An osteogenesis-related transcription factor, core-binding factor A1, is constitutively expressed in the chondrocytic cell line TC6, and its expression is upregulated by bone morphogenetic protein-2

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Abstract

Core-binding factor A1 (Cbfa1), also called *Pebp2aA*/AML3, is a transcription factor that belongs to the *runt*-domain gene family. Cbfa1-deficient mice are completely incapable of both endochondral and intramembranous bone formation, indicating that Cbfa1 is indispensable for osteogenesis. Maturation of chondrocytes in these mice is also disorganized, suggesting that Cbfa1 may also play a role in chondrogenesis. The aim of this study was to examine the expression and regulation of *Pebp2aA*/AML3/Cbfa1 expression in the chondrocyte-like cell line, TC6. Northern blot analysis indicated that Cbfa1 mRNA was constitutively expressed as a 6.3 kb message in TC6 cells and the level of Cbfa1 expression was enhanced by treatment with bone morphogenetic protein-2 (BMP2) in a time- and dose-dependent manner. This effect was blocked by an RNA polymerase inhibitor, 5,6-dichloro-

1- β -D-ribofuranosylbenzimidazole, but not by a protein synthesis inhibitor, cycloheximide. Western blot analysis of the cell lysates using polyclonal antibody raised against Cbfa1 indicated that BMP2 treatment increased the Cbfa1 protein level in TC6 cells. In TC6 cells, BMP2 treatment enhanced expression of alkaline phosphatase and type I collagen mRNAs but suppressed that of type II collagen mRNA. In addition to TC6 cells, Cbfa1 mRNA was also expressed in primary cultures of chondrocytes and BMP2 treatment enhanced Cbfa1 mRNA expression in these cells similarly to its effect on TC6 cells. These data indicate that the *Pebp2aA*/AML3/Cbfa1 gene is expressed in a chondrocyte-like cell line, TC6, and its expression is enhanced by treatment with BMP.

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Introduction

Polyoma enhancer-binding protein (Pebp2)/core-binding factors (Cbfa) belong to a family of transcription factors that form the heterodimeric proteins of two unrelated subunits, comprising of a DNA-binding α subunit and a non-DNA-binding β subunit (Ogawa *et al.* 1993a, Wang *et al.* 1993, Ito & Bae 1997). *Pebp2a*/Cbfa contains a domain homologous to the *Drosophila* segmentation gene, *runt*. Three *runt*-related genes (*Pebp2aA*/AML3/Cbfa1, *Pebp2aB*/AML1/Cbfa2, and *Pebp2aC*/AML2/Cbfa3) have been identified, to date, as members of this family (Bae *et al.* 1993, 1995, Ogawa *et al.* 1993b, Wang *et al.* 1993, Levanon *et al.* 1994, Wijmenga *et al.* 1995). Cbfa1 is involved in the regulation of T cell gene expression (Wang & Speck 1992) and Cbfa2 is homologous to the human AML-1 gene identified during a study on acute myeloid leukemia (Meyers *et al.* 1993, Wang *et al.* 1996). *Pebp2a*s/Cbfa form heterodimers with a non-DNA binding factor,

Pebp2b/Cbfb and this heterodimerization enhances the DNA binding and transactivation activities of *Pebp2a*s/Cbfa *in vitro* (Ogawa *et al.* 1993b, Wang *et al.* 1993, Ito & Bae 1997).

Mice homozygous for the mutation on the Cbfa1 gene completely lack bones and die just after birth due to the inability to breathe. In these mice, mature osteoblasts are hardly observed, indicating that Cbfa1 plays a critical role in the regulation of osteoblastic differentiation (Komori *et al.* 1997). On the other hand, heterozygous mice show specific bone defects that recapitulate the phenotype of the human skeletal disorder named cleidocranial dysplasia (CCD) (Otto *et al.* 1997) and, in fact, Cbfa1 mutations are associated with human CCD (Mundlos *et al.* 1997) as well. While Cbfa1 plays an essential role in osteoblastic differentiation and most cartilaginous primordial tissues can be observed in Cbfa1 knockout mice, the maturation of chondrocytes also appears to be disorganized in Cbfa1-deficient mice (Komori *et al.* 1997). In these mice,

chondrocyte differentiation is impaired around the time of appearance of prehypertrophic chondrocytes in part of the cartilage tissues, although hypertrophic chondrocytes can be observed in the skeletons, including tibia, fibula, radius and ulna. Neither vascular nor mesenchymal cell invasion is observed in calcified cartilage, suggesting that *Cbfa1* may play a role in chondrocyte differentiation, although there is no information regarding the expression and regulation of *Cbfa1* in chondrocytes in culture.

