Activation of Protein Kinase A Is a Pivotal Step Involved in Both BMP-2- and Cyclic AMP-Induced Chondrogenesis

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We studied the roles of protein kinase A (PKA) activation and cyclic AMP response element binding protein (CREB) phosphorylation in chondrogenesis using serum-free chicken limb bud micromass cultures as a model system. We showed the following points: 1) in micromass cultures, activation of PKA enhances chondrogenesis and increases the phosphorylation of CREB; 2) BMP-2, a chondrogenic stimulator, increases PKA activity and the level of phosphorylated CREB (P-CREB); 3) IBMX, a PKA inhibitor, inhibits chondrogenesis; 4) the chondrogenic activities of BMP-2 and cAMP are suppressed by H8; and 5) long-term TPA treatment (a protein kinase C (PKC) modulator) inhibits chondrogenesis and decreases the levels of CREB and P-CREB. These results suggest that activation of PKA is a physiological event during chondrogenesis that is involved in the chondrogenic effects of both BMP-2 and cyclic AMP (cAMP)-dependent pathways. J. Cell. Physiol. 170:153-165, 1997.

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During skeletal development, the loosely arranged mesenchymal cells aggregate to form precartilage condensations (Ede, 1983), which subsequently differentiate into cartilage. Several categories of molecules have been found to regulate the growth control of chondrogenic competent limb bud cells. Extracellular growth factors, such as transforming growth factor (TGF) beta acting on the Ser/Thr kinase receptor pathway and fibroblast growth factor (FGF) family members acting on the receptor tyrosine kinase pathway, have opposite effects (Niswander and Martin, 1993). FGF family members tend to increase the number of chondrogenic competent cells through enhancing cell proliferation (Savage et al., 1993), while TGF beta family members such as the BMPs, TGF beta, and activin tend to promote the formation of precartilage condensations and/or to enhance chondrogenesis (Scheid et al., 1986; Schofield and Wolpert, 1990; Sato and Urist, 1984; Leonard et al., 1991; Jiang et al., 1992; Roark and Greer, 1994; Duprez et al., 1996).

Adhesion molecules such as neutral cell adhesion molecules (NCAM), N-cadherin, fibronectin, and tenascin are also involved in the formation of precartilage condensations (Widelitz et al., 1993; Oberlender and Tuan, 1994; Frenz et al., 1989; Mackie et al., 1987). Growth factors may alter the expression of these adhesion molecules. For example, TGF beta stimulates fibronectin expression (Leonard et al., 1993), while activin enhances NCAM expression and therefore promotes the formation of precartilage condensations (Jiang et al., 1993).

We are interested in determining the intracellular molecular pathways through which extracellular growth factors and other cellular effectors exert their effects on the formation of precartilage condensations. Early studies have indicated that the protein kinase A (PKA) and C (PKC) pathways are involved in chondrogenesis. Dibutyryl cyclic AMP (cAMP) (a PKA agonist) stimulates chondrogenesis (Solursh et al., 1981; Rodgers et al., 1989). A cAMP-dependently phosphorylated nuclear substrate, p35, and tyrosine PKA are identified in the nuclei of precartilage cells but not cartilage cells (Zhang et al., 1996). Long-term phorbol 12-myristate 13-acetate (TPA) (a PKC modulator) treatment suppresses chondrogenesis by depressing PKC activity (So et al., 1992). Staurosirarin (a PKC antagonist) enhances chondrogenesis (Kulik, 1991). However, much remains to be learned about the intracellular chondrogenic pathway.

In this study we focused on PKA-related pathways. We explored the relationship between PKA and BMP-2, cAMP, and NCAM expression using dissociated high density micromass cultures (Ahrens et al., 1977). This model allows us to ask questions using biochemical, pharmacological, and immunological approaches.

MATERIALS AND METHODS

Materials

Chicken eggs were from SPAFAS (Preston, CT). Embryos were staged according to Hamburger and Hamilton (1951). The following reagents were used. Rabbit anti-chicken NCAM was according to Chuong and Edelman (1985). Rabbit antibodies against the cAMP response element binding protein (CREB) and phosphorylated CREB

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Received 23 January 1996; Accepted 20 September 1996
absence of nuclear staining in the anti-PCREB + CREB phosphopeptide (Fig. 1C,D). The experiments that contained an equal amount of albumin instead of P-CREB peptide or CREB protein were used as the controls.

