Cartilage Matrix Protein Forms a Type II Collagen-independent Filamentous Network: Analysis in Primary Cell Cultures with a Retrovirus Expression System

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Cartilage matrix protein (CMP) is expressed specifically in mature cartilage and consists of two von Willebrand factor A domains (CMP-A1 and CMP-A2) that are separated by an epidermal growth factor-like domain, and a coiled-coil tail domain at the carboxyl terminal end. We have shown previously that CMP interacts with type II collagen-containing fibrils in cartilage. In this study, we describe a type II collagen-independent CMP filament and we analyze the structural requirement for the formation of this type of filament. Recombinant wild-type CMP and two mutant forms were expressed in chick primary cell cultures using a retrovirus expression system. In chondrocytes, the wild-type virally encoded CMP is able to form disulfide bonded trimers and to assemble into filaments. Filaments also form with CMP whose Cys⁴⁵⁵ and Cys⁴⁵⁷ in the tail domain were mutagenized to prevent interchain disulfide bond formation. Therefore, intermolecular disulfide bonds are not necessary for the assembly of CMP into filaments. Both the wild-type and the double cysteine mutant also form filaments in fibroblasts, indicating that chondrocyte-specific factors are not required for filament formation. A truncated form of CMP that consists only of the CMP-A2 domain and the tail domain can form trimers but fails to form filaments, indicating that the deleted CMP-A1 domain and/or the epidermal growth factor domain are necessary for filament assembly but not for trimer formation. Furthermore, the expression of the virally encoded truncated CMP in chondrocyte culture disrupts endogenous CMP filament formation. Together these data suggest a role for CMP in cartilage matrix assembly by forming filamentous networks that require participation and coordination of individual domains of CMP.

INTRODUCTION

The unique molecular architecture of the cartilaginous matrix is very evident when the organization of its filamentous network is compared with that of other

tissues. In contrast to tendons that contain straight aligned fibrillar cables and to corneal stroma that has perpendicular layers of fibrils (Birk and Linsenmayer, 1994), the matrix of cartilage contains thin fibrils (5–10 nm) that run in various directions and interconnect with each other to form an integrated network (Mendler *et al.*, 1989; Brewton and Mayne, 1994). Although it has been realized that this unique organization of the matrix in cartilage provides strength and resilience to the tissue, it is not understood how this tissue-specific architecture

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is formed. Cartilage matrix protein (CMP)¹ is one molecule that has been suggested to be involved in the organization and assembly of the cartilage matrix (Paulsson and Heinegård, 1981; Argraves *et al.*, 1987; Winterbottom *et al.*, 1992). CMP is a noncollagenous protein whose gene is expressed specifically during cartilage maturation (Chen *et al.*, 1995). It is distributed in the mature zone and the hypertrophic zone, the two post-mitotic regions of an epiphyseal growth plate during endochondral bone formation. CMP is also found in cartilage from various organs, including tracheal, nasal-septum, xiphisternal, and auricular cartilage (Paulsson and Heinegård, 1982).

The implication of the involvement of CMP in cartilage matrix assembly comes largely from biochemical studies. CMP has been shown to co-fractionate with proteoglycans from cartilage, indicating a possible interaction between these two matrix components (Paulsson and Heinegård, 1979). CMP also distributes along the collagen fibrils of cartilage with a periodicity of 59 nm (Winterbottom *et al.*, 1992). In vitro, CMP is able to bind and concatemerize monomeric type II collagen, the major type of collagen in cartilage (Winterbottom *et al.*, 1992). CMP also has the ability to bind other types of fibrillar collagens such as type I in enzyme-linked immunosorbent assay (Winterbottom *et al.*, 1992). The collagen binding activity of CMP has been localized to the two CMP-A domains of the molecule (Tondravi *et al.*, 1993).

The genomic organization of CMP suggests that the CMP gene is formed through gene duplication and divergence during evolution. Like many extracellular matrix proteins (Engel, 1991), CMP is a mosaic protein that consists of modular units that correspond to the exon structure of the CMP gene (Kiss *et al.*, 1989; Jenkins *et al.*, 1990). CMP is composed of two A domains (CMP-A1 and CMP-A2) located symmetrically at both sides of the central epidermal growth factor (EGF)-like domain, with a signal peptide at the amino-terminal end and a heptad repeat-containing tail at the carboxyl-terminal end. The CMP-A domains and the EGF-like domain occur in other extracellular proteins and extracellular portions of transmembrane proteins, including integrins, collagens, and laminins (reviewed in Engel, 1991; Colombatti *et al.*, 1993). Both A and EGF-like domains have been implicated in protein-protein interactions, however, their precise functions in most cases remain to be determined.

¹ Abbreviations used: CMP, cartilage matrix protein; LP, link protein; CEF, chick embryonic fibroblast; CEC, chick embryonic chondrocyte; PBS, phosphate-buffered saline; RCAS, replication competent avian leukemia virus LTR with a splice acceptor; RCAN, replication competent avian leukemia virus LTR with no splice acceptor; mAb, monoclonal antibody.

