Journal of Biochemistry and Molecular Biology, Vol. 31, No. 4, July 1998, pp. 350–354

JOURNAL OF
Biochemistry and Molecular Biology
© BSRK & Springer-Verlag 1998

Phosphorylation of a 66 kDa Protein, a Putative Protein Kinase C Substrate, is Related to Chondrogenesis of Chick Embryo Mesenchymes *In Vitro*

Sun Ryung Lee[†], Jong Kyung Sonn[‡]*, Byung Je Yoo[†], Young Bin Lim[‡] and Shin-Sung Kang[†]
[†,‡]Department of Biology, [†]College of Natural Sciences and [‡]Teachers' College, Kyungpook National University, Taegu, 702-701, Korea
[†]Department of Biology, College of Natural Sciences, Taegu University, Kyungsan, 712-714, Korea

Received 16 March 1998
Revised 15 April 1998

To understand the role of protein kinase C (PKC) in the regulation of chondrogenesis, we examined proteins which are phosphorylated by PKC. Stage 23/24 chick embryo wing mesenchymes were micromass-cultured to induce chondrogenesis and cell extracts were phosphorylated in a condition that activates PKC. Several proteins including 63 and 66 kDa proteins were phosphorylated. The 66 kDa protein was phosphorylated only in the presence of phorbol 12-myristate 13-acetate (PMA) and phosphatidylserine (PS), and the phosphorylation was almost completely diminished by bisindolylmaleimide, a PKC inhibitor. In addition, partially purified PKC increased the phosphorylation of the 66 kDa protein. Treatment of cultures with lysophosphatidylcholine (LPC) promoted chondrogenesis and phosphorylation of 66 kDa protein, while PMA and thymeleatoxin inhibited both of the two events. Our results suggest that the 66 kDa protein is a putative substrate of PKC, and phosphorylation of the 66 kDa protein, probably by PKCα is required for chondrogenesis.

Keywords: Chondrogenesis, Phosphorylation, Protein kinase C.

Introduction

Chondrogenesis is one of the distinct phenomena in early limb development, and is widely used as a model system for cell differentiation. Mesenchymes derived from chick embryo limb buds spontaneously undergo differentiation into chondrocytes when micromass-cultured at high cell density. Chondrogenic differentiation is characterized by large accumulation of a cartilage-specific extracellular matrix such as type II collagen and sulfated proteoglycans (Goetinck *et al.*, 1974; Dessau *et al.*, 1980; Kosher *et al.*, 1986).

Regulation of cell differentiation is a complicated process, which requires a number of intracellular signaling networks. PKC, a family of related serine/threonine protein kinase, appears to be involved in the regulation of cell differentiation (for review, see Goodnight *et al.*, 1994). Increasing number of evidence also suggests that PKC is involved in the regulation of chondrogenesis. PMA, which induces down-regulation of PKC, inhibits the expression of cartilage-specific type II collagen (Sasse *et al.*, 1983) and the accumulation of type II collagen transcripts (Kulyk, 1991) in cultures of chick limb bud mesenchymes. PKC activity is increased during the differentiation of chick limb bud mesenchyme *in vitro*, and chondrogenesis is inhibited by prolonged treatment of cells with PMA (Sonn and Solursh, 1993).

PKC is a multigene family with 11 known isozymes (Goodnight *et al.*, 1994; Nishizuka, 1995). We have previously identified possible isozymes of PKC which appear to regulate chondrogenic differentiation (Choi *et al.*, 1995). However, a pathway through which PKC regulates chondrogenesis is not clearly understood. PKC might exert its role by phosphorylating a number of proteins. Known PKC substrates include myristoylated, alanine-rich C kinase substrate (MARCKS), lamin B, and GAP-43 (for review, see Jaken, 1996). However, no direct evidence is available on whether these proteins are involved in the regulation of cell differentiation. In this study, we examined possible substrates for PKC in

* To whom correspondence should be addressed.
Tel: 82-53-950-5915; Fax: 82-53-950-6809
E-mail: sonnjk@kyungpook.ac.kr

chondroblasts to elucidate the functional role of PKC in chondrogenesis. The data obtained indicate that a 66 kDa protein is a putative substrate for PKCα and its phosphorylation is closely related to chondrogenesis.

