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Participation of Cbfa1 in regulation of chondrocyte maturation

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Summary

Objective: Cbfa1 is a transcription factor, which is classified into the runt family. The mice lacking this gene display complete loss of bone formation, indicating that Cbfa1 is an essential factor for osteoblast differentiation. The Cbfa1-deficient mice also show an abnormality in cartilage development. Although cartilage anlagens are well formed in these mice, endochondral ossification is blocked, and most of chondrocytes fail to differentiate into their maturation form as characterized by the absence of type X collagen and low levels of alkaline phosphatase activity. It is suggested that Cbfa1 may participate in chondrocyte differentiation. In this study, we have investigated the role of Cbfa1 in chondrocytes during their cytodifferentiation *in vitro*.

Design: To investigate the role of Cbfa1 in regulation of chondrocyte differentiation, we over-expressed Cbfa1 or its dominant negative form in cultured chick chondrocytes using a retrovirus (RCAS)system and examined changes in chondrocyte behaviour induced by the introduced genes.

Results: Mature chondrocytes isolated form the cephalic portion of sterna seemed to express Cbfa1 more prominently than immature chondrocytes isolated from the one-third caudal portion of sterna. Over-expression of Cbfa1 in immature chondrocytes strongly stimulated alkaline phosphatase activity and matrix calcification. In contrast, expression of a dominant negative form of Cbfa1, which lacks the C-terminal PST domain, severely inhibited alkaline phosphatase activity and matrix calcification in mature chondrocytes.

Conclusion: Taken together with the observation that Cbfa1 transcripts dominantly localized in hypertrophic chondrocytes as well as in osteoblasts, it is suggested that Cbfa1 plays an important role in the progression of chondrocyte maturation. © 2001 OsteoArthritis Research Society International

Key words: Cbfa1, Chondrocyte, Endochondral ossification.

Introduction

Endochondral ossification is the essential mode of bone formation during embryonic development[1]. In growth plates, chondrocytes actively proliferate and synthesize abundant extracellular matrix including aggrecan, type IX collagen and type II collagen. As chondrocytes become postmitotic, the cells start to terminally differentiate, which is characterized by expression of type X collagen and high activity of alkaline phosphatase. This process is called hypertrophy or maturation. The cells then induce matrix calcification, and the calcified matrix was invaded by blood vessels and replaced by bone. To date, extensive investigation have revealed that many soluble factors including fibroblast growth factors[2,3], bone morphogenetic proteins[4–6], Indian hedgehog[7,8] and parathyroid hormone related peptide[7,9] regulate or modulate changes in expression of the chondrocyte phenotype during endochondral ossification. However, the mechanism of regulation of chondrocyte differentiation by these factors has remained unknown. It is no doubt that clues to clarify the mechanism of cellular differentiation come from an understanding transcriptional regulation of gene expression. Recently several transcription factors such as Sox 9[10,11], c-fos/c-jun[12–15] and Cbfa1[16–18] have been reported to participate in regulation of gene expression of chondrocytes during cartilage development.

Cbfa1 is one of members of the runt transcription factor family, which proteins share a unique 125-amino acid motif called the 'runt' DNA binding domain[19]. Recently Cbfa1 has become regarded as an essential transcription factor for osteoblast differentiation and bone formation[18,20–22]. The lack of this gene in mice leads to the complete absence of bone formation due to blockage of osteoblast maturation[18,22]. Transfection of Cbfa1 gene in osteogenic cells or non-osteogenic cells enhances or induces osteoblastic phenotype as defined by the expression of alkaline phosphatase, type I collagen and osteocalcin[20,23]. Further, Cbfa1 directly promotes transcription of osteocalcin and osteopontin, which protmotor regions contain binding sites of Cbfa1[23,24]. In addition to its involvement of osteoblast differentiation, this gene is also suggested to be important for chondrocyte differentiation. In the developing limbs, Cbfa1 transcripts initially appear in the regions surrounding cartilaginous condensation, and then more evident in the hypertrophic chondrocytes when the cartilage tissues become well developed[16,20]. In Cbfa1 deficient mice, endochondral ossification as well as intramembranous ossification is also severely disturbed[16–18]. Although cartilage formation normally occurs, most parts of the cartilage

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tissues excluding tibia, fibula, radius and ulna do not become calcified[16–18]. Chondrocytes in the mutant mice show much lower levels than normal of expression of ALPase and type X collagen gene[16,17]. These data suggest that Cbfa1 may regulate chondrocyte maturation. In this study we aimed to investigate the roles of Cbfa1 in chondrocyte differentiation. To do so, we used the retrovirus technique to introduce two types of Cbfa1 with different N-terminal domains, one starting from exon 2 (originally cloned as Pebp2-A)[19] and the other starting from exon 1[20,25] into chick chondrocytes in culture. Further, to inactivate the function of Cbfa1, we also caused over-expression of a dominant negative form of Cbfa1, which lacks the C-terminal portion from the runt domain, in chondrocytes. As a result, expression of Cbfa1 strongly stimulated proteoglycan synthesis, production of ALPase activity and matrix calcification in chondrocytes, and the expression of the dominant negative form of Cbfa1 conversely inhibited these events. These findings strongly suggest that Cbfa1 is a positive regulator of chondrocyte maturation.

