The Phylogenetically Conserved Molluscan Chitinase-like Protein 1 (Cg-Clp1), Homologue of Human HC-gp39, Stimulates Proliferation and Regulates Synthesis of Extracellular Matrix Components of Mammalian Chondrocytes*

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Members of chitinase-like proteins (CLPs) have attracted much attention because of their ability to promote cell proliferation in insects (imaginal disc growth factors) and mammals (YKL-40). To gain insights into the molecular processes underlying the physiological control of growth and development in Lophotrochozoa, we report here the cloning and biochemical characterization of the first Lophotrochozoan CLP from the oyster Crassostrea gigas (Cg-Clp1). Gene expression profiles monitored by real time quantitative reverse transcription-PCR in different adult tissues and during development support the involvement of this protein in the control of growth and development in C. gigas. Recombinant Cg-Clp1 demonstrates a strong affinity for chitin but no chitinolytic activity, as was described for the HC-gp39 mammalian homolog. Furthermore, transient expression of Cg-Clp1 in primary cultures of rabbit articular chondrocytes as well as the use of both purified recombinant protein and conditioned medium from Cg-Clp1-expressing rabbit articular chondrocytes established that Cg-Clp1 stimulates cell proliferation and regulates extracellular matrix component synthesis, showing for the first time a possible involvement of a CLP on type II collagen synthesis regulation. These observations together with the fact that Cg-Clp1 gene organization strongly resembles that of its mammalian homologues argue for an early evolutionary origin and a high conservation of this class of proteins at both the structural and functional levels.

Growth factors orchestrate growth and development in metazoan organisms. Despite the huge variety of growth factors characterized in vertebrates, only a few have been identified in Protostome lineages and are mainly restricted to the Ecdysozoan model organisms Caenorhabditis elegans and Drosophila melanogaster. Thus, the third branch of bilaterian, named Lophotrochozoa, is an obviously understudied group of animals, since none of the major model organisms presently belong to this clade (1). Recent reports show that Lophotrochozoan animals exhibit biological characteristics that are considered ancestral, or at least less derived from the ancestral state than in other established systems that are known (2). Since bivalve mollusks belong to the Lophotrochozoa, they are good candidates to characterize growth factors and then contribute to our understanding of the evolution of growth and developmental regulations in bilaterian animals. Furthermore, a better knowledge of the molecular control of mollusk physiology may help improve the hatchery production of these economically important animals. Unfortunately, the number of growth factor genes identified so far at the molecular level in bivalve mollusks is extremely limited. In the pacific oyster, Crassostrea gigas, only four members of the transforming growth factor-β superfamily and their corresponding receptors have already been described (3–6). This lack of knowledge is above all the consequence of both the paucity of genomic sequence information and the difficulty in developing functional bioassays in these anatomically complex animals. Many growth factors are considered to be phylogenetically conserved between Deuterostomes and Protostomes. Examples include the superfamilies of insulin-related peptides (7), epidermal growth factor-related proteins (8), and transforming growth factor-β-related proteins (6). This opens the possibility of identifying growth factor-like molecules in oysters by homology searching.

Among growth factors, some of them are more attractive, since they are able to promote cell proliferation. Except for one growth factor family isolated in D. melanogaster (9), no proliferating polypeptides have been shown to have direct mitogenic activity in invertebrates. These new soluble mitogenic growth factors named imaginal disc growth factors...
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(IDGFs) cooperate with insulin to stimulate proliferation, polarization, and mobility of imaginal disc cells. These proteins are structurally related to chitinases but show an amino acid substitution that is known to abrogate catalytic activity. This suggests that they may have evolved from chitinases and have subsequently acquired a new growth-promoting function that does not require chitinase catalytic activity. Interestingly, homologues of chitinase-like proteins (CLPs) have also been described in mammals. Among these factors, human HC-gp39 protein (human cartilage glycoprotein-39) and its mammalian orthologues (YKL-40) also display mitogenic activity. Recklies et al. (10) have recently reported that purified HC-gp39 stimulates growth of connective tissue cells in concentration ranges similar to those effective for insulin-like growth factor-1 (10). This mitogenic activity is mediated by signaling through the mitogen-activated protein kinase and the phosphorylinsositol 3-kinase pathways. Prominent sites of HC-gp39 production are observed in the degenerated articular cartilage and inflamed or hyperplastic synovium, fibrotic liver tissue, and gliomas, where a correlation of HC-gp39 production with malignancy has been reported (13). Based on the reported tissue distribution, a role of HC-gp39 in tissue remodeling has been proposed (11, 14). The guinea pig orthologue stimulates synthesis of the cartilage matrix component, aggrecan, in chondrocytes, in addition to cell growth (15), suggesting that this protein acts generally to promote anabolic events in connective tissues. With mammalian and insect homologues, the CLP family seems to be phylogenetically conserved at both the structural and functional levels. Nevertheless, no member of this singular mitogenic growth factor family is identified at the moment in Lophotrochozoa. The characterization of homologous genes in this clade should help decipher the functionalities of this important but poorly understood family of growth factors.

In this paper, we report the structural and functional characterization of the first Lophotrochozoan “chitinase-like protein” from the oyster C. gigas. Cg-Clp1 (C. gigas chitinase-like protein 1) tissue distribution and temporal pattern of expression was established by real time PCR in order to assess its biological function in vivo. Phylogenetic analysis, gene structure comparison with homologues, and biochemical properties of Cg-Clp1 recombinant protein indicate that Cg-Clp1 is more closely related to mammalian YKL-40 than to insect IDGFs. Furthermore, we show that Cg-Clp1 activities on mammalian chondrocytes are identical to its mammalian closest homolog YKL-40. For the first time, the involvement of a CLP on type II collagen synthesis regulation was also demonstrated.

EXPERIMENTAL PROCEDURES

Animals

Adult oysters C. gigas were purchased from a local oyster farm (Normandie, France). Embryo and larval stages were produced in the IFREMER shellfish laboratory of Argenton (France).

