Journal of Cellular Biochemistry 88:713–718 (2003)

ARTICLES

Chondrogenesis Induced by Actin Cytoskeleton Disruption is Regulated Via Protein Kinase C-Dependent p38 Mitogen-Activated Protein Kinase Signaling

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Abstract Disruption of the actin cytoskeleton in subconfluent mesenchymal cells induces chondrogenic differentiation via protein kinase C (PKC) α signaling. In this study, we investigated the role of p38 mitogen-activated protein (MAP) kinase in the chondrogenic differentiation of mesenchymal cells that is induced by depolymerization of the actin cytoskeleton. Treatment of mesenchymal cells derived from chick embryonic limb buds with cytochalasin D (CD) disrupted the actin cytoskeleton with concomitant chondrogenic differentiation. The chondrogenesis was accompanied by an increase in p38 MAP kinase activity and inhibition of p38 MAP kinase with SB203580 blocked chondrogenesis. Together these results suggest an essential role for p38 MAP kinase in chondrogenesis. In addition, inhibition of p38 MAP kinase did not alter CD-induced increased expression and activity of PKCα, whereas down-regulation of PKC by prolonged exposure of cells to phorbol ester inhibited CD-induced p38 MAP kinase activation. Our results therefore suggest that PKC is involved in the regulation of chondrogenesis induced by disruption of the actin cytoskeleton via a p38 MAP kinase signaling pathway. J. Cell. Biochem. 88: 713–718, 2003.

Key words: PKC; p38 MAPK; cytoskeleton; chondrogenesis

During early chondrogenesis of chick limb mesenchymal cells, aggregation of the cells occurs to form precartilage condensation and subsequent cartilage nodules in which chondrocyte-specific type II collagen and proteoglycan are synthesized [DeLise et al., 2000]. Ahrens et al. [1977] developed an in vitro micromass culture system in which cells are plated at confluent density to mimic the in vivo developmental stage when prechondrogenic condensation occurs prior to overt chondrogenesis. Mesenchymal cells can also differentiate into chondrocytes in monolayer cultures when they are converted to a round shape. For example, chondrogenesis was induced by culturing the cells on a three-dimensional structure such as collagen gel [Solursh et al., 1982] or semi-adhesive substratum [poly(HEMA)] [Archer et al., 1982] or by changing cell shape via disrupting the actin cytoskeleton [Zanetti and Solursh, 1984; Loty et al., 1995; Lim et al., 2000]. However, the mechanism by which cell shape change exerts its inductive effect on chondrogenesis remains uncharacterized.

We have shown previously that disruption of the actin cytoskeleton with cytochalasin D (CD) in subconfluent chick limb bud mesenchymal cells can induce chondrogenic differentiation

Abbreviations: MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

Grant sponsor: Korea Science and Engineering Foundation; Grant number: R01-2000-00108.

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Received 30 August 2002; Accepted 24 September 2002
DOI 10.1002/jcb.10389

[Lim et al., 2000]. In this case, chondrogenesis was induced by the increased expression and activity of protein kinase C (PKC) α [Lim et al., 2000]. In addition to PKC, cellular responses to disruption of the actin cytoskeleton are associated with changes in MAP kinase activity. For instance, disassembly of actin filaments inhibited stretch-induced ERK activation [Numaguchi et al., 1999] and abolished insulin-induced phosphorylation of ERK and p38 MAP kinase [Tsakiridis et al., 1998]. Inhibition of the ERK and p38 MAP kinase pathways also affected expression of the matrix metalloproteinases induced by CD treatment [Lambert et al., 2001]. We, therefore, investigated the role of MAP kinase subtypes, ERK-1/-2 and p38 MAP kinase, in PKCα-regulated chondrogenesis of mesenchymal cells induced by cytoskeleton disruption. We report here that chondrogenesis induced by disruption of the actin cytoskeleton is regulated by PKCα via the activation of p38 MAP kinase signaling.