For the spatial distribution, anti-CREB immunostaining showed a nuclear staining pattern with weak cytoplasmic staining. CREB-positive cells were distributed uniformly in the whole culture (Fig. 2A–C). P-CREB-positive cells were sporadically distributed through the cell population (Fig. 2A'–C'). The distribution of P-CREB-positive cells is not restricted to the precartilage condensations but also appears in cells between condensations. Anti-CREB mainly stained the nucleus, but some weak staining is also seen in the cytoplasm. Anti-P-CREB immunostaining was mainly in the nucleus (Fig. 2C,C').

For the temporal expression pattern, we observed an initial high level of expression of CREB that remained high in 2 h, 18 h, and 72 h cultures (Fig. 3A, left column). In contrast, the expression of P-CREB was much more dynamic. The level of P-CREB expression was high at 2 h and 18 h but decreased by 72 h in the differentiation phase (Fig. 3A, left column). The ratio of P-CREB to CREB was high in the precartilage condensation phase (the first day of micromass culture) and low in the differentiation phase (the third day of culture).

Dibutyryl cAMP enhances the expression of P-CREB, while TPA downregulates the expression of both CREB and P-CREB

Dibutyryl cAMP enhanced chondro-differentiation with a dose-dependent response (Kosher et al., 1979). In our serum-free cultures, 1 mM dib-cAMP led to 215% enhancement of chondrogenesis (Table 1). This was the concentration used in subsequent experiments. With

**Fig. 1.** The immunoreactivities of anti-CREB and anti-P-CREB were neutralized by synthetic CREB phosphopeptide and/or CREB control protein. Limb bud cell cultures were fixed and stained with anti-CREB or P-CREB followed by alkaline phosphatase-conjugated secondary antibodies. NBT and BCIP were used as substrates of the alkaline phosphatase. CREB-positive cells were distributed uniformly and showed the positive cytoplasmic and nuclear staining pattern (A). The immunoreactivity of anti-CREB (4,000× dilution) was effectively neutralized by preincubation with CREB phosphopeptide (0.4 mg/ml) and CREB control protein (0.12 mg/ml) extracted from SR-X-MC cells (B). In anti-P-CREB (1,000× dilution) stained cultures, P-CREB-positive cell were sporadically distributed (C). Synthetic CREB phosphopeptide (0.4 mg/ml) was incubated with anti-PCREB for 1 h at room temperature and effectively neutralized the immunoreactivity of anti-P-CREB (D). Bar, 100 μm.
not change significantly at 2 h between the control and treated cultures. The difference was most remarkable at 18 h, and these values were used for calculation of the P-CREB/CREB ratio. At 18 h, exogenous db-cAMP increased P-CREB/CREB ratio 2.5-fold, while the control culture (also at 18 h) remained similar to the level at 2 h (Fig. 3C).

In a dose range of 50–400 nM, TPA inhibits chondro-differentiation and dephosphorylates PKC activity in micromass cultures treated for 1 day or 3 days (Sasse et al., 1983; Sonn and Solursh, 1993). In our serum-free cultures, 200 nM of TPA suppressed chondrogenesis by 50% (Table 1). The level of CREB protein was nearly suppressed to the basal level at day 3 (Fig. 3A,B). The P-CREB level was not altered much at 2 h but rapidly diminished at 18 h. At 72 h, not much CREB and P-CREB was visible (Fig. 3A). When the P-CREB/CREB ratio was calculated on the TPA-treated cultures, the ratio was 43% of the control (Fig. 3C). Although little is known about what factors may modulate PKC activity in vivo, the results suggest that suppression of the CREB level and degree of phosphorylation are among the targets that PKC can act on to suppress chondro-differentiation. The results are also consistent with the notion that phosphorylation of CREB is correlated with chondrogenesis.