Reverse Transcription, Cloning, and Sequencing

Reverse transcription was carried out using oligo(dT)17 as primer, 1 μg of mRNA and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). cDNAs were used as templates for PCR amplifications using two degenerated primers designed to anneal to conserved consensus regions of Drosophila imaginal disc growth factors. The sense primer corresponding to the LK(I/M)L(F/L)(S/T/R/C/W)VGG amino acid sequence was 5’-CTN AAR ATN CTN YTN WSN GTN GGN GG-3’, whereas the antisense primer corresponding to the FDGLDLA amino acid sequence was 5’-GGC NAG RTC NAG GCC RTC RAA-3’ (where Y represents C or T, R is A or G, S is C or G, W is A or T, and N is A, C, G, or T). PCR was performed in a total volume of 50 μl with 10 ng of mantle edge cDNA in 10 mM Tris/HCl, pH 9.0, containing 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTP, 1 μM each primer, 2.5 mM MgCl2, and 1 unit of TaqDNA polymerase (Eurogentec). The reaction was cycled between 94, 50, and 72 °C (45, 60, and 90 s, respectively), followed by an extension step at 72 °C for 5 min. After 40 cycles, a resulting 147-bp fragment was isolated. Full-length cDNA was generated by 5’ and 3’ RACE using the Marathon cDNA amplification kit (Clontech). Double-stranded cDNA from oyster mantle edges was ligated to adaptors, and 25 ng of this template was used to PCR-amplify 5’- and 3’-RACE fragments using adaptor-specific primers and gene-specific primers deduced from the initial 147-bp fragment sequence. PCR products were subcloned into pGEM-T easy vector using a TA cloning kit (Promega) and sequenced using ABI cycle sequencing chemistry.

Phylogenetic Analysis

Sequences used in the phylogenetic analyses were chosen to represent a range of chitinases and chitinase-like family proteins from both Ecdyszoaons and Deuterostomes. The sequences were aligned using ClustalX version 1.81 and by manual inspection. From these alignments, distance-based phylogenetic trees were constructed using the minimum evolution method of the PAUP (Phylogenetic Analysis Using Parsimony) package version 4.0b4a. One thousand bootstrap trials were run using the neighbor-joining algorithm for each node.

Real Time Quantitative PCR

Quantitative RT-PCR analysis was performed using the iCycler apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma) according to the manufacturer’s instructions. After treatment for 20 min at 37 °C with 1 unit of DNase I (Sigma) to prevent genomic DNA contamination, 1 μg of total RNA was reverse transcribed using 1 μg of random hexanucleotidic primers (Protections).
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Cg-Clp1 cDNA coding region (without signal sequence) was generated by PCR using a gene-specific sense primer containing a BamHI site and an antisense primer containing a HindIII site. Both the pQE30 expression vector (Qiagen) and PCR-amplified Cg-Clp1 cDNA fragment were digested by BamHI and HindIII, purified, ligated, and subsequently transfected into a XL1 Blue MRF’ strain (Stratagene). Correct insertion of the coding fragment was checked by sequencing. Selected recombinant colonies were propagated in LB containing 50 μg/ml of ampicillin at 37 °C. When culture grew at logarithmic phase, an optimum concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the Cg-Clp1 gene. After 3 h of induction, the cultured cells were harvested by centrifugation, lysed overnight with a denaturation buffer containing 20 mM NaHPO4/Na2PO4, pH 8, 500 mM NaCl, and 8 mM urea. After centrifugation for 10 min at 10,000 × g, supernatant containing Cg-Clp1 was analyzed by SDS-PAGE.

Eucaryotic Recombinant Expression of Cg-Clp1

Flp-In™-CHO cells (Invitrogen) used in this study harbor in their genome an FRT site, which allows an efficient Flp recombinase-mediated DNA integration of an FRT-tagged construction plasmid. These cells were transfected according to the manufacturer’s instructions in 10-cm² culture dishes with a mixture of Lipofectamine 2000 (10 μl) in serum-free Ham’s F-12 medium and 4 μg of plasmid DNA (3.6 μg of pOG44 Flp recombinase expression vector (Invitrogen) and 0.4 μg of the expression plasmid pSecTag-Cg-Clp1). pSecTag-Cg-Clp1 was obtained by cloning Cg-Clp1 coding sequence (without signal sequence) in frame with the lvg signal sequence of the pSecTag/FRT/V5 His plasmid (Invitrogen).

Selection of a Stable Cell Line

After 7 h, fetal calf serum was added to a final concentration of 10%, and the incubation continued for 24 h, following which hygromycin (500 μg/ml) was added to select Cg-Clp1 recombinant cells. After 20 days of selection, hygromycin-resistant transfected cells were lysed in 20 mM Hepes, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, and 1 mM PMSF by repeated freeze thaw cycles. This cell extract was centrifuged for 10 min at 20,000 × g and analyzed by SDS-PAGE. Recombinant Cg-Clp1 was detected by Western blot analyses using a V5 tag-specific antibody.

Purification of Recombinant Cg-Clp1 Protein

Recombinant Cg-Clp1 protein was purified using the ProBondTM purification system under native conditions according to the manufacturer’s protocol (Invitrogen). About 3 × 10⁸ nontransfected Flp-In™-CHO cells (used as a negative con-
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trol) and 3 × 10⁸ cells expressing Cg-Clp1 protein were each suspended in 11 ml of binding buffer (1 mM PMSF, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4) and lysed by three freeze-thaw cycles using a liquid nitrogen and a 42 °C water bath. For both lysates, DNA was sheared by passing the preparation through an 18-gauge needle several times, and cell debris was spun down at 20,000 × g for 10 min. Clear lysates were incubated with 2 ml of Ni²⁺-nitrilotriacetic acid-equilibrated resin for 20 min. The resin columns were washed three times with 10 ml of A buffer (30 mM imidazole, 1 mM PMSF, 20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0). Finally, the columns were eluted with 12 ml of B buffer (30 mM imidazole, 1 mM PMSF, 20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0), and elution fractions were then monitored by dot blot analyses using a V5 tag-specific antibody. Immunopositive fractions and corresponding fractions from nontransfected cells were each pooled and then desalted, buffer-exchanged to 100 mM acetic acid, and concentrated to one-milliliter volume by ultrafiltration using centrifugal filter membranes (Centricon concentrator YM50; Amicon; Millipore Corp.), which removed molecules smaller than 50 kDa. Concentration of recombinant Cg-Clp1 protein was evaluated by comparing the intensity of the signal in Western blot with those obtained for given quantities of a control protein (PositopeTM; Invitrogen).