Materials and methods

IN SITU HYBRIDIZATION

Digoxigenin-11 UTP-labelled single strand RNA probes were prepared using DIG RNA labelling kit (Roche Diagnostics Mannheim Gemany) used according to the manufacture's instructions. Hybridization was carried out as described by Nomura *et al*.[26]. An 0.6 kb PstI-Hind III fragment of Cbfa1 cDNA was used to generate an antisense probe.

CELL CULTURES

Chicken embryo fibroblasts were obtained from the torso of virus-free White Leghorn 11 day-old embryos (line M; Nisseiken, Yamanashi Japan) and cultured in medium 199 containing 10% fetal bovine serum (FBS). Upper sterna (US) and lower sterna (LS) chondrocytes were isolated from the cephalic portion and one-third caudal portion, respectively, of the sternum of 17-day-old embryos (line M) and cultured in high-glucose DMEM containing 10% FBS as described previously[27,28].

RT-PCR

Total RNA was prepared from chondrocyte cultures by the method of Smale and Sasse[29] with minor modifications[30]. 1 μg of whole cellular RNA was reverse-transcribed by Superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD)[30]. Subsequent amplification was carried out with Elongase (Gibco BRL) and gene-specific primers under the following conditions: 95°C for 10 sec and 60°C for 1 min. Primers for PCR were as follows: 5′-GCA TTC CTC ATC CCA GTA TGA GA-3′ and 5′-GTA AAG GTG GCT GG(G/A) TAG TGC A-3′ for Cbfa1 cDNA; 5′-GC(A/G) TCG TGA TT(A/G) GCG ATG ATG A-3′ and 5′-GTC (A/G)AG GGC (A/G)TA TCC AAC AAC A-3′ for HPRT cDNA. Chick Cbfa1 primers were designed from the consensus sequence of human (L40992) and mouse Cbfa1 (AF010284) cDNAs. The amplified products were sequenced to verify the accuracy of the PCR reaction in each RT-PCR experiments (data not shown). According to the sequence of chick RT-PCR products for Cbfa1, the homology between chick and human Cbfa1 was 92%. The entire structure of chick Cbfa1 cDNA will be published elsewhere.

CONSTRUCTION AND EXPRESSION OF RECOMBINANT RETROVIRUSES ENCODING CBFA1 ISOFORMS AND A DOMINANT NEGATIVE FORM OF CBFA1

cDNAs including entire coding sequences of mouse Cbfa1/Pebp2 A and Osf2/Cbfa1/til-1, in which the Kozak consensus sequence CCACC is attached to the 5′ of ATG and which were recently used for the functional analysis of Cbfa1 isotypes, were employed. The two isotypes of Cbfa1 cDNAs and the dominant negative form of Cbfa1 cDNAs were each subcloned into the RCAS (A) retroviral vector[31–33]. The dominant negative form of Cbfa1 which lacks the PST domain, one of the transactivation domains[24] was generated by subcloning a BamHI/ Hind III fragment of the mouse Cbfa1 coding sequence (AA 1-225 of D14636) into modified pBluescript that contains a myc-epitope (EQKLISEEDL) and stop codon. The tagged DN-Cbfa1 cDNA fragment was then subcloned into RCAS (B) retroviral vector. Chick fibroblasts were transfected with the vector constructs wiht the constructed vectors by use of FuGENE6 transfection reagent according to the manufacturer's protocol (Roche Diagnostics, Mannheim). The recombinant virus in the medium was concentrated by centrifugation (2500 rpm for 3 h) and used to infect freshly isolated chondrocytes.

NUCLEAR EXTRACTION AND IMMUNOBLOT

Nuclear extracts were prepared form the virus-infected cultures (P1 cultures) according to the method previously described[34]. The μg of nuclear extract was separate on a 10.0% gel by SDS-PAGE and transferred to a PVDF membrane (Millipore Japan, Tokyo, Japan). After the membrane had been blocked with 10% horse serum overnight at 22°C, it was incubated with polyclonal rabbit antibodies, αA1Cl7 and αA1N35 against C-terminal and N-terminal domains of mouse Cbfa1/Pebp2aA, respectively[35] and then with peroxidase-conjugated anti-rabbit IgG goat antibody (Biomedical Technologies Inc., Stoughton, MA).

