Mutations in human cartilage oligomeric matrix protein (COMP) have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia; however, the functions of both wild-type and mutant COMP in the skeletogenesis remain unknown. In an effort to define the biological functions of COMP, a functional genetic screen based on the yeast two-hybrid system was performed. This led to the identification of granulin-epithelin precursor (GEP), an autocrine growth factor, as a COMP-associated partner. COMP directly binds to GEP both in vitro and in vivo, as revealed by in vitro pull down and co-immunoprecipitation assays. GEP selectively interacts with the epidermal growth factor repeat domain of COMP but not with the other three functional domains of COMP. The granulin A repeat unit of GEP is required and sufficient for association with COMP. COMP co-localizes with GEP predominantly in the pericellular matrix of transfected rat chondrosarcoma cell and primary human chondrocytes. Staining of musculoskeletal tissues of day 19 mouse embryo with antibodies to GEP is restricted to chondrocytes in the lower proliferative and upper hypertrophic zones. Overexpression of GEP stimulates the proliferation of chondrocytes, and this stimulation is enhanced by COMP. In addition, COMP appears to be required for GEP-mediated chondrocyte proliferation, since chondrocyte proliferation induced by GEP is dramatically inhibited by an anti-COMP antibody. These findings provide the first evidence linking the association of COMP and GEP and identifying a previously unrecognized growth factor (i.e. GEP) in cartilage.

Cartilage oligomeric matrix protein (COMP) is a noncollagenous component of the extracellular matrix. It is expressed in cartilage, ligament, tendon, bone (osteoblasts only), and synovium (1–3). COMP is a 524-kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each) (4, 5). Although COMP has been implicated in the regulation of chondrogenesis in a micromass culture of mesenchymal stem 10T1/2 cells and in limb development in vivo (6, 7), its function remains largely unknown. COMP binds to chondrocytes in vitro through an arginine-glycine-aspartic acid (RGD) sequence in COMP and the integrin receptors α5β1 and αV3 (8). COMP has been shown to be up-regulated after traumatic knee injury (9) and has been implicated in the pathogenesis of rheumatoid arthritis and osteoarthritis (10–12). Monitoring COMP levels in either joint fluid or serum can be used to assess the presence and progression of arthritis (13–18). Mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia, autosomal-dominant forms of short limb dwarfism characterized by short stature, normal facies, epiphyseal abnormalities, and early onset osteoarthritis (19–25).

During mouse development, COMP staining has been described in the pericellular matrix of maturing articular chondrocytes (25), and during rat development it has been associated mainly with the growth plate (3). These in vivo data suggested that COMP may play important roles in chondrogenesis and cartilage development. One of the aims of the present study, therefore, was to isolate the proteins that associate with COMP in order to elucidate its biological functions in skeletogenesis. A yeast two-hybrid screen using the EGF domain of COMP as bait led to the isolation of granulin-epithelin precursor (GEP) as a COMP-binding growth factor.

GEP, also referred to as progranulin, proepithelin, PC cell-derived growth factor, or acrogranin, is a 68.5-kDa secreted growth factor. It is heavily glycosylated and appears as an ~90-kDa protein on SDS-PAGE. Structurally, it belongs to none of the well established growth factor families. GEP is secreted in an intact form (26, 27) or undergoes proteolysis, leading to the release of its constituent peptides, the granulins (28–30). Individual granulins have an approximate molecular mass of 6 kDa and are structurally defined by the presence of 12 cysteines arranged in a characteristic motif: \(X_2-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5-CX_5\) (31).

The abbreviations used are: COMP, cartilage oligomeric matrix protein; EGF, epidermal growth factor; GEP, granulin-epithelin precursor; GST, glutathione S-transferase; Y2H, yeast two-hybrid; RCS, rat chondrocyte sarcoma; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; aa, amino acids.
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parision of the biosynthetic origin of granulin peptides in various mammals reveals that all are commonly derived from a precursor composed of one amino-terminal half (p) followed by seven (A–G) nonidentical copies of the granulin motif (32).