CMP occurs as a trimer linked covalently by disulfide bonds. In electrophoretic analysis on SDS-PAGE, CMP migrates as a 200-kDa trimer that is shifted to a 54-kDa monomer upon reduction (Paulsson and Heinegård, 1981; Winterbottom *et al.*, 1992). In images from electron microscopy, CMP is seen as three ellipsoid subunits connected at one end (Hauser and Paulsson, 1994). It has been shown that the carboxyl terminal CMP tail contains heptad repeats (Hauser and Paulsson, 1994) that are important for the formation of trimer through α -helical coiled-coil interaction (Haudenschild *et al.*, 1995). The 11th and 12th cysteines located at the beginning of the coiled-coil domain of the mature CMP (Cys⁴⁵⁵ and Cys⁴⁵⁷) are responsible for covalently connecting the three subunits through disulfide bonds (Haudenschild *et al.*, 1995).

To study the role of CMP in cartilage matrix assembly, in the present study, we examined the appearance of CMP in the matrix of primary chondrocyte cultures. CMP is present in two types of filaments, one that contains type II collagen as we previously observed (Winterbottom *et al.*, 1992), and another that does not contain type II collagen. To analyze the structure-function relationship of CMP in matrix assembly, we expressed recombinant wild-type and mutant CMP in primary cultures using a retrovirus system. Our data have assigned different roles for the individual domains of CMP in matrix organization and suggest that CMP is a "bridge" molecule that connects both collagenous and noncollagenous filaments in cartilage matrix.

MATERIALS AND METHODS

Construction of Retroviral Expression System with Recombinant CMP Transgenes

The retroviral expression system used is the one described in detail by Morgan and Fekete (1994). It consists of two vectors: an adaptor vector SLAX-myc (Hofer *et al.*, 1994), which is a derivative of SLAX 12 (Morgan and Fekete, 1994) and a proviral vector RCAS BP(A) (RC, replication competent; A, avian leukemia virus LTR; S, with a splice acceptor) (Hughes *et al.*, 1987; Petropoulos and Hughes, 1991). The myc-tag fused to the COOH-terminal end of the recombinant protein (Hofer *et al.*, 1994) consists of a 10-amino acid epitope of human c-myc, which is recognized by the monoclonal antibody (mAb) 9E10 (Evan *et al.*, 1985). The myc-tag allows the distinction of the protein encoded by a transgene from the endogenous protein. CMP cDNA was also cloned in the control vector RCAN, which is identical to RCAS except that it does not contain an RNA splice acceptor sequence before the cDNA insertion site (Hughes *et al.*, 1987). Therefore, no protein is expressed from the gene inserted in RCAN, although the replication-competent virus is still formed when RCAN is transfected into cells. This vector serves as a control for possible effects of retroviral transfection and infection.

Two recombinant CMP mutants were generated. In one mutant, CMP-C11/C12-RCAS, Cys⁴⁵⁵ (TGC) and Cys⁴⁵⁷ (TGT) of wild-type CMP were converted to Ser⁴⁵⁵ (TCT) and Ser⁴⁵⁷ (TCC) by site-directed mutagenesis according to the protocol from Bio-Rad (Melville, NY). The second mutant, mini-CMP-RCAS, contains only the COOH-terminal half of the molecule (CMP-A2 domain and the tail domain) fused directly to the CMP signal sequence. This mutant

was constructed by the polymerase chain reaction (PCR)-based method of gene splicing by overlap extension (Horton *et al.*, 1989).

For the cloning of the recombinant CMP into retroviral vectors, cDNAs encoding wild-type and mutant CMP in pcDNA-1 were amplified by PCR using two primers that covered the translation start codon (primer 1) and the stop codon (primer 2) of CMP cDNA, respectively. The two primers were designed in such a way that primer 1 contained a *NcoI* site and primer 2 contained a *NarI* site (primer 1, 5'-GGGTACCATGGACGGG-3'; primer 2, 5'-TGTTCTGGGGCGCCGATGATC-3'). After PCR amplification, all the PCR products were digested with *NcoI* and *NarI*, and they were directionally cloned into the adaptor vector SLAX-myc that had been digested with *NcoI* and *NarI*. The *ClaI* fragment of SLAX-myc that contained the insert was excised and cloned into RCAS or RCAN. The orientation of the insert in RCAS or RCAN was verified by restriction mapping. The entire coding region of each recombinant CMP construct was sequenced to confirm the desired mutations and to exclude possible spontaneous mutations that resulted from PCR amplification.

