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# Assembly of a Novel Cartilage Matrix Protein Filamentous Network: Molecular Basis of Differential Requirement of von Willebrand Factor A Domains

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Cartilage matrix protein (CMP) is the prototype of the newly discovered matrilin family, all of which contain von Willebrand factor A domains. Although the function of matrilins remain unclear, we have shown that, in primary chondrocyte cultures, CMP (matrilin-1) forms a filamentous network, which is made up of two types of filaments, a collagen-dependent one and a collagen-independent one. In this study, we demonstrate that the collagen-independent CMP filaments are enriched in pericellular compartments, extending directly from chondrocyte membranes. Their morphology can be distinguished from that of collagen filaments by immunogold electron microscopy, and mimicked by that of self-assembled purified CMP. The assembly of CMP filaments can occur from transfection of a wild-type CMP transgene alone in skin fibroblasts, which do not produce endogenous CMP. Conversely, assembly of endogenous CMP filaments by chondrocytes can be inhibited specifically by dominant negative CMP transgenes. The two A domains within CMP serve essential but different functions during network formation. Deletion of the A2 domain converts the trimeric CMP into a mixture of monomers, dimers, and trimers, whereas deletion of the A1 domain does not affect the trimeric configuration. This suggests that the A2 domain modulates multimerization of CMP. Absence of either A domain from CMP abolishes its ability to form collagen-independent filaments. In particular, $Asp^{22}$ in A1 and $Asp^{255}$ in A2 are essential; double point mutation of these residues disrupts CMP network formation. These residues are part of the metal ion–dependent adhesion sites, thus a metal ion–dependent adhesion site–mediated adhesion mechanism may be applicable to matrilin assembly. Taken together, our data suggest that CMP is a bridging molecule that connects matrix components in cartilage to form an integrated matrix network.

## INTRODUCTION

In a connective tissue such as cartilage, extracellular matrix (ECM) molecules mediate cell–matrix and matrix–matrix interactions, thereby providing tissue integrity. Matrilins, a novel ECM protein family with undefined functions, consist at least of four members (Wagener *et al.*, 1998). Cartilage matrix protein (CMP), the first member of the matrilin family, is expressed exclusively during cartilage maturation (Chen *et al.*, 1995a). Although matrilin-3 is also expressed in cartilage, matrilin-2 is absent from cartilage but present in bone, uterus, and heart (Deak *et al.*, 1997). Matrilin-4 is prominent in lung, with weak expressions in brian, sternum, kidney, and heart (Wagener *et al.*, 1998). All

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Abbreviations used: CEF, chick embryonic fibroblast; CEC, chick embryonic chondrocyte; CMP, cartilage matrix protein; ECM, extracellular matrix; EGF, epidermal growth factor; MIDAS, metal ion–dependent adhesion site; RCAN, replication-competent avian leukemia virus long terminal repeat with no splice acceptor; RCAS, replication-competent avian leukemia virus long terminal repeat with a splice acceptor.

the members of matrilin family contain von Willebrand factor A domains, epidermal growth factor (EGF)-like domains, and a heptad repeat coiled-coil domain at the C-terminal end. Although CMP (matrilin-1) and matrilin-2 and -4 contain two A domains (A1 and A2) separated by EGF-like domains (Wagener *et al.*, 1998), matrilin-3 lacks the A2 domain (Wagener *et al.*, 1997). It is not known whether the lack of one A domain from a matrilin affects its assembly.

The assembly of CMP, the prototype of the matrilin family, consists of two steps, as demonstrated by our mutational analyses (Chen *et al.*, 1995b; Haudenschild *et al.*, 1995). At the first step, the CMP trimer is formed by the C-terminal coiled-coil domain acting as nucleation sites. The trimers are subsequently stabilized through disulfide bonds involving two cysteines at the beginning of the coiled coil ($Cys^{432}$ and $Cys^{435}$; numbering of amino acids refers to the mature form of avian CMP) (Haudenschild *et al.*, 1995; Beck *et al.*, 1996). In support of this evidence, the CMP trimer is seen by electron microscopy as three compact ellipsoids connected at one end (Winterbottom *et al.*, 1992; Hauser and Paulsson, 1994). At the second step, the CMP trimers interact with ligands in the cartilage matrix through all three subunits to build a network. The N-terminal half of the molecule including the A1 domain and the EGF domain is necessary for the formation of the network (Chen *et al.*, 1995b). However, it is not known in which domain the CMP matrix adhesion sites reside.

Recent data suggest that the matrix adhesion sites of CMP may reside in one or both of the A domains. The A domain is a domain present in many molecules that are involved in cell–cell, cell–matrix, and matrix–matrix interactions. The A domain is found in plasma proteins such as von Willebrand factor, transmembrane proteins such as the $\alpha$ subunits of seven integrins, in which it is also called the I domain, and ECM proteins such as the microfibrillar collagen type VI ($\alpha$1, $\alpha$2, and $\alpha$3), the anchoring fiber collagen type VII ($\alpha$1), the fibril-associated collagens type XII ($\alpha$1) and type XIV ($\alpha$1), and the matrilin family (for review, see Colombatti *et al.*, 1993; Lee *et al.*, 1995b). The A domains have been shown to bind a variety of ligands, including collagen, laminin, and the glycosaminoglycans heparin and hyaluronan (Kielty *et al.*, 1992; Colombatti *et al.*, 1993; Calderwood *et al.*, 1997; Dickson *et al.*, 1997). In this study, we test whether the A domains in CMP are essential for its assembly.

The adhesive property of the A domain from the $\alpha$ subunit of integrin CR3 has been proposed to be mediated by a specific metal ion–dependent adhesion site (MIDAS) within the domain. The MIDAS motif may consist of five amino acids (DXSXS, T, and D) that contribute to divalent cation ($Mg^{2+}$) coordination. In addition, the sixth amino acid (E) from an A domain of another molecule may also participate in the coordination (Lee *et al.*, 1995a,b; Qu and Leahy, 1995). The mutagenesis data of the $\alpha$ subunit of the integrins support this model. Mutating either one of the two aspartate (D) residues within the MIDAS motif destroys the cation binding as well as the ligand binding (Michishita *et al.*, 1993). Although this model is supported by data from different laboratories (Goodman and Bajt, 1996; Puzon-McLaughlin and Takada, 1996), some A domains do not necessarily have this function (Baldwin *et al.*, 1998). In this study, we test whether MIDAS motifs are involved in CMP assembly by point mutations within these motifs of CMP.

In a previous study, we observed that CMP forms a filamentous network in a primary chondrocyte culture system (Chen *et al.*, 1995b). This network consists of at least two types of CMP filaments; one associates with the type II collagen-containing fibrils, and the other does not. In this study, we show that the collagen-independent CMP filaments are rich in the pericellular region, connecting a chondrocyte to its interstitial matrix. The A domains are essential for the CMP assembly process. Deletion of either A domain abolishes the filament formation. In addition, the A2 domain plays a role in regulating the multimerization of CMP. The lack of this domain converts the trimeric configuration into a mixture of monomers, dimers, and trimers. The mutations within the A domains suggest that CMP may use an adhesion mechanism similar to the one that is responsible for integrin–ligand interactions.

## MATERIALS AND METHODS

### *Primary Cultures of Chondrocytes and Skin Fibroblasts*

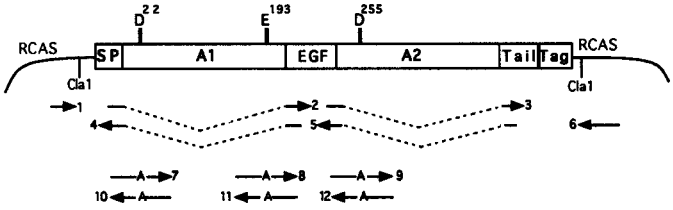
Primary cultures of chick embryonic chondrocytes (CECs) were established as follows. Sterna from 15-d embryonic chicks were subjected to enzymatic treatment with 0.1% trypsin (Sigma, St. Louis, MO), 0.3% collagenase (Worthington, Freehold, NJ), and 0.1% type 1 testicular hyaluronidase (Sigma) (dissociation 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 1 h. Chondrocytes were resuspended in Ham's F-12 medium containing 10% FBS (Life Technologies, Grand Island, NY) and 0.01% testicular hyaluronidase. After culturing overnight, the medium was replaced with fresh medium without hyaluronidase. Medium was changed every other day.