GEP is abundantly expressed in rapidly cycling epithelial cells, in cells of the immune system, and in neurons (30, 33–35). High levels of GEP expression have been reported from several human cancers and are believed to contribute to tumorigenesis in breast cancer, clear cell renal carcinoma, invasive ovarian carcinoma, glioblastoma, adipocytic teratoma, multiple myeloma (28, 32, 36–41), and osteosarcoma. The role of GEP in the regulation of cellular proliferation has been well characterized through the cell cycle (42). In contrast, GEP is the only known growth factor able to bypass the requirement for the insulin-like growth factor receptor gene (RΔ cells). These cells are unable to proliferate in response to insulin-like growth factor and other growth factors (EGF and platelet-derived growth factor) necessary to fully progress through the cell cycle (42). Increasing evidence has also implicated GEP in the regulation of differentiation, development, and pathological processes. It has been isolated as a differentially expressed gene from mesothelial differentiation (44), sexual differentiation of the brain (45), macrophage development (46), and synovium of rheumatoid arthritis and osteoarthritis (47). Remarkably, GEP was also shown to be a crucial mediator of wound response and tissue repair (38, 48).

The aim of this study is to characterize the interaction between GEP and COMP and to investigate the biological significance of this interaction in regulating chondrocyte proliferation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Yeast expression vectors pDBleu and pPC86 (both from Invitrogen) are fusion vectors for the linkage of proteins to the Gal4 DNA binding domain and to the VP16 transactivation domain, respectively. The fragments encoding the four functional domains (i.e. the N-terminal domain (aa 20–83), EGF repeat domain (aa 84–261), type III repeat domain (aa 266–520), and C-terminal domain (aa 521–755; GenBankTM accession number AF257516)) of mouse COMP were amplified by PCR and cloned in frame into the SalI/NotI sites of pDBleu (pDB-COMP-NT, pDB-COMP-epidermal growth factor, pDB-COMP-type III, and pDB-COMP-CT) to serve as bait in yeast two hybrid screens. The mammalian expression vectors pDBleu and pPC86 (both from Invitrogen) are fusion vectors for the linkage of proteins to the Gal4 DNA binding domain and to the VP16 transactivation domain, respectively. The fragments encoding the four functional domains (i.e. the N-terminal domain (aa 20–83), EGF repeat domain (aa 84–261), type III repeat domain (aa 266–520), and C-terminal domain (aa 521–755; GenBankTM accession number AF257516)) of mouse COMP were subcloned in frame into the BamHI/EcoRI sites of pGEX-3X to generate the plasmids pGEX-N-term, pGEX-EGF, pGEX-type III, and pGEX-C-term, respectively. The bacterial expression plasmid pBAD TOPO vector (Invitrogen) was used to produce His-tagged proteins in E. coli.

cDNA inserts encoding different fragments (Table 1) of GEP (GenBankTM accession number NM_017113.1) were subcloned into the pBAD TOPO vector per the manufacturer’s instructions to generate the indicated plasmids.

The mammalian expression pEGFP-GEP construct was kindly provided by Dr. Mathews at University of Medicine and Dentistry of New Jersey. The mammalian expression pDsRed1-N1 (BD Biosciences Clontech) was used to produce recombinant protein. A cDNA fragment encoding mouse full-length COMP was amplified by PCR and subcloned in frame into the EcoRI/KpnI sites of pDsRed1-N1 to produce plasmid pDsRed1-N1-COMP, which expresses RED fusion proteins in the mammalian cell line.

All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (available on the World Wide Web at ncbi.nlm.nih.gov/blast/).

Generation of Stable Lines in RCS Cells

Rat chondrosarcoma cells (RCS cell) were cultured in tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. RCS cells were cultured for 1 day before transfection using Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions, at a density of 1.5 × 10^6 cells/30-mm plate. The plasmid pEGFP-GEP, pDsRed1-N1-COMP, and pEGFP-GEP plus pDsRed1-N1-COMP or the empty pEGFP vector were transfected into RCS cells to generate RCS-GEP, RCS-COMP, RCS-GEP plus COMP, and RCS-control cell line. Two days after transfection, cells were divided into 100-mm dishes at a density of 10^6 cells/dish in 10 ml of DMEM containing 1,000 µg/ml of G418. After 14 days in selective medium (medium changed every 3 days), cells were expanded in DMEM containing 500 µg/ml G418.