Cell Cultures

RAC were prepared from the shoulders and the knees of 3-week-old rabbits, as previously described (19–21). Cells were seeded at 2 × 10⁴ cells/cm² in either 6-well plates, 100-mm dishes, or 75-, 150-, and 175-cm² flasks and cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and Fungizone (0.25 µg/ml) in a 5% CO₂ atmosphere. The medium was changed twice a week.

Transfection Experiments

Chondrocytes seeded at a density of 2 × 10⁴ cells/cm² in 6-well plates were transiently transfected at 80% confluence by the calcium phosphate precipitation method (22, 23). Different amounts of pSegTag-Cg-Clp1 expression vector (Invitrogen) and the insertless corresponding vector used as a complement to 15 µg were cotransfected with a pSV40 β-galactosidase expression vector (2 µg) as an internal control of transfection efficiency and, in some experiments, with 15 µg of COL2A1-luciferase reporter plasmid. After overnight transfection, the medium was replaced by a fresh one, and cells were harvested 24 h later. Protein amount, β-galactosidase, and luciferase activities were determined. Luciferase activity was measured on total cell extracts (kit from Promega) in a luminometer (Berthold Lumat LB 9501). β-Galactosidase activity was assayed with a colorimetric assay (24), whereas the protein amount was determined by the Bradford colorimetric method (Bio-Rad). Luciferase activities were normalized to protein amount and expressed in relative luciferase activity as the mean ± S.D. of three independent samples.

DNA Constructions

The COL2A1-luciferase reporter vectors (pGL2–3.774kb, pGL2–0.387kb, and pGL2–0.110kb) have been previously described (25).

Nuclear Extracts and Gel Retardation Assays

Nuclear extracts from transfected RAC were prepared by a minipreparation procedure (26), and gel retardation assays were performed with the following nucleotides: +2392/+2415 α1(II), 5’-AGCCCCATTCATGAGAGACGAGGT-3’ (SOX9 binding site is indicated in italic type); +2817/+2845 α1(II), 5’-AGGCAGAGCTGGCCCAGCAGCGCGCGC-GTCGGACCGGGCGGGCCGGCAGCGGCGGCGGCGGCGG-3’ (known to contain Sβ-responsive elements (22, 27). They were end-labeled with [γ-³²P]dATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Invitrogen). RAC nuclear extracts (7 or 15 µg) were incubated for 30 min at room temperature with the probe (2 fmol) in 20 µl of a specific binding buffer (for +2392/+2415 probe, 20 mM HEPES, pH 7.9, 50 mM KCl, 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.05% (v/v) Nonidet P-40; for +2817/+2845 probe, 10% glycerol, 0.5 mM DTT, 0.05% Nonidet P-40, 0.05 mM EDTA, 25 mM HEPES, pH 7.9, 50 mM NaCl) and in the presence of 1 µg of poly(dG-dC)poly(dG-dC) (Amersham Biosciences), used as DNA nonspecific competitor. Samples were fractionated by electrophoresis for 1.5 h at 150 V on a 5% polyacrylamide gel (acylamide/bis at 30:1) in 0.5 × TBE (45 mM Tris borate, 1 mM Na₂EDTA) and visualized by autoradiography.

Glycosaminoglycan Synthesis

The total amount of labeled GAG/PG was estimated after [³⁵S]sulfate (Amersham Biosciences) labeling as previously described (28).

Preparation of Conditioned Medium

Chondrocytes seeded at a density of 2 × 10⁶ cells/55-cm² culture plates were transiently transfected at 80% confluence by the calcium phosphate precipitation method with 10 µg of pSegTag-Cg-Clp1 expression vector (Invitrogen) or 10 µg of the insertless corresponding vector as a negative control. The resulting conditioned media (5 ml/55 cm²) were collected 24 h later and stored at −80 °C until used for their effect on the neosynthesis of collagen.

Collagen Labeling and Assay

A measurement of total collagen neosynthesis was performed as previously described (22). In addition to transfection experiments, both nickel-purified recombinant Cg-Clp1 and conditioned medium were tested. Recombinant Cg-Clp1 was added to the [³H]proline-labeled culture medium at a final concentration of 1 mM (60 ng/ml). As a negative control, a corresponding protein extract purified from nontransfected cells was used. Alternatively, the labeled culture medium was prepared with conditioned medium (1.5 ml/9.6 cm²) instead of fresh culture medium.

[³H]Thymidine Incorporation

Tritiated thymidine (74.5 Ci/mmol; PerkinElmer Life Sciences) was added to the culture medium of proliferating chondrocytes at
a final concentration of 1 µCi/ml. After 24 h, the medium was removed, and the monolayers were rinsed with PBS. This rinse medium was aspirated, and the culture dishes were placed on an ice block while 1 ml of ice-cold 5% trichloroacetic acid was added slowly. The culture dishes were placed in a cold room at 4 °C for 30 min, whereupon the trichloroacetic acid was removed, and the fixed monolayer was carefully rinsed three additional times with ice-cold 5% trichloroacetic acid. Then 0.5 ml of 0.1 M NaOH was added.
added to each well for 1 h at 50 °C to solubilize the precipitated material. Aliquots of 0.4 ml of NaOH extracts were removed from each well for liquid scintillation counting.

Western Blotting

Western blot analysis of type II collagen was performed on RAC as previously described (22). 15 µg of cell layer-associated proteins/well was used instead of 50 µg. Anti-type II collagen antibody was from Novotec (Lyon, France).

Chitinolytic and Chitin Binding Assays

Zymogram Detection—Chitinolytic enzyme activities were examined after polyacrylamide gel electrophoresis. A chitinase assay was carried out as described by Filho et al. (29). To visualize the active enzyme, 0.01% glycol chitin was incorporated into 6% (w/v) polyacrylamide minigels. After electrophoresis, the gel was incubated in 1% Triton X-100, 0.1 mM sodium acetate, pH 5.0, at 37 °C for 3 h at room temperature. The gel was transferred in a fresh solution of 0.01% calcofluor white M2R (Sigma), a specific fluorescent chitin stain, in 0.5 M Tris-HCl, pH 9.0. After 5 min, the gel was removed and washed several times in distilled water for 1 h. Areas of activity with digested chitin were visualized as dark bands on a fluorescent background under a UV transilluminator and photographed.

