Animal Cells and Systems 13: 289-295, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Activation of CREB by PKA Promotes the Chondrogeneic Differentiation of Chick Limb Bud Mesenchymal Cells

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Abstract: Cyclic AMP-mediated signaling pathways regulate a number of cellular functions. In this study, we examined the regulatory role of cAMP signaling pathways in chondrogenesis of chick limb bud mesenchymal cells *in vitro*. Forskolin, which increases cellular cAMP levels by the activation of adenylate cyclase, enhanced chondrogenic differentiation. Inhibition of PKA with specific inhibitors (H89 or KT5720) blocked pre-cartilage condensation stage, indicating that chondrogenesis is regulated by the increase in cellular cAMP level and subsequent activation of PKA. Downstream signaling pathway of PKA leading to gene expression was investigated by examination of several nuclear transcription factors. Forskolin treatment increased transcription level for a cartilage-specific marker gene Sox9. However, inhibition of PKA with H89 led to restore expression of Sox9, indicating PKA activity was required to regulate the expression of Sox9 in chondrogenesis. In addition, CREB was highly phosphorylated at early stage of mesenchyme culture, and followed by progressive dephosphorylation. CBP and ATF, another CRE related proteins were transiently expressed at the early stage of chondrogenesis with a pattern similar to CREB phosphorylation. Electrophoretic mobility shift assays confirmed that the binding activity of CREB to the CRE is closely correlated to the phosphorylation pattern of CREB. Therefore, cAMP-mediated signal transduction to nuclear events for the induction of genes appeared to be required at the early stage of chick limb bud chondrogenesis.

Key words: chondrogenesis, PKA, Sox9, CREB

INTRODUCTION

Like many other developmental processes, chondrogenesis is regulated by environmental signals apparently including those transmitted through the extracellular milieu and those received by direct surface contact with other cells (Solursh, 1988). Protein kinase C (Chang et al., 1998; Lim et al., 2000), MAP kinase (Oh et al., 2002; Bobick and Kulyx, 2004), PKA (Lee and Chuong, 1997; Yoon et al., 2000a), and growth factors (Chen et al., 1993; Yoon et al., 2000b) are among those molecules that have been implicated in the cell signaling events during chondrogenic differentiation in chick embryo limb bud.

Cyclic AMP appears to play an important role in the regulation of limb bud cartilage differentiation. During the critical cellular condensation process at the onset of chondrogenesis, cellular cAMP levels are transiently increased. In the condensation period, prechondrogenic cells become closely juxtaposed and interact with one another prior to initiating extracellular matrix deposition (Biddulph et al., 1988). The elevated intracellular cAMP level has been shown to increase the steady-state levels of mRNAs for cartilage-characteristic type II collagen and the core protein of cartilage-specific proteoglycan in limb bud mesenchymal cells (Rodgers et al., 1989).

Intracellular levels of cAMP are regulated primarily by adenylate cyclase which is modulated by extracellular stimuli. cAMP, in turn, binds cooperatively to two sites on the regulatory subunits of cAMP-dependent protein kinase (PKA), releasing the active catalytic subunits (Lalli and Sassone-Corsi, 1994). The activated catalytic subunits are released and used to phosphorylate a number of cytoplasmic and nuclear proteins. In the nucleus, PKA-mediated phosphorylation ultimately influences the transcriptional regulation of various cAMP-inducible genes (Borrelli et al., 1992). The cAMP response element (CRE) was being identified first as, an inducible enhancer of genes that can