Materials and Methods

Preparation of micromass culture Wing buds of HH-stage 23/24 chick embryo (Hamburger and Hamilton, 1951) were dissected and collected in Ca^{2+}/Mg^{2+}-free saline G. Micromass culture was performed using the wing bud mesenchymes as described by Ahrens *et al.* (1977). Briefly, cells were dissociated by 0.1% trypsin-collagenase at 37°C for 10 min. Single cells were obtained by filtering the cell suspension through 2 layers of Nytex 20. The cells were resuspended in F12 culture medium containing 10% fetal calf serum at a density of 2×10^7 cells/ml. The cells were inoculated onto 35-mm culture dishes and were allowed to attach to the dishes by incubating at 37°C for 1 h. The cells were maintained in 1.5 ml culture media containing LPC (100 μM), PMA (100 nM), or thymeleatoxin (10 nM) with replacement everyday.

Fixation and staining Cultures were rinsed with saline G and fixed in Kahle's fixative for 10 min. To localize the cartilage area, cultures were stained with Alcian blue at pH 1.0 which stains the sulfated glycosaminoglycans in the extracellular matrix (Lev and Spicer, 1964). Hematoxylin was used as a counterstain to demonstrate the nonchondrogenic regions of the cell layer.

Partial purification of PKC PKC was purified as previously described (Choe *et al.*, 1991). Briefly, chick brains were homogenized in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 0.5 mM PMSF, 2 μg/ml leupeptin) using an Elvehjem homogenizer. The homogenate was centrifuged at $100{,}000 \times g$ for 1 h and the supernatants were applied to a DEAE-cellulose column which was previously equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol). The column was washed with buffer B and eluted with a NaCl gradient from 20 mM to 300 mM. PKC activity was measured as described previously (Sonn and Solursh, 1993). Fractions that contain PKC activity were pooled, dialyzed against buffer C (20 mM KH_2PO_4, pH 7.5, 1 mM dithiothreitol, 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol), and concentrated by ultrafiltration with a YM 30 Amicon cell. The enzyme solution was applied to a hydroxyapatite column previously equilibrated with buffer C. The column was eluted with buffer C containing 300 mM KH_2PO_4, dialyzed against buffer C, and concentrated with the Amicon cell.

Preparation of cell extracts Micromass-cultured cells were rinsed three times with saline G and phosphate buffered saline. Cells were harvested by scraping, and extracted in a buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1% Triton X-100) by sonication. The extracts were centrifuged at $100{,}000 \times g$ for 1 h and the supernatant was used as total cell extracts. To obtain cytosolic and membrane fractions, the cells were scraped into the above buffer without Triton X-100. The cells were disrupted by sonication, centrifuged at $100{,}000 \times g$ for 1 h, and the supernatant was saved as a cytosolic fraction. The pellets were resuspended with the above extraction buffer supplemented with 3% Triton X-100, centrifuged at $100{,}000 \times g$ for 1 h, and the supernatant was used as a particulate membrane fraction.

Protein phosphorylation *in vitro* Proteins were phosphorylated as previously described (Shin *et al.*, 1993). Proteins from total cell extracts, cytosolic, or membrane fraction were used for phosphorylation in a reaction mixture (50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 μM $CaCl_2$, 1 mM DTT, 0.28 mg/ml phosphatidylserine, 1 μM PMA) at 30°C for 3 min. Phosphorylation was initiated by adding 0.5 μCi of [γ-^{32}P]ATP in 5 μM ATP to the reaction mixture. Effects of bisindolylmaleimide (10 μM), an inhibitor of PKC, KT5720 (1 μM), an inhibitor of cAMP-dependent protein kinase, and KN-62 (40 μM), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase, on the protein phosphorylation were examined by adding them to the cell extracts 30 min prior to the beginning of reaction. The reaction was stopped by the addition of electrophoresis sample buffer and boiling for 2 min. The reaction mixture was separated by 7.5% SDS-PAGE and the phosphorylated proteins were detected by autoradiography.