Establishment of Primary Cultures of Chondrocytes and Skin Fibroblasts with CMP Transgenes in Retroviral Constructs

Primary cultures of chick embryonic chondrocytes (CEC) were established as described previously (Schmid and Conrad, 1982). Sterna from 15-day embryonic chicks were subjected to enzymatic treatment with 0.1% trypsin (Sigma, St. Louis, MO), 0.3% collagenase (Worthington, Freehold, NJ), and 0.1% type 1 testicular hyaluronidase (Sigma) (dissociation medium). After an incubation of 30 min at 37°C, the dissociation medium was removed and replaced with fresh dissociation medium and incubated at 37°C for an additional hour. Chondrocytes were resuspended in plating medium plus 0.01% testicular hyaluronidase (plating medium: 10% fetal bovine serum in Ham F-12 medium [Life Technologies, Grand Island, NY]). After culturing overnight, the medium of the chondrocyte culture was replaced with fresh medium without hyaluronidase. Medium was changed every other day. Cultures were incubated either in the presence or absence of ascorbic acid (100 µg/ml; Sigma) as indicated.

For primary cultures of chick embryonic fibroblasts (CEF), pieces of skin from the dorsal region of 9-day embryonic chick were dissociated in 0.3% collagenase (Worthington, Freehold, NJ) for 1 h at 37°C. Cells were washed and plated in DMEM containing 10% fetal bovine serum (Life Technologies).

Primary cell cultures that produced retrovirus that harbored transgenes were established as described (Morgan and Fekete, 1994). Briefly, confluent fibroblasts were split 1:5 and incubated overnight. Six micrograms of plasmids of retroviral constructs were transfected into fibroblast monolayer culture using the calcium phosphate method (Sambrook *et al.*, 1989). Cultures were incubated for 1 wk. Transfection of the cultures was assessed by Western blot or by immunostaining using mAb 3C2 (Potts *et al.*, 1987) for the detection of the viral gag protein.

For the preparation of recombinant virus the medium of a confluent culture of transfected fibroblasts was replaced by a thin layer of fresh medium. After 24 h, the culture supernatant was collected and debris was removed by centrifuging at 3,750 rpm for 5 min. The supernatant was filtered through a 0.45 micron filter and stored frozen at -80°C. The virus was titrated by the standard method (Morgan and Fekete, 1994).

Infection of primary cultures of CECs or CEFs with recombinant virus was achieved as follows. Confluent cultures were split 1:5 and plated overnight. Cells were infected with a thin layer of filtered culture supernatant that contained the virus. After 1 h of incubation, medium was added to the normal level (15 ml per 100-mm dish). The infected culture was incubated for 1 wk to allow the infection to spread before further characterization.

Analysis of CMP Transgene Expression with Immunofluorescent Histochemistry and Western Blot

Western blot analysis was performed on both the medium and cell extracts. Twenty milliliters of conditioned medium of transfected or infected cultures was collected directly from each plate. After removal of the medium, the cells were rinsed with phosphate-buffered-saline (PBS). One milliliter of extraction buffer (3.8 M urea, 50 mM Tris, pH 8.5, 0.1 mM phenylmethylsulfonyl fluoride) was added to the plates and cells were scraped off the dishes and passed through 21-gauge needles to shear DNA. After incubation for 30 min on ice, the cell extract was centrifuged for 20 min at 4°C and the supernatant was used for electrophoretic analysis on SDS-PAGE. For nonreducing condition, cell extracts or medium were mixed with standard 2× SDS gel-loading buffer (Sambrook *et al.*, 1989). For reducing conditions, the loading buffer contained 5% β-mercaptoethanol and 0.05 M dithiothreitol. Samples were boiled for 10 min before being loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, 15% methanol. The membranes were blocked in 2% bovine serum albumin fraction V (Sigma) in PBS for 30 min and then probed with antibodies. Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (H+L) (Bio-Rad), diluted 1:3,000, was used as a secondary antibody. Visualization of immunoreactive proteins was achieved using the ECL Western blotting detection reagents (Amersham, Arlington Heights, IL) and exposing the membrane to Kodak X-Omat AR film (Rochester, NY).

For immunofluorescence staining, cultured cells were fixed at -20°C with 70% ethanol, 50 mM glycine, pH 2.0, for 20 min. Slides were then washed with PBS and incubated with primary antibodies. After washing with PBS, affinity-purified fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated donkey anti-mouse or donkey anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) were applied with or without Hoechst nuclear dye (0.5 mg/ml). Slides were washed and mounted in 95% glycerol in PBS. Single or multiple exposure photography was performed with a microscope from Nikon (Melville, NY).

The mAbs used were 1H1, which recognizes CMP, and 4B6, which recognizes link protein (LP). Both mAbs were generated as described in detail by Binette *et al.* (1994). mAb II-II6B3 was directed against a helical epitope in type II collagen (Linsenmayer and Hendrix, 1980). mAb 3C2 was raised against the viral matrix protein encoded by gag in avian retrovirus (Potts, 1987). mAb 9E10 was raised against an epitope in human c-myc (Evan *et al.*, 1985). Two polyclonal rabbit antibodies against CMP were used. RC2 was generated against purified chicken CMP, and D2/1496 was made against a synthetic peptide whose sequence is derived from the CMP-A2 domain (Haudenschild *et al.*, 1995). Both antibodies were purified by chromatography on a CMP affinity column and they were characterized in Western blots to recognize CMP specifically.