Spectrophotometric Enzyme Activity Measurement—N-Acetyl-β-D-glucosaminidase activity was assayed spectrophotometrically by using p-nitrophenyl-β-D-glucosaminide (pNPG; Sigma) as a soluble substrate. The reaction was carried out at 37 °C in 20 mM Tris-HCl (pH 5.2) containing 1 mM pNPG. The p-nitrophenol formed was determined spectrophotometrically at 405 nm.

Chitin Binding Assays—Lysed extracts of C. gulficlaid producing bacteria were refolded by stepwise dilutions of the denaturation buffer up to a concentration of 2 M urea and dialyzed four times against 40 volumes of 100 mM acetic acid, 1 mM PMSF for 48 h at 4 °C. The last dialysis step was performed against a binding buffer (20 mM Hepes, pH 8.0, 500 mM NaCl, and 0.1 mM EDTA, 1 mM PMSF). Renatured bacterial extract was incubated for 3 h at 4 °C on a 360° rotary shaker with 0.25 ml of affinity matrix of chitin beads (New England Biolabs), previously equilibrated with the binding buffer. Samples were centrifuged (5 min at 14,000 × g), the supernatants containing the unbound proteins were collected, and the chitin beads were extensively washed with 10 bead volumes of binding buffer containing 0.1% Triton X-100. A second washing step was carried out with 20 mM Hepes, pH 8.0, 1 M NaCl, 0.1 mM EDTA, and 50 mM DTT. Proteins bound to the chitin beads were eluted with SDS loading buffer and boiled for 5 min before being subjected to 12% SDS-PAGE under reducing conditions. Total proteins were visualized with Coomassie Blue staining.

Chitin binding was also examined using chitin particles as described by Renkema (30). 15 × 10^6 Flp-In™-CHO C. gulficlaid-expressing
cells were lysed in 1 ml of 20 mM Hepes, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, and 1 mM PMSF by repeated freeze thaw cycles. This cell extract was centrifuged for 10 min at 20,000 \( \times g \), and the supernatant was incubated with 25 mg of chitin particles (Sigma) overnight at 4 °C under gentle agitation. Particles were washed and eluted as described above. Protein fractions were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel, and Cg-Clp1 was detected by Western blot using V5 tag antibody.

### RESULTS

#### Isolation and Sequence Analysis of Cg-Clp1 Full-length cDNA

PCR with degenerate primers whose design was based on the conserved amino acid sequences of the catalytic domain of members of family 18 glycosyl hydrolase (GH) resulted in the amplification of an expected 147-bp sequence. Cloning and sequencing revealed an open reading frame showing amino acid sequence similarity to members of family 18 GH. Subsequently, specific primers deduced from the 147-bp fragment were used to perform 5' and 3' RACE-PCR to obtain the full-length cDNA. The complete 2182-bp cDNA obtained (EMBL Nucleotide Sequence Data base, accession number AJ971241) revealed an open reading frame of 1416 bp, starting with an ATG at position 423 and ending with a TAA at position 1839. This open reading frame encodes a protein composed of 472 amino acids with a putative N-terminal 21-amino acid signal peptide. The predicted secreted protein, named Cg-Clp1 contains two potential recognition sites for N-linked oligosaccharide at residues 256 and 397 of the mature protein.

#### Sequence Homology with Other Proteins

A comprehensive search of the amino acid data bases revealed significant homology with chitinases and CLPs of family 18 GH. The strongest identities were observed with the two human chitinases named AMCase (acidic mammalian chitinase) (26.5%) and chitotriosidase (26.5%), with the chitinase MF1 (26%) of the nematode Brugia malayi, and with three mammalian CLPs named Ym1 (24.5%), HC-gp39 (24%), and YKL-39 (24%). The optimal alignment of Cg-Clp1 with some of these proteins revealed regions of homology (Fig. 1). In particular, the catalytic center of family 18 GH is highly conserved. However, the glutamate residue in the putative active site, which is known to be critical for chitinase activity (31), was replaced by a glutamine residue, suggesting that Cg-Clp1 may lack chitinolytic activity. Moreover, four cysteine residues are present in conserved positions. These cysteines are known to be implicated in the formation of two disulfide bridges in the Drosophila and human CLP homologues, IDGF2 and HC-gp39, respectively.

#### Phylogenetic Analysis

Graphical representation of the phylogenetic relationships between metazoan members of family 18 GH clearly shows the existence of two subfamilies: the insect CLP members named IDGFs and all of the others, including both chitinases and mammalian CLPs. With a high level of bootstrap (99.9%), Cg-Clp1 clusters unambiguously within the chitinase/mammalian CLP subgroup and appears to be closer to an ancestor of this subgroup (Fig. 2).

#### Genomic Organization and Comparison with Homologues

The gene consists of eight exons with sizes ranging from 112 up to 722 bp and seven introns with sizes from 88 to 753 bp. Exon and intron sizes and the sequences at the boundaries between exons and introns are summarized in Table 1 (EMBL accession number AJ971240). All splice junctions contain the expected GT splice donor and AG splice acceptor. The first exon contains the 5'-untranslated region and the first in-frame ATG and the sequence corresponding to the putative 21-amino acid signal peptide; the last exon comprises the stop codon and the entire 3'-untranslated region. The sequence corresponding to highly conserved regions thought to constitute the catalytic center are located in exon III. Intron/exon organization (Fig. 3) is highly similar to that of human HC-gp39 and the nematode Bombyx mori chitinase (accession number AB048355), except for the bound-