Abbreviations: CRE, cAMP response element; CREB, CRE-binding protein; PKA, cAMP-dependent protein kinase; CBP, CREB-binding protein; ATF, activating transcription factor; TBS, Tris buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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be transcribed in response to an increased cAMP level (Roesler et al., 1988). The 43-kDa CRE-binding protein (CREB) was initially isolated from rat brain tissue and found to express ubiquitously. CREB binds the consensus motif 5'-TGACGTCA-3' and activates transcription (Montminy and Bilezikjian, 1987). One of the known mechanisms of CREB transcriptional activation is the phosphorylation of Ser^{133} by the catalytic subunit of PKA (Gonzalez and Montminy, 1989). Phosphorylation at Ser appears to induce CREB activity by promoting its association with a second protein, CREB-binding protein (CBP) (Kwok et al., 1994). The phosphorylation of CREB and its association with CBP facilitates the assembly of the basic transcriptional machinary and leads to the activation of CREB-regulated genes (Kwok et al., 1994). In addition to CREB, there are several other proteins that bind to the CRE sequence such as activating transcription factors (ATFs) (Habener, 1990).

In this study, we examined whether the regulation of chondrogenesis by cAMP involves PKA and also determined the downstream signaling pathway of PKA leading to regulation of gene expression. The data obtained indicate that cAMP-induced activation of PKA is required for chondrogenesis and PKA appears to regulate activity of CREB by phosphorylation. Furthermore, during the early stage of chondrogenesis, other nuclear factors such as CBP and ATF are transiently expressed in similar patterns to the phosphorylation of CREB.

MATERIALS AND METHODS

Materials

Fertilized white Leghorn chicken eggs were obtained from Singi hatchery (Taegu, Korea). Adenosine 3',5'-cyclic monophosphate (cAMP), guanidine-HCl, and forskolin were purchased from Sigma Chemical Co. (St. Louis, MO). H89 and KT5720 were from Calbiochem (La Jolla, CA). Cell culture media and equipments were obtained from Gibco BRL (Gaithersburg, MD). Antisera against CREB, phosphorylated CREB, and CBP were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-CREB and anti-ATF antibodies were obtained from Santa Cruz (Santa Cruz, CA)

Cell culture

Micromass culture of chick limb bud mesenchymal cells of Hamburger-Hamilton stage 23/24 was carried out as described previously (Yoon et al., 2000b). Briefly, the cells were suspended at a density 2×10^7 cells/mL in Ham's F-12 medium containing 10% fetal calf serum. Ten microliter drops of cell suspension were inoculated onto 35 mm Corning (Corning, NY) plastic culture dishes. The cells were incubated for 1 hr at 37°C in a humidified CO_2 incubator to allow attachment and then cultured either in the absence or presence of various reagents as described in each experiment. Stock solutions of forskolin, H89, and KT5720 were prepared in dimethyl sulfoxide and diluted in media just before the addition to cultures. Quantitative analysis of chondrogenesis was carried out according to the method described previously (Chang et al., 1998). Alcian blue bound to sulfated glycosaminoglycan was extracted with 4 M guanidine-HCl and quantified by measuring the absorbance at 600 nm.

Preparation of total cell extract and nuclear extract

The cells were sonicated for 30 sec in 50 mM Tris-HCl buffer (pH 7.5, 5 mM $MgCl_2$, 5 mM NaCl, and 5%(v/v) glycerol). After centrifugation at 10,000×g for 30 min, supernatants were collected and referred to cell extract. Nuclear extracts were prepared by the method of Zheng et al. (1993). Briefly, cell pellets were lysed by the addition of Nonidet P-40 (final concentration of 0.6%(v/v)). The homogenate was centrifuged for 30 sec in a microcentrifuge (12,000×g). The nuclear pellet was resuspended in 50 mL of 20 mM HEPES buffer (pH 8.0, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). This suspension was agitated at 4°C for 15 min, followed by centrifugation at 12,000×g for 5 min. The resulting supernatant was stored in small aliquots at −70°C. Protein concentration was measured by using the Bradford protein dye reagent (Bio-Rad, Hercules, CA).

Immunoblotting

Total cell lysates or nuclear fractions from chondroblasts at the various indicated time were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. The proteins were transferred electrophoretically onto nitrocellulose membranes (0.2 mm, Schleicher and Schuell). The membranes were blocked with 3% non-fat dry milk and 0.1% Tween 20 in Tris buffered saline (TBS). The membranes were subsequently probed with primary antibody in TBS containing 3% non-fat dry milk and 0.1% Tween 20. The antibody-antigen complexes were detected by using goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates, followed by the ECL system.