Expression and Purification of GST and His-tagged Proteins

For expression of GST fusion proteins, the appropriate plasmid pGEX-N-term, pGEX-EGF, pGEX-typeIII, or pGEX-C-term was transformed into E. coli DH5α (Invitrogen). Fusion proteins were affinity-purified on glutathione-agarose beads, as described previously (49). His-GnA, His-GnC, His-GnD, His-GnE, His-GnACDE, and His-GEP were purified by affinity chromatography using a HiTrap chelating column (Amerham Biosciences). Briefly, bacteria lysates supplemented with 20 mM HEPES (pH 7.5) and 0.5 M NaCl were applied to the

### Table 1

| Fragment Plasmid |
|------------------|
| Granulin A (aa 278–333) pGEP-(278–333) |
| Granulin C (aa 361–413) pGEP-(361–413) |
| Granulin D (aa 438–492) pGEP-(438–492) |
| Granulin E (aa 512–567) pGEP-(512–567) |
| Granulin ACDE (aa 278–588) pGEP-(278–588) |
| GEP (aa 1–588) pGEP-(1–588) |

3 Y. Zhang and C. Liu, unpublished data.
HiTrapp chelating column; the column was washed with HSB buffer (40 mM HEPES, pH 7.5, 1 mM NaCl, and 0.05% Brij 35) containing 10 mM imidazole; and the His-GrnA, His-GrnC, His-GrnD, His-GrnE, and His-GrnACDE were eluted with HSB buffer containing 300 mM imidazole.

**Yeast Two-hybrid (Y2H) Library Screen**

Plasmid pDB-COMP-epidermal growth factor (see above) was used as bait to screen the Y2H rat brain cDNA library (Invitrogen) according to a modified manufacturer’s protocol. Briefly, bait plasmid was introduced into a yeast MAV203 strain that contained three reporter genes, *HIS*<sup>+</sup>, *ura*<sup>-</sup>, and *lacZ* (Invitrogen), and transformants were selected on defined medium lacking leucine. The rat brain cDNA library in the vector pPC86 was then transformed into the resultant Leu<sup>+</sup> strain MAV203 with the selected plasmids and 2) selective SD-leu<sup>-</sup> and growth phenotypes on growth phenotypes on 30 °C, colonies were screened for histidine, and uracil but containing 25 mM 3-amino-1,2,4-triazole that can specifically inhibit the activity of HIS3 gene product and block the basal concentration of HIS3 in yeast (SD-leu<sup>-</sup>/trp<sup>-</sup>/his<sup>-</sup>/ura<sup>-</sup>/3AT<sup>+</sup>). After incubation for 7–10 days at 30 °C, colonies were screened for β-galactosidase activity by a filter lift assay (24). Individual pPC86 recombinant plasmids that were identified in the initial screen were further verified for interaction with bait by repeating the Y2H assay.

**Assay of Protein-Protein Interactions Using the Y2H System**

Three independent colonies were analyzed for interaction in yeast of two proteins, one of which was fused to the Gal4 DNA binding domain and the other to the VP16 transactivation domain. The procedures of Vojtek et al. (50) and Hollenberg et al. (51) were followed for 1) growing and transforming the yeast strain MAV203 with the selected plasmids and 2) β-galactosidase activity and growth phenotypes on growth phenotypes on selective SD-leu<sup>-</sup>/trp<sup>-</sup>/his<sup>-</sup>/ura<sup>-</sup>/3AT<sup>+</sup> plates.

**In Vitro Binding Assay**

For examination of the binding of COMP to GEP in vitro, glutathione-Sepharose beads (50 μl) preincubated with either purified GST (0.5 μg, serving as control) or GST-EGF-like domain of COMP and Ni<sup>2+</sup>-nitrilotriacetic acid-Sepharose preincubated with either His or His-tagged GEP were incubated with purified His-tagged Grn-ACDE or COMP (purified from HEK293 cells stably transfected with an expression plasmid encoding full-length human COMP), respectively. Bound proteins were resolved by 12% SDS-PAGE and detected by Western blotting with anti-His antibodies and polyclonal rabbit anti-COMP antiserum.