MEASUREMENT OF SULPHATED GLYCOSAMINOGLYCAN CONTENT, DNA CONTENT AND ALPASE ACTIVITY

Cultures were harvested in saline solution containing 0.2% TX-100 and 0.02 N NaOH and then sonicated. Cell lysates were centrifuged thereafter, and the supernatant was used for determination of DNA and sulphated glycosaminoglycan (GAG) contents by the fluorometric procedure of[36] and direct spectrophotometric microassay of Farndale *et al*.[37], respectively. ALPase activity associated with the cell layer was measured by a modification of Bessey *et al*.[38] using p-nitrophenyl phosphate (pNP) as a substrate as described previously[28].

ALIZARIN RED STAINING

To detect calcium accumulation, the cultures were washed with saline and fixed with 95% ethanol. Alizarin red staining was then carried out.

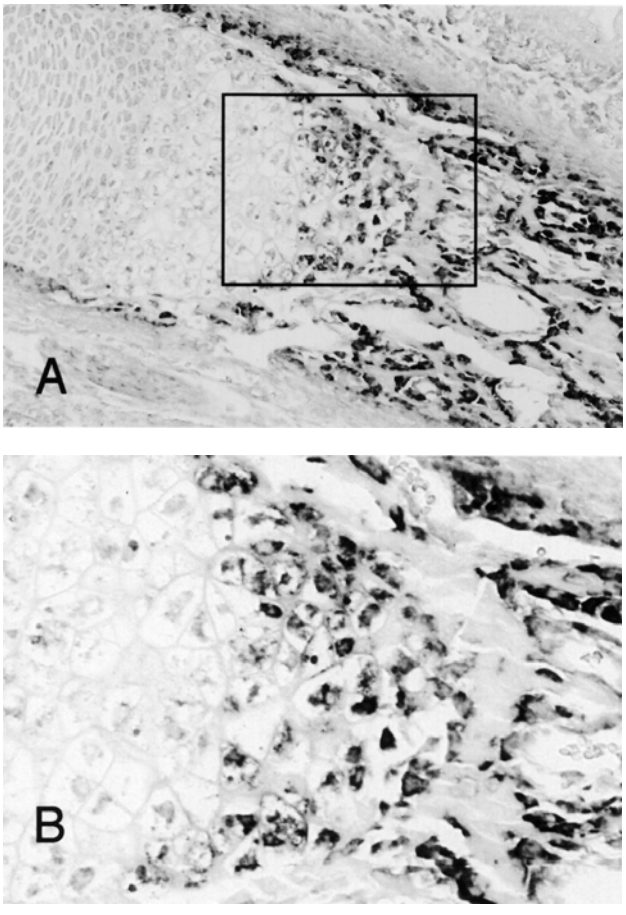

Fig. 1. *In situ* hybridization of Cbfa1 in tibia of E16.5 mouse. Cbfa1 is strongly expressed in hypertrophic chondrocytes as well as osteoblasts. The boxed region in (A) is magnified in (B).

Results

EXPRESSION OF CBFA1 IN CHICK CHONDROCYTES IN CULTURE

Cbfa1 transcripts were found in hypertrophic chondrocytes as well as osteoblasts in the growth plates of the mice[16,17]. Figure 1A showed the localization of Cbfa1 transcripts in the tibiotarsus of Day 16.5 mouse embryo. Particularly intense signals were detected in the hypertrophic chondrocytes near the vascular invasion front (Fig. 1B). To elucidate the function of Cbfa1, we decided to introduce Cbfa1 or its dominant negative form into chondrocytes by using a chick retrovirus (RCAS) system. Before these experiments, however, we first investigated the expression profile of the Cbfa1 gene in chick chondrocytes. We isolated chondrocytes from two parts of chick sternal cartilage, one being the caudal one-third portion of the sternum (LS) and the other part, the cephalic portion of the sternum (US). The chondrocytes isolated from the LS show immature chondrocyte phenotype, whereas those isolated from the US display mature one. The LS cells are smaller in size and more actively proliferate than US cells (Fig. 2A). The rate of proteoglycan synthesis in the LS chondrocytes was less than that in the US ones, as judged by determining sulphated glycosaminoglycan contents (Fig. 2B). As regards expression of maturation phenotype, the US cells showed high levels of alkaline phosphatase (ALPase) activity (Fig. 2B) and type X collagen expression[4,39,40] while LS cells contained very low levels of ALPase activity (Fig. 2B) and no expression of type X collagen at the start of the culture period.

To examine expression of Cbfa1, we made degenerative primers based on the sequence of mouse Cbfa1 and carried out RT-PCR using total RNA prepared from LS and US chondrocyte cultures. After 30 cycles of amplification, RT-PCR products were observed in reaction mixtures containing RNA from either LS or US cultures. The sequence of the amplified product had 92% homology to the corresponding region of human Cbfa1. The signal for Cbfa1 in the case of US cultures seemed stronger than that obtained with LS cultures (Fig. 2C, Cbfa1). The results of RT-PCR for hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Fig. 2C, HPRT) showed no differences in the intensity of the amplified products between the two cultures.