In the present paper, we show that *Cbfa1* is expressed at a low level in the chondrocyte-like cell line, TC6, which is derived from knee articular cartilage of transgenic mice harboring SV40 large T-antigen gene (Mataga *et al.* 1996), and that *Cbfa1* expression is upregulated by bone morphogenetic protein-2 (BMP2) treatment. Furthermore, *Cbfa1* is also expressed in the primary cultures of chondrocytes and is upregulated by treatment with BMP2.

Materials and Methods

Reagents

Human recombinant BMP2 was a kind gift from J Wozney (Genetics Institute, Cambridge, MA, USA). Recombinant human transforming growth factor (TGF)- β 1 and insulin-like growth factor (IGF) were purchased from R&D Systems (Minneapolis, MN, USA).

Cell cultures

TC6 cells were established using the cells derived from the articular cartilage of the distal ends of femora of 2-month-old transgenic mice (C57BL/6J mouse) harboring a tsSV40 large T-antigen gene as described previously (Mataga *et al.* 1996). The cells express cartilage specific genes, such as type II collagen, aggrecan and link protein and the cells also retain their expression of cartilaginous phenotypes in an *in vivo* environment (Takazawa *et al.* 1999). For maintenance, the cells were cultured as described previously (Mataga *et al.* 1996) at 33 °C in α -minimum essential medium (α -MEM; Sigma Chemical Co., St Louis, MO, USA) supplemented with 0.5% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD, USA) in a humidified atmosphere of 5% CO₂. During experiments, TC6 cells were plated at 1.8×10^4 cells/cm² (Costar, Cambridge, MA, USA) and were cultured in α -MEM supplemented with 0.5% FBS at 33 °C. After reaching subconfluency, the media were replaced with fresh ones supplemented with 0.1% FBS containing BMP2 or other agents.

Primary chondrocytes were prepared from the rib cartilages of 1-day-old ICR mice. The rib cartilages were digested with 3 mg/ml collagenase in Dulbecco's minimum essential medium (DMEM; GIBCO BRL) for 30 min at 37 °C, rinsed with phosphate-buffered saline

and then digested with 3 mg/ml collagenase in DMEM at 37 °C in the atmosphere of 5% CO₂ for 5 h, as previously described (Lefebvre *et al.* 1994). An average of 4×10^6 chondrocytes was obtained per mouse. Cells were plated at 0.8×10^5 cells/cm² (Costar) and were cultured in DMEM supplemented with antibiotics (penicillin (50 U/ml) and streptomycin (50 μ g/ml); GIBCO) and 10% FBS. After 4 days in culture, the media were replaced with fresh ones with or without BMP2.