### Table 1

| Exon | Exon size (bp) | Motif | 5'-splice donor | Intron size (bp) | 3'-splice acceptor | Amino acids interrupted |
|------|---------------|-------|-----------------|-----------------|-------------------|----------------------|
| I    | >637          | 5'UTR + signal peptide | ATCCATGGAGgttggtaaa-753-attatgacagAATTCACTCAA | G-F             |
| II   | 175           |       | ACATGCCACTgtatgtctata-169-tgggttaaggGCCACAGAGG | H-P             |
| III  | 253           | Catalytic center | CTTCTAGGATaatgaagag-705-tctccctacagATAATGAGGG | E/I             |
| IV   | 125           |       | GACTGCCAGGcaaggagag-738-tatatttttagAGAGFPGAC | A-E             |
| V    | 112           |       | GATAAACATCTatatccoca-448-ttatttttaagAAATCCTTGG | I/N             |
| VII  | 171           |       | TGCTCAAATAAagaagctaa-184-tatatggccagATATGTCATT | K/I             |
| VIII | 120           |       | GCGGAGAAAAGGcaagaataa-88-attattcttagGCATGGTG | K/A             |

* The sequence region that corresponds to putative active sites in chitinases (although no chitinase or N-acetyl-\( \beta \)-\( \delta \)-glucosaminidase activities were measured for Cg-Clp1).
aries of one and two of the introns, respectively. In contrast, there is no common intron/exon junction with the insect relative IDGF2.

Expression of Cg-Clp1 mRNA during Development and in Adult Tissues—To gain insights into possible physiological functions of Cg-Clp1, determination of tissue distribution and temporal pattern during development was performed by real time RT-PCR (Fig. 4). Cg-Clp1 transcripts were expressed only at moderate levels in all embryonic and larval stages. Remarkably, message for Cg-Clp1 was about 10-fold up-regulated during metamorphosis. In adult tissues, Cg-Clp1 mRNAs were found to be most abundant in mantle edge and digestive gland. To investigate which type of cells expressed Cg-Clp1 in mantle edge, in situ hybridization was performed. Cg-Clp1 transcripts were expressed both in epithelial and conjunctive cells of the mantle.

Since Cg-Clp1 was mostly expressed in mantle edge, transcripts levels were measured by real time RT-PCR after shell breaking (Fig. 5). This level was significantly up-regulated 2 weeks after shell breaking.

Production and Activity of Recombinant Cg-Clp1 Proteins—To investigate the biochemical properties of Cg-Clp1, recombinant proteins were expressed in E. coli and the CHO-K1 cell line.

Lack of Chitinase Activity—Cg-Clp1 belongs to the GH18 family, which is predominantly composed of chitinases. Moreover, a mammalian CLP named Ym1, displaying the same amino acid substitution as Cg-Clp1 in the putative catalytic center, possesses N-acetyl-β-D-glucosaminidase activity. Therefore, chitinase and N-acetyl-β-D-glucosaminidase activities were both tested by a zymogram and the use of pNPG asubstrate, respectively. Although degradation of glycol chitin on the gel is observed for an
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E. coli protein of 28 kDa, no chitinolytic activity corresponding to Cg-Clp1 was detectable (Fig. 6A).

Similarly, no N-acetyl-β-D-glucosaminidase activity was demonstrated when assayed against pNPG as substrate (Fig. 6B) in Cg-Clp1 elution fractions 4 and 5. The activity measured in fractions 2 and 3 was most likely due to endogenous CHO-K1 N-acetyl-β-D-glucosaminidase (11).

**Chitin Affinity Assays**—Because HC-gp39, the human homolog of Cg-Clp1, is a chitin-specific lectin, in vitro affinity assays were performed with chitin beads and chitin particles from crab shell. Unpurified lyzates from both prokaryotic and eukaryotic production systems were used for binding experiments. As shown in Fig. 6, C and D, Cg-Clp1 protein binds specifically and tightly to chitin, since neither 0.1% Triton X-100 nor 1 M NaCl with 50 mM DTT could disrupt the Cg-Clp1-chitin interaction. Cg-Clp1 protein could only be eluted from chitin by boiling for 5 min in a 2% SDS solution.

**Activity of Recombinant Cg-Clp1 Protein on Articular Chondrocytes**—To elucidate whether Cg-Clp1 can fulfill functions similar to its closest vertebrate counterpart YKL-40, transient expression of Cg-Clp1 was carried out in RAC (Fig. 7A). Activity of Cg-Clp1 was tested by measuring DNA, PG/GAG, total collagen, and type II collagen levels of synthesis. Activities were normalized only to protein amounts, because it was observed that forced expression of Cg-Clp1 always decreased the β-galactosidase activity in a dose-dependent manner (Fig. 7B).

**Cg-Clp1 Increases DNA and PG/GAG Syntheses**—Chondrocyte proliferation was estimated by measuring the incorporation of [3H]thymidine into DNA for 24 h. The level of relative DNA synthesis increased significantly from 0.5 μg up to 5 μg of Cg-Clp1 expression vector used for transfection (Fig. 7C). At 0.5 and 1 μg, Cg-Clp1 stimulated DNA synthesis by 95%. The ability of Cg-Clp1 to induce the synthesis of proteoglycans and glycoaminoglycans by cultured chondrocytes was evaluated by measuring the incorporation of [35S]sulfate into glycan chains for 24 h. Cg-Clp1 stimulated total PG/GAG synthesis by RAC from 0.5 μg up to 15 μg of Cg-Clp1 expression vector (Fig. 7D). Maximal (383%) Cg-Clp1-increased PG/GAG synthesis was observed for 10 μg of expression vector.

**Cg-Clp1 Triggers Inhibition of Total Collagen Synthesis**—The effect of Cg-Clp1 on total collagen synthesis was assayed by measuring the incorporation of [3H]-
FIGURE 7. Activities of recombinant Cg-Clp1 protein on Cg-Clp1-expressing RAC. A, transient transfection of Cg-Clp1 in RAC. Western blot was probed with an anti-V5 tag antibody to determine the expression levels of recombinant Cg-Clp1 resulting from transfection of chondrocytes with different amounts of Cg-Clp1 expression vector. For each amount of Cg-Clp1 expressed, the insertless pSeqTag/V5/His vector was used as a complement to 15 μg of H9262 g.

B, Cg-Clp1 down-regulates -galactosidase expression level in a dose-dependent manner. Primary RAC (9.6-cm² dishes) cultures were transiently transfected with different amounts of Cg-Clp1 expression vector, in the presence of 2 μg of pSV40-β-galactosidase expression vector, used as an internal control for transfection efficiency. β-Galactosidase activity was measured in cell extracts as described under “Experimental Procedures” by measuring the absorbance at 572 nm after incubation with the -galactosidase substrate, resorufin-D-galactopyranoside (23). The values represent the mean ± S.D. of three independent samples of a representative experiment.