MATERIALS AND METHODS

Cell Culture

Mesenchymal cells were obtained from Hamburger-Hamilton stage 23/24 chick wing buds [Hamburger and Hamilton, 1951] by digestion at 37°C for 10 min in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 0.1% trypsin and 0.1% collagenase, as described previously [Lim et al., 2000]. The cells were plated at a density of 6×10^6 cells/60 mm tissue culture dish or 6×10^5 cells in each well of 24 well culture plates. Cells were grown in F-12 medium supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air with daily changing of the culture medium.

Determination of Chondrogenesis

Chondrogenesis of mesenchymal cells was determined by Western blot analysis of type II collagen expression, and quantified by measuring the accumulation of sulfated proteoglycan in Alcian blue-stained cells. Briefly, cells were rinsed with phosphate buffered saline (PBS) and fixed with Kahle's fixative for 5 min. The cells were stained with Alcian blue in 0.1 N HCl overnight and bound dye was extracted using 4 M guanidium HCl overnight at 4°C. The optical density of the extracted dye was measured at 595 nm with a microplate reader (Bio-Rad, Hercules, CA).

Cell Fractionation

Cytosolic and particulate membrane fractions of cells were prepared as described previously [Lim et al., 2000]. Briefly, the cells were sonicated in buffer A (20 mM Tris, pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate) and centrifuged for 10 min at $1{,}000 \times g$ to remove cell debris. The supernatants were then centrifuged at $100{,}000 \times g$ for 30 min at 4°C, and the resulting supernatants were saved as the cytosolic fractions. Proteins in the pellets were extracted with 0.3 ml of buffer B (20 mM Tris, pH 7.5, 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate). Following centrifugation at $100{,}000 \times g$ for 30 min at 4°C, the supernatants were saved as the particulate membrane fractions.

Immunoprecipitation

Cells were washed in ice-cold PBS and lysed in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate, 0.25% sodium deoxycholate, and 1% NP-40. The cell lysates were precleared by incubating with protein A-agarose for 1 h at 4°C. The lysates were then incubated with anti-phosphotyrosine antibody (1 μg) at 4°C for 1 h, followed by 25 μl of protein A-agarose at 4°C for 1 h.

Preparation of Cell Lysates and Western Blot Analysis

To prepare a total cell lysate, cells were washed in ice-cold PBS and lysed in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate, 0.25% sodium deoxycholate, and 1% NP-40. The supernatants were collected after centrifugation for 10 min at $10{,}000 \times g$. Protein samples were size-fractionated by electrophoresis and the proteins were electrotransferred to nitrocellulose membrane. The membranes were immunoblotted with primary antibodies followed by horseradish peroxidase-conjugated anti-mouse IgG. The

immunoblotted proteins were visualized by a chemiluminescence ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The primary antibodies were purchased from the following sources: anti-type II collagen from Developmental Studies Hybridoma Bank (University of Iowa), anti-PKC α, γ/ι, and ϵ antibodies from BD Transduction Lab (Lexington, KY), anti-phospho ERK-1/-2 from Cell Signaling (Beverly, MA), anti-p38 MAP kinase from Santa Cruz Biotech (Santa Cruz, CA), and anti-phosphotyrosine antibody from ICN (Costa Mesa, CA).

p38 Kinase Assay

p38 immunoprecipitates obtained as described above were washed three times with lysis buffer and once with a kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, and 2 mM DTT). The immune complexes were mixed with 10 μl of the kinase buffer containing 20 μg of myelin basic protein as a substrate and 20 μM [γ-^{32}P]ATP (20 Ci/mmol; Amersham). After incubation for 30 min at 30°C, the reaction was stopped by the addition of SDS sample buffer and by boiling for 5 min. Phosphorylation of the substrate was determined by autoradiography.