Results and Discussion

Proteins extracted from cells cultured for three days were used for *in vitro* phosphorylation under the condition that activates PKC, i.e., in the presence of Ca^{2+} and PMA/PS. Under the condition, several proteins including 63 and 66 kDa proteins were phosphorylated, as shown in Fig. 1. When Ca^{2+} and PMA/PS were omitted in the reaction buffer, phosphorylation of the 66 kDa protein disappeared while that of the 63 kDa protein was not affected. Addition of bisindolylmaleimide, a specific inhibitor of PKC, completely inhibited phosphorylation of the 66 kDa protein. The inhibition of PKC also considerably reduced the phosphorylation of 63 kDa protein. On the other hand, neither KT5720 nor KN-62 inhibited the phosphorylation of the the 66 kDa protein. The results indicate that these proteins are specifically phosphorylated by PKC. To further examine the role of PKC in the phosphorylation of proteins, partially purified PKC by DEAE-cellulose and hydroxyapatite column chromatography was applied to the reaction buffer. The addition of PKC increased phosphorylation of several proteins including 63 and 66 kDa proteins, while bisindolylmaleimide reduced PKC-induced phosphorylation of 63 and 66 kDa proteins. Distribution of the putative PKC substrates between the cytosolic and particulate membrane fractions was examined. As shown in Fig. 1, phosphorylation of 63 and 66 kDa proteins was prominent in the particulate membrane fraction. We next examined the changes in the protein phosphorylation pattern during chondrogenesis in an attempt to determine the functional role of the phosphoproteins (Figs. 2 and 3). Phosphorylation of the 66 kDa protein appeared on day 2 of culture and increased on day 3, while phosphorylation of the 63 kDa protein was

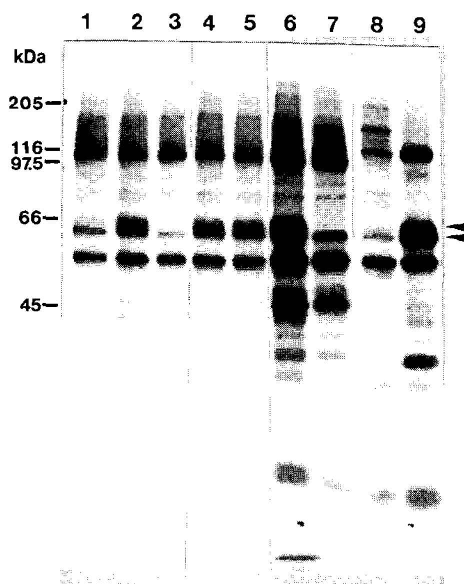

Fig. 1. Protein phosphorylation by PKC *in vitro* in the cultures of chick embryo wing mesenchymes. Proteins in cell extracts were phosphorylated in the absence (lane 1) or presence of Ca^{2+} and phosphatidylserine (lanes 2–9). To the reaction buffer, bisindolylmaleimide (lane 3), KT5720 (lane 4), KN-62 (lane 5), partially purified PKC (lane 6), or partially purified PKC and bisindolylmaleimide (lane 7) was added. The cells were fractionated into cytosolic (lane 8) and particulate membrane fractions (lane 9). Samples were separated by 7.5% SDS-PAGE followed by autoradiography. Arrow heads indicate 63 and 66 kDa proteins.

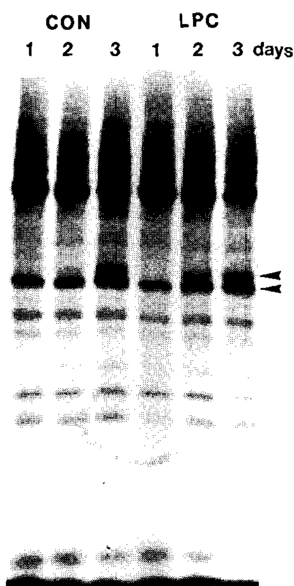

Fig. 2. Effects of LPC on the protein phosphorylation during chondrogenesis *in vitro*. Cells were cultured in the absence (CON) or presence of LPC (LPC) for 1, 2, or 3 days. Cell extracts were phosphorylated in a PKC-activating condition as described in Materials and Methods. Samples were separated by 7.5% SDS-PAGE followed by autoradiography. Arrow heads indicate 63 and 66 kDa proteins.