RESULTS

CMP Forms Two Distinct Pools of Filaments in Chondrocyte Culture

To study the role of CMP in cartilage matrix assembly, we examined the appearance of CMP in the matrix deposited by CEC in primary cultures. CECs were isolated from 15-day embryonic chick sterna and cultured in the presence of 10% fetal bovine serum and 100 µg/ml ascorbate. An immunohistochemical analysis of the cell layer on the 7th day of

culture with anti-CMP mAb 1H1 indicates that CMP filaments exist in several forms that are found in different locations in the cultures (Figure 1, A and B). One form consists of strings of multiple interconnected filaments that link different islands of chondrocyte colonies. These filaments (arrow in Figure 1A) span a considerable distance of up to 50 μm , which contains no cells. Another form makes up a network of fine CMP filaments that are most prominent around and between the chondrocyte colonies (arrow in Figure 1B), but are not detected in the extracellular spaces distant from the colonies. Filaments stem directly from the colonies of cells (Figure 1, A and B), suggesting that the filaments originate from the chondrocytes.

Fibrillar collagen, including type II collagen, can form fibers connecting colonies of cells (Stopak and Harris, 1982) and because we have shown previously that CMP is associated with type II collagen

(Winterbottom *et al.*, 1992), we determined whether the observed CMP filaments were associated with type II collagen. Double immunofluorescent staining with polyclonal antiserum RC2 against CMP, and the mAb II-II6B3 against type II collagen, indicates that there are two pools of extracellular CMP filaments (Figure 1, C and D). One type of filament contains both CMP and type II collagen (yellow filaments pointed by blank arrowheads). Another type of filament contains CMP without type II collagen (green filaments pointed by filled arrowheads). The type II collagen-containing CMP filaments are seen connecting cells whereas the type II collagen-independent CMP filaments form the finer pericellular networks (Figure 1D). The two pools of CMP filaments are connected and they form an integrated continuous network (see Figure 1, C and D).

Figure 1. Two distinct pools of CMP in chondrocyte culture. (A and B) Immunostaining of chondrocyte cultures with a mAb against CMP, 1H1. The secondary antibody was rhodamine-coupled donkey anti-mouse antiserum (red). Arrows point to CMP filaments. (C and D) Double immunostaining of chondrocyte culture with a mAb against type II collagen, II-II6B3 (rhodamine; red), and a polyclonal Ab against CMP, RC2 (fluorescein; green). Overlapping of type II collagen (red) and CMP (green) results in a yellow color. Nuclear Hoechst dye staining (blue) indicates location of cells. Open arrow heads point to the type II collagen containing CMP filaments (yellow), and filled arrowheads point to type II collagen-independent CMP filaments (green). Bar, 20 μm .

Figure 2. Two pools of CMP filaments can be separated by the presence or absence of ascorbate in chondrocyte cultures. (A and B) Micrographs from the same field of chondrocytes cultured in the presence of 100 $\mu\text{g}/\text{ml}$ ascorbate. (C and D) Micrographs from the same field of chondrocytes cultured in the absence of ascorbate. Panels A and C are staining patterns with a mAb against type II collagen (II-II6B3), and panels B and D are staining patterns with a polyclonal Ab against CMP (RC2). Bar, 40 μm .

Separation of the Two Pools of CMP Filaments by Metabolic Regulation

To separate the two pools of CMP filaments present in cell culture effectively, CECs were cultured either in the presence or absence of ascorbate. Because the secretion and deposition of collagen fibrils are dependent on the presence of ascorbate (Pacifci, 1990), we reasoned that the type II collagen-containing fibrils would be eliminated from ascorbate-deficient CEC cultures. Indeed, in the presence of ascorbate, both type II collagen fibrils (Figure 2A) and CMP filaments (Figure 2B) are present. In the absence of ascorbate, type II collagen is seen intracellularly, and there are no extracellular type II collagen fibrils detected (Figure 2C). However, the finer pericellular CMP filaments are present in ascorbate-deficient cultures (Figure 2D), confirming the existence of type II collagen-independent CMP filaments. The type II collagen-dependent CMP filaments require ascorbate whereas the type II collagen-independent CMP filaments do not. Therefore, the two pools of CMP filaments can be separated experimentally in cell culture by either including or excluding ascorbate in the medium. All the experiments that follow were performed with cells cultured in the absence of ascorbate to analyze the assembly of the type II collagen-independent CMP filaments.