RESULTS

Activation of p38 MAP Kinase is Required for CD-Induced Chondrogenesis of Mesenchymal Cells

Treatment of mesenchymal cells cultured at subconfluent density with CD causes disruption of the actin cytoskeleton [Lim et al., 2000] with concomitant induction of chondrogenesis, as determined by Alcian blue staining (Fig. 1A). To determine the possible involvement of p38 MAP kinase in this CD-induced chondrogenesis, changes in p38 MAP kinase activity during chondrogenesis were monitored by measuring tyrosine phosphorylation and by a direct kinase assay. Tyrosine phosphorylation of p38 MAP kinase was low in control cultures but gradually increased with the time of culture in CD-treated cells (Fig. 1B). An in vitro kinase assay also indicated that p38 MAP kinase activity increased significantly in cultures treated with CD, whereas the levels remained constant in control cultures (Fig. 1C). To determine whether the increased p38 MAP kinase activity in cells

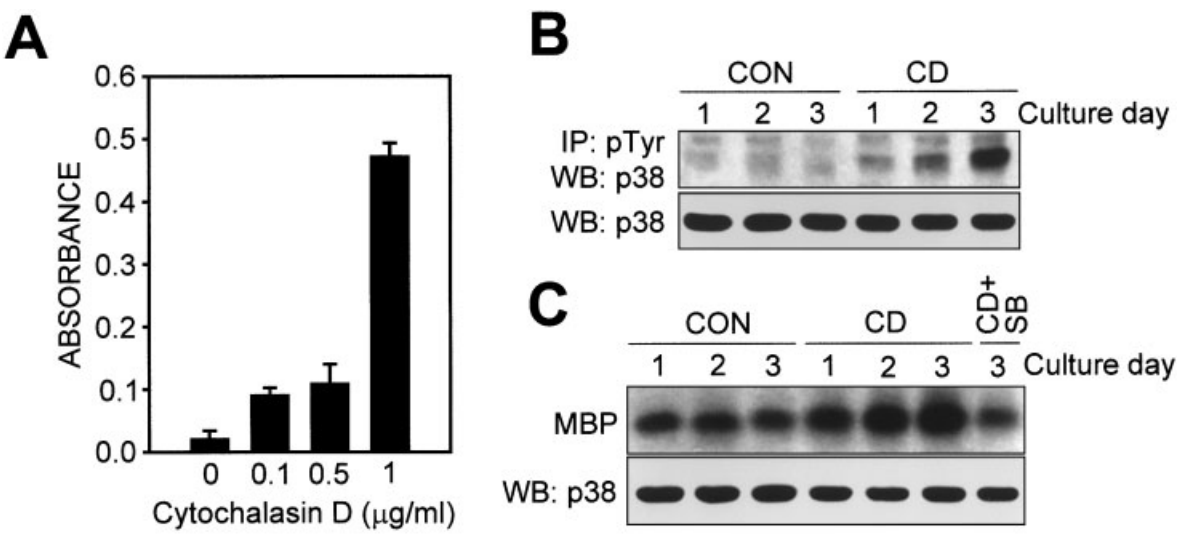

Fig. 1. Cytochalasin D (CD)-induced chondrogenesis accompanies an increase in p38 MAP kinase activity. **A**: Mesenchymal cells were incubated with the indicated concentrations of CD for three days and accumulation of sulfated proteoglycan was determined by Alcian blue staining. The average values from three independent experiments are shown (mean ± SE). **B**: Mesenchymal cells were either untreated as a control (CON) or treated with 1 μg/ml CD for the indicated time periods. Tyrosine phosphorylation of p38 MAP kinase was determined by Western blot analysis of p38 kinase immunoprecipitates. Levels of p38 MAP kinase from whole cell lysates were determined by Western blot analysis. **C**: Mesenchymal cells were cultured in the absence (CON) or presence of 1 μg/ml CD alone or CD and 10 μM SB203580 (CD+SB). p38 MAP kinase activity was determined by a kinase binding assay using MBP as a substrate and analysed by Western blot analysis. The data shown in B and C represent typical results from at least four independent experiments.