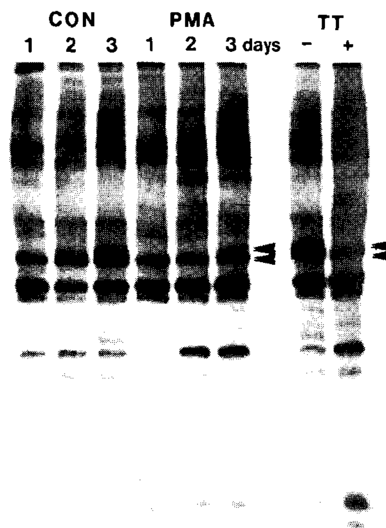

Fig. 3. Effect of PMA and thymeleatoxin on the protein phosphorylation during chondrogenesis *in vitro*. Cells were cultured in the absence (CON) or presence of PMA (PMA) for 1, 2, or 3 days. For the thymeleatoxin experiment (TT), cells were cultured in the absence (−) or presence (+) of thymeleatoxin for 3 days. Cell extracts were phosphorylated in a PKC-activating condition as described in Materials and Methods. Samples were separated by 7.5% SDS-PAGE followed by autoradiography. Arrow heads indicate 63 and 66 kDa proteins.

detected on day 1 of culture and remained at a constant level throughout the culture period.

PKC is a multigene family composed of 11 isozymes (Goodnight *et al.*, 1994; Nishizuka, 1995). PKC can be grouped depending on the activation mechanism: calcium-dependent conventional PKC (cPKCα, βI, βII, γ), calcium-independent new PKC (nPKCδ, ε, η, θ), atypical PKC (aPKCξ, λ/ι), and PKCμ. Activation of cPKC requires Ca^{2+}, diacylglycerol (DG), and PS, while members of nPKC group do not require Ca^{2+} but depend on DG/PS or DG/PMA for activation. Activation of aPKCs is independent of both Ca^{2+} and DG/PS. The result that the 66 kDa protein is phosphorylated only in the presence of Ca^{2+} and PMA/PS indicates that this protein is phosphorylated by cPKC isozymes. The 63 kDa protein seems to be phosphorylated by aPKCs because phosphorylation of the protein remained unchanged regardless of the absence or presence of Ca^{2+} and/or PMA/PS.

We next examined the effects of LPC, a hydrolysis product of phosphatidylcholine by phospholipase A_2, on the protein phosphorylation pattern, because the metabolite is reported to exert its physiological effect through the PKC pathway (Takahara *et al.*, 1996; Huwiler *et al.*, 1997) and is also known to be responsible for sustained activation of PKC and cell differentiation (Asaoka *et al.*, 1992). The

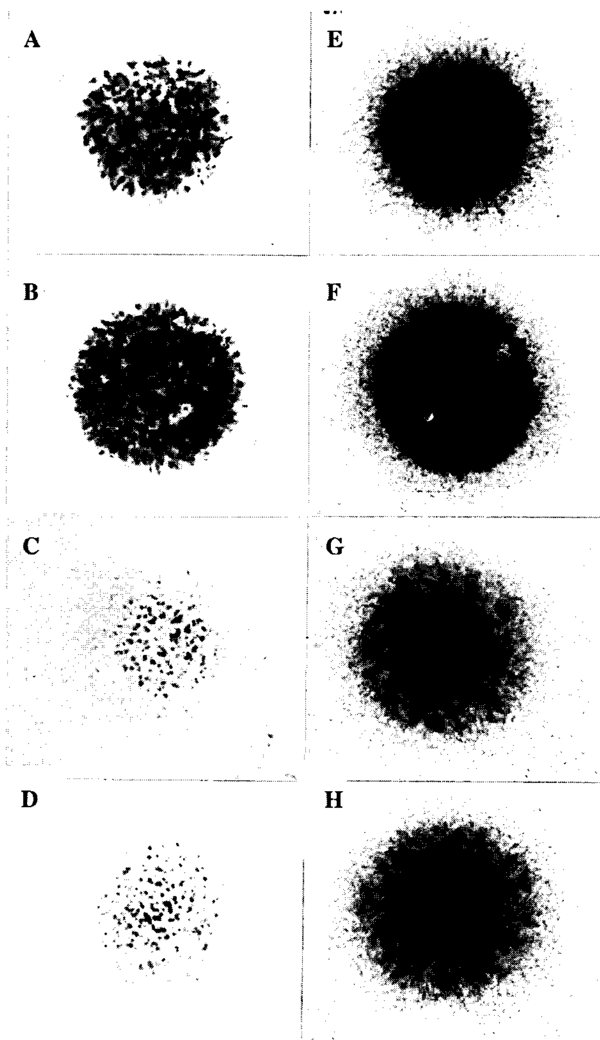

Fig. 4. Effect of LPC, PMA, and thymeleatoxin on the chondrogenesis *in vitro*. Cells of chick embryo wing buds were micromass-cultured in the absence (A, E) or presence of LPC (B, F), PMA (C, G), or thymeleatoxin (D, H). After fixation with Kahle's fixative, cultures were stained with Alcian blue at pH 1.0 (A, B, C, D) and counterstained with hematoxylin to visualize the nonchondrogenic areas of the culture (E, F, G, H). Photomicrographs were taken at ×25.