INTRODUCTION OF CBFA1 INTO IMMATURE CHONDROCYTES

The data above indicate that chick chondrocytes also expressed Cbfa1 gene, and its expression was likely to increase in hypertrophic chondrocytes. Therefore, we forcedly expressed Cbfa1 gene in immature chondrocytes to see whether over-expression of Cbfa1 would change the phenotype of immature chondrocytes. Cbfa1 has two isotypes with different N-terminal domains, one starting from exon 2 which we called Type-I Cbfa1 (originally cloned as Pebp2αA)[19] and the other starting from exon 1, which we called Type-II Cbfa1 (originally cloned Osf2/Cbfa1 or til-1)[20,25]. Freshly isolated LS chondrocytes were infected with RCAS viruses endocing mouse Type-I or Type-II Cbfa1. The infection with either Type-I or Type-II Cbfa1 viruses induced dramatic changes in cell shape (Fig. 3A). The cells in Type-I or Type-II Cbfa1-infected cultures (Fig. 3A, Type-I or Type-II) were much bigger in size, and more refractile than those in the control cultures treated with the virus encoding the vector alone (Fig. 3A, Control). When we carried out immunoblotting to confirm that Cbfa1 proteins were produced in these infected cultures, we found, as shown in Fig. 3B, that the antibody recognizing the C-terminal portion of both Type-I (lane 2) and Type-II Cbfa1 (lane 3) reacted with the nuclear extracts to form a single band. The size of the band was around 56 kDa for both Cbfa1's and corresponded well to the expected size[35], indicating that Cbfa1 virus-infected cultures expressed the constructed gene products.

To evaluate the changes in phenotype expression in the virus-treated chondrocytes, we first examined the effects of Cbfa1 on proteoglycan synthesis by measuring the content of sulphated glycosaminoglycan (GAG) which is a component of cartilage proteoglycan. In the Type-I or Type-II Cbfa1 virus-infected cultures, the content of sulphated GAG had increased twofold (Fig. 3C). We next investigated whether Cbfa1 stimulated expression of the phenotype of the hypertrophic chondrocytes. Expression of either Cbfa1 strongly stimulated ALPase activity in the chondrocytes, whereas the control cultures exhibited only low levels of ALPase activity (Fig. 3C). Further, the cultures infected with either type of Cbfa1 showed extensive matrix calcification, whereas the control cultures had no matrix calcification (Fig. 3D). These data suggest that Cbfa1 stimulated proteoglycan synthesis and expression of hypertrophic phenotype in chondrocytes. The activities of Type-I and Type-II Cbfa1 to stimulate GAG synthesis, ALPase activity and matrix calcification were comparable.