Characterization of Recombinant CMP Expressed in Primary Cell Cultures by a Retroviral System

Viral constructs carrying recombinant wild-type CMP (CMP-RCAS), the two mutant forms of CMP (CMP-C11/C12-RCAS and mini-CMP-RCAS) (Figure 3A),

and the control viral construct CMP-RCAN were transfected into cultured CECs that synthesize endogenous CMP, and into cultured CEFs that do not synthesize endogenous CMP. All viral constructs actively produce virus in cultures of transfected CEFs as indicated from the positive reactivity for an 80-kDa viral matrix protein encoded by gag (Figure 3B, arrow). This is in contrast to the mock (no DNA constructs) transfected cultures that are negative for this protein. Our unpublished data show the identical results from mock transfection of CECs.

The recombinant CMP expressed by transfected CEFs was characterized by its reactivity with antibodies against CMP and by its electrophoretic mobility in denaturing SDS gels (Figure 3C). Recombinant wild-type CMP migrates as a trimer of 200 kDa under nonreducing conditions, and as a monomer of 54 kDa under reducing conditions (Figure 3C, CMP-RCAS). This electrophoretic behavior is identical to that of CMP purified from cartilage (Figure 3C, purified CMP) demonstrating that the retrovirus system can be used to produce proteins with properties that are identical to those purified from tissue. The mini-CMP-RCAS-transfected cells synthesize a transgene product with an electrophoretic mobility of 100 kDa and 30 kDa under nonreducing and reducing conditions, respectively (Figure 3C, mini-CMP-RCAS), indicating that the truncated CMP forms trimers. Therefore, the information for trimer formation resides in the CMP-A2 domain and in the coiled-coil tail that make up the mini-CMP. The cells transfected with CMP C11/C12-RCAS produced a band of CMP around 54 kDa under both reducing and nonreducing conditions

Figure 3. (A) Recombinant CMP constructs in retrovirus. 1. CMP, myc-tagged wild-type CMP; 2. CMP-C11/C12, myc-tagged CMP in which the codons for the 11th and 12th cysteines were converted to those for serines through site-directed mutagenesis; and 3. mini-CMP, myc-tagged truncated CMP in which the A1 domain and the EGF domain were spliced out. (B) Production of retrovirus in cultures of CEFs. Extracts of CEFs were separated by SDS-PAGE under reducing condition, blotted to membranes, and probed with a mAb 3C2 against a viral gag protein (Potts, 1987). Seven and one-half microliters of extracts were loaded in each lane. The arrow points to the 80-kDa gag protein, which is present in cultures of CEFs transfected with retroviral constructs, but is absent in mock-transfected CEFs. (C) Expression of CMP transgenes by CEFs. Western blot of cell lysates from cultures of transfected CEFs. Seven and one-half microliters of cell lysates were loaded in each lane. The antibody used to detect CMP was 1H1 in all the cultures except in mini-CMP RCAS-transfected cell culture, where D2/1476 was used. Arrows point to the translational products of CMP transgenes recognized by anti-CMP antibodies. (D) Expression of CMP transgenes in cultures of both CEFs and CECs. Samples were subject to electrophoresis under nonreducing conditions. Seven and one-half microliters of cell lysates or conditioned medium were loaded in each lane. Left panel, extracts of cell lysates from cultures of CEFs; central panel, conditioned medium from cultures of CEFs; and right panel, conditioned medium from cultures of CECs.

(Figure 3C, CMP-C11/C12-RCAS). This indicates that these two cysteines are responsible for forming intermolecular disulfide bonds to covalently link CMP into trimers. In both CMP-RCAN or mock-transfected CEFs, no CMP is detected (Figure 3C). Therefore, CEFs do not synthesize endogenous CMP. The recombinant CMP synthesized by transfected CEFs was also secreted by them. This is shown by the presence of recombinant CMP in the conditioned medium of transfected CEFs (Figure 3D, compare CEF anti-CMP extract and medium). Because each lane contains the same volume of medium (7.5 ml) and the densities of the three recombinant CMPs are similar (Figure 3D, CEF anti-CMP medium), we conclude that all three

recombinant CMPs are present in the conditioned medium at a similar level.

Recombinant CMP was also expressed successfully by transfected CECs, which synthesize endogenous CMP. The transgene encoded CMP was distinguished from the endogenous CMP by using the anti-myc mAb 9E10, which only detects myc-tagged recombinant CMP, but not the endogenous CMP (Figure 3D, CEC anti-Myc tag medium). Our unpublished data show the identical results from the extracts of transfected CECs. The fact that the electrophoretic behavior of the proteins encoded by the transgenes is indistinguishable from that of CMP extracted from tissues indicates that the myc-tag at the C-terminal end of the

Figure 4. Micrographs of immunofluorescent analysis of cell cultures with a mAb against the myc-tag 9E10. (A–D) Chondrocyte cultures. (E–H) Fibroblast cultures. Note the difference of cell shape between CECs and CEFs. (A and E) CMP-RCAS transfected; (B and F) CMP C11/C12-RCAS transfected; (C and G) mini-CMP-RCAS transfected; (D and H) CMP-RCAN transfected. Bar, 40 μ m.

virally encoded proteins does not interfere with either protein secretion, or structural properties of CMP such as trimerization.