RT-PCR analysis

Total RNA was isolated from cultured mesenchymal cells using the TRI reagent (Molecular Research Center, Inc.) method. RNA amounts were quantified by absorbance at 260 nm. The following PCR conditions were employed: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and an additional extension step for 5 min at 72°C as the last cycle. Amplified PCR products were analyzed by

agarose gel electrophoresis and ethidium bromide staining. The following primer sequences were used: for chicken Sox9 5'-CCC CAA CGC CAT CTT CAA-3' and 5'-CTG CTG ATG CCG TAG GTA-3', and chicken GAPDH 5'-GAG AAC GGG AAA CTT GTC AT-3' and 5'-GGC AGG TCA GGT CAA CAA-3'.

Electrophoretic mobility shift assays

For gel shift assays, consensus CRE oligonucleotide was labeled with T4 polynucleotide kinase. Nuclear extracts (10 mg of protein) were incubated in 25 μL of reaction buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 2.5 μg of poly(dI-dC) for 15 min at 15°C. The ^{32}P-labeled CRE probe was then added to the reaction mixture and incubated for 20 min at room temperature. Competition analysis was performed by including 100-molar excess of unlabeled CRE in the binding reaction. In supershift experiments, 2 μg of antibody against CREB was pre-incubated with nuclear extracts for 30 min at room temperature. The reaction products were analyzed by electrophoresis in a 6% polyacrylamide gel with 0.25×TBE buffer (22.3 mM Tris, 22.2 mM borate, and 0.5 mM EDTA). The gel was dried and analyzed by autoradiography.

RESULTS

cAMP and PKA are required for chondrogenesis

Several studies have been implicated cAMP as a positive effector of chondrogenesis. Endogenous levels of cAMP have been reported to increase in cells undergoing chondrogenesis *in vitro* (Rodgers et al., 1989). The effect of perturbing the cAMP levels on chondrogenesis was examined by treating cells with forskolin, a diterpene that activates adenylate cyclase. To quantify the effects of forskolin on chondrogenesis, we measured the absorbance proteoglycan accumulation based on the Alcian blue bound to the cartilage matrix. During the first 1 day of culture between the cells treated with forskolin and dimethyl sulfoxide as vehicle alone, no distinctive difference in proteoglycan accumulation was observed (Fig. 1A). However, forskolin enhanced the deposition of Alcian-blue positive cartilage matrix after 2 days of culture. The condensation grew larger and progressed to form cartilage nodule on day 4 of culture, as indicated by Alcian blue staining (Fig. 1B).

Since it has been known that increased intracellular cAMP exerts cellular responsiveness by activating PKA, we examined whether PKA is involved in chondrogenesis by using PKA specific inhibitors, H89 and KT5720 (Chijiwa et al., 1990; Gadbois et al., 1992). Initial experiments evaluated the effects of various concentrations of H89 and KT5720 on the process of chondrogenesis in micromass