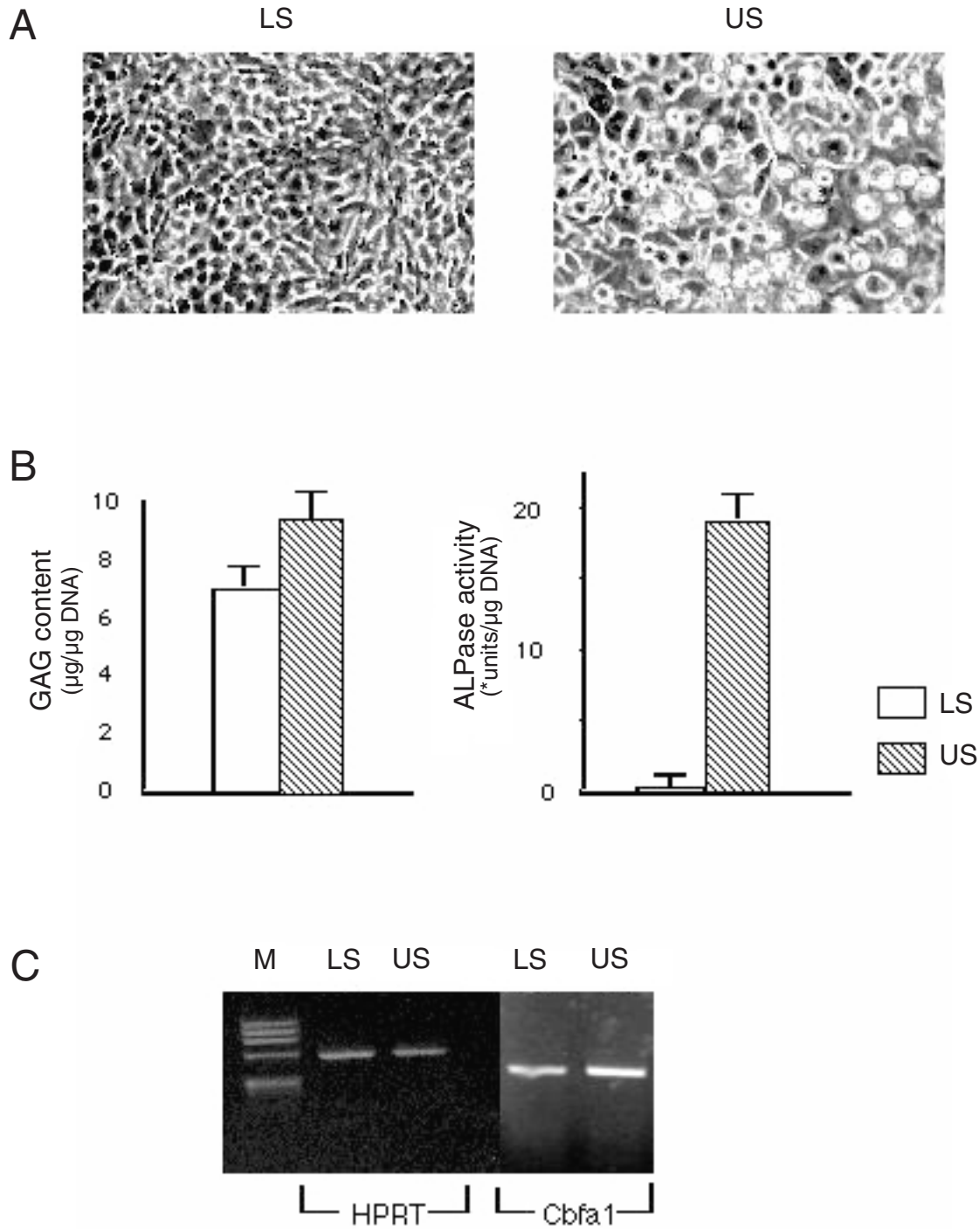

Fig. 2. The differences in cell shape, GAG synthesis, ALPase activity, and Cbfa1 expression between LS and US chondrocyte cultures. LS and US chondrocytes were isolated as described in **Materials and methods** and plated at the density of 30×10^4 cells per 35-mm dish. A and B: Photographs were taken (A), and sulphated GAG contents and ALPase activity were measured (B) on Day 5. C: On Day 7, total RNA was extracted from the cultures, and expression of Cbfa1 and HPRT was analysed by RT-PCR.

INTRODUCTION OF A DOMINANT NEGATIVE FORM OF CBFA1 INTO MATURE CHONDROCYTES

We next asked how inactivation of Cbfa1 would affect chondrocyte function. Cbfa1 contains a QA domain, runt DNA binding domain and PST domain[41,42]. The QA domain, a glutamine and alanine-rich one, has been reported to be responsible for transactivation and heterodimerization[24]. The PST domain, in the C-terminal portion of Cbfa1 is also necessary for transactivation of the target genes[24,43]. So we constructed a dominant negative form of Cbfa1 (DN-Cbfa1) by deletion of the PST domain from mouse Type-I Cbfa1 and subcloned into the RCAS vector. The RCAS virus encoding DN-Cbfa1 was used to infect US chondrocytes. We first examined whether the DN-Cbfa1 virus-infected cells expressed the constructed

