Chondrocyte-derived ezrin-like domain containing protein (CDEP), a rho guanine nucleotide exchange factor, is inducible in chondrocytes by parathyroid hormone and cyclic AMP and has transforming activity in NIH3T3 Cells


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Summary

Objective: The purpose of this study is to investigate stage- and hormone-dependent expression of chondrocyte-derived ezrin-like domain containing protein (CDEP), a putative guanine nucleotide exchange factor (GEF) for Rho in chondrocytes, and demonstrate the guanine nucleotide exchange activity of this protein in vitro, as well as the transforming activity in NIH3T3 cells.

Methods: The expression of CDEP mRNA in growth plate chondrocytes in vivo and in vitro was examined by RT-PCR Southern analysis. The guanine nucleotide exchange activity was determined using a recombinant CDEP peptide containing the DH and PH domains in Sf9 cell lysates. The transforming activity was examined using NIH3T3 cells transiently transfected with a truncated CDEP cDNA.

Results: CDEP mRNA was expressed at the highest level in the hypertrophic (terminal) stage of chondrocytes in vivo and in vitro. Parathyroid hormone (PTH) elicited a biphasic increase of CDEP mRNA in chondrocytes. The CDEP mRNA level increased within 1 h, then decreased nearly to the control level at 3 h. Thereafter the mRNA level started to increase at 6 h, reaching a plateau at 24 h. Dibutyryl cyclic AMP had a similar effect on CDEP expression in chondrocytes. The dissociation of [3H]GDP from RhoA was stimulated dose-dependently by Sf9 cell lysates containing the CDEP peptide. Furthermore, transfection of a truncated CDEP cDNA induced focus formation in NIH3T3 cultures.

Conclusions: CDEP is a novel GEF for Rho family GTPases with the transforming activity. CDEP may play a role in mediating or modulating the action of cAMP-elevating hormones on maturing chondrocytes. © 2001 OsteoArthritis Research Society International

Key words: Chondrocyte, GEF, PTH, Rho.

Introduction

Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHRP), acting through the same receptors, play a crucial role during endochondral bone formation. This action of PTH/PTHRP on chondrocytes is mediated by cAMP. The mechanism by which PTH/PTHRP/cAMP modulates chondrocyte differentiation is unknown. To address this issue, we established a primary culture system for human embryo chondrocytes. In this system, chondrocytes synthesize cartilage-specific proteins, including type II collagen, matrilin-1 and aggrecan only in the presence of dibutyryl cyclic AMP (DBcAMP). Using the subtraction method, we isolated a cDNA for CDEP (chondrocyte derived ezrin-like domain containing protein) that was expressed in DBcAMP-exposed, well-differentiated chondrocytes but not in DBcAMP-free dedifferentiated cells. CDEP had the ezrin-like domain in the amino-terminal region, and DH (Dbi homology) and PH (pleckstrin homology) domains in the middle region. The DH and PH domains are found in all known members of the guanine nucleotide exchange factors (GEFs) for Rho-like GTPase, including Dbl, Ost, Ect2, Lbc, and FGD1. Dbl, Ost, Ect2 and Lbc are oncogene products, whereas FGD1 is the gene responsible for faciogenital dysplasia. The DH domain catalyses GTP-GDP exchange for Rho proteins, and the PH domain is necessary for binding to the membrane or cytoskeleton. Furthermore, these domains are indispensable for the transforming activity of Rho GEF.

In this study, we examined the changes in CDEP expression during chondrocyte differentiation and the effect of PTH or cAMP on CDEP mRNA expression in chondrocytes. Further, we investigated whether CDEP has the guanine nucleotide exchange activity for RhoA and the transforming activity in focus formation assays.

Materials and methods

Materials

Human recombinant PTH[1-84] was supplied from Dr N. Kubota (Chugai Pharmaceutical Co., Tokyo, Japan). α-Modified Eagle’s medium (α-MEM), fetal bovine serum and DBcAMP were obtained from Sanko Pharmaceutical.
CULTURES OF CHONDROCYTES AND MRC5 CELLS

Chondrocytes were isolated from the growth plates of the rib cartilage of 4-week-old male Japanese white rabbits, as previously described. The chondrocytes or human embryonic lung fibroblasts (MRC5) (Riken Gene Bank, Tsukuba, Japan) were seeded at 1 × 10^6 cells per 22.7-mm plastic tissue culture dish. These cells were maintained in 3 ml of α-MEM containing 10% fetal bovine serum, 50 μg/ml of ascorbic acid, 32 units/ml of penicillin and 40 μg/ml of streptomycin.