In the case of the binding assay for dissecting the repeat unit of GEP required for interaction with COMP, glutathione-Sepharose beads (50 μl) preincubated with either purified GST (0.5 μg, serving as control) or the GST-EGF domain of COMP were incubated with purified His-tagged Grn-ACDE, GrnA, GrnC, GrnD, or GrnE, respectively. Bound proteins were processed as described above.

**Coimmunoprecipitation**

Approximately 500 μg of cell extracts prepared from isolated human chondrocytes were incubated with anti-GEP (25 μg/ml, acrogranulin, N-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or control rabbit IgG (25 μg/ml) antibodies for 1 h, followed by incubation with 30 μl of protein A-agarose (Invitrogen) at 4 °C overnight. After washing five times with immunoprecipitation buffer, bound proteins were released by boiling in 20 μl of 2× SDS loading buffer for 3 min (52). Released proteins were examined by Western blotting with anti-COMP antibodies (3, 53–55), and the ECL chemiluminescent system (Amersham Biosciences).

**Co-localization Assays of COMP and GEP**

**Ectopic Expression of Fluorescent GEP and COMP in Living Cells—**To examine whether co-expressed GEP and COMP colocalize in the living cells, GFP-linked GEP and red fluorescent protein-fused COMP were co-transfected into RCS chondrocytes, and 48 h later the culture was directly observed under a fluorescence microscope with appropriate optical filters. Microscopic images were captured using the Image Pro program (Media Cybernetics) and an Olympus microscope. Images were arranged using the Adobe Photoshop program.

**Immunostaining for COMP and GEP in Primary Human Chondrocytes—** Cultures of isolated human chondrocytes were plated on glass coverslips coated with polylysine and grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen) under an atmosphere of 5% CO<sub>2</sub> at 37 °C. After reaching 80% confluence, the cells were fixed with cold acetone/methanol (1:1) for 20 min and air-dried. After rehydration in phosphate-buffered saline and blocking with 20% goat serum in phosphate-buffered saline for 30 min, the cells were incubated with primary antibodies (i.e. mouse monoclonal anti-COMP antibodies; Santa Cruz Biotechnology; diluted 1:50) and polyclonal goat anti-GEP antibodies (Santa Cruz Biotechnology; diluted 1:50) at room temperature for 1 h. After being washed with phosphate-buffered saline, the coverslips were incubated with secondary antibodies (i.e. goat anti-mouse IgG conjugated with rhodamine (Santa Cruz Biotechnology; diluted 1:100) and chick anti-goat IgG conjugated with fluorescein isothiocyanate (Santa Cruz Biotechnology; diluted 1:400) for 50 min. The specimens were observed, and the images were processed as described above.

**Immunohistochemistry**

4-μm-thick formalin-fixed paraffin sections of 19-day-old embryonic murine limbs were immunostained for GEP. The sections were pretreated with chondroitinase (Sigma) for 30 min at 37 °C followed by protein block (Dako serum-free protein block) for 10 min at room temperature to reduce nonspecific staining. Polyclonal goat anti-human GEP (Santa Cruz Biotechnology) was diluted at 1:200 and incubated overnight at 4 °C. Binding of primary antibodies was detected using biotinylated anti-goat secondary antibody (Jackson Laboratories) diluted at 1:800 and incubated for 30 min at 37 °C followed by alkaline phosphatase (Vector) at 37 °C for 30 min and developed with Vector Red (Vector) for 2 min at room temperature. Sections were counterstained with Mayer’s hematoxylin (Dako). The primary antibody was substituted with Negative Control SuperSensitive goat serum (BioGenex) for the negative control section. In the case of the assay for examining the COMP expression, the same tissue was used, and the same pro-
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tocol was followed, except the anti-GEP was replaced by anti-COMP antibody, and the sections were not pretreated with chondroitinase.