For primary cultures of chick embryonic fibroblasts (CEF), pieces of skin from the dorsal region of 9-d embryonic chick were dissociated in 0.3% collagenase (Worthington) for 1 h at 37°C. Cells were washed and plated in Dulbecco's modified Eagle's medium containing 10% FBS (Life Technologies). Both CECs and CEFs were incubated either in the presence or absence of ascorbate acid (100 $\mu$g/ml; Sigma) as indicated.

### *Expression of CMP Transgenes in Cell Culture*

To express CMP transgenes in primary cultures of fibroblasts and chondrocytes we used the retroviral expression system described previously (Chen *et al.*, 1995b). It consists of two vectors: an adaptor vector, SLAX-myc (Haudenschild *et al.*, 1995), which is a derivative of SLAX 12 (Morgan and Fekete, 1994), and a proviral vector, replication-competent avian leukemia virus long terminal repeat

A




B

Primer List:

1. CCTCGCGTACCACTGTGGCA
2. GTCTGGGGGGCTTGCAGTGGT
3. ACCTGCAGTGAGGAAGATCCATGCGAATGT
4. ACTGCAAGCCCCCCAGACTCCACAGCTCTG
5. ATCTTCCTCACTGCAGGTCTTCCCATCATT
6. TACAACCGAAGCCCCGCT
7. CATCATTGCCAGCTCTCGGA
8. AGTTTCAAGCAGCGTTCTGT
9. CCTGATCGCTGGCTCCAAGA
10. TCCGAGAGCTGGCAATGATG
11. ACAGAACGCTGCTTGAAACT
12. TCTTGGAGCCAGCGATCAGG

C

| Construct Name | Primer A | Primer B | Primer C | Primer D | Template Name | Mutations in Construct |
|---|---|---|---|---|---|---|
| Wild-type CMP | N/A | N/A | N/A | N/A | From cDNA | none |
| CMPΔA1 | 1 | 4 | 2 | 6 | Wild-type CMP | A1-Less |
| CMPΔA2 | 1 | 5 | 3 | 6 | Wild-type CMP | A2-Less |
| CMP-D1 | 1 | 10 | 7 | 6 | Wild-type CMP | $D^{22}$ to A |
| CMP-D2 | 1 | 12 | 9 | 6 | Wild-type CMP | $D^{255}$ to A |
| CMP-E1 | 1 | 11 | 8 | 6 | Wild-type CMP | $E^{193}$ to A |
| CMP-D1D2 | 1 | 12 | 9 | 6 | CMP-D1 | $D^{22}$ to A,$D^{255}$ to A |
| CMP-D1E1 | 1 | 11 | 8 | 6 | CMP-D1 | $D^{22}$ to A, $E^{193}$ to A |
| CMP-D2E1 | 1 | 12 | 9 | 6 | CMP-E1 | $D^{255}$ to A, $E^{193}$ to A |
| CMP-D1D2E1 | 1 | 12 | 9 | 6 | CMP-D1E1 | $D^{22}$ to A, $D^{255}$ to A,$E^{193}$ to A |

**Figure 1.** Construct production and point mutations. (A) The relative locations of the primers used to produce the various CMP constructs are shown underneath the schematic model of CMP. The amino acid residues in MIDAS were mutated to alanine residues indicated by A. (B) The sequences of all primers used in 5′ to 3′ orientation are indicated. The primers are numbered as in A. The mutated codons are listed in bold. (C) The primer pairs, templates, and introduced mutations for the single or recombinant PCRs are listed.

with a splice acceptor (RCAS) BP(A) (Hughes *et al.*, 1987; Petropoulos and Hughes, 1991). The myc tag fused to the C-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 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 replication-competent avian leukemia virus long terminal repeat with no splice acceptor (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.

## *Construction of Mutant CMP Transgenes*

Two groups of recombinant CMP mutants were generated. In the first group, certain domains were deleted, and in the second group, point mutations were introduced. All the mutants were generated by single-step PCR or two-step recombinant PCR with overlapping primers (Horton *et al.*, 1989).

Figure 1A represents diagrammatically the CMP cDNA and identifies the location of the primers used and the single letter amino acid substitution for the mutagenized residues. Figure 1B provides the 5′ to 3′ nucleotide sequence of all the primers used. Figure 1C names the various constructs and shows the primer pairs used to generate them. In the two-step recombinant PCR, primers A and D are sequences flanking the *Cla*I sites in the RCAS vector. Primers B and C are overlapping primers from the coding region of CMP that also code for mutations. Step 1 PCR used either a wild-type CMP or a CMP mutant in RCAS as a template (see Figure 1C). Primer pair A and B was used for the "left" PCR. Primer pair C and D was used for the "right" PCR. Step 2 PCR used 1 $\mu$l each of the left and right reactions as a template and primer pair A and D. A denaturation followed by a 5-min extension at 72°C was used to anneal the left and right products to each other so that they can subsequently serve as templates for primer pair A and D.

The coding region of each recombinant CMP construct was sequenced to confirm the desired mutations and to exclude the possibility of undesired mutations resulting from PCR amplification.

## *Transfection and Infection of CMP Transgenes*

Primary cell cultures that produced retrovirus that harbor 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 3750 rpm for 5 min. The supernatant was filtered through a 0.45-$\mu$m filter and stored frozen at −80°C. Virus was titrated by standard methods (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/100-mm dish). The infected culture was incubated for 1 wk to allow the infection to spread before further characterization.

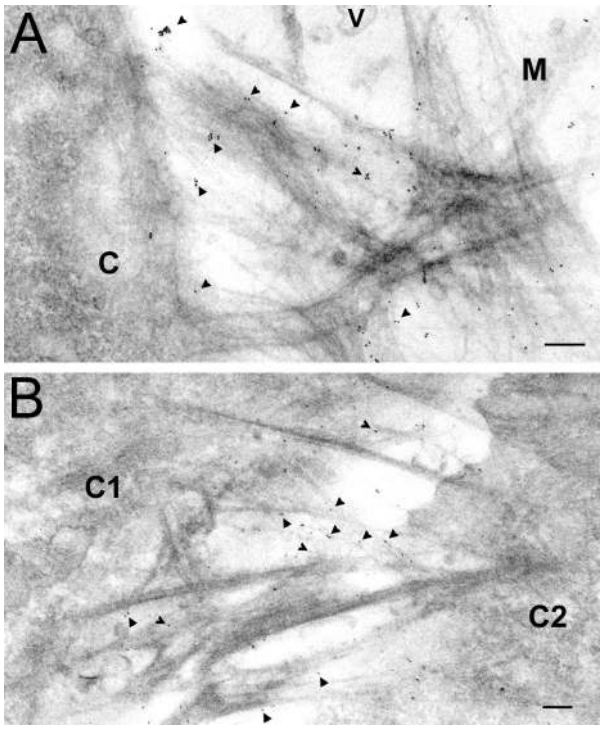



**Figure 2.** Electron microscopy of CMP filamentous networks. Chondrocytes were cultured in the absence of ascorbate to prevent collagen secretion, before immunogold electron microscopy (see Figure 5I for the absence of extracellular Col II in the culture without ascorbate). (A) CMP filaments connect a chondrocyte to matrix. Notice the gold particles decorate the matrix filaments protruding from the cell membrane. C, cell; M, matrix; V, matrix vesicles. Arrows point to some of the gold particles coupled to a mAb against CMP. Bar, 120 nm. (B) CMP filaments connect a chondrocyte to another chondrocyte. C1, cell 1; C2, cell 2. Notice the gold particles decorate the matrix filament bundles connecting neighboring cells. Bar, 120 nm. See Figure 3C for higher magnification of gold-labeled CMP filaments.

## *Immunofluorescent Cytochemistry and Western Blot*

For immunofluorescence staining, cultured cells were fixed at −20°C with 70% ethanol and 50 mM glycine, pH 2.0, for 20 min. Some of the cultures were treated with 5% type 1 testicular hyaluronidase (Sigma) for 20 min at 37°C before immunostaining. Slides were then washed with PBS and incubated with primary antibodies. After washing with PBS, affinity-purified FITC- or TRITC-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).