RNA preparation and Northern blot analysis

Total cellular RNA was extracted according to the acid guanidium (isothiocyanate–phenol–chloroform) method (Chomczynski & Sacchi 1987). Aliquots of 10 μ g of the total RNA/lane were fractionated in agarose gel (1% agarose, 20 mmol/l MOPS pH 7.0, 0.5 mmol/l EDTA, 0.66 mol/l formaldehyde) and then transferred onto nylon filters (Gene Screen; NEN Research Products, Boston, MA, USA) by electroblotting. The filters were prehybridized for 3–7 days at room temperature. A NcoI fragment of murine *Pebp2aA/AML3/Cbfa1* cDNA, which contains almost all of the C-terminal part of the coding region (1294–2568 bp *Pebp2aA/Cbfa1* cDNA) was labeled with ³²P-deoxycytidine triphosphate using the BcaBEST random primer labeling kit (Takara Shuzo Co. Ltd, Tokyo, Japan) and was used for hybridization. Hybridization was performed at 42 °C for 24 h in a fresh hybridization buffer containing 3×10^6 c.p.m./ml of the labeled probe. Filters were washed in $1 \times$ SSC–0.1% SDS three times at room temperature for 5 min each, followed by rinsing for 20 min in $0.5 \times$ SSC, 0.1% SDS at 60 °C. The filters were subjected to autoradiography using X-ray films (Fuji Photo Film Co., Minamishigara, Japan) and intensifying screens (New England Nuclear–DuPont, Boston, MA, USA) at –80 °C for several days. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the control. The bands in the Northern blot autoradiograms were quantitated by densitometry using a laser densitometer (Zero D Scan Image Analysis System), and each value was normalized against that of the GAPDH band in the corresponding lane. The normalized values obtained were used to calculate fold induction.

Western blot analysis

For Western blot analysis, TC6 cells grown to subconfluence in 145 cm² dishes were cultured in modified α -MEM medium with or without BMP2 at 500 ng/ml for 24 h. The cells were then lysed in a buffer containing 0.0625 M Tris–HCl, 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% Bromophenol blue (BPB). The protein concentration was determined according to the method of Bradford (1976), and 10 μ g of each sample was separated by 15% SDS-PAGE (Laemmli 1970). The

proteins were electrophoretically transferred to nitrocellulose filters (Protran Nitrocellulose; S & S, Dassel, Germany) as described previously (Burnette 1981). The filters were then blocked for 2 h in 3% non-fat dried milk in Tris-buffered saline and transferred proteins were incubated for 1 h in the presence of 1:500 dilution of polyclonal antibody raised against Cbfa1 (Y Ito, Kyoto University, Kyoto, Japan). The proteins on the filters were visualized by using an ECL-Plus Western blotting detection system (Amersham) as follows. The filters were first incubated with biotinylated anti-rabbit IgG (Vector Laboratories) and then the coloring was developed using 0.045 units 3,3'-diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl, pH 7.5, containing 0.03% H₂O₂. As a control for protein loading, the same aliquots of proteins loaded in parallel gels were stained with Coomassie Brilliant Blue G 250 (Fluka Chemie AG CH-9471 Buchs, Germany).

Statistical analysis

Statistical evaluations of the data were conducted by using Student's *t*-test for per-comparison analysis. The data are presented as means $\pm$ s.d. The results obtained from two to four independent experiments were analyzed.

Results

We first examined whether Cbfa1 is expressed in the chondrocytic cell line, TC6 cells. Northern blot analysis indicated that Cbfa1 mRNA is constitutively expressed as a 6.3 kb message in TC6 cells (Fig. 1A, control). The level of Cbfa1 expression was enhanced by treatment with 500 ng/ml BMP2 (Fig. 1A). Treatment with IGF-I or TGF- β did not enhance the expression of Cbfa1 (Fig. 1B), indicating that BMP specifically enhanced the expression of Cbfa1. GAPDH levels served as control (Fig. 1A and B).

The time-course of the effect of BMP2 is shown in Fig. 2. BMP2 treatment upregulated Cbfa1 mRNA expression within 12 h and this effect lasted at least up to 48 h in TC6 cells (Fig. 2). TC6 cells were also treated for 24 h with BMP2 at various concentrations ranging from 50 to 500 ng/ml. The BMP2 effect on Cbfa1 mRNA level was dose-dependent, maximal at 500 ng/ml (Fig. 3). The abundance of GAPDH mRNA was not affected by BMP2, indicating the specificity of the effect of BMP2 on Cbfa1 gene expression.