presence of LPC in the culture medium promoted chondrogenesis *in vitro* as determined by Alcian blue staining (Fig. 4B). Treatment of cells with LPC also increased the phosphorylation of 66 kDa protein on day 2 of culture (Fig. 2). Thus, the enhancement of chondrogenic differentiation of chick embryo wing mesenchymes by LPC appears to be related to the increased phosphorylation of the 66 kDa protein.

Prolonged treatment of cells with PMA, an activator of PKC, is known to inhibit chondrogenesis *in vitro* and depress PKC activity in the cultures of chick embryo wing mesenchyme (Sonn and Solursh, 1993). To confirm the relationship between chondrogenesis and phosphorylation of the 66 kDa protein, we used PMA to down-regulate PKC and observed the phosphorylation of the 66 kDa protein. Addition of PMA to the culture medium repressed chondrogenic differentiation, as shown by reduced staining of the sulfated glycosaminoglycans in the extracellular matrix (Fig. 4C), and inhibited the phosphorylation of 66 kDa protein after day 2 of culture (Fig. 3). Reduction of Alcian blue staining of PMA-treated cultures (Fig. 4C), is not due to the cytotoxic effect of PMA, because hematoxylin staining shows lots of cells in the cultures (Fig. 4G). As compared with hematoxylin staining of control (Fig. 4E) or LPC-treated cultures (Fig. 4F), the weak hematoxylin staining of PMA-treated cultures is considered to be the result of decrease in DNA content by PMA treatment (Sonn and Solursh, 1993). These results support the idea that phosphorylation of the 66 kDa protein by PKC is closely related to chondrogenesis.

PKC isozymes seem to be relatively nonspecific ser/thr kinases because they phosphorylate a motif XRXX/TXRX of several known substrates such as histones H1, myelin basic protein (MBP), and protamine. However, there are some different activities of PKC isozymes for the same substrates. For instance, PKCα, βI, βII, and γ have higher activity for histone IIIs and protamine than PKCγ, while nPKCs have low activity for histone IIIs or MBP (for review, see Hug and Sarre, 1993). Furthermore, site-selective preferences of PKCα and δ for phosphorylation of a common substrate result in distinct functional consequences (Noland *et al.*, 1996). Thymeleatoxin, a highly selective activator of cPKC (Ryves *et al*, 1991), is known to down-regulate PKCα and inhibit chondrogenesis *in vitro* (Yang *et al.*, 1998). When thymeleatoxin was treated to the cultures of chick embryo mesenchyme, it inhibited chondrogenic differentiation (Fig. 4D) and suppressed the phosphorylation of the 66 kDa protein (Fig. 3). Once again, there was no significant cytotoxic effect by thymeleatoxin as shown in Fig. 4H. Because PKCα is the only cPKC expressed in mesenchyme of chick embryo limb bud, and is down-regulated by thymeleatoxin (Yang *et al.*, 1998), these results indicate that the 66 kDa protein is phosphorylated by PKCα.

Taken together, our results suggest that the 66 kDa protein is a putative substrate of cPKCs and its phosphorylation is required for chondrogenic differentiation in chick embryo mesenchymes *in vitro*.

Acknowledgements The authors wish to thank Dr. Jang-Soo Chun for careful reading of the manuscript and Dr. Young-Sup Lee for technical advice. This work was supported by grants from the Korea Ministry of Education through Research Fund (BSRI-4402) and from the Korea Science and Engineering Foundation (951-0507-049-2).

References

Ahrens, P. B., Solursh, M. and Reiter, R. S. (1977) Stage-related capacity for limb chondrogenesis in cell culture. *Dev. Biol.* **60**, 69–82.

Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y. and Nishizuka, Y. (1992) Role of lysophosphatidylcholine in T-lymphocyte activation: involvement of phospholipase A2 in signal transduction through protein kinase C. *Proc. Natl. Acad. Sci. USA* **89**, 6447–6451.