Western blot analysis was performed on both the medium and cell extracts. Twenty milliliters of conditioned medium of transfected or infected cultures were collected directly from each plate. After removal of the medium, the cells were rinsed with PBS before extraction. In some experiments, 4% paraformaldehyde in PBS was added into each dish for 20 min on ice without perturbing the cells. The monolayers were then washed with PBS for three times before adding 0.1 M glycine in PBS for 20 min. After another three washes with PBS, the monolayers were extracted with 1 ml of extraction buffer (4 M urea, 50 mM Tris, pH 8.5, and 0.1 mM PMSF), and the 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 14,000 rpm at 4°C. The supernatant (soluble fraction) or the pellet (insoluble fraction) 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 DTT. Protein concentration in each sample was determined by BCA protein assay (Pierce, Rockford, IL). Samples were boiled for 10 min before being loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred onto an Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 15% methanol. The membranes were blocked in 2% BSA fraction V (Sigma) in PBS for 30 min and then probed with antibodies. HRP-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin G (heavy and light chain; Bio-Rad, Melville, NY), diluted 1:3000, 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 (Rochester, NY) X-Omat AR film. The exposed film was scanned by a laser densitometer from Molecular Dynamics (Sunnyvale, CA). The protein signal intensity was quantified using Discovery Series Quantity One software from Protein Databases (Huntington, NY).

The mAbs used were 1H1, which recognizes CMP, and 3H8, which recognizes link protein. Both mAbs were generated as described in detail by Binette *et al.* (1994). mAb 9E10 was against an epitope in human c-myc (Potts *et al.*, 1987). mAb II-II6B3 and I-BA1 were directed against an epitope in avian type II and type I collagen, respectively (Linsenmayer *et al.*, 1979).

## *Purification of CMP from Cartilage*

CMP was purified according to a modified method from Winterbottom *et al.* (1992). Briefly, chick sterna were homogenized and extracted in extraction buffer (4 M GnCl and 50 mM Tris, pH 7.5) for 1 h. The homogenate was centrifuged at 10 kg for 15 min. The supernatant was applied to an Octyl-Sepharose CL-4B column (Pharmacia, Piscataway, NJ) and eluted with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid in extraction buffer. The eluted sample was further separated in a Sephacryl-400 column and applied to a MonoQ column. The flow-through that contained CMP in PBS was examined and quantified with SDS-PAGE and Western blot (see RESULTS).

## *Electron Microscopy*

Purified CMP (94 μg/ml) in PBS was applied onto the carbon-coated grids for 5–30 min either at 37°C or at room temperature. In some experiments, EDTA stock solution was added to the purified CMP to the final concentration of 10 mM. The excess solution was removed from the grid after incubation, and the grid was let dry for 5 min at room temperature. Four percent solution of phosphotungstic acid, pH 7.4 (adjusted with 1 M KOH), was applied to the grid for 2 min. The excess staining solution was blotted with filter paper. The negatively stained samples on grids were viewed with a JEOL (Tokyo, Japan) 100B transmission electron microscope.

Immunoelectron microscopy using ultrathin cryostat sections was performed as previously described (Chen *et al.*, 1992). Briefly, the monolayer chondrocytes were fixed in paraformaldehyde–lysine–periodate fixative, infiltrated with sucrose at 4°C, mounted on stubs, and frozen. One hundred-nanometer sections were cut with an ultracryotome (FC4; Reichert Jung, Vienna, Austria), placed on Formvar-coated grids, and reacted with antibodies complexed to colloidal gold particles. mAbs used were anti-CMP 1H1 (Binette *et al.*, 1994), anti-Col II II-II6B3, and anti-Col I IBA1 (Linsenmayer *et al.*,

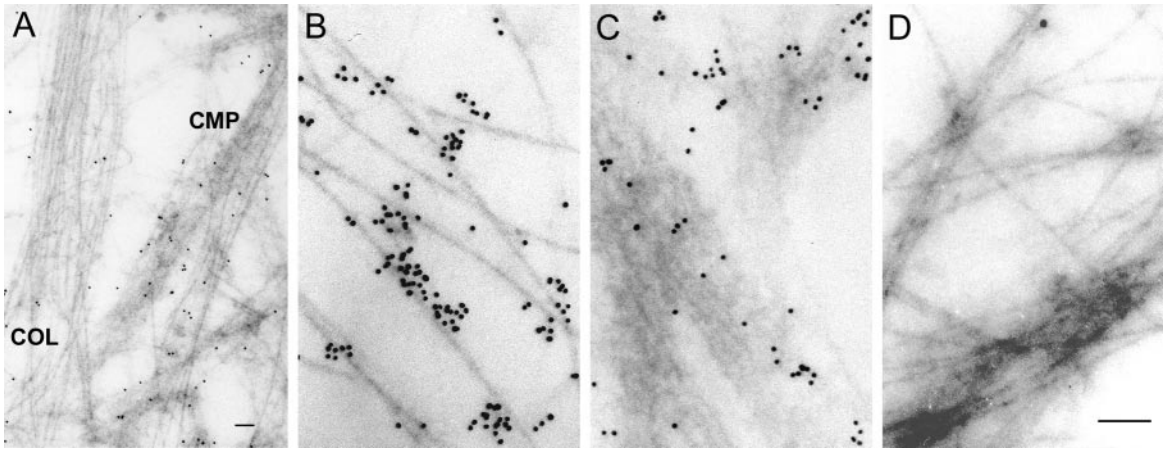



**Figure 3.** Immunogold electron microscopy. (A, B, and D) Chondrocytes were cultured in the presence of ascorbate to allow secretion of collagen fibrils. (C) Chondrocytes were cultured in the absence of ascorbate. (A) Two populations of filaments. COL, collagen fibrils; CMP, CMP filaments. Section reacted with a gold-coupled mAb against CMP. Notice the morphological difference between the two populations of filaments. Bar, 75 nm. (B) Collagen fibrils. Section reacted with a gold-coupled mAb against type II collagen. (C) CMP filaments. Section reacted with a gold-coupled mAb against CMP. (D) Control for immunogold electron microscopy. Section reacted with a gold-coupled mAb against type I collagen. Bar (B–D), 180 nm.

1979). Sections were viewed in a Philips (FEI, Hillsboro, OR) CM10 electron microscope.

## RESULTS

### *Immunoelectron Microscopy*

Our previous study has shown that CMP forms a filamentous network in a primary chondrocyte culture. To determine the location and morphology of the CMP filaments, immunoelectron microscopy was performed with an anti-CMP mAb coupled to 15-nm gold. Chondrocytes were incubated in an ascorbate-deficient medium to prevent secretion and deposition of fibrilar collagens. CMP-positive filaments were seen extending directly from chondrocyte membranes, connecting cells with ECM networks (Figure 2A). CMP-rich filaments were also seen connecting two neighboring chondrocytes (Figure 2B). Thus, CMP is part of the filamentous network that is responsible for chondrocyte–chondrocyte and chondrocyte–matrix interactions.

To compare the morphology of collagen-independent CMP filaments with that of collagen fibrils, chondrocytes were incubated in the presence of ascorbate to allow secretion and deposition of collagen fibrils. At least two populations of filaments were observed. One is typical cartilage collagen fibrils with regular banding patterns and uniform diameters between 10 and 20 nm (Figure 3A, COL). The identity of the collagen fibrils was confirmed by their positive reaction to a mAb against type II collagen (Figure 3B). The other is electron-dense mats enriched with CMP (Figure 3A, CMP). CMP filaments with similar morphology were also present in ascorbate-free culture conditions (Figure 3C). These mats do not have collagen-characteristic banding patterns or uniform diameters. In the presence of ascorbate, these two populations of filaments may intertwine with each other (Figure 3A).