To investigate the mode of BMP2 action in the regulation of Cbfa1 gene expression, the cells were treated for 24 h with BMP2 in the presence or absence of inhibitors for transcription or protein synthesis. As shown in Fig. 4, we first analyzed the effect of BMP2 on Cbfa1 mRNA levels in the presence of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) which inhibits mRNA synthesis, and found that BMP2 enhancement of

Figure 1 Cbfa1 expression and its regulation by BMP2 and other agents in TC6 cells. TC6 cells at subconfluence were treated for 24 h with (A) BMP2 (500 ng/ml) and (B) TGF- β (2 ng/ml) and IGF-I (50 ng/ml). Total RNA (10 μ g/lane) was fractionated on 1% agarose gel and Northern blot analysis was conducted to examine the Cbfa1 level, as described in Materials and Methods. GAPDH was used as control. The data represent one of three independent experiments with similar results. Arrows and arrowheads indicate the positions for Cbfa1, GAPDH, 28S and 18S rRNAs. The relative Cbfa1 mRNA levels normalized against GAPDH mRNA levels for control and BMP2 were 1 and 2.23 respectively in (A). The relative Cbfa1 mRNA levels normalized against GAPDH mRNA levels for control, TGF- β and IGF-I were 1, 0.34 and 0.22 respectively in (B).

Figure 2 Time-course of the effects of BMP2 on the levels of Cbfa1 mRNA in TC6 cells. TC6 cells at subconfluence were cultured for the indicated periods of time (hours) in the absence (-) or presence (+) of 500 ng/ml BMP2. Total RNA (10 μ g/lane) was prepared as described in Materials and Methods and subjected to Northern blot analysis. The same filters were hybridized with ³²P-labeled Cbfa1 and then GAPDH probes later. The data represent one of three independent experiments with similar results. Arrows and arrowheads indicate the positions for Cbfa1, GAPDH, 28S and 18S RNAs. The relative Cbfa1 mRNA levels normalized against GAPDH mRNA levels for 0-h control, 12-h control, 12-h BMP2, 24-h control, 24-h BMP2, 48-h control and 48-h BMP2 were 1, 1.19, 2.25, 0.56, 1.28, 1.42 and 2.28 respectively.

the levels of Cbfa1 mRNA was blocked by DRB (Fig. 4A). The BMP2 enhancement of Cbfa1 mRNA levels was still observed in the presence of a protein synthesis inhibitor, cycloheximide, indicating the direct effect of

Figure 3 Dose-dependent effects of BMP2 on the levels of Cbfa1 mRNA in TC6 cells. TC6 cells at subconfluence were treated for 24 h with BMP2 at various concentrations ranging from 50 to 500 ng/ml. Total RNA (10 µg/lane) was fractionated on 1% agarose gel and Northern blot analysis was conducted to examine the Cbfa1 level as described in Materials and Methods. GAPDH was used as the control. The data represent one of three independent experiments with similar results. Arrows and arrowheads indicate the positions for Cbfa1, GAPDH, 28S and 18S rRNAs. The relative Cbfa1 mRNA levels normalized against GAPDH mRNA levels for control, 100, 200, 400 and 500 ng/ml BMP2 were 1, 1.08, 1.14, 1.20 and 1.77 respectively.

BMP2 on Cbfa1 gene expression without requirement for new protein synthesis (Fig. 4B).

To examine whether the effect of BMP2 on Cbfa1 mRNA expression is translated into Cbfa1 protein levels, TC6 cells were treated with BMP2 at 500 ng/ml for 24 h and Western blot analysis was conducted. We

found that treatment with BMP2 enhanced the level of Cbfa1 protein, which migrated as a 65 kDa band (Fig. 5A). In addition to the 65 kDa band, Western blot analysis indicated induction of 53 and 75 kDa bands, though the natures of these bands have not yet been identified. These bands were not recognized by non-immune antiserum. As a control for protein loading, the same aliquots of proteins used for the Western blot were loaded in the parallel gels and Coomassie Brilliant Blue staining indicated similar levels of the proteins in each of the lanes (Fig. 5B).

We also examined the effects of BMP2 on the expression of genes encoding phenotype-related proteins in TC6 cells. BMP2 treatment enhanced alkaline phosphatase and type I collagen mRNA expression (Fig. 6A and B) while it suppressed type II collagen mRNA expression (Fig. 6C) in these cells.