Formation of Filamentous Network by Virally Encoded Wild-Type CMP

To determine whether the recombinant CMP forms filaments, we performed immunofluorescence analysis on transfected CECs in culture. The recombinant wild-type CMP assembles into filaments in primary culture of CECs (Figure 4A). These recombinant CMP filaments resemble the endogenous filaments (Figure 1). This observation demonstrates that the retroviral expression system can be used to study ECM assembly in cell culture. To examine whether the CMP synthesized endogenously by the chondrocytes is required for the assembly of the virally expressed CMP, we examined the cultures of transfected CEFs for the formation of CMP filaments. Although CEFs do not synthesize endogenous CMP (Figure 3, C and D), filaments also form in these cultures (Figure 4E). Thus, type II collagen-independent CMP filaments can form in the absence of any endogenous CMP, and no chondrocyte-derived factors are necessary for their formation.

Intermolecular Disulfide Bonds Are Not Required for the Formation of CMP Filaments

CECs transfected with CMP-C11/C12-RCAS form type II collagen-independent filaments (Figure 4B) that resemble those that result from the assembly of wild-type CMP (Figure 4A). This observation suggests that covalent inter-chain disulfide bonds in full-length CMP are not required for filament formation. The inclusion of C11/C12 CMP into filaments in chondro-

cyte cultures, however, could result from interactions of the mutant protein with the endogenous wild-type CMP synthesized by the chondrocytes. This possibility was tested by expressing CMP-C11/C12 mutant protein in cultures of CEFs that do not synthesize any endogenous CMP (Figure 3, C and D). In such cultures CMP filaments were also detected (Figure 4F). This result indicates that stable disulfide-bonded trimers are not necessary for the formation of type II collagen-independent CMP filaments in a nonchondrocyte cellular environment.

The CMP-A1 Domain and EGF-like Domain Are Necessary to Form CMP Filaments

In contrast to the full-length wild-type CMP and the full-length C11/C12 mutant CMP, the mini-CMP fails to form filaments in the cultures of either transfected CECs (Figure 4C) or transfected CEFs (Figure 4G), although the cells actively synthesize mini-CMP as demonstrated by intense intracellular staining (Figure 4, C and G) and from the presence of mini-CMP in the medium (Figure 3D). Therefore, the deleted CMP-A1 domain and the EGF-like domain are necessary for CMP assembly into filaments and the formation of trimers is not sufficient for filament assembly.

Mini-CMP Is a Dominant Negative Mutation of CMP Filament Formation

Because mini-CMP does not assemble into filaments but is able to form trimers, we tested the effect of mini-CMP on the formation of endogenous CMP filaments by chondrocytes. Over-expression of the mini-CMP transgene could have a dominant negative effect on the function of CMP by sequestering wild-type CMP and disrupting endogenous filament assembly.

Figure 5. Mini-CMP disrupts the formation of endogenous CMP filaments. (A–D) micrographs of immunofluorescent analysis of cultures of CECs with a mAb against CMP, 1H1. (A) CMP-RCAS transfected; (B) CMP-C11/C12-RCAS transfected; (C) Mini-CMP-RCAS transfected; and (D) CMP-RCAN transfected. Bar, 40 μm. (E) Mini-CMP forms heterotrimers with endogenous full-length CMP. Western blot of conditioned medium from cultures of CECs transfected with mini-CMP-RCAS (mini-CMP) and mock-transfected (mock). Seven and one-half microliters of conditioned medium were loaded in each lane. Band 1, (CMP)₃; band 2, (CMP)₂(mini-CMP); band 3, (CMP)(mini-CMP)₂; and band 4, (mini-CMP)₃.

To test this hypothesis, CECs transfected with mini-CMP-RCAS were examined by immunostaining with mAb against CMP, 1H1. Because the epitope recognized by 1H1 is localized in the EGF-like domain, the mAb does not react with mini-CMP, but detects endogenous full-length CMP synthesized by chondrocytes (Figure 3A). In the culture of CECs transfected with mini-CMP RCAS, no endogenous CMP filament network is seen (Figure 5C). However, CMP filaments are detected in CECs transfected with CMP-RCAS (Figure 5A), with CMP C11/C12-RCAS (Figure 5B), and mock-transfected CECs (no DNA) (Figure 5D). Thus, mini-CMP disrupts the assembly of endogenous CMP into filaments. These data demonstrate that mini-CMP behaves as a dominant negative mutation of CMP filament assembly.

