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cAMP INDUCES PHOSPHORYLATION OF A 40-kDa NUCLEAR PROTEIN WHICH IS DISTINCT FROM CREB DURING CHONDROGENESIS OF CHICK LIMB BUD MESENCHYMAL CELLS *IN VITRO*

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SUMMARY : We examined the effect of cAMP on the phosphorylation of intracellular proteins in cultured chondroblasts to understand the stimulatory role of intracellular cAMP in chondrogenesis of chick limb bud mesenchymal cells. A 40-kDa protein was remarkably phosphorylated by cAMP and the phosphorylation was completely blocked by an inhibitor of cAMP-dependent protein kinase. The phosphorylation of the 40-kDa protein was maximum at early stage of chondrogenesis (i.e., 24 hr of culture) which is consistent with the changes in the level of intracellular cAMP. The 40-kDa phosphoprotein was exclusively located in the nuclear parts of chondroblast but distinct from cAMP response element binding protein.

Chondrogenesis is an essential event in development of the vertebrate embryonic limb. Like many other developmental processes, it is regulated by environmental signals apparently including those transmitted through the extracellular milieu and those received by direct surface contact with other cells (1). A variety of studies indicate that a key event in limb chondrogenic differentiation is a cellular condensation process which involves cell-extracellular matrix and cell-cell interactions. The cellular condensation appears to be regulated by the increase in cellular cAMP levels (2-6).

It is well recognized that regulation of enzyme activities by reversible phosphorylation plays important roles in many signal transduction system. The presence of cAMP-dependent protein kinase (PKA) in cultured chick limb bud mesenchymal cells suggests a regulatory role of cAMP-PKA signal transduction system in the differentiation of limb tissues (7,8).

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Therefore, we investigated the effects of cAMP on the phosphorylation of intracellular proteins in cultured chick chondroblasts. The data obtained indicate that cAMP induces the phosphorylation of a 40-kDa protein by the activation of PKA during the early stage of chondrogenesis. The 40-kDa phosphoprotein is exclusively located in nuclear part but distinct from CREB, a cAMP response element binding protein (9).

MATERIALS AND METHODS

Materials. Fertilized white Leghorn chicken eggs were obtained from Singi hatchery (Taegu, Korea). Adenosine 3',5'-cyclic monophosphate (cAMP), PKA, and PKA-inhibitor (synthetic peptide) were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham. Most of the cell culture media and equipments were obtained from Gibco.

Cell culture and preparation of cell extract. The micromass culture of chick limb bud mesenchymal cells of Hamburger-Hamilton stage 23/24 were carried out as described (10). Chondroblasts, cultured for indicated time periods, were washed three times with ice-cold PBS, and harvested by centrifugation. The cells were sonicated for 30 sec in 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 5 mM NaCl, and 5% (v/v) glycerol. After centrifugation at 10,000 x g for 30 min, supernatants were collected and referred to cell extract. Crude nuclear pellet of chondroblasts was isolated by a pulse centrifugation and resuspended in 20 mM HEPES buffer, pH 8.0, containing 10% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride as described (11). Nuclei were sonicated for 10 sec and clarified by centrifugation.

***In vitro* Phosphorylation**. Phosphorylation of cell extracts were performed by the addition of 100 μg of cell extract to the reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.5, 0.1 mM [γ-^{32}P]ATP, and 5 mM $MgCl_2$. Following incubation of the mixture at 20°C for 1 min, the reaction was terminated by the addition of SDS sample buffer (12). Effects of cAMP, PKA, or PKA-inhibitor on phosphorylation were examined by preincubating them with assay mixture for 10 min prior to the addition of ATP. The samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and phosphorylated proteins were detected by autoradiography.

RESULTS AND DISCUSSION

Increased cellular cAMP level is known to enhance chondrogenesis of chick limb bud mesenchymal cells (2-6). We, therefore,

investigated whether phosphorylation of proteins by cAMP-dependent pathway is involved in chondrogenic differentiation. We first examined changes in the phosphorylation pattern of cellular proteins during differentiation of cultured chick chondroblasts. As shown in Fig. 1, several proteins were phosphorylated as determined by *in vitro* phosphorylation and the phosphorylation pattern was changed during differentiation of chondroblasts. In particular, phosphorylation of 30-, 47- and 53-kDa proteins increased during the early period of differentiation, while that of a 65-kDa protein declined gradually along with time of culture.

To identify proteins phosphorylated in a cAMP-dependent manner, extracts of chondroblast cultured for 72 hr were incubated in the