While TC6 cells are derived from cartilage, their response to BMP could be different from authentic chondrocytes since TC6 cells harbor SV40 large T-antigen gene. We therefore examined whether Cbfa1 was expressed in chondrocytes freshly isolated from the rib cartilage of normal mice. In the Northern blot analysis using RNAs prepared from primary cultures of chondrocytes, Cbfa1 mRNA was expressed as a 6.3 kb message in these chondrocytes and its level was enhanced by treatment with 500 ng/ml BMP2 (Fig. 7A). BMP2 treatment also enhanced alkaline phosphatase mRNA expression in these cells (Fig. 7B).

Figure 4 Effects of DRB and cycloheximide on BMP2-induced enhancement of Cbfa1 mRNA in TC6 cells. TC6 cells at subconfluence were treated with vehicle (–) or 500 ng/ml BMP2 (+) for 24 h in the presence or absence of (A) 0.2 µg/ml DRB or (B) 20 µg/ml cycloheximide (CHX). Total RNA was isolated as described in Materials and Methods and was subjected to Northern blot analysis. Quantitation of Cbfa1 mRNA levels was conducted by using a densitometer and the values obtained were normalized against those of GAPDH mRNA levels. Data were obtained from three independent experiments and are presented as means ± s.d. * $P < 0.05$. There was no difference between the vehicle and BMP2 in the presence of DRB (A).

Figure 5 BMP2 enhancement of Cbfa1 protein production in TC6 cells. (A) Subconfluent TC6 cells in 145 cm² dishes were cultured for 24 h in the absence (–) or presence (+) of 500 ng/ml BMP2. Aliquots containing 10 µg protein were used for Western blot analysis as described in Materials and Methods. (B) Coomassie Blue staining of the gels; molecular sizes are in kDa and the position of Cbfa1 is indicated. The data represent one of two experiments with similar results.

Discussion

Our present study demonstrates that Cbfa1 mRNA is constitutively expressed in the chondrocyte-like cell line, TC6, and that it is a specific target of BMP2 action.

Furthermore, we also showed that Cbfa1 is expressed in primary cultures of chondrocytes, and BMP2 again enhances the expression of Cbfa1 in these cells. Mesenchymal cell differentiation into specific lineages of cells, including chondrocytes, is under the control of certain sets

Figure 6 Effects of BMP2 on the expression of alkaline phosphatase and collagen genes in TC6 cells. TC6 cells at subconfluence were treated for 24 h with BMP2 (500 ng/ml). Total RNA (10 µg/lane) was fractionated on 1% agarose gel and Northern blot analysis was conducted to examine the (A) alkaline phosphatase (ALP), (B) type I collagen (Coll I) or (C) type II collagen (Coll II) mRNA levels, as described in Materials and Methods. GAPDH was used as the control. The data represent one of two independent experiments with similar results. Arrows and arrowheads indicate the positions for Cbfa1, GAPDH, 28S and 18S RNAs. The relative levels of alkaline phosphatase mRNA levels normalized against GAPDH mRNA levels for control and 500 ng/ml BMP2 were 1 and 1.93 respectively. The relative levels of the type I collagen mRNA normalized against GAPDH mRNA levels for control and 500 ng/ml BMP2 were 1 and 1.94 respectively. The relative levels of the type II collagen mRNA normalized against GAPDH mRNA levels for control and 500 ng/ml BMP2 were 1 and 0.26 respectively.

Figure 7 Cbfa1 gene expression and its regulation by BMP2 in primary cultures of chondrocytes. Primary cultures of chondrocytes were prepared as described in the Materials and Methods. The cells were treated for 24 h with BMP2 (500 ng/ml). Total RNA (10 µg/lane) was prepared, fractionated on 1% agarose gel and subjected to Northern blot analysis to examine (A) Cbfa1 and (B) alkaline phosphatase (ALP) expression and the effect of BMP2 on its expression. GAPDH was used as the control. The data represent one of two independent experiments with similar results. Arrows and arrowheads indicate the positions for Cbfa1, alkaline phosphatase, GAPDH, 28S and 18S rRNAs. The relative Cbfa1 mRNA levels normalized against GAPDH mRNA levels for control and 500 ng/ml BMP2 were 1 and 2.97 respectively. The relative alkaline phosphatase mRNA levels normalized against 28S rRNA levels for control and 500 ng/ml BMP2 were 1 and 16.74 respectively.

of transcription factors (Aubin *et al.* 1993). Although Cbfa1 is a prerequisite for osteogenesis, it is expressed in chondrocytes as we have shown here and also in several non-osteoblastic cells, at least in culture. These observations support the previous notion that a relatively wide range of cell types are potentially capable of differentiating into osteoblasts if the cells are kept under certain appropriate conditions *in vivo* or *in vitro*.