Assays for Chondrocyte Proliferation

1.0 × 10^5 of stable transfected RCS-control (GFP vector, serving as control), RCS-COMP, RCS-GEP, and RCS-COMP plus GEP cells were cultured in DMEM supplemented with 5% heat-inactivated fetal calf serum, 0.2 mM l-glutamine, and antibiotics in 6-well dishes, and the viable cells were counted every day until day 5. Each group was repeated three times. In the case of the antibody-blocking experiment, we first generated affinity-purified anti-COMP antibodies in order to study the dosage response in a controlled condition. Briefly, the anti-COMP serum (3, 53–55) was incubated with Affi-Gel-10 beads (Bio-Rad) to which purified COMP was covalently linked. The bound antibodies were eluted from the beads with 0.15 M glycine buffer (pH 2.5) and immediately neutralized with 1.5 M Tris-HCl buffer (pH 8.0) (56). Then various doses of the purified anti-COMP (2.5, 5.0, or 10.0 μg/ml) or the control antibody (10.0 μg/ml) were added into the cultures of the 6.0 × 10^4 RCS cells transfected with a plasmid encoding GEP in 24-well plates, and the cell proliferation was assayed as above.

RESULTS

Isolation of GEP as a COMP Binding Partner—to better understand the biological functions of COMP, we performed a Y2H screen. Briefly, we linked the four functional domains of COMP (the N-terminal pentamerizing domain (aa 20–83), the EGF-like domain (aa 84–261), type 3 repeats (aa 266–520), and the C-terminal (aa 521–755)) to the Gal4 DNA-binding domain in the plasmid pDBleu. We used the respective constructs as bait to screen a library of rat brain cDNA expressed as fusion proteins to the VP16 acidic activation domain in the vector pPC86.

A Y2H rat cDNA library was screened with the construct encoding the EGF-like repeats of COMP. We screened ~2.5 million clones and identified 21 that activated the three reporter genes. Further tests involved the retransformation of yeast with the purified target plasmids and bait. Only 12 of the original 21 yeast clones expressed hybrid proteins that still interacted with the EGF-like domain bait (not shown). Two of the positive clones encoded two N-terminal truncated mutants (aa 228–588 and aa 334–588) of a secreted growth factor GEP (accession number NM_017113.1).

Confirmation of Interaction between COMP and GEP in Yeast—the Y2H assay was repeated to verify the interaction between the EGF-like domain of COMP and the C terminus of GEP (aa 228–588). The plasmid encoding the EGF-like domain of COMP linked to the Gal4 DNA-binding domain (above the line in Fig. 1) and the plasmid encoding C-terminal of GEP fused to the VP16 acidic activation domain (below the line in Fig. 1) were used to cotransform the yeast. Like the c-Jun/c-Fos pair, which is known to interact and used as a positive control, our assays indicated that COMP interacts with GEP in yeast, based on the activation of the lacZ reporter gene (left) and growth phenotypes on SD-leu^-/trp^-/his^-/ura^-/3AT^- plates (right).

COMP Directly Binds to GEP—to verify the interaction between COMP and GEP that was first identified in yeast, a GST pull-down assay was first performed (Fig. 2A) to test whether the EGF-like domain of COMP binds to the C-terminal of GEP (aa 228–588, Gnr-ACDE) in vitro. GST did not pull down Gnr-ACDE protein (lane 2), whereas GST-EGF pulled down purified recombinant His-tagged Gnr-ACDE protein (lane 1), indicating binding of COMP to GEP in vitro. An opposite pull-down assay in which His-tagged GEP was conjugated to the beads was also per-
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The EGF-like domain of COMP by GEP was further verified by a GST pull-down assay (Fig. 3, C and D).

Fragment GrnA of GEP Is Required and Sufficient for Interaction with COMP—Since the C-terminal region of GEP (aa 228–518) was isolated as a COMP-binding protein in a Y2H screen, followed by a confirmatory in vitro pull-down assay, we generated various constructs that expressed various His-tagged GEP C-terminal repeat unit fusion proteins in E. coli, to narrow down the binding domain and to dissect the COMP-binding repeat unit in GEP. The proper folding of purified His-tagged GEP C-terminal repeat unit was verified by SDS-PAGE analysis of recombinant proteins with or without dithiothreitol (not shown). Results from in vitro pull-down assays (Fig. 4B) of all of these mutants are summarized in Fig. 4A. The unit C (GrnC), the unit D (GrnD), and the unit E (GrnE) failed to bind COMP; however, the unit A (GrnA) did bind to COMP. Our conclusion is that the granulin A unit of GEP is required and sufficient for its interaction with COMP.