### *Negative Staining Electron Microscopy*

We tested whether the collagen-independent CMP filaments could derive from self-assembly of CMP molecules. Native CMP was purified to homogeneity from chick sterna by urea extraction and affinity chromatography. After the final purification step, a single protein of 220 kDa is present at nonreducing conditions (Figure 4A). This protein band is shifted to 54 kDa under reducing conditions (Figure 4A, SDS-PAGE). These two bands (220 and 54 kDa) correspond to the trimeric and the monomer form of CMP, respectively (Chen *et al.*, 1995b). Western blot with a mAb against CMP, 1H1, confirms their identity as CMP (Figure 4A, Western).

The highly purified CMP was tested for its ability to self-assemble under physiological conditions. Purified CMP was incubated in PBS before being negatively stained for electron microscopy. A filamentous network was seen (Figure 4B). Its appearance resembles that of the collagen-independent filamentous network seen in cell culture (Figures 2 and 3). Individual ellipsoids representing the subunits of CMP are visible, with values of 7.6 nm for the longer and 5.6 nm for the shorter axis. These values are consistent with measurements of individual CMP by electron microscopy published previously (Winterbottom *et al.*, 1992; Hauser and Paulsson, 1994). Interactions of CMP subunits are depicted in a model (Figure 4D).

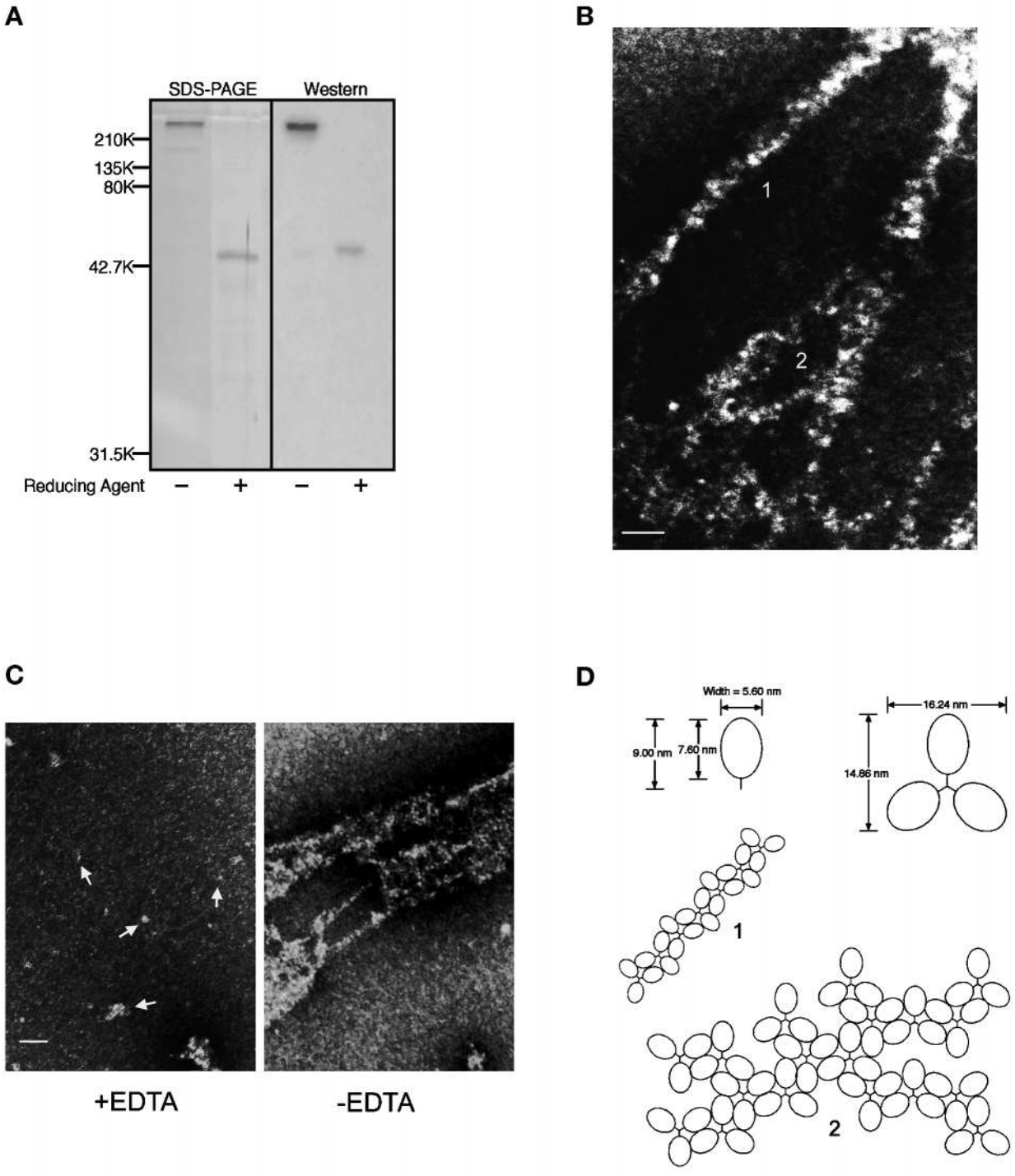



**Figure 4.** Purified CMP forms a filamentous network in vitro. (A) Biochemical analysis of purified CMP by SDS-PAGE (lanes 1 and 2), and by Western blot (lanes 3 and 4). (B) Electron micrograph of negatively stained specimens. Bar, 25 nm. (C) Self-assembly of the CMP filamentous network is dependent on cation. Electron micrograph of negatively stained purified CMP incubated either in the presence or absence of EDTA. Arrows indicate disrupted multimeric structures or individual CMP trimeric molecules. Bar, 50 nm. (D) Model of CMP filament formation. (1) Straight filament; (2) branched filaments. Measurements are marked. The models of these two types of filaments are based on original observations (1 and 2) in B. Note the model predicts that each CMP subunit should have at least two matrix adhesion sites to form a filament.

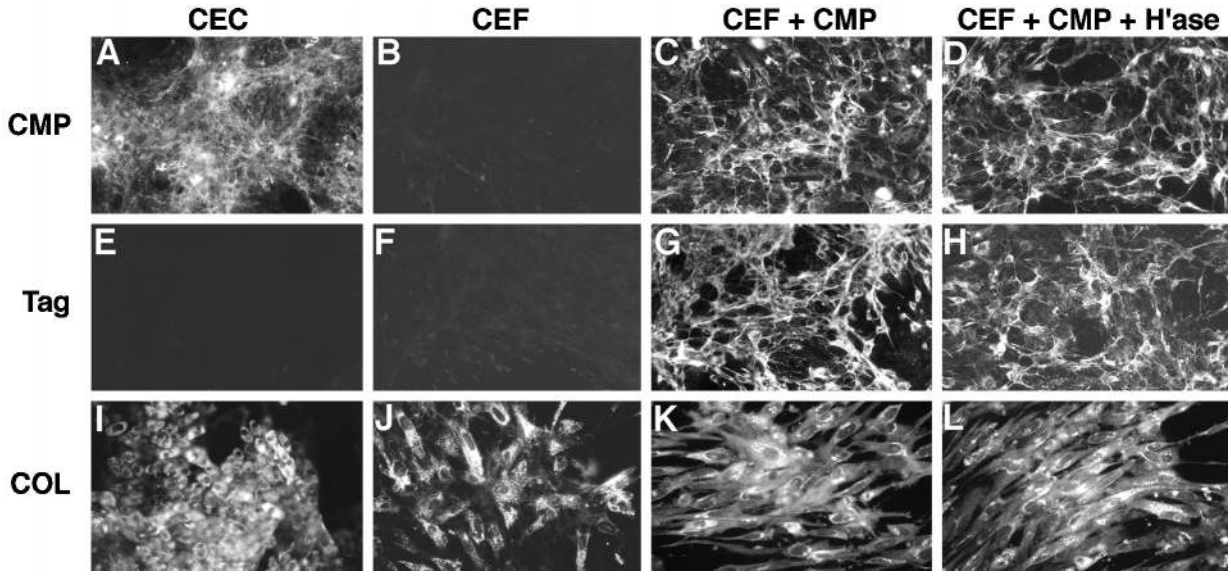



**Figure 5.** Formation of a filamentous network by expression of a wild-type CMP in cell cultures. A–D were reacted with a mAb against CMP (1H1); E–H were reacted with a mAb against the myc tag in recombinant CMP (9E10); I was reacted with a mAb against type II collagen (II-II6B3); J–L were reacted with a mAb against type I collagen (IBA1). Bar, 40 $\mu$m. (A, E, and I) CECs. (B, F, and J) CEFs. (C, G, and K) CEFs transfected with a wild-type CMP transgene. (D, H, and L) Wild-type CMP-transfected CEF culture after testicular hyaluronidase treatment.