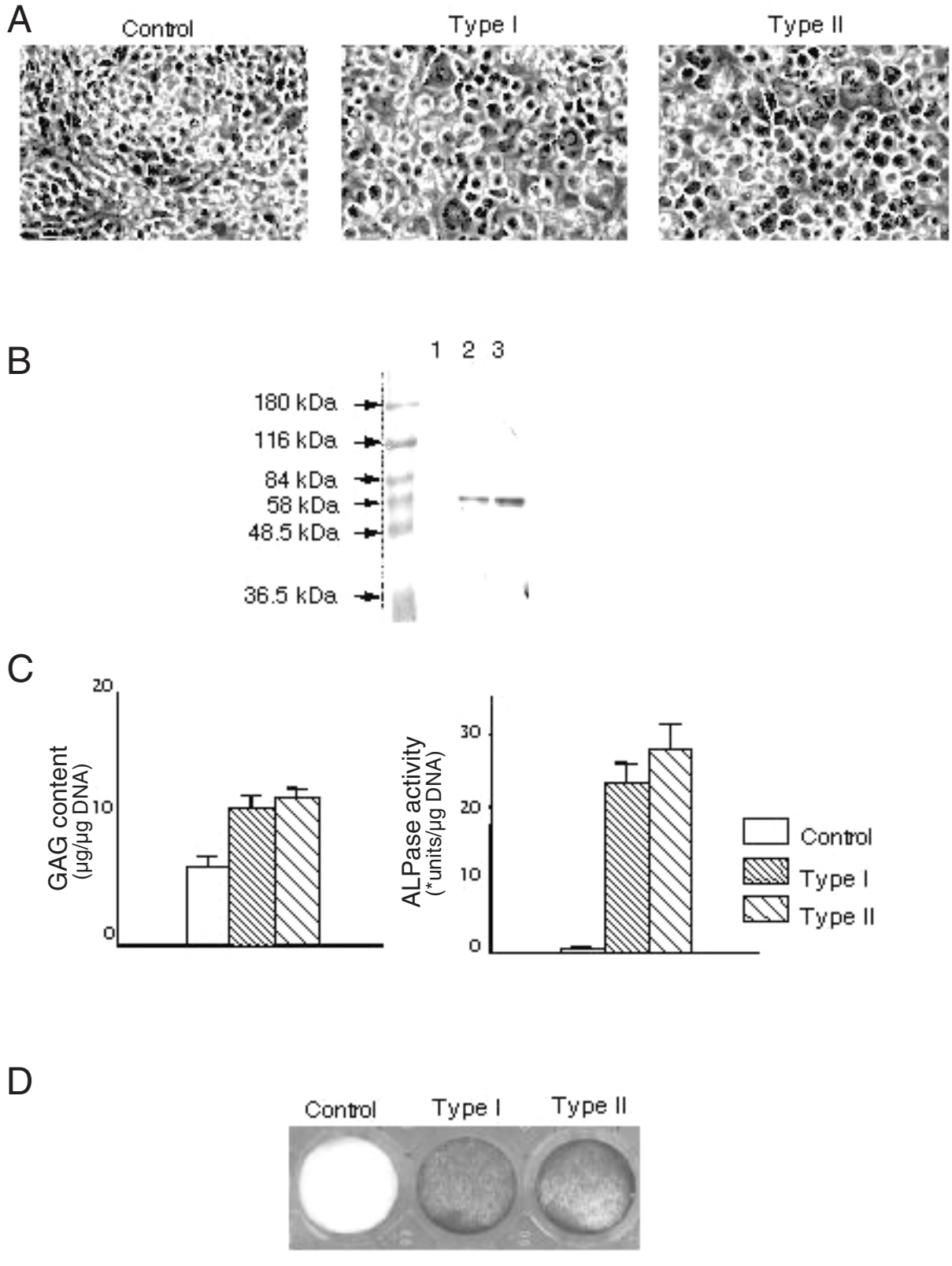

Fig. 3. Type-I and Type-II Cbfa1 stimulated expression of hypertrophic phenotype in immature chondrocytes. Freshly isolated LS chondrocytes were infected by RCAS virus encoding Type-I Cbfa1, Type-II Cbfa1 or vector alone. The confluent cultures were passaged and replated at the density of 15×10^4 cells (A and C) per 35-mm dish, 200×10^4 cells per 100-mm dish (B) or 40×10^4 cells per 16-mm well (D). The cultures were maintained in DMEM containing 10% FBS and 10 μg/ml of ascorbic acid. (A and B): On Day 4 photographs were taken (A) and sulphated GAG contents and ALPase activity were measured (B). (C): Nuclear proteins were extracted form the cultures infected with the virus encoding vector alone (lane 1), Type-I Cbfa1 (lane 2) or Type-II Cbfa1 (lane 3) on Day 6 and analysed by immunoblot. (D): For detection of mineral deposition, the cultures were stained with alizarin red on Day 10.