To examine whether the dominant negative mutation effect of the mini-CMP results from the formation of heterotrimers with endogenous full-length CMP, the conditioned medium of the culture of CECs transfected with mini-CMP was collected and analyzed with antibodies against CMP on a nonreducing Western blot. With mAb 1H1, which recognizes only full-length CMP but not the mini-CMP (see Figure 3A), one band representing the 200-kDa full-length CMP homotrimer is seen in mock-transfected CECs (Figure 5E, 1H1 mock). In CECs transfected with mini-CMP, two extra bands of lower molecular mass are seen (Figure 5E, 1H1 mini-CMP). They represent the heterotrimer of two full-length CMP with one mini-CMP and another heterotrimer of one full-length CMP with two mini-CMP. The homotrimer of mini-CMP is not detected with 1H1, because the 1H1 epitope region is not included in mini-CMP (see Figure 3A). However, this 100-kDa band can be detected with D2/1476, an antibody against an epitope in CMP-A2 domain (Figure 5E, D2 mini-CMP). The intensity of this band reflects the overexpression of the mini-CMP transgene

and suggests that the dominant negative effect is due to its over-expression. The over-expression of mini-CMP, however, does not affect the expression of other proteins by CECs, such as another major glycoprotein in cartilage, the 45-kDa LP (Figure 5E, LP 4B6).

DISCUSSION

In the present study we describe and characterize the CMP filamentous network in primary chondrocyte culture. This network consists of two major types of filaments. One contains type II collagen and the other does not. These two types of filaments can be separated experimentally by manipulating the ascorbic acid content in the chondrocyte culture medium. In the absence of ascorbate, only the type II collagen-independent CMP filaments are deposited because type II collagen is not secreted by the chondrocytes under such conditions (Pacifi, 1990; Shapiro *et al.*, 1991). Although there are two different types of CMP filaments in the matrix deposited by chondrocytes, they form a continuous, connected, and integrated filamentous network (Figure 1). The common component shared by these two types of filaments is CMP. Therefore, CMP may play a role in integrating the collagenous filament network with the noncollagenous filament network in cartilage matrix.

The role of CMP proposed here is consistent with the temporal and spatial expression pattern of CMP during development. We have shown previously that the CMP gene is not transcribed at the initiation of cartilage formation when other genes encoding major components of cartilage matrix such as type II collagen, aggrecan core protein, and LP begin to be transcribed (Stirpe and Goetinck, 1989). Furthermore, we have observed a correlation between initiation of CMP synthesis with cessation of chondrocyte proliferation and initiation of cartilage maturation (Chen *et al.*, 1995). Taken together, our data suggest that one of the functions of CMP is to modify and integrate pre-

existing nascent type II collagen filaments and noncollagenous filaments in cartilage matrix. Therefore, CMP may play an important role of stabilizing matrix during cartilage maturation in skeletal development.

The recombinant CMPs were expressed in primary cell cultures using a retroviral expression system to evaluate the role of CMP in matrix assembly. A similar retroviral system has been used for *in vivo* studies such as cell lineage analysis (Cepko, 1992), tissue transplantation (Fekete and Cepko, 1993), and ectopic expression of homeobox genes and the sonic hedgehog gene in chick limb buds (Morgan *et al.*, 1992; Riddle *et al.*, 1993). We demonstrate here that the retrovirus expression system can be used successfully for studying protein assembly in cell culture. In comparison to other expression systems, the retroviral expression system has several advantages. It has higher transfection/infection efficiency in primary cell cultures, and the recombinant gene of interest that has been studied in cell culture can be further analyzed in specific tissues *in ovo*, through the injection of virus particles into chicken embryos (Cepko, 1992).

Two mutant CMPs were generated and expressed to analyze the structural requirement of CMP filament assembly. In one mutant, the construct was designed in such a way that the truncation occurred in the middle of the molecule, at the boundary between exon 4 and exon 5 of the CMP gene (Kiss *et al.*, 1989), and the amino-terminal half of the molecule is deleted. To ensure that the truncated CMP (mini-CMP) is secreted by the cells, the portion encoding the signal peptide was included with the construct. Western blot analysis of lysate and conditioned culture medium of infected cells indicates that the mini-CMP is successfully expressed and secreted. The mini-CMP forms trimers of a smaller size compared with trimers of wild-type full-length CMP. The mini-CMP trimers become mini-CMP monomers under denaturing and reducing conditions (Figure 3). This indicates that the CMP-A2 domain and the heptad repeat domain that make up the mini-CMP are sufficient for the formation of trimers linked by intermolecular disulfide bonds.

In the second CMP mutant, the 11th and 12th cysteines in the mature CMP located at the beginning of the heptad repeat domain were replaced by serines. The C11/C12-CMP mutant migrated as a monomer under both reducing and nonreducing conditions. Similar results were obtained when such mutant forms were expressed transiently in COS cells (Haudenschild *et al.*, 1995). This indicates that these two cysteines are responsible for forming intermolecular disulfide bonds connecting the three subunits. Such pairs of closely spaced cysteines are also found at the borders of coiled-coil domains of laminins, tenascins, and fibrinogens (Beck *et al.*, 1992). Our data here suggest that CMP forms disulfide rings between three chains through C11/C12, in a similar arrangement as

the "disulfide-knobs" proposed for the fibrinogen trimer (Doolittle, 1984).