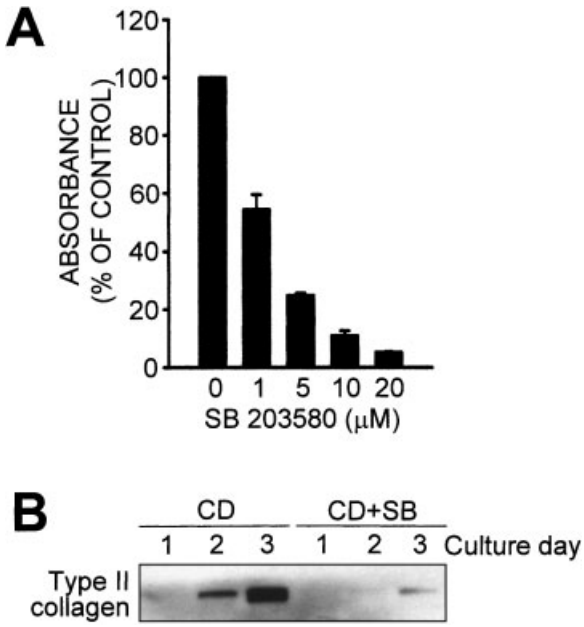

Fig. 2. CD-induced activation of p38 kinase is required for chondrogenesis. **A**: Mesenchymal cells were treated with the indicated concentrations of SB203580 for three days in the presence of 1 μg/ml CD and accumulation of sulfated proteoglycan was determined by Alcian blue staining. The average values of three independent experiments are shown (mean ± SE). **B**: Mesenchymal cells were cultured in the presence of CD alone or CD and 10 μM SB203580 (CD+SB) for three days and expression of type II collagen was determined by Western blot analysis. The data represent typical results from at least four independent experiments.

treated with CD is necessary for chondrogenesis, mesenchymal cells were treated with the p38 MAP kinase inhibitor, SB203580 [Cuenda et al., 1995] prior to CD treatment. SB203580 effectively inhibited the CD-induced activation of p38 MAP kinase (Fig. 1C). This result was confirmed by the reduction in both Alcian blue staining (Fig. 2A) and type II collagen expression (Fig. 2B). The above results indicate that CD-induced activation of p38 MAP kinase is required for the chondrogenic differentiation of mesenchymal cells.

p38 MAP Kinase Mediates PKC Regulation of Chondrogenesis Induced by CD Treatment

We previously demonstrated that increased expression and activity of PKCα is necessary for CD-induced chondrogenesis of mesenchymal cells [Lim et al., 2000]. In this study, we, therefore, aimed to determine the functional relationship between PKCα and p38 MAP kinase signaling in CD-induced chondrogenesis. Consistent with our previous report [Lim et al., 2000], chondrogenesis of mesenchymal cells by CD treatment was accompanied by increased expression of PKCα and a slight decrease in the expression of PKCε and γ/ι (Fig. 3A). Inhibition of p38 MAP kinase with SB203580 did not significantly alter the increased PKCα expression (Fig. 3A) or translocation of cytosolic PKCα to the particulate membrane fraction (Fig. 3B), even though chondrogenic differentiation was dramatically blocked (Fig. 2). In contrast, CD-induced activation of p38 MAP kinase was significantly decreased by both down-regulation of PKC following prolonged treatment with phorbol 12-myristate 13-acetate (PMA) and inhibition of PKC with GF109203X (Fig. 4A). The inhibitory effects of PMA on p38 MAP kinase activity were dose-dependent as shown in Fig. 4B. Inhibition or down-regulation of PKC also blocked chondrogenesis as indicated by the reduction in Alcian blue staining (Fig. 4C). Taken together, the above results suggest that PKC regulates CD-induced chondrogenesis by activating p38 MAP kinase signaling.