RT-PCR/SOUTHERN BLOT ANALYSIS

The guanidine thiocyanate/cesium trifluoroacetate method extracted total RNA from cartilage slices or chondrocyte cultures. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using total RNA as a template and the oligonucleotide primers. PCR primers for CDEP, type X collagen, alkaline phosphatase (ALPase), aggrecan and GAPDH are as follows: 5'-AGTCTGAGGTGACCTGAGCC-3' and 5'-ACAGCTCGGAAAGTGAGAGGG-3' for CDEP, 5'-CCAACACGACGAGCATG-3' and 5'-ATCACCTTTGATGCTTGCT-3' for type X collagen, 5'-AGCTTGAGATGGACACATGTT-3' and 5'-TGTTGCAATTCGCTCCTGCTT-3' for ALPase, 5'-GGACACACGAGACCTATG-3' and 5'-CCCGAGCTCGACCGAGAAGCC-3' for aggrecan, as well as 5'-GTGAAGGCTGGAGAACCAGG-3' and 5'-TCACCCCTGCTGTA for GAPDH.

The PCR products were analysed by electrophoresis on a 1% agarose gel containing 1 μg/ml ethidium bromide. Some samples were transferred to Hybond-N membranes (Amersham International plc, Buckinghamshire, UK). A CDEP cDNA fragment was labelled with [32P]dCTP and served as a hybridization probe. The membranes were washed at 65°C for 30 min with 0.2× SSC containing 0.5% SDS. The washed membranes were exposed to BioMax X-ray films (Eastman Kodak Co., Rochester, NY, USA). The radioactivity was measured using a laser image analyser (Fuji, BAS 2000).

Results

HIGH LEVEL EXPRESSION OF CDEP mRNA IN THE HYPERTROPHIC STAGE OF CHONDROCYTES IN VITRO AND IN VIVO

To examine the changes in the CDEP-mRNA level during chondrocyte differentiation, we used sequential slices of cartilage as described previously (R, resting cartilage; G1, the proliferating zone; G2, the matrix-forming zone; G3, the hypertrophic zone of the growth plate). CDEP was expressed at a higher level in the hypertrophic zone than in any other zones in vivo (Fig. 1). In addition, we established growth-plate chondrocyte cultures in which the cells undergo proliferation (day 6), cartilage-matrix synthesis (days 10–14), hypertrophy (days 14–26) and calcification (day 26). Collagen type X and alkaline phosphatase (ALPase) were expressed in the hypertrophic stage (Fig. 2). The CDEP mRNA level was low in the proliferating and matrix-forming stages, and markedly increased in the hypertrophic and calcifying stages (Fig. 2). The mRNA level of GAPDH was almost consistent throughout the culture period.

EFFECTS OF PTH AND DBCAMP ON CDEP EXPRESSION IN CHONDROCYTES AND FIBROBLASTS

The CDEP was expressed at a high level in well-differentiated chondrocytes exposed to DbcAMP for several days, but not detectable in dedifferentiated cartilage cells maintained without DbcAMP. In this study, we examined whether PTH, a potent activator of adenylate cyclase as well as DbcAMP induces CDEP mRNA in dose-, time- and stage-dependent manners.

In growth-plate chondrocyte cultures exposed to 10−7 M PTH, the CDEP mRNA level markedly increased within 1 h, and then decreased to the control level at 3 h. Thereafter, it increased gradually until 24 h. The high CDEP level was sustained at least until 48 h (Fig. 3A). This effect could be
detected at a PTH concentration of $10^{-10}$ M, and reached a plateau at $10^{-8}$ M (Fig. 3B). During the matrix-forming and hypertrophic stages, PTH increased the CDEP mRNA level, but not in the proliferating stage (data not shown). PTHrP[1-34] that binds to a common PTH/PTHrP receptor also increased CDEP expression in chondrocytes on day 14 (data not shown).

DBcAMP at 1 mM increased the CDEP mRNA level in chondrocytes on day 14, and the extent of induction was similar to that obtained with PTH (data not shown).