COMP and GEP Co-localized in vivo—Next, we examined the subcellular localization of COMP and GEP to determine whether these two proteins overlap in the same cell. We first transfected RCS cells with plasmids encoding GFP-linked GEP and RED-fused COMP. As revealed in Fig. 5A (a–c), in the living chondrocytes, GEP is clearly expressed and overlaps with COMP, an extracellular matrix protein of chondrocytes. The co-localization between COMP and GEP was further verified with immunostaining for COMP and GEP in primary human chondrocytes. As shown in Fig. 5B (d–f), COMP also co-localizes with GEP predominantly in the pericellular matrix of isolated adult human chondrocytes. These findings are in agreement with the physical interactions detected in the yeast two-hybrid and confirmed by pull-down and co-immunoprecipitation assays and suggest that in chondrocytes, the membrane binding of GEP in chondrocytes may be mediated, at least in part, by the COMP protein.

COMP and GEP Co-localize in the Chondrocytes of the Growth Plate in Vivo—We next examined the in vivo expressions of COMP and GEP and aimed to determine whether these two proteins also show overlapping expression patterns in vivo using immunohistochemistry assays on the 19-day-old embryonic murine limbs. In line with previous findings on COMP expression in vivo, COMP was expressed in both chondrocytes and also osteoblasts (Fig. 6, C and D).
Expression of GEP, however, was localized exclusively to the lower proliferative and upper hypertrophic zones of the growth plate chondrocytes and was absent in osteocytes, osteoblasts, periosteum, and perichondrium (Fig. 6, A and B). Expression of GEP in musculoskeletal tissues appears to be restricted to chondrocytes and is concentrated in areas where ossification will occur (Fig. 6, A and B). Although COMP is also expressed in the chondrocytes of growth plate, it demonstrates a broader expression pattern. Although GEP functions primarily as a secreted growth factor, it was also found to be localized intracellularly and to directly modulate intracellular activities (35, 57–59). Here GEP was also revealed to be intracellular localization in the growth plate chondrocytes of developing cartilage (Fig. 6B).

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FIGURE 4. The GrnA unit of GEP is required and sufficient for interaction with COMP. A, schematic diagram of GEP constructs used to map those of its repeat units that bind to COMP. GEP consists of seven (A–G) and a half (p) tandem repeats of a 12-cysteine motif. Closed boxes, granulin repeats; open boxes, intervening spacer regions; hatched box, signal sequence. Interactions between COMP and GEP units are summarized and indicated by plus or minus signs. B, in vitro GST pull-down assay was used to test interaction between purified His-tagged GEP units and COMP. Purified proteins and GST serve as positive and negative control, respectively. Note that the lower band in lane 1 is a degraded product of purified His-Grn-ACDE.

FIGURE 5. COMP and GEP co-localize in the cell surface of chondrocytes. The top row (a–c) shows the pericellular matrix expression of COMP and GEP in the transfected RCS cells with plasmids encoding red fluorescent protein-linked COMP and GEP-fused GEP, and the bottom row (d–f) shows pericellular matrix staining of COMP and GEP with anti-COMP and anti-GEP antibodies in the isolated human chondrocytes, revealing colocalization of COMP and GEP in specific punctate areas on the cell surface.