Choe, M., Kim, J.-D. and Ju, J.-S. (1991) Effect of dietary fats on protein kinase C activity in mouse epidermal cell. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **24**, 331–336.

Choi, B., Chun, J.-S., Lee, Y.-S., Sonn, J.-K. and Kang, S.-S. (1995) Expression of protein kinase C isozymes that are required for chondrogenesis of chick limb bud mesenchymal cells. *Biochem. Biophys. Res. Commun.* **216**, 1034–1040.

Dessau, W., von der Mark, H., von der Mark, K. and Foscier, S. (1980) Changes in the pattern of collagenase and fibronectin during limb bud chondrogenesis. *J. Embryol. Exp. Morphol.* **59**, 51–60.

Goetinck, P. R., Pennypacker, J. P. and Royal, P. D. (1974) Proteochondroitin sulfate synthesis and chondrogenic expression. *Exp. Cell Res.* **87**, 241–248.

Goodnight, J., Mischak, H. and Mushinski, J. F. (1994) Selective involvement of protein kinase C isozymes in differentiation and neoplastic transformaiton. *Adv. Cancer Res.* **64**, 159–209.

Hamburger, V. and Hamilton, H. W. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92.

Hug, H. and Sarre, T. F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291**, 329–343.

Huwiler, A., Staudt, G., Kramer, R. M. and Pfeilschifter, J. (1997) Cross-talk between secretory phospholipase A_2 and cytosolic phospholipase A_2 in rat renal mesangial cells. *Biochem. Biophys. Acta* **1348**, 257–272.

Jaken, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168–173.

Kosher, R. A., Gay, S. W., Kamantiz, J. R., Rodgers, B. J., Sai, S., Tanaka, T. and Tanzer, M. L. (1986) Cartilage proteoglycan core protein gene expression during limb cartilage differentiation. *Dev. Biol.* **118**, 112–117.

Kulyk, W. M. (1991) Promotion of embryonic limb cartilage differentiation *in vitro* by staurosporine, a protein kinase C inhibitor. *Dev. Biol.* **146**, 38–48.

Lev, R. and Spicer, S. S. (1964) Specific staining of sulfate groups with alcian blue at low pH. *J. Histochem. Cytochem.* **12**, 309.

Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**, 484–496.

Noland, T. A. Jr., Raynor, R. L., Jideama, N. M., Guo, X., Kazanietz, M. G., Blumberg, P. M., Solaro, R. J. and Kuo, J. F. (1996) Differential regulation of cardiac actomyosins S-1 MgATPase by protein kinase C isozyme-specific phosphorylation of specific sites in cardiac troponin I and its phosphorylation site mutants. *Biochemistry* **35**, 14923–14931.

Ryves, W. J., Evans, A. T., Olivier, A. R., Parker, P. J. and Evans, F. J. (1991) Activation of the PKC-isotypes α, $\beta 1$, γ, δ and ε by phorbol esters of different biological activities. *FEBS Lett.* **288**, 5–9.

Sasse, J., von der Mark, K., Pacifici, M. and Holtzer, H. (1983) An immunological study of cartilage differentiation in cultures of chick limb bud cells: Influence of a tumor promoter (TPA) on chondrogenesis and on extracellular matrix formation; in *Limb Development and Regeneration. Part B*, Kelly, R. O., Goetinck, P. F. and MacCabe, J. A. (eds.), pp. 159–166, Alan R. Liss, New York

Shin, P. G., Kim, G. D., Cheong, Y. H., Lee, S. Y., Bahk, J. D., Hong, J. C. and Cho, M. J. (1993) Characterization of a Ca^{2+}/calmodulin dependent phosphorylated protein expressed at the early stages of soybean germination. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **26**, 125–131.

Sonn, J. K. and Solursh, M. (1993) Activity of protein kinase C during the differentiation of chick limb bud mesenchymal cells. *Differentiation* **53**, 155–162.

Takahara, N., Kashiwagi, A., Maegawa, H. and Shigeta, Y. (1996) Lysophosphatidylcholine stimulates the expression and production of MCP-1 by human vascular endothelial cells. *Metabolism* **45**, 559–564.

Yang, M.-S., Lee, Y.-S., Sonn, J. K., Kang, S.-S., Park, T.-K. and Chun, J.-S. (1998) Regulation of chondrogenic differentiation of mesenchymes by protein kinase Cα. *Mol. Cells* **8**, in press.