We also found that interactions of CMP subunits are dependent on the presence of cations. The addition of EDTA during the incubation period prevents the formation of the filamentous network (Figure 4C).

### *Cell Transfection*

To characterize the molecular requirement for CMP filament assembly, we used chick embryonic skin fibroblast culture as our system for transfection. In contrast to the presence of an endogenous CMP filamentous network in a chondrocyte culture (Figure 5A), such filaments are absent in fibroblast cultures (Figure 5B) because of the lack of CMP synthesis by fibroblasts (Chen *et al.*, 1995b). By transfecting fibroblasts with a myc-tagged full-length wild-type CMP cDNA, CMP filaments can be detected in fibroblast cultures (Figure 5, C and G), consistent with our previous data (Chen *et al.*, 1995b). Extracellular recombinant CMP filaments are resistant to hyaluronidase digestion (Figure 5, D and H). Consistent with our electron microscopic data (Figures 2 and 3), CMP filaments are present (Figure 5, C, D, G, and H) in the absence of extracellular collagen fibrils (Figure 5, K and L). Fibril-forming collagens, type II in chondrocytes (Figure 5I) and type I in fibroblasts (Figure 5, J–L), remain intracellular under our culture conditions, which lack ascorbate in the medium. Thus organization of CMP filaments does not depend on the presence of collagens or chondroitin sulfate glycosaminoglycans.

### *Deletion of the A Domain*

To determine the role of the A domains in the formation of filaments, we transfected fibroblasts with two deletion constructs, CMPΔA1 and CMPΔA2 (Figure 6A). Neither CMPΔA1 nor CMPΔA2 is capable of forming extracellular filaments (Figure 6B, CMPΔA1-RCAS and CMPΔA2-RCAS). Strong intracellular staining of recombinant CMP is seen in the cells transfected with truncated CMP (Figure 6B), indicating that the cells are actively synthesizing recombinant proteins.

To examine whether cells secrete recombinant CMPs, conditioned medium from transfected cultures was collected and analyzed by Western blot. Both recombinant proteins are present in the medium of their respective cultures (Figure 6C). Under nonreducing conditions, CMPΔA1 exists as a trimer, as the wild-type CMP. However, CMPΔA2 is a mixture comprising trimers, dimers, and monomers (Figure 6C). The multimers were shifted to monomers under reducing conditions (Figure 6C, Reduced). Thus, CMPΔA1 is secreted as a trimer, and CMPΔA2 is secreted as a mixture of trimers, dimers, and monomers.

### *Sequence Analysis*

To identify the amino acid residues within the A domains that could be important for interaction with matrix ligands, the CMP A-domain sequences were examined for the presence of the MIDAS motif. Exam-

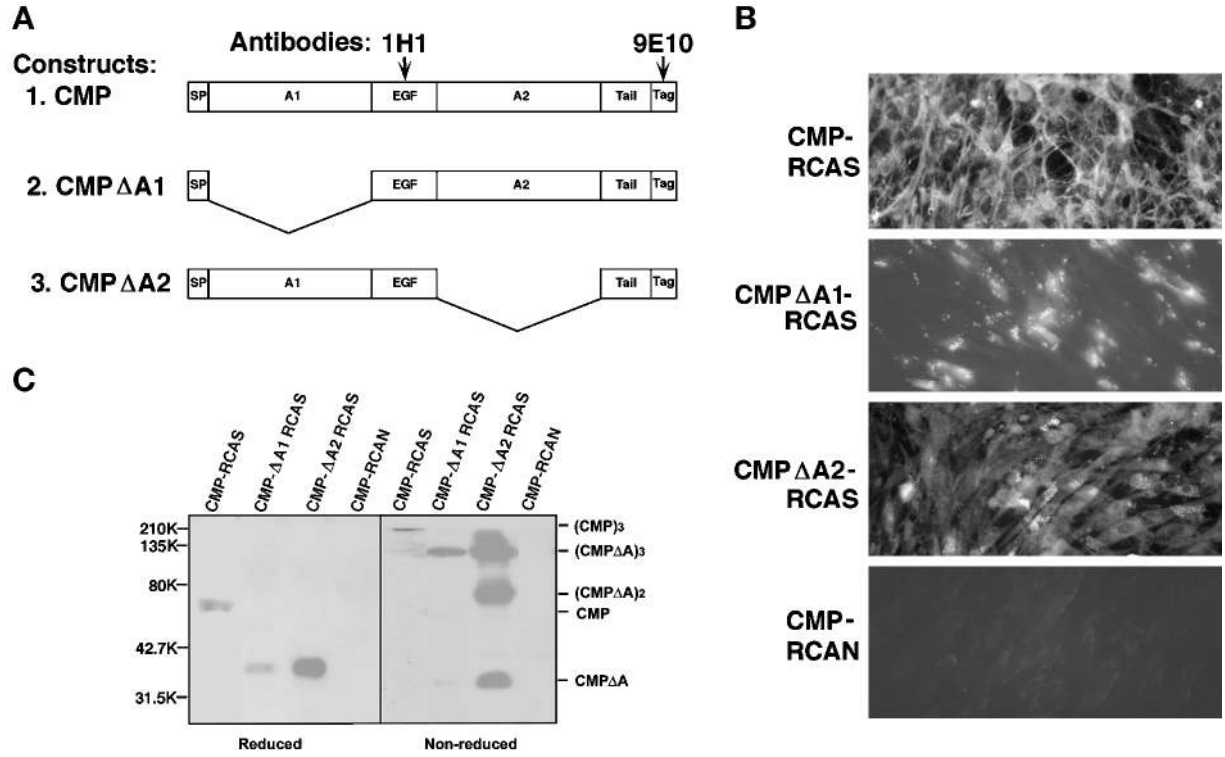



**Figure 6.** The A1 and A2 domains of CMP are necessary for the formation of the filamentous network. (A) Constructs of domain-deleted CMP mutants in retrovirus. (1) CMP: myc-tagged wild-type CMP; (2) CMPΔA1: myc-tagged CMP in which the first A domain is deleted; (3) CMPΔA2: myc-tagged CMP in which the second A domain is deleted. The mAbs detecting recombinant CMPs and the positions of their epitopes are indicated. (B) Micrographs of immunofluorescent analysis of transfected fibroblast cultures with a mAb against the myc tag, 9E10. (A) CMP-RCAS transfected; (B) CMPΔA1-RCAS transfected; (C) CMPΔA2-RCAS transfected; (D) CMP-RCAN transfected. (C) Expression of CMP transgenes by CEFs. Western blot of conditioned medium from cultures of transfected CEFs. Seven and a half microliters of conditioned medium were loaded in each lane. Reducing conditions were indicated. The antibody used to detect CMP was 1H1.

ination of the protein sequences of CMP from chick, mouse, and human reveals that both the A1 and A2 domains contain the five amino acid residues of the MIDAS motif (Figure 7B). The coordinating E residue (putative *trans* site) is conserved in the A1 domain among all three species but is not conserved in the A2 domain from all three species (Figure 7B). Thus each A domain in CMP contains a potantial MIDAS motif, with a *cis* site and a *trans* site in the A1 domain and a *cis* site only in the A2 domain.

### *Mutations within the A Domain*

Three groups of mutations (single, double, and triple) were made to mutate one, two, or three amino acid residues of the MIDAS motifs in a CMP monomer (Figure 8A). The single mutations convert the first aspartic acid residues in the *cis* sites of the MIDAS motifs ($D^{22}$ in A, or $D^{255}$ in A2) or the glutamic acid residue in the *trans* site of the MIDAS motif ($E^{193}$ in A1) to an alanine residue, respectively, by site-directed mutagenesis (Figure 8A). Three double mutants were generated in which two of the single mutations were combined in all three possible combinations. Finally, a triple mutant was generated in which all three single mutants were combined. Previous studies have shown that a conversion of either aspartic acid residue to alanine in the MIDAS *cis* site abolishes its cation-dependent ligand-binding property (Michishita *et al.*, 1993). All seven mutants (three single, three double, and one triple) were expressed in fibroblast cultures. The Western blot analysis shows that all seven recombinant CMPs are synthesized and secreted successfully by transfected cells (Figure 8B). None of the point mutations alters the trimeric organization of CMP.