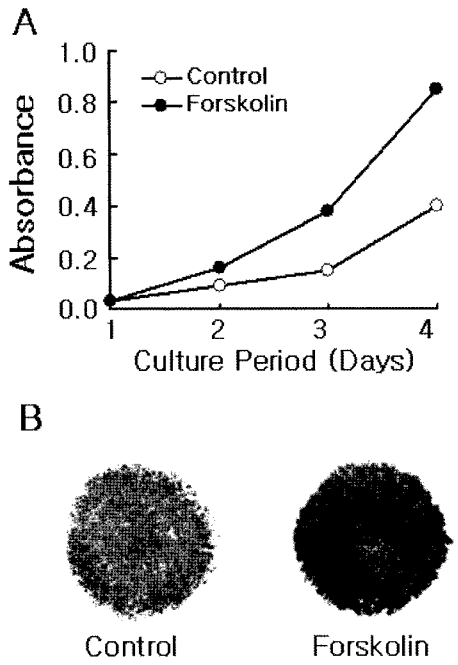

Fig. 1. Promoting effects of forskolin on chondrogenesis *in vitro*. (A) Mesenchymal cells were micromass cultured in the presence of vehicle alone as a control or 10 μM forskolin for the first 24 hr. The cells were washed and maintained without forskolin for the rest of culture. At the indicated time periods, the cells were stained with Alcian blue and the absorbance of extractable Alcian blue with 4 M guanidine-HCl was measured at 600 nm. (B) Mesenchymal cells were treated with 10 μM forskolin for 4 days. The cells were stained with Alcian blue to determine chondrogenesis. The data represent a result of typical experiment performed four times with similar patterns.

cultures of mesenchymal cells. Control cultures of chondrogenic competent cells formed numerous, discrete Alcian-blue positive cartilage nodules at 4 days of culture. In contrast, the accumulation of proteoglycan that stained with Alcian blue was strongly inhibited by treatment of 1 μM KT5720 or completely blocked by 20 mM H89 (Fig. 2A). To investigate which stage of chondrogenesis was affected by PKA, H89 was treated at various stages of micromass culture. As shown in Fig. 2B, the most prominent inhibitory effects of H89 was observed when chondroblasts were treated with H89 for the first 24 hr. Inhibition of the accumulation of cartilage matrix became less effective when H89 was treated at later stage of chondrogenesis. Taken together, chondrogenesis appears to be regulated by increase in cellular cAMP level and subsequent activation of PKA at the early period of micromass culture.

Expression of transcription factor Sox9 is increased by activation of PKA

At pre-cartilage condensation stage of chondrogenesis, the cells surround themselves with an abundant layer of extracellular matrix. Expressions of type II collagen and

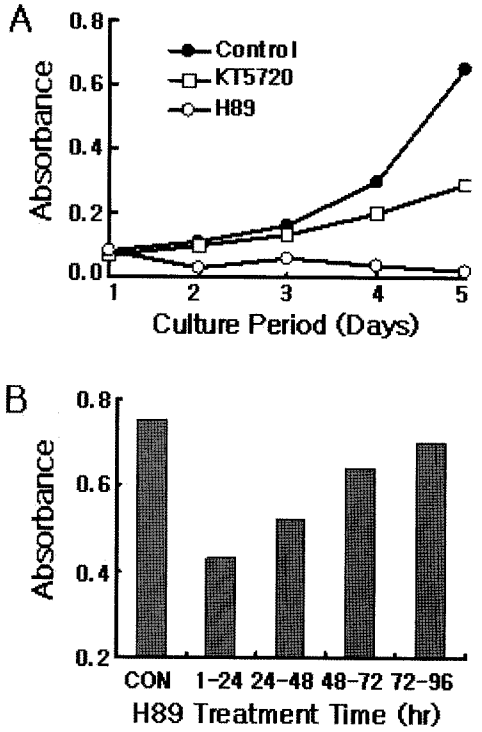

Fig. 2. Inhibition of chondrogenesis by PKA inhibitors. (A) Mesenchymal cells were cultured for 4 days in the absence (control) or presence of 20 μM H89 or 1 μM KT5720 to inhibit PKA. (B) Cells were cultured in the presence of 20 μM H89 for 24 hr for the indicated periods or in the absence of H89 as a control. Following H89 treatment, the cells were washed extensively with Ham's F12 medium and kept in culture up to 96 hr. The absorbance was measured as described in the legend of Fig. 1. The data represent the mean values of three experiments.