C, effects of Cg-Clp1 on cell proliferation. Chondrocyte proliferation was assayed by measuring the incorporation of [3H]thymidine into DNA. Primary RAC (9.6-cm² dishes) cultures were transiently transfected with different amounts of Cg-Clp1 expression vector. 24 h after transfection, cells were incubated for 24 h in DMEM plus 10% FCS and 1 μCi/ml tritiated thymidine. At the end of the experiment, the amount of radiolabeled macromolecules was assayed. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, represent the mean ± S.D. of triplicate dishes and are expressed as the relative level of DNA synthesis related to the level of DNA synthesis with 0 μg of Cg-Clp1 expression vector.

D, effects of Cg-Clp1 on PG/GAG synthesis. The ability of Cg-Clp1 to induce the synthesis of PGs/GAGs by cultured chondrocytes was evaluated by measuring the incorporation of [35S]sulfate into glycan chains for 24 h. Primary RAC (9.6-cm² dishes) cultures were transiently transfected with different amounts of Cg-Clp1 expression vector. 24 h after transfection, cells were incubated for 24 h in DMEM plus 10% FCS and 3 μCi/ml [35S]sulfate. At the end of the experiment, the amount of radiolabeled macromolecules was assayed. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, represent the mean ± S.D. of triplicate dishes and are expressed as the relative level of GAG/PG synthesis related to the level of GAG/PG synthesis with 0 μg of Cg-Clp1 expression vector.

E, effects of Cg-Clp1 on total collagen neosynthesis. Primary RAC cultures at 80% confluence were transiently transfected with different amounts of Cg-Clp1 expression vector, in DMEM plus 10% FCS supplemented with 50 μg/ml ascorbic acid. 24 h after transfection, cells were incubated for 24 h in DMEM, 10% FCS, 50 μg/ml ascorbic acid, 100 μg/ml β-aminopropionitrile, 2 μCi/ml tritiated proline. At the end of the experiment, the amount of radiolabeled collagen was assayed in both the medium and cell layer as collagenase-digestible radioactivity. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, represent the mean ± S.D. of triplicate dishes and are expressed as the relative level of collagen synthesis related to the level of collagen synthesis with 0 μg of Cg-Clp1 expression vector.

F, effects of Cg-Clp1 on type II collagen mRNA levels. Real time RT-PCR was performed to examine the effect of transfection of different amounts of Cg-Clp1 expression vector on type II collagen mRNA levels. Each value represents the mean ± S.D. of three independent samples. G, Cg-Clp1 inhibits type II collagen production in proliferating chondrocytes. Primary RAC (9.6-cm² dishes) cultures were transiently transfected with different amounts of Cg-Clp1 expression vector in DMEM with 10% FCS. After 24 h, protein extracts were prepared and used in Western blotting experiments as described under “Experimental Procedures” to detect type II procollagen using a specific antibody.
located immediately upstream of the transcription start mRNA synthesis, real-time RT-PCR was performed. As shown fresh DMEM supplemented with nickel-purified recombinant Cg obtained from To exclude a nonspecific effect due to general affects of an Cg supplemented with 50 total collagen neosynthesis. OCTOBER 6, 2006•7 proline in collagenase-digestible proteins. As shown in Fig. FIGURE 8. Effect of the addition of purified recombinant Cg-Clp1 protein and conditioned medium on total collagen neosynthesis. Primary RAC cultures at 80% confluence were grown in DMEM plus 10% FCS supplemented with 50 μg/ml ascorbic acid. 24 h after, cells were incubated for 24 h in culture medium containing 10% FCS, 50 μg/ml ascorbic acid, 100 μg/ml β-aminopropionitrile, 2 μCi/ml tritiated proline, and either fresh DMEM supplemented with nickel-purified recombinant Cg-Clp1 (1 nM) (A) or conditioned DMEM obtained from Cg-Clp1-expressing RAC (B). At the end of the experiment, the amount of radiolabeled collagen and noncollagenous proteins were assayed in both the medium and cell layer as collagenase-digestible and non-collagenase-digestible radioactivity, respectively. The values, normalized to the amount of total protein assayed by the Bradford colorimetric method, represent the mean ± S.D. of at least three independent wells and are expressed as the relative level of collagen and noncollagenous protein neosyntheses related to their levels of synthesis with either DMEM supplemented with the nickel-purified protein extract from nontransfected CHO (Control, panel A) or conditioned DMEM from RAC transfected with insertless expression vector (Control, panel B).

proline in collagenase-digestible proteins. As shown in Fig. 7E, total collagen synthesis was decreased by transfection of Cg-Clp1 expression plasmid in a dose-dependant manner. To exclude a nonspecific effect due to general affects of an extraneous protein expression in RAC, conditioned medium obtained from Cg-Clp1-expressing RAC and the direct addition of nickel-purified recombinant Cg-Clp1 to cell culture medium (at a final concentration of 1 nM) were also tested. As shown in Fig. 8, Cg-Clp1-induced inhibition of collagen synthesis was unequivocally confirmed by the use of both conditioned medium and nickel-purified recombinant protein. Moreover, this decrease of collagen synthesis appears to be specific, since the levels of noncollagenic protein synthesis are not affected in these experiments (Fig. 8). Since the predominant collagen of the cartilage extracellular matrix is type II, we further examined the effect of Cg-Clp1 on type II collagen production in RAC.

Type II Collagen mRNA and Protein Levels Are Decreased by Cg-Clp1—To measure the effect of Cg-Clp1 on type II collagen mRNA synthesis, real-time RT-PCR was performed. As shown in Fig. 7F, the level of COL2A1 mRNA was decreased by transfection of Cg-Clp1 expression plasmid from 0.1 μg up to 10 μg. At 2.5 μg, Cg-Clp1 decreased type II collagen mRNA synthesis by 80%. This inhibition of type II collagen mRNA synthesis was confirmed at the protein level by Western blotting (Fig. 7G).

