vivo and directly regulates BMP-2 gene transcription.
in a chondrocyte cell line in vitro. In the −2712/+165 region of the BMP-2 gene, two NF-κB response elements were identified. Mutations of the NRE-1 and NRE-2 sites decreased BMP-2 promoter activity and responses to expression of mutant IκBα proteins in chondrocytes. In p50/p52 dKO mice, BMP-2 mRNA expression and cell proliferation were dramatically reduced in growth plate chondrocytes. These findings demonstrate that NF-κB is a transcriptional activator for BMP-2 gene transcription in chondrocytes. Of note, it has been previously reported that the NF-κB response element serves as a positive regulatory element when it is located adjacent to a Sp1 site (21). Indeed, in BMP-2 promoter, next to NRE-1 site, two overlapped Sp1 sites were found (Table I). Therefore, it is likely that NF-κB and Sp1 transcription factors interact to coordinate BMP-2 gene regulation.

NF-κB is a family of transcription factors, which are composed of five subunits, p65, c-Rel, RelB, p50, and p52, among which p50 and p52 are two highly homologous members and are the most frequent partners among family members (14). In our gel shift assays, we only detect binding of p50 and p65 to the NRE-1 site of our gel shift assay, we only detect binding of p50 and p65 to the NRE-1 site of NF-KB gene transcription, and in NF-KB/H9260 growth and shortened long bones (15). Decreases in BMP-2 expression of the BMP-2 gene但由于其间接效应通过破骨细胞，组织-或细胞-specific approaches are required, such as generating transgenic mice in which NF-κB signaling is disrupted specifically in chondrocytes. Nevertheless, reduction in cell proliferation in dKO mice suggests that NF-κB-regulated BMP-2 expression does play an important role in chondrocyte function.

In addition to direct regulation of BMP-2 gene transcription, NF-κB may also regulate BMP-2 gene expression through indirect mechanisms and other transcription factors may also play a role in BMP-2 gene transcription in growth plate chondrocytes. The summation of these transcriptional inputs determines the spatial and temporal expression pattern of BMP-2 gene in growth plate chondrocytes in vivo.

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