The ability for the triple mutant to form an extracellular multimeric network was compared with that of the wild-type CMP. Monolayer cells that express wild-type CMP and CMP-D1D2E1 were fixed without permeating the cells. CMP that has been incorporated into the matrix would be cross-linked into high-molecular forms and become relatively insoluble in the urea extracts of the monolayers. Western blot analysis indicates that most of the wild-type CMP is in the cross-

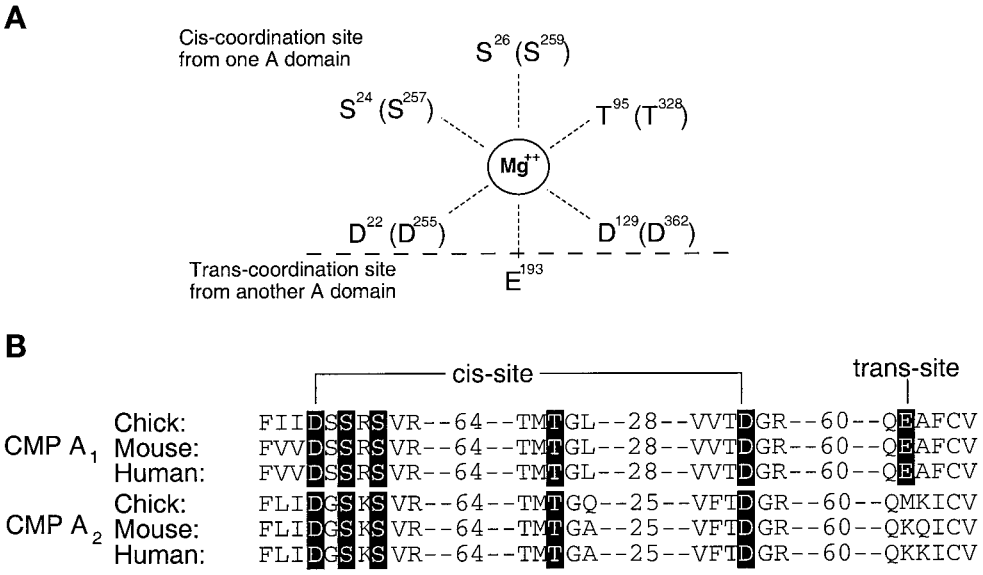



**Figure 7.** (A) Diagram depicting the putative *cis* and *trans* sites of the MIDAS interaction. The numbers indicate the position of the residues within CMP amino acid sequence. The letters within parentheses represent the MIDAS residues in CMP-A2 domain. (B) Sequence comparison of the MIDAS motifs in CMP from chick, mouse, and human. Shaded letters indicate conserved residues within MIDAS. The numbers indicate the numbers of amino acid residues that are not shown in the sequences.

linked high-molecular form, at the interface of the stacking and separating gel (Figure 9A, solid arrow). Furthermore, the amount of CMP-D1D2E1 associated with the urea-insoluble matrix is <10% of that from wild-type CMP. Thus point mutations within the MIDAS motif greatly reduce the ability for CMP to associate with the matrix network. In the urea-soluble fraction comprising intracellular un–cross-linked CMP, both wild-type CMP and CMP-D1D2E1 contain monomeric CMP (Figure 9A), indicating cells are synthesizing recombinant CMP. Immunofluorescent analysis of transfected cells confirms this finding (Figure 9B). An extracellular CMP network is seen with the wild-type CMP (Figure 9B, B), and only intracellular CMP is seen with the triple mutant (Figure 9B, A).

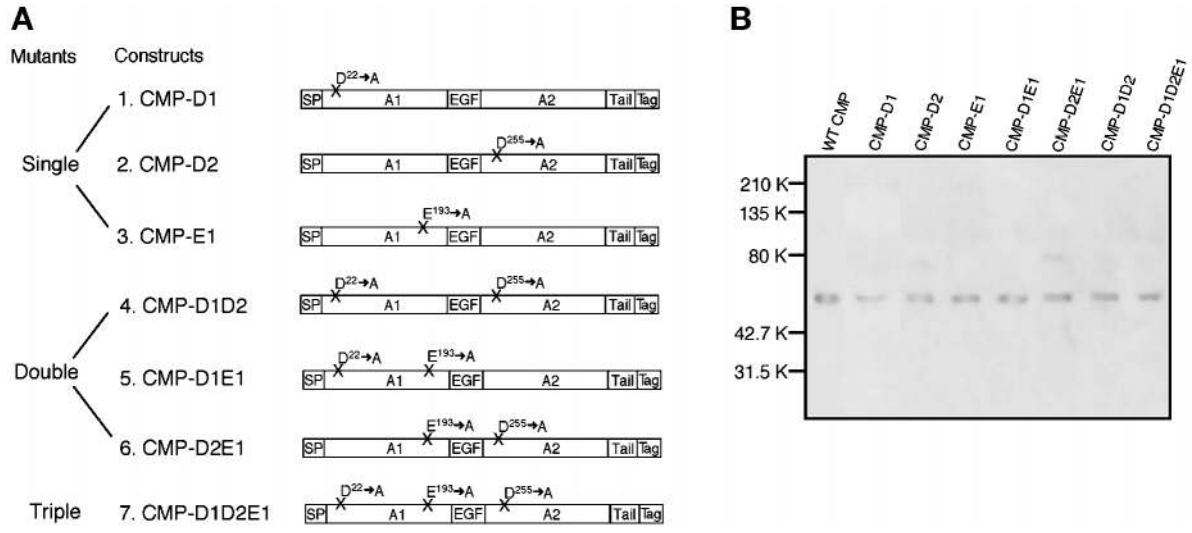



**Figure 8.** Retroviral expression of CMP transgenes with point mutations. (A) Constructs of CMP transgenes with point mutations. (1) Single mutations: CMP-D1, CMP-D2, and CMP-E1; (2) double mutations: CMP-D1D2, CMP-D1E1, and CMP-D2E1; (3) triple mutation: CMP-D1D2E1. X indicates the position where the mutation is made. (B) Expression of CMP transgenes by CEFs. Western blot of cell lysates from cultures of transfected CEFs. Seven and a half microliters of cell lysates were loaded under reducing conditions in each lane. The antibody used to detect CMP was 9E10, which detects the myc-tagged recombinant protein. Note the amount of synthesized recombinant CMP in cell lysates is similar in all of the constructs, including wild-type and mutants. Same results were obtained from conditioned medium from cultures of transfected CEFs.