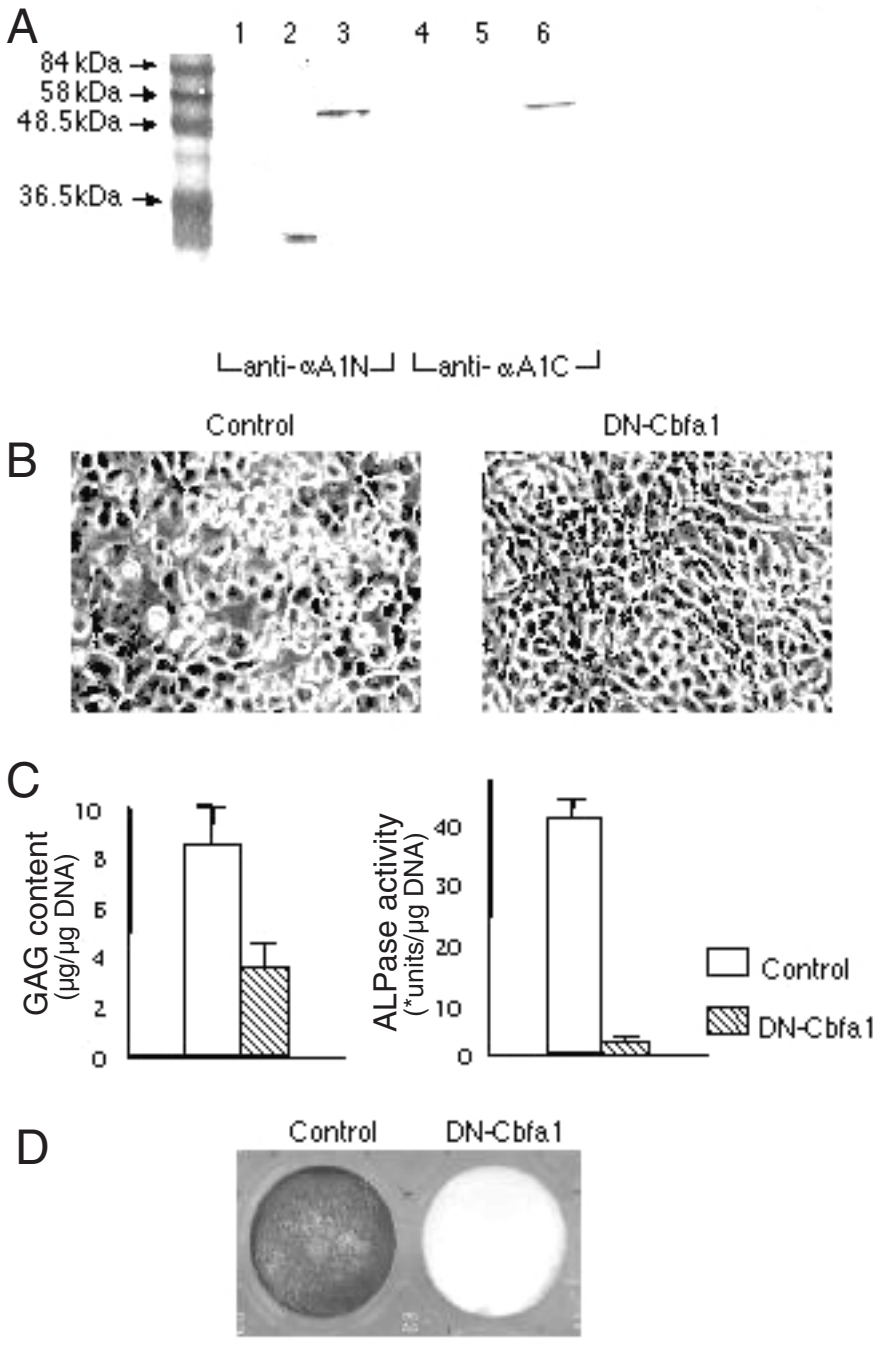

Fig. 4. A dominant negative form of Cbfa1 inhibited expression of hypertrophic phenotype in mature chondrocytes. Freshly isolated US chondrocytes were infected by RCAS virus encoding DN-Cbfa1 or vector alone. The confluent cultures were passaged and replated at the density of 200×10^4 cells per 100-mm dish (A), 15×10^4 cells (B and C) per 35-mm dish or 40×10^4 cells per 16-mm well (D). The cultures were maintained in DMEM containing 10% FBS and 10 μg/ml of ascorbic acid. (A): Nuclear proteins were extracted form the cultures infected with the virus encoding vector alone (lanes 1 and 4), DN-Cbfa1 (lanes 2 and 5) or Type I Cbfa1 (lanes 3 and 6) on Day 6. The immunoblot was carried out using the antibody αA1C17 (anti-αA1C) or αA1N35 (anti-αA1N). (B and C): On Day 4 photographs were taken (B) and sulphated GAG contents and ALPase activity were measured (C). (D): For detection of mineral deposition, the cultures were stained with alizarin red on Day 10 (D).

gene. The antibody against the N-terminal portion of Type-I Cbfa1 recognized a 30 kDa band in the nuclear extracts of the DN-Cbfa1 virus-infected cultures (Fig. 4A, lane 2) and a 56 kDa one in those of the Type-I Cbfa1 virus-infected cultures (Fig. 4A, lane 3). The antibody against the C-terminal portion of Type-I Cbfa1 only recognized a 56 kDa band in the nuclear extracts of the Cbfa1 virus-infected cultures (Fig. 4A, lane 4–6). These data indicated that the virus-infected cells expressed the expected encoded gene products. When US chondrocytes were infected with DN-Cbfa1 virus, the cells actively proliferated; and the cell size was smaller than that of the control (Fig.

4B, DN-Cbfa1) whereas the control cultures slowly grew and contained large cells (Fig. 4B, Control). The feature of the cell morphology in the DN-Cbfa1-expressing cultures was very similar to that in LS cultures (compare Fig. 4B, DN-Cbfa1 with Fig. 3A, Control). Further, the sulphated GAG content in the DN-Cbfa1 cultures was half of the control value (Fig. 4C). The similarity of characteristic between the DN-Cbfa1 virus-infected US cultures and the LS control cultures was also observed with respect to the expression of hypertrophic phenotype. The DN-Cbfa1-expressing cultures exhibited very low levels of ALPase activity (Fig. 4C) and never underwent matrix calcification (Fig. 4D, DN-Cbfa1), whereas the control US cultures displayed high levels of ALPase activity and matrix calcification (Fig. 4C and D, Control).