Cg-Clp1-induced Inhibition of Type II Collagen Expression Is Mediated At Least by a Short 63-bp Promoter Fragment Located Immediately Upstream of the Transcription Start Site—To further investigate the molecular mechanisms whereby Cg-Clp1 down-regulates COL2A1 mRNA levels, the transcriptional activity of the human COL2A1 gene was assayed using the luciferase reporter construct pGL2-basic. To delineate the sequences implicated in that effect, transient cotransfections were performed in RAC cultures, using three constructs containing deletions in both the promoter and/or first intron regions of the COL2A1 gene. As shown in Fig. 9, Cg-Clp1 inhibits the transcriptional activity of all of these constructs, from the largest one (pGL2-3.77kb), covering 1 kb of the promoter and ~90% of the first intron region, to the shortest, which contains only a proximal 63-bp promoter. These experiments suggest that the Cg-Clp1-induced inhibition of the COL2A1 gene is mediated at least by a 63-bp proximal promoter.

SOX9-, Sp1-, and Sp3-binding Sites Mediate the Cg-Clp1-induced Transcriptional Inhibition of the COL2A1 Gene—To investigate whether characteristic enhancer sequences found in the first intron involved in the tissue specific expression of the COL2A1 gene are implicated in Cg-Clp1-induced inhibition of that gene, nuclear extracts from Cg-Clp1-treated RAC were tested in gel retardation assays using +2392/+2415 and +2817/+2845 labeled probes. As shown in Fig. 10, the SOX9-DNA and both the Sp1/Sp3-DNA and Sp3-DNA complexes were significantly decreased in Cg-Clp1-stimulated cells.

DISCUSSION

In the present study, we identified in C. gigas the first Lophotrochozoan member of the GH18 family. The GH18 family is phylogenetically conserved, since members are found in almost every group of life kingdoms including bacteria, fungi, plants, nematodes, insects, and mammals. In addition to a vast majority of chitinases, this family contains proteins named CLPs that are closely related to chitinases but are lacking this enzymatic activity (32).

Since we have not been able to demonstrate any chitinase or any N-acetyl-β-d-glucosaminidase activity associated with recombinant Cg-Clp1, this protein may not be catalytically active but may use some of the structural elements of the chitinases to mediate its own function, such as binding to specific carbohydrates. The glutamic acid present in the catalytic core of chitinase is replaced by glutamine in Cg-Clp1, and this substitution is known to abolish catalytic activity in bacterial chitinase (31). Moreover, a similar substitution probably explains the lack of chitinase activity observed for other CLPs like...
**Oyster Chitinase-like Protein (Cg-Clp1)**

![Graph showing relative luciferase activity](image)

**FIGURE 9. Cg-Clp1 down-regulation of type II collagen expression is mediated at least by a short 63-bp promoter fragment located upstream of the transcription start site.** Primary RAC, at 80% confluence, were transiently cotransfected with 15 μg of different COL2A1 reporter plasmids together with different amounts of Cg-Clp1 expression vector and 2 μg of pSV40-β-gal. 24 h later, the samples were harvested; protein content, β-galactosidase, and luciferase activities were assayed. Each series of transfections was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity, after correction for the protein amount.

HC-gp39 (11) or IDGFs (9), but it may also confer strong chitin-binding properties, as was demonstrated for human chitinotriosidase (30). Therefore, to explain the significant capacity of recombinant Cg-Clp1 to bind tightly to chitin beads and particles, we hypothesized that the slightly modified catalytic center of Cg-Clp1 might be responsible for its chitin-specific lectin property.

Phylogenetic and comparative sequence analyses show Cg-Clp1 to be more closely related to chitinases and mammalian CLPs than to insect IDGFs. Comparison of Cg-Clp1 genomic structure with that of homologous genes confirms this result and reveals the existence of a single common ancestral gene for Cg-Clp1, chitinases, and mammalian CLPs. Both the phase and the position of the five last exon boundaries of the Cg-Clp1 gene and HC-gp39 gene are strictly conserved. This suggests that the ancestral gene already displayed this organization. Such a conservation over a time period as long as the 600 million years from the divergence of Deuterostomes (vertebrates) and Protostomes (Ecdysozoa and Lophotrochozoa) indicates a low acceptance of mutational changes on this gene section during evolution. The protein domains encoded by this gene region might have functional importance, despite lack of information on this protein region. In contrast, the singular IDGF gene structure may be explained by gene reorganization peculiar to Ecdysozoa.

Since C. gigas cell cultures are not available, we assayed for Cg-Clp1 activities on a heterologous system. Since YKL-40 promotes the growth of connective tissue cells, we chose to measure Cg-Clp1 activity on RAC after transient expression of the corresponding recombinant protein in these cells. Alternatively, we tested nickel-purified recombinant Cg-Clp1 and conditioned medium obtained from Cg-Clp1-expressing RAC. The expression of Cg-Clp1 induces RAC proliferation, in addition to an increase of PG/GAG synthesis and a decrease of type II collagen synthesis. Both the use of conditioned medium and the direct addition of recombinant protein to cell culture medium confirm Cg-Clp1 activity on collagen synthesis. Thus, our results are in agreement with those obtained for YKL-40 on guinea pig and rabbit chondrocytes (15). The authors show that YKL-40 induces proliferative events in cultured chondrocytes and increases GAG synthesis in chondrocytes. In a similar manner, the results presented here suggest an involvement of Cg-Clp1 in anabolic events in connective tissues.