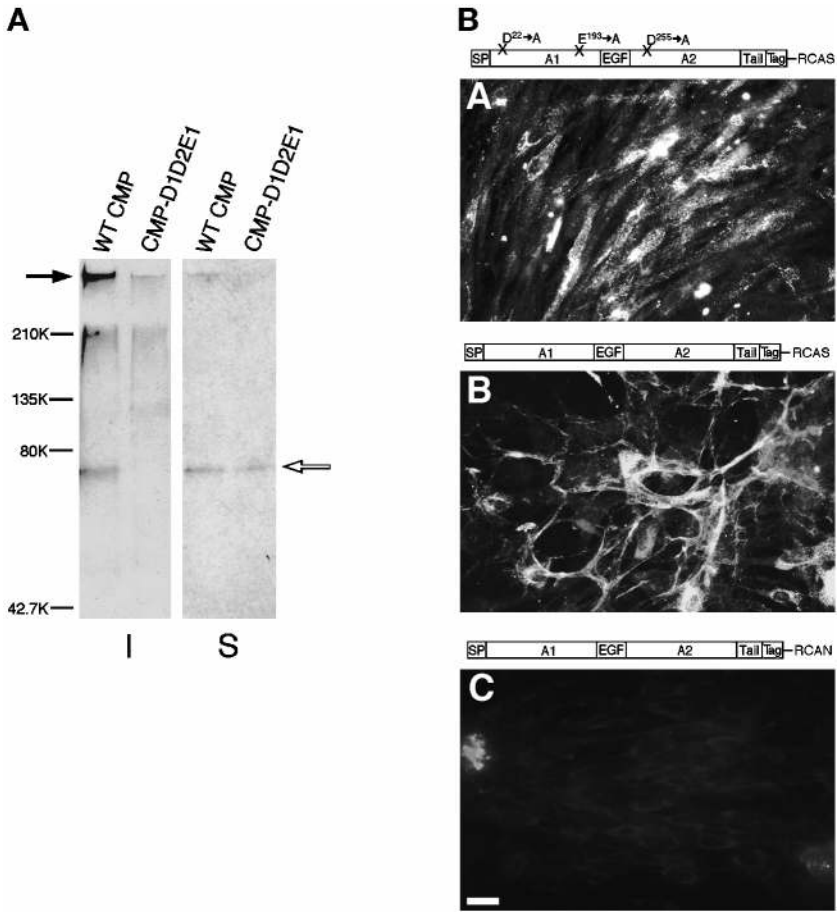



**Figure 9.** MIDAS motifs are involved in the formation of the CMP filamentous network. (A) Western blot of extracts from cross-linked monolayer cultures of transfected CEFs. From the insoluble fraction (I) of the urea extracts, 2.5 $\mu$g of total protein were loaded under reducing conditions in each lane of a 5% SDS-PAGE gel. From the soluble fraction (S) of the urea extracts, 2 $\mu$g of total protein were loaded in each lane. The antibody used to detect CMP was 1H1. The filled arrow points to the cross-linked high-molecular form of CMP at the stacking and separating gel interface. The empty arrow points to the monomeric CMP. (B) Micrographs of immunofluorescent analysis, with the mAb against the myc tag, 9E10, of fibroblast cultures transfected with CMP-D1D2E1 (A), CMP-RCAS (B; positive control), and CMP-RCAN (C; negative control). Bar, 40 $\mu$m.

Next, we examined whether all three residues, which are distributed in the MIDAS motif in both A domains, are required to form a matrix network. Immunofluorescent analyses were performed to examine the formation of CMP filaments with single and double MIDAS mutants. CMP filaments are seen in all the cultures transfected with a CMP single mutant, CMP-D1, CMP-D2, and CMP-E1 (Figure 10, A–C), and with two of the double mutants, CMP-D1E1 and CMP-D2E1 (Figure 10, E and F). Thus these mutations cannot abolish the formation of the network. In contrast, CMP filaments are absent in cultures that express the double MIDAS mutant, CMP-D1D2 (Figure 10D). Thus, formation of the CMP network requires at least one MIDAS motif (*cis* site) from one of the A domains in CMP.

### *Dominant Negative Inhibition of Filament Formation*

If the MIDAS-deficient mutant is defective in matrix interactions, it should achieve a dominant negative inhibition of filament formation by sequesting endogenous wild-type CMP via coiled-coil interactions. To test this, CMP-D1D2E1 was transfected into primary cultures of chondrocytes (Figure 11, B, F, and J). The triple MIDAS mutant CMP does not form extracellular filaments (Figure 11F), similar to its behavior in fibroblast cultures (Figure 9B, A). Furthermore, the endogenous extracellular CMP filaments are absent, as shown by detection with an anti-CMP mAb (Figure 11B). In contrast, extracellular CMP filaments are seen in a chondrocyte culture without any exogenous recombinant CMP (Figure 11A).

The inhibition of the endogenous CMP filament formation by the triple MIDAS mutant CMP is specific, because the endogenous hyaluronan–aggrecan–link protein network is not affected by the expression of CMP-D1D2E1 (Figure 11J). The dominant negative result is not due to a nonspecific effect resulting from the transfection and infection of a transgene in cell culture. The expression of a single MIDAS mutant, CMP-D1 (Figure 11, C, G, and K), and of a double MIDAS mutant, CMP-D1E1 (Figure 11, D, H, and L) by the same retroviral vector does not inhibit the formation of the extracellular CMP filaments.

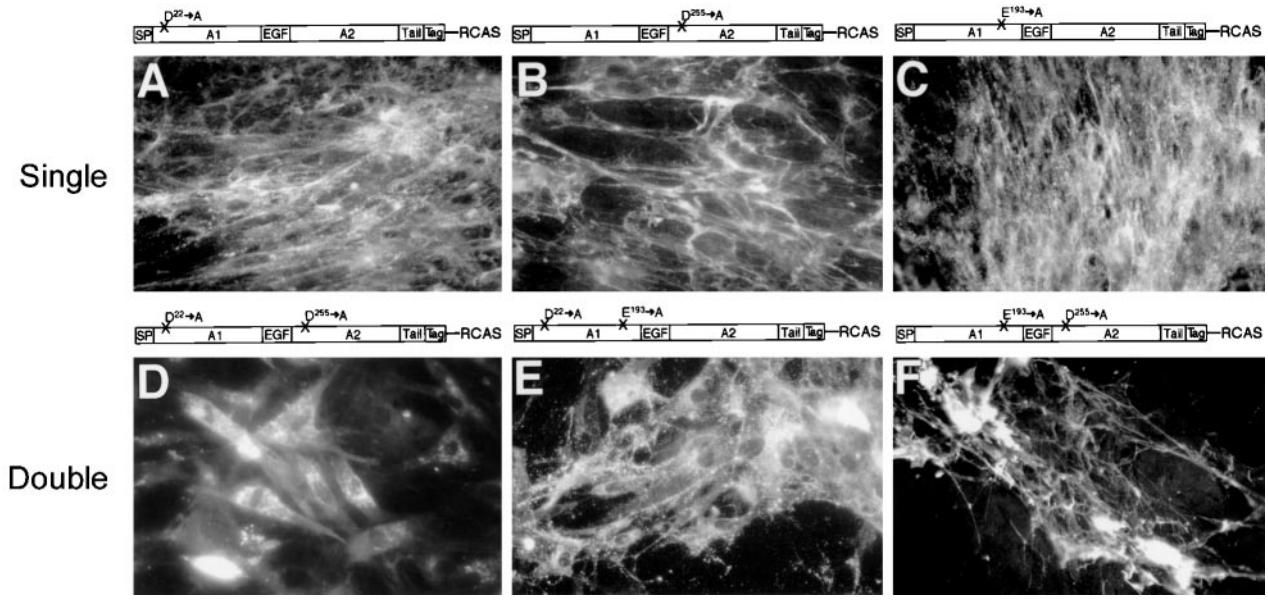



**Figure 10.** Micrographs of immunofluorescent analysis, with the mAb against the myc tag, 9E10, of fibroblast cultures transfected with: CMP-D1-RCAS (A), CMP-D2-RCAS (B), CMP-E1-RCAS (C), CMP-D1D2-RCAS (D), CMP-D1E1-RCAS (E), and CMP-D2E1 (F). Bar, 40 $\mu$m.