DISCUSSION

Several lines of evidence suggest that MAP kinase subtypes are important regulators of

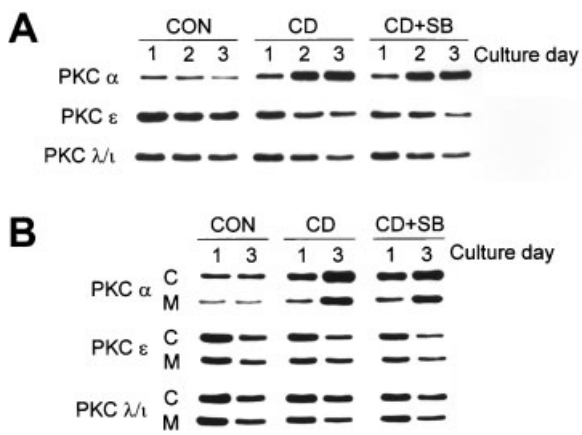

Fig. 3. Inhibition of p38 MAP kinase does not affect PKCα signaling. Mesenchymal cells were cultured in the absence (CON) or presence of 1 μg/ml CD or CD and 10 μM SB203580 (CD+SB). Levels of PKC isoforms in whole cell lysates were determined by Western blot analysis (**A**). Alternatively, the cells were fractionated into cytosolic (C) and particulate membrane (M) fractions and levels of PKC isoforms in each fraction were determined by Western blot analysis (**B**). The data represent result of typical experiments from at least four independent experiments.