GEF ACTIVITY OF CDEP FOR RHO IN VITRO

A recombinant GST-CDEP peptide containing the DH and PH domains was expressed in Sf9 cells using a baculovirus expression system (Fig. 4A). GEF activity was determined by measuring dissociation of $^3$H-GDP from $^3$H-GDP-RhoA bound to nitrocellulose. The dissociation of $^3$H-GDP from $^3$H-GDP-RhoA was stimulated by Sf9 cell lysates containing an N-truncated CDEP fragment dose-dependently, but not by control lysates (Fig. 4B).

TRANSFORMATION OF NIH3T3 CELLS BY EXPRESSION OF AN N-TRUNCATED CDEP PEPTIDE

Most Rho GEFs can induce malignant transformation when N-truncated forms of RhoGEF containing the DH and PH domains are transfected into fibroblasts. However, the transforming activity of Rho GEFG alone is not prominent in most assays. In this study, an N-truncated form of CDEP containing the DH and PH domains (Fig. 5A) was expressed in transiently transfected NIH3T3 cells. In cultures of the CDEP-transfected cells, foci appeared 5–7 days after transfection. The size of focus increased with time. Activated c-H-Ras also induced focus formation. The size and morphology of CDEP- and activated c-H-Ras-transformed cell-foci resembled each other (Fig. 5B). The average number (±SD) of foci 17 days after transfection was 19±2, 14±2, 6±1 and 3±1 per 10-cm dish for CDEP cultures, activated c-H-Ras cultures, cultures expressing the vector alone and mock-transfected cultures, respectively. The foci-like structure formed in the control cultures appears to represent cellular aggregation.

Discussion

Rho regulates many cell functions including adhesion, migration, proliferation, differentiation, secretion, and malignant transformation. Rho is inactivated by Rho GDI (GDP dissociation inhibitor), activated by Rho GEF and returns to the inactive form by Rho GAP. In resting cells, Rho GEFs are usually maintained as an inactive form. Some Rho GEF proteins are thought to be activated by protein–protein interactions or phosphorylation in response to extracellular stimuli. On the other hand, N-truncated forms of Rho GEFs containing the DH and PH domains show constitutive GEF activity, perhaps because of the absence of the inhibitory action of the N-terminal region. We demonstrated here that an N-truncated form of CDEP has the Rho GEF activity in vitro. It is noteworthy that the N-terminal region of CDEP contains an ezrin-like domain.
Ezrin is necessary to release Rho GDI from the Rho-Rho GDI complex before the activation of Rho by Rho GEF. Rho GDI renders most of Rho proteins cytosolic and blocks the GEF action. Thus the ezrin-like domain in CDEP may be useful to release Rho GDI form the complex in vivo. In other words, CDEP could be more potent in activating Rho in vivo than other Rho GEFs.

Another significant finding of this study is the transformation of NIH3T3 cells transfected with a truncated form of CDEP. Previous studies have shown that several Rho GEF fragments containing the DH and PH domains can induce transformation of NIH3T3 cells, although the transforming activity of Rho GEF is usually very low. Fortunately, we could observe the transforming activity of the truncated CDEP in focus formation assays. Perhaps the constitutively active form of CDEP induced transformation via the activation of Rho family members.

The importance of CDEP in chondrocyte differentiation is also suggested by the findings that CDEP was expressed stage-dependently, and that PTH/PTHrP and cAMP induced CDEP expression within 1 h. DBcAMP and PTH elicited a similar pattern of changes in CDEP mRNA expression, indicating that PTH/PTHrP induces CDEP via an increase in the intracellular cAMP concentration.

There have been no studies reporting that hormones or growth factors enhance Rho GEF mRNA expression, although some extracellular stimuli have been shown to increase the enzyme activity of pre-existing Rho GEF proteins. Our findings suggest that hormones/growth factors/cAMP can modulate differentiation of some cells such as chondrocytes through an increase in the protein level of some Rho GEFs including CDEP.

PTH/PTHrP is crucial for chondrocyte differentiation particularly at the terminal (hypertrophic) stage. The CDEP expression was up-regulated in the hypertrophic stage, and further enhanced by PTH/PTHrP. These findings suggest that CDEP is involved in the control of chondrocyte phenotypic expression as a downstream regulator of the PTH/PTHrP/cAMP signalling pathway. However, CDEP is ubiquitously expressed in many tissues in vivo, and DBcAMP induced CDEP in fibroblasts (unpublished data). Thus CDEP may be involved in mediating or modulating the action of cAMP-elevating hormones in various cells.

References


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