Although bone is totally absent in Cbfa1 null mice, cartilage is mostly observed in limb and axial skeletons such as vertebrae. However, detailed examination of the chondrocytes revealed an alteration in their morphology. Our observations on the expression and BMP regulation of Cbfa1 suggest that Cbfa1 may play a certain role in chondrocytes *per se* as well. Observations on the minor change in chondrocytes compared with osteoblasts in Cbfa1 null mice suggest that lack of Cbfa1 could be compensated by, at least in part, another as yet unidentified transcription factor(s). Alternatively, the role of Cbfa1 in chondrocytes may be limited to specific stages of chondrocyte differentiation such as the hypertrophic stage

of the cell. It may also be possible that, at least under culture conditions, certain fractions of chondrocytes could become undifferentiated and these cells could generate cells with relatively immature fibroblastic phenotypes, and thus are capable of differentiating into osteoblasts upon stimulation by certain signals such as BMP2.

The expression of osteoblast phenotype-related alkaline phosphatase and type I collagen genes in the chondrocyte-like TC6 cells was enhanced by the treatment with BMP2, while expression of the chondrocyte-related type II collagen gene was suppressed. These observations suggest that BMP2 might suppress expression of the genes encoding cartilage-related phenotype and/or it might shift chondrocytic cells to become more osteoblast-like cells. Alternatively, BMP may promote maturation of chondrocytes. This possibility is still open, as alkaline phosphatase mRNA expression, a marker of both osteoblasts and hypertrophic chondrocytes, was enhanced by BMP in both TC6 cells and primary cultures of chondrocytes. However, we did not detect type X collagen expression even after BMP2 treatment (data not shown). Whether

the upregulation of Cbfa1 by BMP2 causes such an alteration in the expression of phenotype-related genes needs further analysis. Our preliminary experiments indicated that the cells prepared from Cbfa1 knockout mice are unable to increase type I collagen mRNA expression even after treatment with BMP (data not shown).

It has been indicated that BMPs enhance expression of Cbfa1 in calvaria-derived osteoblast-enriched cells (Komori *et al.* 1997). BMPs also enhance expression of phenotype-related genes in osteoblast-like MC3T3-E1 cells (Hiraki *et al.* 1991), rat calvaria-derived osteoblast-enriched cells (Vukicevic *et al.* 1989, Chen *et al.* 1991a) and osteoprogenitor cells (Yamaguchi *et al.* 1991). In addition, other studies have suggested that BMPs affect chondrogenesis as well (Carrington *et al.* 1991, Chen *et al.* 1991b, Hiraki *et al.* 1991, Iwasaki *et al.* 1994). These diverse results may be due to the differences in the responses to BMPs based on the difference in the sets of transcription factors and cofactors operating in the individual cellular backgrounds and/or under culture conditions.

The actions of BMP have been shown to be mediated via Sma+mad (Smad) and/or TGF β -activated kinase (TAK) systems in osteoblasts and fibroblasts actions. Whether similar adapter protein systems are operating in chondrocytes requires further elucidation of BMP signaling pathway in chondrocytes. The role of Cbfa1 in chondrocytes could be to balance the levels of expression of the genes in the skeletal cells, depending on which direction the cells differentiate into, although elucidation of the mechanisms of the tuning of this balance needs further investigations. In the process of fracture repair, it is thought that certain fractions of chondrocytes redifferentiate into osteoblasts directly. BMP2 regulates Cbfa1 during the healing process of fractures, the drugs or treatment targeted to enhance BMP as well as Cbfa1 expression would be beneficial for the healing of the fractures.

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