FIGURE 6. Immunohistochemistry of COMP and GEP in the section of long bone from a 19-day-old mouse embryo. A, low power microphotograph of a section stained with anti-GEP polyclonal antibody (red) and counterstained with Mayer’s hematoxylin (blue). Immunostaining for GEP demonstrates localization of strongly immunopositive chondrocytes in the lower proliferative/upper hypertrophic zones of the growth plate. B, high power microphotograph of section in A. C, low power microphotograph of section stained with anti-COMP polyclonal antibody (red) and counterstained with Mayer’s hematoxylin (blue); immunostaining reveals positive staining in chondrocytes. D, high power microphotograph of section in C. S, resting chondrocytes; P, proliferating chondrocytes; H, hypertrophic chondrocytes; M, bone metaphysis. Bar, 100 µm.
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that both are true. Briefly, RCS chondrocytes were transfected with different constructs encoding COMP, GEP, or vector only and selected with G418. The overexpressions of GEP and COMP in the resultant stable transfectants were verified using an immunoblotting assay (Fig. 7A), and the stable lines were used for a cell proliferation assay in serum-starved conditions (Fig. 7B). Overexpression of COMP alone produced negligible effects on cell growth, whereas overexpression of GEP resulted in an increase in cell proliferation of 43% by day 3, and robust stimulation was observed by day 4 (1.8-fold) and day 5 (3.5-fold). Intriguingly, this GEP-mediated stimulation was further enhanced by coexpression of COMP. The enhancement was observed as early as day 2 (2.3-fold) and continued through day 5 (2.1-fold increase by day 3, 1.7-fold increase by day 4, and 1.5-fold increase by day 5).

We next examined whether endogenous COMP in chondrocytes is required for GEP-mediated stimulation of cell growth by performing an antibody blocking assay. When compared with the control antibody, the addition of affinity-purified anti-COMP antibody dramatically inhibited GEP-mediated stimulation of chondrocyte proliferation in a dose-dependent manner, clearly indicating that natively expressed COMP is needed for the action of GEP in stimulating cell proliferation (Fig. 7C).

**DISCUSSION**

Yeast two-hybrid screening has proven to be an effective tool in identifying protein interaction (54, 60, 61). To identify protein interaction partners of COMP, an extracellular matrix protein that has been implicated in the regulation of chondrogenesis and cartilage development (3, 6, 53), we screened the yeast expression cDNA library using the EGF repeat domain of COMP as bait and identified the GEP, a growth factor that has not been previously described in cartilage, as a direct binding protein of COMP.

GEP itself is a secreted growth factor with high molecular weight that is involved in various biological and pathological processes, including mesothelial differentiation (44), sexual differentiation of the brain (45), macrophage development (46), rheumatoid arthritis and osteoarthritis (47), and wound response and tissue repair (38, 48). In some cases, GEP may be processed into small 6-kDa peptides, which may be stored in vesicles in the neutrophil. At present, only granulin A/epithelin 1 and granulin B/epithelin 2 have been shown to have biological activities. The actions of the other granulins (GrnC, GrnD, GrnE, GrnF, or GrnG) are unknown (32). Granulin A/epithelin 1 stimulates the proliferation of murine keratinocytes in culture; granulin B/epithelin 2 has no reported proliferative effects, but, at an ~10-fold molar excess, it antagonizes the mitogenic action of granulin A/epithelin 1 (62). Our in vitro binding assay showed that the granulin A (GrnA) unit of GEP is required and sufficient for interaction with COMP, suggesting that COMP might also affect the activity of granulin A under some conditions (Fig. 4).

GEP is a multirepeat glycoprotein that has the potential to interact with other extracellular matrix proteins or cell surface receptors. It is conceivable that important biological functions of GEP are mediated by protein-protein interactions between the functional domains of GEP and its binding proteins. Several GEP-associated partners have been reported and have been found to affect GEP action in various processes. One example of this is the secretory leukocyte protease inhibitor. Elastase digests GEP exclusively in the interepithelin linkers, resulting in the generation of granulin peptides, suggesting that this protease may be an important component of a GEP convertase. Secretory leukocyte protease inhibitor blocks this proteolysis either by directly binding to elastase or by sequestering epithelin peptides from the enzyme (48). It was found that GEP can modulate transcription activities by interacting with human cyclin T1, a component of positive transcription elongation factor b (58), and Tat-positive transcription elongation factor b (57). GEP was also found to interact with perlecan, a heparan sulfate proteoglycan. The perlecan-GEP interaction was suggested to modulate tumor growth (37). Our global screen led to
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the isolation of GEP as a novel binding growth factor of COMP, and the interaction between these two molecules appears to regulate chondrocyte proliferation (Figs. 1, 2, and 7).