Discussion

CBFA1 STIMULATED EXPRESSION OF HYPERTROPHIC PHENOTYPE IN CHONDROCYTES

Two lines of evidence support the hypothesis that Cbfa1 is a positive regulator of chondrocyte differentiation. One is the demonstration, by analysis of *in situ* hybridization in normal mice, that Cbfa1 is expressed in hypertrophic chondrocytes as well as in osteoblasts[16,17]. Further, an analysis of Cbfa1 expression by use of a lacZ reporter is consistent is with the results of *in situ* hybridization for Cbfa1[17]. The other evidence is that Cbfa1 deficient mice display severe blockage of chondrocyte differentiation[16,17]. In such mice, only restricted cartilage elements including those in the tibia, fibula, radius and ulna become calcified[16–18]. In the other parts of cartilage including humerus and phalanges, calcification does not proceed; and the expression of Indian hedgehog and type X collagen is inhibited, indicating that the lack of Cbfa1 gene impairs maturation of chondrocytes[16,17]. In the present study we demonstrated that forced expression of Cbfa1 in chondrocytes stimulated expression of the hypertrophic phenotype such as production of ALPase activity and induction of matrix calcification. These findings provide additional evidence on favour of the hypothesis stated above, and further suggest that Cbfa1 directly regulates chondrocyte maturation.

The Cbfa1 gene has been predicted to produce at least three types of isoforms[25,44]. One isoform, originally identified as Pebp2 A starts from exon2[19]. The other two isoforms, originally reported as OSF2/Cbfa1/til-1 are translated from two different tranlational sites of the same mRNA starting from exon 1[21,25,45]. All three isoforms of Cbfa1 have the ability to activate osteocalcin and osteopontin gene expression[23]. However, differences in the translational activity among the three isoforms have been demonstrated from the results of promotor analysis and those of the stable transfection experiments[23,46]. For the forced expression of Cbfa1 in chondrocytes in this study, we used two isoforms: one was Pebp2αA starting from exon 2[19], which we have referred to as Type-I Cbfa1; and the other was a shorter isoform starting from exon 1 originally cloned OSF2/Cbfa1/til-1[20,25] and here called Type-II Cbfa1. We found that both isoforms strongly stimulated ALPase activity and matrix calcification, and we did not detect any clear difference in the stimulatory activity between the two isoforms. Using specific probes for each isoform, we have found that both isoforms were expressed in growth plates, particularly in hypertrophic chondrocytes (unpublished results). Therefore, functional differences in chondrocyte differentiation between the two isoforms remain unclear at this moment and should be further investigated.

CBFA1 IS REQUIRED FOR CHONDROCYTE MATURATION

Ducy *et al*.[43] reported that expression of DNA binding domain of Cbfa1 inhibited transactivation function of Cbfa1 in a dominant negative manner. Further, they showed that osteoblasts expressing the DNA binding domain of Cbfa1 showed less ALPase activity and poorly performed matrix calcification *in vitro*[43]. We made a dominant negative form of Cbfa1 in a similar way except for the remainder of the N-terminal portion of the molecule. We only deleted the C-terminal portion from the nuclear location signal sequence just after the runt domain of Cbfa1. This constructed gene product, named DN-Cbfa1, was also competitively inhibited the DNA binding of Cbfa1 to OSE2 elements (data not shown). Expression of DN-Cbfa1 in mature chondrocytes changed the phenotype of the cells to an immature state, as judged from cell shape, proliferation activity, and lack of expression of hypertrophic markers, suggesting that dysfunction of Cbfa1 inhibits progression of chondrocyte maturation and that Cbfa1 is required for chondrocyte maturation. The decrease in GAG synthesis and ALPase activity in DN-Cbfa1 expressing chondrocytes was partially recovered by the addition of bone morphogenetic protein-2 (BMP-2) (manuscript in preparation). It is likely that chondrocytes have a Cbfa1-independent BMP signalling pathway to stimulate their maturation. This may be a reason why some of cartilage elements including tibia and ulna proceed to terminal differentiation and exhibit matrix calcification in Cbfa1-deficient mice[16,18].

When DN-Cbfa1 virus was infected into LS cells, the cells remarkably decreased GAG synthesis and became fibroblastic, suggesting that Cbfa1 is also important for the maintenance of the differentiated phenotype of chondrocytes. However, this is contrary to the observation that Cbfa1-deficient mice showed normal cartilage formation. The discrepancy may be explained in several ways. One is that chondrocytes may require the Cbfa1 signal to maintain their phenotype when transferred to an *in vitro* setting. Another possibility is that other runt-related proteins may exert important functions in the expression of the differentiated phenotype in chondrocytes. DN-Cbfa1 competitively inhibits the DNA binding by the runt domain, and the sequence of this domain is highly conserved among the runt family members[47]. Therefore, DN-Cbfa1 might inhibit the function of the other subtypes of runt gene products. Indeed, chicken homologues to Cbfa2, another runt domain protein, have been cloned from a chondrocyte library and suggested to be implicated in chondrogenic differentiation[48].

Acknowledgments

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