The type II collagen-independent CMP filaments were analyzed in primary cell cultures by transfecting myc-tagged recombinant CMP into chondrocytes. The C11/C12-CMP mutant that migrated as a monomer on denaturing SDS-PAGE, assembled into filaments in culture of chondrocytes just like the wild-type recombinant CMP. There are several possible explanations for the existence of the C11/C12-CMP filaments in chondrocyte cultures. C11/C12-CMP may exist as a monomer that either associates with the endogenous CMP filamentous network, or forms filaments without trimer formation. This does not seem to be the case because, first, the CMP monomers that lack the α -coiled-coil tails fail to form filaments (Chen *et al.*, unpublished observations), and second, the translation product of the C11/C12-CMP transgene assembles successfully into filaments in culture of fibroblasts that do not synthesize endogenous CMP (Figure 4). Alternatively, CMP may trimerize through the α -coiled-coil tail, and the intermolecular disulfide bonds only stabilize the trimeric structure. Thus the trimeric C11/C12-CMP is able to assemble into a filamentous network just like wild-type CMP. This hypothesis is consistent with the observations that the formation of a CMP trimer is disrupted when the coiled-coil α -helix-containing tail is deleted and the 11th and 12th cysteines are still present (Haudenschild *et al.*, 1995), and that the trimeric structure of CMP is retained after complete reduction under native conditions (Hauser and Paulsson, 1994). Our data demonstrate that the intermolecular C11/C12 disulfide bonds are not necessary for the formation of the CMP filaments, although they could still be important for stabilizing the filamentous network.

In culture of fibroblasts, mini-CMP fails to form filaments, although it is synthesized and secreted by the cells, as demonstrated by the intensive intracellular staining and the presence of the product in the culture medium. This indicates that the deleted CMP-A1 domain and the EGF-like domain located at the amino-terminus of CMP, are necessary to interact with noncollagenous ligands to form filaments. When the mini-CMP transgene is expressed in chondrocytes that synthesize endogenous CMP, mini-CMP not only fails to form its own network, but also disrupts the formation of the endogenous CMP network. This occurs by mini-CMP sequestering endogenous full-length CMP through formation of CMP heterotrimers (Figure 5). Thus, mini-CMP would be an ideal dominant negative mutant to test the function of CMP filament network *in vivo*. Dominant negative mutations involving similar mechanisms of faulty assembly were also found to occur naturally in other extracellular matrix molecules such as type I collagen in osteogenesis imperfecta (OI). In some of the OI patients,

Figure 6. Model of two step assembly of CMP filaments. Step 1, CMP monomers assemble into a trimer; step 2, CMP trimers assemble into CMP filamentous networks.

the three propeptide chains of type I collagen are assembled through interactions of the normal carboxyl-terminal domains, but are defective in their functions because of deletions or mutations in their triple helical domains at the amino end (Prockop and Kivirikko, 1984).

The type II collagen-independent CMP filaments could result from the self-assembly of CMP. The formation of such a CMP filamentous network in cultures of both chondrocytes and fibroblasts that harbor the CMP transgene, two types of cells with very different synthetic repertoires, seems to support this notion. The CMP filaments may also interact with other matrix components. For example, some A-domain-containing proteins are able to interact with hyaluronic acid, heparin, and adhesive glycoproteins (Kielty *et al.*, 1992; Colombatti *et al.*, 1993). Our unpublished results indicate that the CMP filamentous network overlaps partially with both fibronectin and LP-containing matrix networks, suggesting a possible interaction between these networks. The CMP filamentous network is resistant to hyaluronidase treatment.

Based on our data here, we propose a model for supramolecular assembly of CMP (Figure 6). In the first step of assembly, CMP forms a trimer. This step is facilitated by the carboxyl-terminal half of the molecule, which is sufficient to form a trimer. The C11/C12 disulfide bonds would further stabilize the trimer, but may not be required for its formation (Hauser and Paulsson, 1994; Haudenschild *et al.*, 1995). In the second step, the CMP trimers interact with the ligands in the cartilage matrix through all three subunits to build

a three dimensional network. The amino-terminal half of the molecule is necessary for CMP to interact with matrix ligands, which may include both collagenous (Winterbottom *et al.*, 1992) and noncollagenous matrix components (Paulsson and Heinegård, 1979). Thus, CMP could serve as a "three-way bridge" molecule connecting collagenous to collagenous filaments, noncollagenous to noncollagenous filaments, and collagenous to noncollagenous filaments within the cartilage matrix. To achieve its bridge function, CMP requires the participation and coordination of its different domains—the carboxyl-terminal domains to form a trimer, and the amino-terminal domains to interact with ligands in the matrix.

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