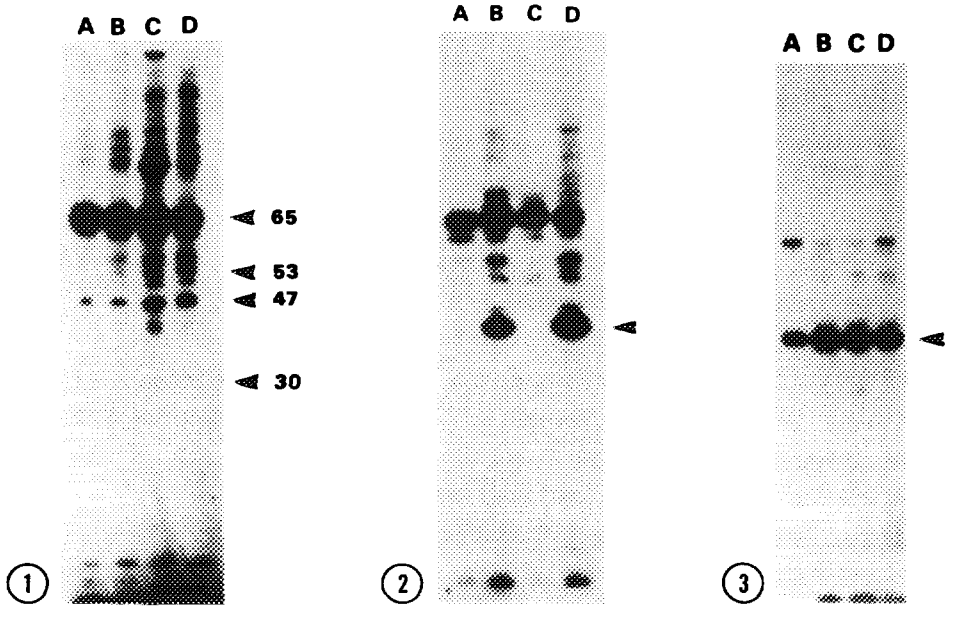

Fig. 1. Changes in the pattern of protein phosphorylation during differentiation of chondroblasts. Chick mesenchymal cells were cultured for 4 hr (A), 24 hr (B), 48 hr (C), and 72 hr (D). At the indicated times, cells were extracted and the aliquots of extracts (100 μg) were used to determine phosphorylation of proteins *in vitro*. The numbers and arrows indicate the molecular weights (kDa) and the proteins showing significant changes in the phosphorylation states, respectively.

Fig. 2. A 40-kDa protein is phosphorylated in a cAMP-dependent manner in chondroblasts. Extracts were prepared from chick mesenchymal cells cultured for 72 hr and assayed for protein phosphorylation in the absence (A) or presence of 2 mM cAMP (B), 2 mM cAMP and 100 units of PKA-inhibitor (C), or 2 mM cAMP and 10 μg of PKA (D). The arrowhead indicates the position of 40-kDa protein.

Fig. 3. Changes in the pattern of 40-kDa protein phosphorylation during chondrogenesis. Chondroblasts were cultured for 4 hr (A), 24 hr (B), 48 hr (C), and 72 hr (D). The cells were extracted and phosphorylated in the presence of 2 mM cAMP.

presence of 2 mM cAMP for 10 min prior to the initiation of phosphorylation. Addition of cAMP to the reaction mixture induced remarkable phosphorylation of a 40-kDa protein (Fig. 2, lane B). Phosphorylation of the 40-kDa protein induced by cAMP was completely blocked by an inhibitor of PKA, Walsh inhibitor (13) (Fig. 2, lane C). The 40-kDa protein was more phosphorylated by exogenous catalytic subunit of PKA (Fig. 2, lane D). The results indicate that the 40-kDa protein is phosphorylated specifically by the PKA. Addition of Ca^{2+}, phorbol myristate acetate, and phosphatidylserine or Ca^{2+} and calmodulin to the reaction mixture had little or no effects on the phosphorylation pattern of the 40-kDa protein (data not shown) indicating that neither protein kinase C nor Ca^{2+}/calmodulin-dependent proein kinase is involved.

Extracts of cells cultured for various periods were used for the *in vitro* phosphorylation to examine whether phosphorylation of the 40-kDa protein changes during chondrogenesis. Phosphorylation of the 40-kDa protein was detected at the early stage of differentiation (i.e., 4 hr), reached at maximum level at 24 hr of culture, and followed by progressive decrease (Fig. 3). The result was closely related to the pattern of changes in intracellular cAMP levels, which were transiently increased at the early stage of chondrogenesis *in vitro* (5).

The 40-kDa phosphoprotein was exclusively located in nuclear part as determined by cell fractionation (Fig. 4A). We, therefore, examined whether 40-kDa phosphoprotein is related to 43-kDa CREB, a nuclear protein phosphorylated by PKA that is linked to induction of

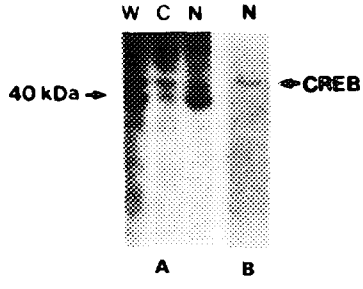

Fig. 4. Localization of the 40-kDa phosphoprotein in chondroblasts. (A) Cell extracts from 24 hr-cultured chondroblasts were phosphorylated in the presence of 2 mM cAMP and 10 μg pf PKA, W:whole cell extract; C:cytosolic proteins; N:nuclear proteins. (B) Western blotting of nuclear proteins using anti-CREB (06-244, UBI).

cAMP-regulated gene expression. Chick chondroblasts express CREB as determined by Western blotting (Fig. 4B). CREB is predominantly located in the nuclear part of chondroblast which is consistant with previous report (9). The molecular size of 40-kDa phosphoprotein is apparently different from that of CREB (Fig. 4). In addition, the protein recognized by anti-CREB antibody was much less phosphorylated compared with the 40-kDa protein in the presence of cAMP. The results suggest that promotion of chondrogenesis of chick chondroblasts by cAMP is closely associated with phosphorylation of the 40-kDa nuclear protein which is apparently distinct from CREB.

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