## DISCUSSION

The functions of matrilins, a novel ECM protein family, are poorly defined. In the present study, we characterize the assembly of CMP, the prototype of matrilins, with a combination of methods, including viral expression of transgenes, gene mutagenesis, protein purification, and electron microscopy. Immunoelec-

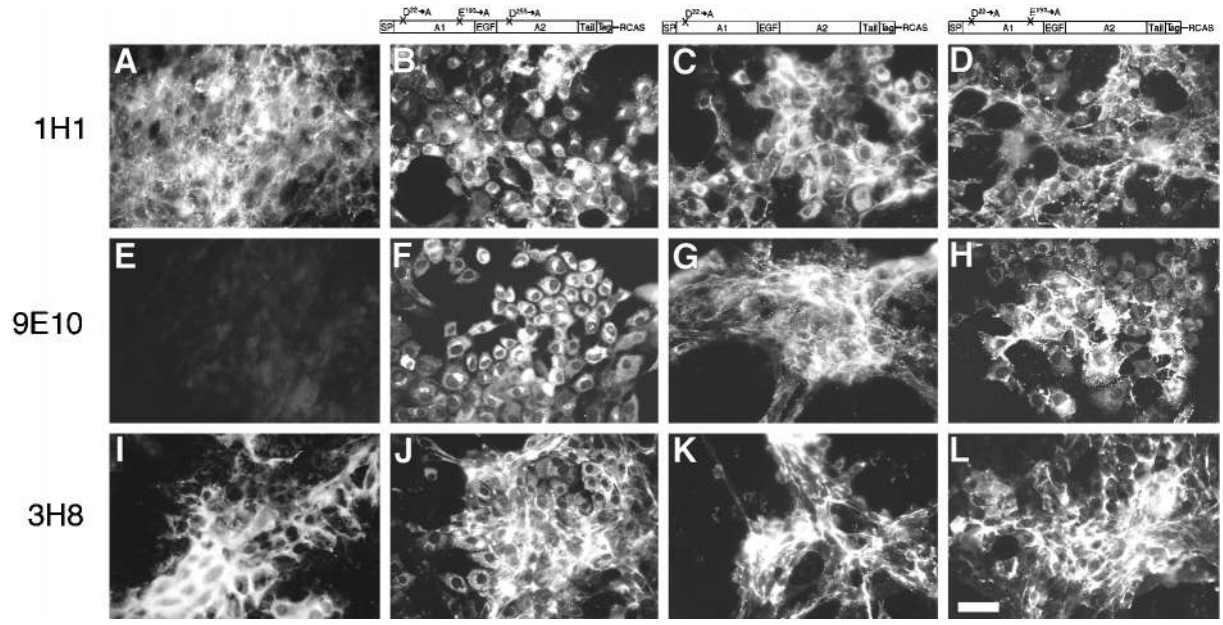



**Figure 11.** Triple MIDAS mutant CMP-D1D2E1 specifically inhibits the formation of endogenous CMP filaments by chondrocytes. Micrographs of immunofluorescent analysis of cultures of CECs, with mAb1H1 to detect CMP (A–D), with a mAb 9E10 to detect a tag of transfected CMP (E–H), and with mAb 3H8 to detect link protein (I–L). (A, E, and I) Chondrocytes with no transfection. (B, F, and J) Chondrocytes transfected with CMP-D1D2E1-RCAS; (C, G, and K) Chondrocytes transfected with CMP-D1-RCAS; (D, H, and L) Chondrocytes transfected with CMP-D1E1-RCAS. Bar, 35 $\mu$m.

tron microscopy reveals that CMP is part of the filamentous network that bridges neighboring chondrocytes and connects chondrocytes to ECM networks. The morphology of collagen-independent CMP filaments was examined in the absence of collagen fibrils by excluding ascorbate from culture medium. It was then compared with that of collagen fibrils side by side by including ascorbate in the medium.

The collagen-independent CMP filaments have a different morphological appearance than collagen fibrils. They lack regular banding patterns and uniform fibril diameters. Instead, they form a network-like structure. We show that at least part of this network-like structure may derive from self-assembly of CMP. The assembly would involve interactions between CMP subunits in a cation-dependent manner, because addition of EDTA, a chelater of cations, disrupts supramolecular assembly of CMP. This is consistent with the observation that EDTA treatment of cartilage tissue extracts CMP from the tissue (Hauser and Paulsson, 1994). Our data have shown that the lack of collagens and proteoglycans from cell culture does not affect CMP self-organization; thus the association of CMP with collagens (Winterbottom *et al.*, 1992) and proteoglycans (Hauser *et al.*, 1996) is an event parallel to or following the self-assembly of CMP. This suggests that CMP is able to connect different matrix components over a distance with its filaments to form an integrated network. Thus CMP may play a role in stabilizing matrix structure in cartilage. It would be intriguing to test whether this is a specific function of matrilins in other tissues as well.

We have characterized the functions of the two A domains in CMP during assembly. All the full-length matrilins contain two A domains separated by EGF repeats, with the notable exception of matrilin-3, which lacks the A2 domain. The surprising finding here is that the two highly homologous A domains in CMP serve different roles in trimerization of the molecule. The C-terminal A2 domain modulates this process, whereas the N-terminal A1 domain has no effect. The deletion of the A2 domain converts a trimeric CMP into a mixture of trimer, dimer, and monomer. The C-terminal A2 domain is adjacent to the coiled-coil domain, which is essential for determining the trimeric state of CMP (Beck *et al.*, 1996). Examination of the amino acid sequence of the coiled-coil domain reveals that it possesses a mixture of characteristics to form either a trimer or a dimer (Figure 12). We propose that the A2 domain may modulate multimerization of CMP by influencing the adjacent coiled-coil domain. It is particularly interesting to note that, although CMP forms a trimer by itself, it forms a heterotetramer by linking a CMP dimer with disulfides to a matrilin-3 dimer, which lacks the A2 domain (Wu and Eyre, 1998).

$$[\overset{*}{a} - b - c - \overset{*}{d} - e - f - g]_n$$

| | | |
|---|---|---|
| | Val (β) | 1 |
| Ile (β) | Leu | 2 |
| Leu | Val (β) | 3 |
| Ile (β) | Leu | 4 |
| Ile (β) | | 5 |

*: hydrophobic residues

β: β-branched residues (Val, Ile)

**Figure 12.** Amino acid residues at the "a" and "d" positions of the heptad repeat of the coiled-coil domain of chick CMP. According to Harbury *et al.* (1993), the heptad repeat with Leu residues at the d position would form a double helix coiled coil, and the one with β-branched residues such as Val and Ile at the d position would form a triple-helix coiled-coil. The chick CMP contains two Leu and two β-branched residues at the d position. n, number of heptad repeats.

Our data strongly suggest that the matrix adhesion sites reside within both A domains of CMP. Both A domains in CMP are necessary for the formation of the filamentous network. Deletion of either A domain abolishes the collagen-independent network formation. If both A domains are required for matrilin network assembly, one could predict that matrilin-3, which lacks the A2 domain, would not form a network by itself. Instead, it may become a part of the matrix network by forming heteromultimers with CMP. Recent identification of a heterotetramer $(CMP)_2(matrilin\text{-}3)_2$ in cartilage supports this hypothesis (Wu and Eyre, 1998). In contrast, matrilins that possess both A domains may form a filamentous network, with matrilin-2 in bone, uterus, and heart and matrilin-4 in lung, similar to the function of CMP in cartilage.

Our mutagenesis data suggest that a CMP monomer is bivalent in its association with matrix networks. Single point mutations in either A domain are not sufficient to eliminate network formation. However, simultaneous mutations of $Asp^{22}$ in the A1 domain and $Asp^{255}$ in the A2 domain are sufficient to abolish the network. Because these two residues are part of the *cis* sites of the MIDAS motif, we conclude that one *cis* site in A1 and another in A2 are involved in CMP interactions with matrix. Because all the residues comprising the MIDAS *cis* site are conserved in all of the A domains from matrilins (Wagener *et al.*, 1998), the MIDAS adhesion mechanism may be applicable to supramolecular assembly of the matrilin family. In

contrast, the putative *trans* site, which is not always conserved in A domains from CMP, is not required for matrix network formation, as we have shown here.

Together, the deletion and mutagenesis studies indicate that supramolecular assembly of CMP may have at least two requirements. First, the CMP subunits from neighboring molecules should have compatible conformations for interaction. The elimination of an entire A domain from the molecule may cause a failure of assembly because of a change of the overall conformation of the molecule, even if the other A domain may still contain an adhesion site. Second, this interaction is stabilized by the adhesion sites within the A domains, which may use an adhesion mechanism similar to the MIDAS mechanism proposed for integrin–ligand interactions (Lee *et al.*, 1995b). The dominant negative effects of the MIDAS motif–deficient CMP support this hypothesis.

In summary, our study suggests that CMP is capable of connecting chondrocytes and matrix by forming a filamentous network. The A domains within the molecule play multiple roles in this assembly process, with the A2 domain modulating the oligomeric state of the molecule and both A domains involved in adhesive interactions with matrix. This assembly process is regulated by the number and position of the A domain present in CMP. This finding has strong implications for understanding the functions of the matrilin family, whose members have variations in the number and position of the A domains within the molecule.

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