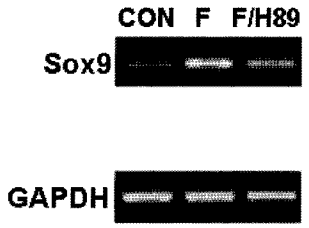

Fig. 3. Effects of forskolin treatment on the expression of Sox9. Cells were incubated for 24 hr in medium containing forskolin (F), or forskolin and H89 (F/H89) or vehicle alone (CON). Total RNA was isolated from cells, and RT-PCR was performed. PCT products were separated on 1.2% agarose gel and photographed. GAPDH was also examined as a reference. Experiments were performed four times with similar patterns.

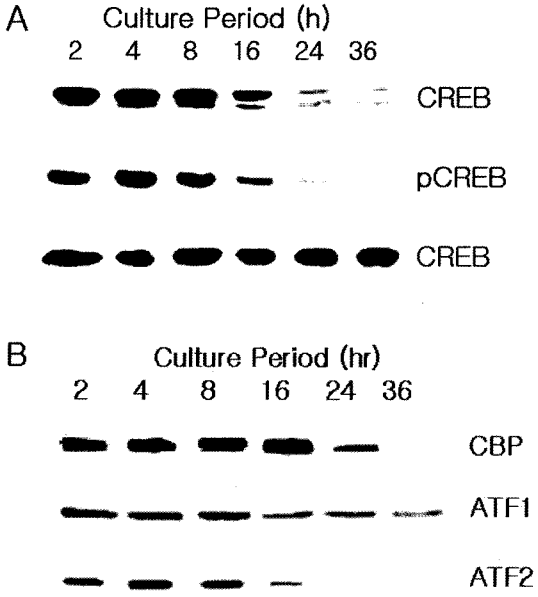

Fig. 4. Expressions of transcription factors at the early stage of chondrogenesis. Nuclear extracts (30 μg) from mesenchymal cells cultured for the indicated periods were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. (A) The nitrocellulose membranes were reacted with anti-CREB (*upper panel*) which recognizes both the unphosphorylated and phosphorylated form of CREB, anti-phosphorylated CREB (*middle panel*), or anti-total CREB (*lower panel*). (B) The membranes were probed with anti-ATF-1, ATF-2, or anti-CBP, and detected with ECL kit

core protein of aggrecan are controlled by Sox9, one of the major transcription factors regulating cartilage differentiation (Akiyama et al., 2002; Kulyk et al., 2000). To better define the effects of PKA on chondrogenesis, we employed RT-PCR analysis to determine whether forskolin and H89 treatments influenced expression of the cartilage-characteristic marker gene, transcription factor, Sox9. As shown in Fig. 3, treatment with forskolin significantly increased Sox9 mRNA levels in cultures of pre-chondrogenic mesenchymal cells isolated from the distal tips of chick embryo limb buds. However, co-treatment with both forskolin and PKA inhibitor H89 restored cartilage-specific gene transcript expression to control levels in distal limb mesenchyme culture.

Modulation of nuclear factors at the early stage of chondrogenesis

cAMP-induced activation of PKA is known to regulate gene expression by the phosphorylation of CREB. Therefore, to further understand the mechanisms underlying cAMP regulation in chondrogenesis *in vitro*, the effects of cAMP on the expression and phosphorylation of CREB was examined. Nuclear extracts of cells cultured for various period of time were used to determine the changes of CREB expression. The anti-CREB antibody we used, that detected two bands of proteins as shown in Fig. 4A (*upper panel*). The levels of lower band did not show changes throughout the culture periods, while the levels of upper band protein were decreased dramatically as chondrogenesis proceeds. Since it is possible that the upper bands represent phosphorylated form of CREB, we used an antibody which recognizes the phosphorylated form of CREB. The levels of phosphorylated CREB was high at the early stage of chondrogenesis (i.e., within 8 hr of micromass culture), and