COMP has been reported to interact with multiple protein partners, and these interactions are important for its physiologic functions and cytoplasmic processing and transport. COMP appears to mediate chondrocyte attachment via an integrin receptor (1, 8), and several reports suggest that COMP may function to stabilize the articular cartilage extracellular matrix by specific cation-dependent interactions with matrix components, including collagen types II and IX, fibronectin, aggrecan, and matrilin-1, -3, and -4 (16, 63–66). COMP has also been shown to associate with several chaperone proteins, including BiP, calreticulin, protein disulfide, ERp72, Grp94, HSP47, and calnexin, and it has been proposed that these associations facilitate the processing and transport of wild-type COMP in normal chondrocytes and in the retention of mutant COMP in pseudoachondroplasia chondrocytes (67–69). In addition to the interactions between COMP and its protein partners, the five-stranded N-terminal domain of COMP forms a complex with vitamin D₃, illustrating that COMP has a storage function for hydrophobic compounds, including prominent cell-signaling molecules (70). Very recently, we reported that ADAMTS-7 and ADAMTS-12, two members of the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) that share similar domain structure and organization, bind to the same domain of COMP to which GEP binds and degrade COMP in vitro (54, 55). The levels of ADAMTS-7 and ADAMTS-12 are significantly up-regulated in the cartilage and synovium of patients with arthritic diseases (54, 55). It remains to be determined whether GEP competes with ADAMTS-7 or ADAMTS-12 for binding to COMP and thus inhibits their COMP-degrading activities. It also would be worthwhile to determine whether and how GEP, ADAMTS-7, ADAMTS-12, and COMP form a protein-protein interaction network in the regulation of COMP degradation and chondrogenesis.

COMP and GEP co-localize in the extracellular matrix of both transfected RCS cells and primary human adult chondrocytes (Fig. 5, A and B), as well as in growth plate chondrocytes in the day 19 mouse embryo (Fig. 6). These results suggest an in vivo association between COMP and GEP. It is interesting to note that the supercellular localization of GEP in chondrocytes depends on the developmental stage and location, since it is intracellular in the early hypertrophic zone and not in the matrix in the growth plate of 19-day-old embryonic murine limbs (Fig. 6), whereas it is primarily in the pericellular matrix in the chondrocytes isolated from adult human articular cartilage (Fig. 5B). In line with the previous findings, COMP was also primarily intracellular in the early hypertrophic zone but was in the pericellular matrix in the calcifying zone chondrocytes (3, 53, 64). Distinct localization of GEP and COMP in various chondrocytes suggests that they may play important roles at various stages in skeletogenesis.

Overexpression of COMP alone showed negligible effect on cell growth, whereas overexpression of GEP significantly stimulated chondrocyte proliferation; in addition, the GEP-mediated stimulation was further enhanced by coexpression of COMP (Fig. 7A). Intriguingly, blocking COMP activity using antibodies directed against this molecule significantly reduced the GEP-stimulated cell growth; however, stimulation of proliferation by GEP was not totally abolished (Fig. 7B). This finding suggests that other GEP-associated extracellular matrix molecules (possibly perlecan, which has been shown to interact with GEP (37)) may be also involved in the regulation of GEP-mediated chondrocyte proliferation. Although the molecular mechanisms underlying the role of COMP/GEP interaction in the modulation chondrocyte proliferation remain unclear, it is speculated that COMP may act as the co-factor of the GEP cell surface receptor(s) and may present GEP to its receptor, followed by the activations of GEP-mediated signal transduction and gene regulation pathways.

In conclusion, we have first identified GEP in cartilage as a COMP-binding protein, and subsequent characterization of this novel association as well as the functional assays showing that the stimulation of chondrocyte proliferation by GEP growth factor is mediated by COMP extend our understanding of the actions of growth factors in cartilage biology and also provide us a potential target for developing and optimizing the therapeutic application in cartilage repair and arthritic disorders.

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