Furthermore, we showed for the first time that a CLP may exert a control on type II collagen synthesis. Since Cg-Clp1 presents the same activities on chondrocytes compared with its closest homolog (i.e. YKL-40), we postulate a great conservation of the tertiary structure between these two CLPs. Thus, the inhibition of type II collagen production induced by Cg-Clp1 might be observed for YKL-40 as well. Type II collagen is considered as a critical phenotypic marker gene for analysis of molecular events involved in the chondrogenesis process as well as in chondrocyte phenotype maintenance. Alteration of type II collagen expression in cartilage may be due to a variety of genetic, inflammatory, or degenerative circumstances and may lead to a variety of chondrodysplasias and joint diseases, such as osteoarthritis. In osteoarthritis, chondrocytes undergo dedifferentiation and synthesize type I and III collagens at the expense of type II (22). A high level of YKL-40 has been found in sera and synovial fluids of patients with inflammatory and degenerative arthritis (33). Thus, the decrease of type II collagen synthesis promoted by Cg-Clp1 pleads for the implication of YKL-40 in chondrocyte dedifferentiation. These effects on RAC have already been reported with transforming growth factor-β1 (25). Indeed, transforming growth factor-β1 is a multifunctional cytokine that enhances...
GAG production (34) and inhibits type II collagen production in proliferating chondrocytes (25). The authors suggest that the inhibitory effect of transforming growth factor-β1 on type II collagen expression could be related to an increase in the Sp3/Sp1 transcription factor ratio that prevents Sp1-induced transcription-activating effects. The same indirect effect of Cg-Clp1 on COL2A1 gene expression could also be suggested to explain how Cg-Clp1-induced inhibition is mediated by the bp −63/+47 proximal promoter. In this respect, it must be noted that the expression of β-galactosidase, used here as an internal control of transfection efficiency in all experiments but not employed for normalization, was constantly inhibited when Cg-Clp1 was transfected, whatever the amount used (Fig. 7B). The SV40 promoter contains several binding sites for Sp1/Sp3 factors, as is also the case for the bp −266/+1 sequences of the COL2A1 gene that bear seven Sp1/Sp3 binding sites, one being located in the bp −63/+1 segment. Thus, we may suggest that Cg-Clp1-induced inhibition is mediated by the Sp1/Sp3 transcription factor ratio. Furthermore, the decrease in the DNA binding activity of SOX9, Sp1, and Sp3 to the tissue-specific intronic enhancer of COL2A1 in Cg-Clp1-expressing cells suggests that Cg-Clp1-induced inhibition is mediated at least by two regions of the COL2A1 gene: the promoter, which is necessary for the mediation of the enhancer effects, and also the enhancer (i.e. +2392/+2415 and +2817/+2845). Moreover, the SOX9 transcription factor has been shown to directly transactivate the COL2A1 gene through the +2392/+2415 sequence of the intronic specific enhancer (35). Therefore, Cg-Clp1-induced inhibition of type II collagen gene expression may be explained, at least in part, both by the decrease of Sox9 and Sp1 binding activities.

Since Cg-Clp1 was tested on RAC, the biological activities of Cg-Clp1 in C. gigas are still an open question. However, we surmise that Cg-Clp1 biological activities on RAC can be applied to some C. gigas cell types having close characteristics. In this respect, mantle cells are good candidates, since they produce various and abundant organic matrix components (36). Furthermore, in vivo Cg-Clp1 mitogenic activity seems to be all the more plausible, since this property appears to be one of the hallmarks of several CLPs identified so far, like human YKL-40, guinea pig YKL-40, and Drosophila IDGF1/2 as well.

We suppose that Cg-Clp1 is secreted and transported to target tissues via the hemolymph, as is proposed for insect IDGFs. However, Cg-Clp1 may have local as well as systemic functions. The analysis of mRNA distribution during development shows that Cg-Clp1 is expressed at a very low level until metamorphosis. This critical period involves sudden changes in body plan and metabolism. In C. gigas, metamorphosis is characterized by the degeneration of larval tissues, such as the velum and the foot, and the remodeling of larval tissues to produce adult tissues (i.e. the development of the gills and the production of an adult shell), which is accompanied by an important growth of the soft body parts (37). In adults, Cg-Clp1 is mostly expressed in the mantle edge and digestive gland. The mantle edge governs shell formation and body growth by the secretion of extracellular components and by proliferation of mantle cells. Besides its role in nutrition, the digestive gland of bivalve mollusks is a constantly reorganizing organ in which proliferation and differentiation of digestive stem cells regularly and actively proceeds to counteract the high rate of degeneration of functional digestive cells (38). Considering Cg-Clp1 activities on RAC and its pattern of expression in oyster, this protein may be considered as a growth factor involved in the control of cell proliferation and tissue remodeling. Moreover, the significant increase of Cg-Clp1 mRNA levels in the mantle edge after shell breaking strongly suggests that this protein could orchestrate secretion of extracellular components and/or proliferation of mantle cells, both processes occurring during shell repair.

The present realization that Cg-Clp1 acts as a chitin lectin may be of particular importance. In mollusks, the presence of chitin in shell is well documented (39). Mollusk shell is composed of an organic matrix associated with an inorganic fraction. The relationship between the mineral phase and the organic phase implies a high level of molecular recognition. In this biocomposite, due to its interaction with shell proteins, chitin has a crucial role in the hierarchical control of the biomineralization processes (39, 40). Since Cg-Clp1 possesses chitin binding activity and a high expression level in mantle edge, this protein may be a shell protein involved in the control of biomineralization.

As a chitin-specific lectin with probable mitogenic and extracellular matrix remodeling functions, Cg-Clp1 is potentially implicated in processes of oyster growth. Moreover, we assume that Cg-Clp1 could also be involved in immune homeostasis, as is the case for its mammalian (e.g. YKL-40, Ym1) and insect (i.e. IDGFs) counterparts (41–44). This hypothesis is in agreement with the recent demonstration that mantle edge behaves as an
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immune tissue, since it expresses antimicrobial peptides (45) and overexpresses cytokine receptors (5) as a result of bacterial lipopolysaccharide challenge.

CONCLUSION

Our results showed a noteworthy conservation between a mammalian and a mollusk CLP at the gene structure and most likely at the protein structure levels. Moreover, Cg-Clp1 possesses the same biochemical activities as YKL-40 (i.e. chitin-binding and no chitinolytic activities) and presents an expression pattern during development and in adult tissues that strongly points to a tissue remodeling role, as is assumed for YKL-40. Thus, this work strongly suggests a high structural and functional conservation of two phylogenetically distant CLPs. This emphasizes an important physiological role for this subfamily of CLPs, whose precise functions remain to be unraveled.

Furthermore, this is the first report of a possible control of type II collagen level of expression by a CLP. Comparative studies on biological activities of Cg-Clp1 and its counterparts may pave the way for a better understanding of YKL-40 implication in degenerating diseases, especially osteoarthritis.

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