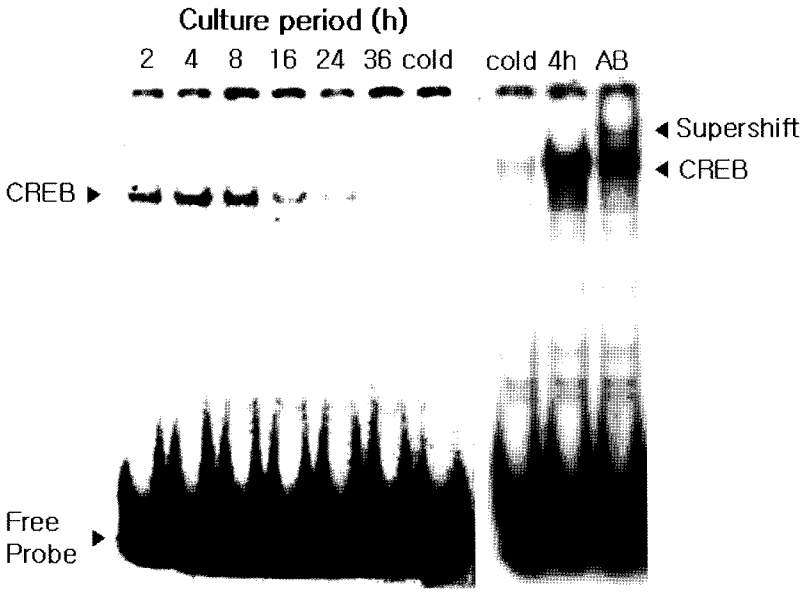

Fig. 5. Electrophoretic mobility shift assay of CRE binding activity. Nuclear extracts were isolated from mesenchymal cells cultured for the indicated times, incubated with consensus ^{32}P-labeled CRE oligonucleotide, and then electrophoresed on a 6% non-denaturing polyacrylamide gel. The gels were dried and exposed to x-ray film. Specificity of CREB binding was determined by the antibody against CREB (AB) or 100-fold excess unlabeled (cold) CRE oligonucleotide.

followed by progressive decrease (Fig. 4A, *middle panel*).

Regulation of cAMP-inducible gene expression by CREB involves CBP, a CREB coactivator. We, therefore, examined the expression of CBP during chondrogenesis. CBP was highly expressed at the early period of culture (within 16 hr of culture). CBP expression was decreased progressively, and not detected after 36 hr of culture (Fig. 4B). The expression pattern of CBP was closely related to the phosphorylation of CREB (Fig. 4A) indicating that CBP expression could be involved in CREB function in the mesenchyme differentiation of limb bud. In addition, we examined expressions of another CRE-binding protein, ATFs. ATFs were detected immediately after seeding of the cells, expressed at relatively high levels until 8 hr of culture, and followed by progressive down regulation (Fig. 4B). Although the function of ATFs in chondrogenesis was not determined, expression pattern of ATF was quite similar to the patterns of CBP expression and CREB phosphorylation.

To investigate the functional significance of CREB phosphorylation, CRE-binding analysis was performed. Consensus oligonucleotide containing CRE sequence was radiolabeled and used for the analysis of CREB binding activity to CRE by electrophoretic mobility shift assays. As shown in Fig. 5A, there is a single band of complex bound to CRE only at the early period of micromass culture. The shifted bands were specific for CRE since the addition of 100-fold excess unlabeled (cold) CRE oligonucleotide to the nuclear extracts abolished the shifted band (Fig. 5). The protein bound to CRE site was CREB because the addition of antibody against CREB reduced the amount of shifted band and caused further gel retardation, supershift (Fig. 5). The binding activity of CREB to CRE site was high within 8 hr after seeding and then progressively decreased (Fig. 5). This binding activity was closely related to the phosphorylation pattern of CREB (Fig. 4A). From these results, cAMP-inducible gene expression during chondrogenesis appears to be regulated by several nuclear transcription factors including CREB, CBP, and ATFs. These factors might be involved at the early stage of chick limb bud mesenchymal development.