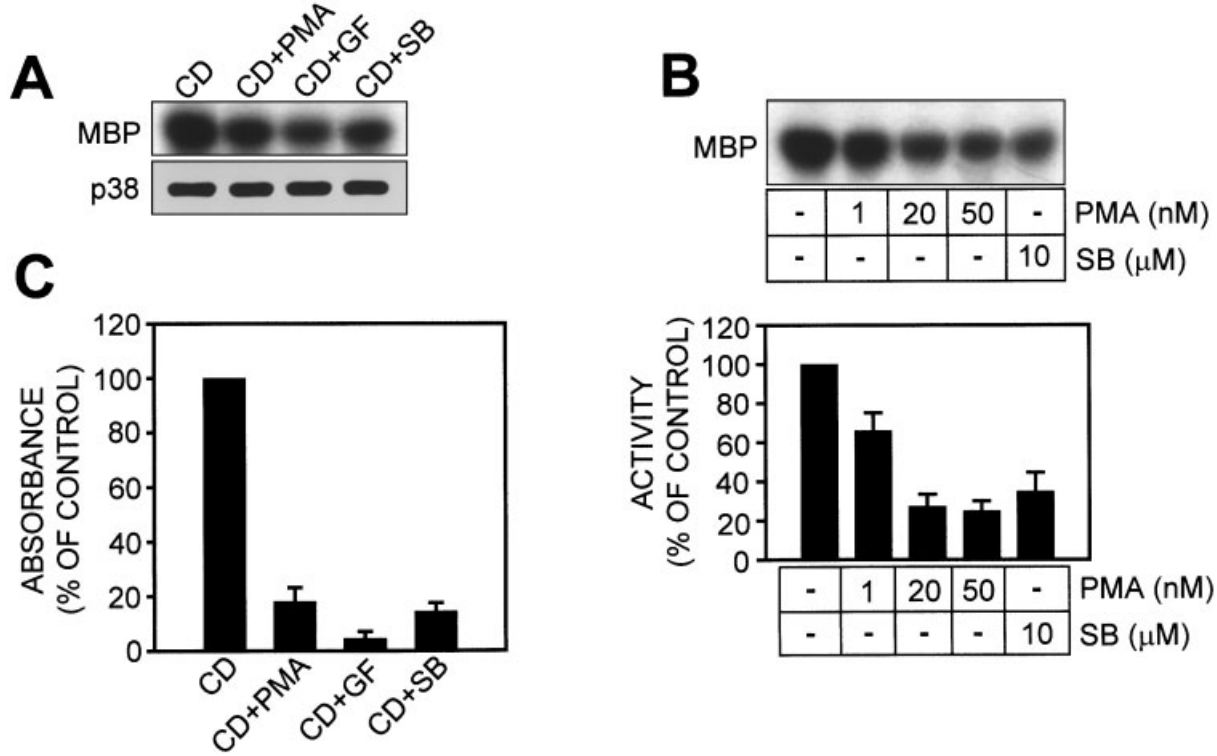

Fig. 4. PKC regulates p38 MAP kinase during CD-induced chondrogenesis. **A**: Mesenchymal cells were cultured in the presence of 1 μg/ml CD, CD and 10^{-7} M PMA (CD+PMA), CD and 5 μM GF109203X (CD+GF), or CD and 10 μM SB203580 (CD+SB). p38 MAP kinase activity was determined by a kinase assay using MBP as a substrate. Levels of p38 MAP kinase in whole cell lysates were determined by Western blot analysis. **B**: Mesenchymal cells were treated with the indicated concentrations of PMA or SB203580 in the presence of 1 μg/ml CD. p38 MAP kinase activity was determined by a kinase assay using MBP as a substrate (**upper panel**), and the relative phosphorylation of MBP was quantified by densitometry (**lower panel**). **C**: Mesenchymal cells were cultured in the presence of 1 μg/ml CD, CD and 10^{-7} M PMA (CD+PMA), 5 μM GF109203X (CD+GF), or 10 μM SB203580 (CD+SB) for three days. Accumulation of sulfated proteoglycan was determined by Alcian blue staining. The average values of three independent experiments are shown (mean ± SE).

chondrogenesis that is induced by micromass culture of mesenchymal cells. For example, phosphorylation of ERK-1 is decreased during micromass culture-induced chondrogenesis and inhibition of ERK activity with PD98059 promotes chondrogenic differentiation [Chang et al., 1998]. ERK activity is also inhibited following an increase in the expression and activity of PKCα [Chang et al., 1998]. In contrast, p38 MAP kinase activity was increased during micromass culture-induced chondrogenesis while inhibition of p38 MAP kinase suppressed chondrogenesis [Oh et al., 2000]. The ability of p38 MAP kinase to induce chondrogenesis is independent of PKCα signaling [Oh et al., 2000]. In addition, p38 MAP kinase also positively regulates cartilage nodule formation and expression of type II collagen induced by growth/differentiation factor-5 in ATDC5 cells [Nakamura et al., 1999].

This study demonstrates that activation of p38 MAP kinase is necessary for CD-induced chondrogenesis. This accords with the chondrogenesis induced by micromass culture of mesenchymal cells [Oh et al., 2000]. However, an important difference in terms of signaling pathways is that p38 MAP kinase activation is independent of PKC signaling during micromass culture-induced chondrogenesis [Oh et al., 2000], whereas we have shown here that CD-induced activation of p38 MAP kinase is regulated by PKC signaling. During micromass culture-induced chondrogenesis, increased expression and activity of PKCα causes chondrogenesis by inhibiting ERK-1/-2 signaling [Chang et al., 1998]. Although the decrease of ERK-1/-2 activity was also observed in our system, inhibition of ERK with PD98059 did not affect the CD-induced chondrogenesis [Lim et al., 2000], suggesting that the decrease in

ERK-1/-2 activity is not critical for CD-induced chondrogenesis. In this study, we observed that inhibition of PKC with GF109203X or down-regulation of PKC with PMA did not affect the CD-induced inhibition of ERK-1/-2 activity (data not shown). In addition, inhibition of ERK-1/-2 with PD98059 did not recover the inhibition of CD-induced chondrogenesis induced by prolonged treatment with PMA or GF109203X (data not shown). Taken together, our data clearly suggest that p38 MAP kinase mediates the PKC involvement in regulating CD-induced chondrogenesis, whereas ERK-1/-2 mediates the PKC regulation of micromass culture-induced chondrogenesis of mesenchymal cells.

In summary, p38 MAP kinase activity was increased by disruption of the cytoskeleton with CD and its activity is required for CD-induced chondrogenesis. Inhibition or down-regulation of PKC blocked CD-induced p38 MAP kinase activation, whereas inhibition of p38 MAP kinase did not influence the expression and activity of PKC, indicating that a PKC-p38 MAP kinase pathway is associated with the induction of chondrogenesis by disruption of the cytoskeleton.

ACKNOWLEDGMENTS

This work was supported by a grant from the Korea Science and Engineering Foundation (R01-2000-00108). The antibody against type II collagen developed by Dr. T. Linsenmayer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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