**BMP-2 enhances the phosphorylation of CREB and PKA activity**

What are the physiological factors that activate PKA during chondrogenesis? Extracellular signaling factors are possible candidate molecules. In this study, we focused on BMP-2, a TGF beta family member known to induce chondrogenesis (Sato and Urist, 1984) and examined its effects on the levels of P-CREB and PKA activity. In serum-free limb micromass cultures, BMP-2 also stimulated chondrogenesis in a dose-dependent manner. At a dose of 250 ng/ml, BMP-2 enhanced chondrogenesis by 200%.

The expressions of CREB and P-CREB in 18 h cultures were examined. In BMP-2 (250 ng/ml)-treated

**Fig. 3.** Expression of CREB and P-CREB in limb bud micromass cultures and modulation by PKA and PKC modulators. Duplicate chicken limb bud micromass cultures were fixed at 2 h, 18 h, and 72 h. The cultures in each dish were then stained with rabbit anti-CREB (left culture) and P-CREB (right culture), followed by alkaline phosphatase (Alk-P)-conjugated secondary antibodies. The color was developed using NBT and BCIP as substrates according to the procedure recommended by the manufacturer (Promega). A: Left column, control; middle column, 1 nM db-cAMP treatment; right column, 200 nM TPA treatment. Note the enhancing effect of db-cAMP and the inhibitory effect of TPA. Although mature (after 4 days of culture) micromass cultures exhibit endogenous Alk-P, Alk-P activity is not detected before day 3 under our experimental conditions. Control experiments without primary antibodies but with secondary antibodies and Alk-P substrates are negative (not shown). The three micromass cultures marked by asterisks are enlarged and shown in Fig. 2A and Fig. 4C. The expression levels of CREB and P-CREB were determined by densitometric analysis. CREB expression remains high over the first 3 days of cultures with or without db-cAMP. The CREB level is suppressed by TPA (B). The relative amount of P-CREB is increased by db-cAMP and BMP-2 (250 ng/ml) particularly at 18 h. The ratio of P-CREB/CREB at 18 h is normalized to the control at the 2 h time point. This ratio is increased to three times the control value by db-cAMP and to about two times the control by BMP-2 (C). A decreased P-CREB/CREB ratio is observed in the presence of TPA (C). Values (ratio of P-CREB/CREB), mean ± s.d., shown are results from two independent experiments.
Figure 4.
other than CREB are likely to be involved in chondrogenesis too. The evidence is strengthened by the newly identified p35, a nuclear substrate for PKA, as being involved in chondrogenesis (Leonard and Newman, 1987; Zhang et al., 1996). In addition, the PKC pathway may interact with cAMP-dependent signaling. Long-term TPA treatment suppresses PKC activity in micromass cultures (Sonn and Solursh, 1993). In our results, similar treatment suppressed the phosphorylation of CREB and the amount of CREB protein (Fig. 3). Thus, different intracellular signaling pathways probably interact with each other during chondrogenesis. Among them, the regulation of PKA activity apparently is a pivotal step.

**Chondrogenic effect of BMP-2 involves PKA activation**

If activation of PKA is pivotal to chondrogenesis, what are the likely upstream extracellular modulators that can activate PKA, either directly or indirectly, in the mesenchymal cell? Since BMP-2 has a profound effect on chondrogenesis, we examined the effect of BMP-2 on PKA. BMP-2 is a member of the TGF beta
Fig. 8. A working model. This is a summary of our efforts to link extracellular signaling, intracellular signaling, adhesion molecules, and cartilage phenotypes together. The molecules examined in this report are shown in bold characters. There is a PKA -> P-CREB -> NCAM -> precartilage condensation pathway. To modulate this pathway, BMP-2 works as a positive regulator. Long-term TPA (a PKC modulator) treatment works as a negative regulator. Other upstream regulatory signals remain to be determined. Molecular events mediated by adhesion molecules such as N-cadherin and fibronectin, which were involved in the formation of precartilage condensations, are likely to be involved in the later stage. The arrows may represent more than one step.

BMP-2-treated cultures formed sheet-like condensations with homogeneous NCAM expression and cartilage differentiation. In contrast, db-cAMP caused large nodular condensations showing high NCAM expres-
chondrogenesis: Phosphorylation of the pre-cartilage 35.5-kDa domain-specific chromatin protein and its regulation by cyclic AMP. Dev. Biol., 129:92–100.