DISCUSSION

When micromass cultured at high-density, mesenchymal cells spontaneously develop numerous cartilage nodules in which prechondrogenic mesenchymal cells aggregate, initiate cartilage-specific gene expression, and elaborate an Alcian-blue positive cartilage matrix predominantly composed of type II collagen and the cartilage-specific proteoglycan. It has been suggested that an elevation of cellular cAMP levels during the condensation phase of chondrogenesis triggers the differentiation of the cells (Biddulph et al., 1988; Rodgers et al., 1989). In this study, we showed that increasing intracellular cAMP level by the treatment with forskolin promotes chondrogenesis of chick limb bud mesenchymal cells in a dose-dependent manner (Fig. 1). The results are consistent with reports by others which showed the stimulatory effects of cAMP derivative, dibutyryl cAMP, on chondrogenesis (Biddulph and Dozier,

1989).

A principle effect of elevated cAMP levels is the activation of PKA in most cells. Based on observations, cAMP mediated signaling pathway appears to involve PKA in chondrogenesis, because PKA inhibitors such as H89 and KT5720 blocked chondrogenesis (Fig. 2). Our results also suggest that PKA might function at the early stage of cartilage differentiation because the inhibitory effects of PKA inhibitor was more evident when treated for the first 24 hr of culture (Fig. 2). The requirement of PKA activity at the early stage of chondrogenesis *in vitro* is supported by other reports which demonstrated the elevation of cellular cAMP level and activation of PKA (Lee and Chuong, 1997; Yoon et al., 2000a). The regulatory role of PKA in chondrogenesis of limb buds is not still clear. One of the possible roles of PKA is phosphorylation of cellular proteins which might be involved in cellular response. We previously showed that activation of PKA in chick chondroblasts caused phosphorylation of 40-kDa nuclear protein which was distinct from CREB (Park et al., 1995). Another possible role of PKA is the involvement in the expression of cartilage-specific extracellular matrix proteins. PKA is known to regulate expressions of cAMP-inducible genes via the modulation of nuclear transcription factors such as CREB (Gonzalez and Montminy, 1989). PKA-induced phosphorylation event is required for CREB to activate the transcription of genes containing CREB binding site.

The requirement of high cell density for cartilage differentiation suggests extensive cell-cell interactions and involvement of molecules responsible for building of the extracellular matrix. The transcription factor Sox9 stimulates the transcriptional activity of cartilage matrix-specific genes such as type II collagen and core protein of aggrecan (Kulyk et al., 2000). The activity of Sox9 protein is regulated by phosphorylation of a serine residue which is a target of PKA (Huang et al., 2000). In our results, we also observed that the efficacy of the transcription promoting activity of Sox9 is increased by PKA (Fig. 3).

Expression of cAMP-inducible genes by CREB is primarily regulated by the phosphorylation of CREB. Like other cell types, CREB is constitutively expressed in chick limb bud mesenchymal cells (Widnell et al., 1994) but the activity is regulated by phosphorylation and dephosphorylation (Fig. 4A). According to the electrophoretic mobility shift assay, CREB was phosphorylated at the early stage of chondrogenesis and this phosphorylation is associated with the binding of CRE (Figs. 4A and 5). Several reports indicated that the decrease in the rate of transcription was due to, in part, the dephosphorylation of CREB (Wadzinski et al., 1993; Wheat et al., 1994). It has been postulated that phosphorylation of CREB increases the affinity of the transactivation domain for the basal transcription machinery of cofactor, CBP, that mediates its interaction with the initiation complex (Chrivia et al., 1993). In addition to CREB, there are several other proteins such as ATFs that bind to the CRE sequence (Habener, 1990). The expressions of CBP and ATF-2, phosphorylation and activation of CREB, and inhibition of chondrogenesis by PKA inhibitor (H89) were effective only at the early condensation period of cartilage differentiation.

Therefore, we demonstrated that cAMP signal pathway to nuclear events via activation of CREB for the induction of cAMP-responsive genes appeared to be required at the early developmental stage of limb bud mesenchymal cells.

ACKNOWLEDGMENT

This work was supported by the Korea Research Foundation Grant (KRF-2005-005-J00102).

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[Received August 17, 2009; accepted September 18, 2009]