The Role of Coiled-coil α-Helices and Disulfide Bonds in the Assembly and Stabilization of Cartilage Matrix Protein Subunits

A MUTATIONAL ANALYSIS*

(Received for publication, May 19, 1995, and in revised form, July 20, 1995)

Dominik R. Haudenschildt, M. Mehrdad Tondravi§, Urs Hofer, Qian Chen, and Paul F. Goetinck†

From the Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129

Cartilage matrix protein (CMP) exists as a disulfide-bonded homotrimer in the matrix of cartilage. Each monomer consists of two CMP-A domains that are separated by an epidermal growth factor-like domain. A heptad repeat-containing tail makes up the carboxyl-terminal domain of the protein. The secreted form of CMP contains 12 cysteine residues numbered C1 through C12. Two of these are in each of the CMP-A domains, six are in the epidermal growth factor-like domain, and two are in the heptad repeat-containing tail. Two major categories of mutant CMPs were generated to analyze the oligomerization process of CMP: a mini-CMP and a heptad-less full-length CMP. The mini-CMP consists of the CMP-A2 domain and the heptad repeat-containing tail. In addition, a number of mutations affecting C9 through C12 were generated within the full-length, the mini-, and the heptad-less CMPs. The mutational analysis indicates that the heptad repeats are necessary for the initiation of CMP trimerization and that the two cysteines in the heptad repeat-containing tail are both necessary and sufficient to form intermolecular disulfide bonds in either full-length or mini-CMP. The two cysteines within a CMP-A domain form an intradomain disulfide bond.

The macromolecular composition of the matrix of cartilage results from the expression of a unique repertoire of genes by chondrocytes. The matrix macromolecules synthesized by the chondrocytes have multiple domains that permit interactions with other matrix molecules or with cell surface components. These complex interactions determine the structure and the integrity of cartilage. The major components of the cartilage extracellular matrix are collagens, proteoglycans, and noncollagenous proteins. Cartilage matrix protein (CMP) is one of the most abundant noncollagenous extracellular proteins in cartilage (1, 2) and has been shown to associate with the cartilage collagen fibril that consists of collagen types II, IX, and XI (3) as well as with proteoglycans (4).

The deduced amino acid sequence of CMP reveals that a CMP monomer is made up of a unique combination of structural domains (4–6). Two highly homologous domains, CMP-A1 and CMP-A2, are separated from each other by a domain with homology to epidermal growth factor (EGF). The last domain is the carboxyl-terminal tail, which contains a series of heptad repeats (7). Each domain has significant sequence or structural homology to portions of other proteins. Homology to the CMP-A domains is found in soluble proteins including von Willebrand factor, the complement components C2 and B, matrix proteins such as collagen types VI, VII, XII, and XIV, undulin, transmembrane proteins such as the α-chains of the integrins VLA-1, VLA-2, LFA-1, Mac-1, p150,95, and a Caenorhabditis elegans protein involved in muscle attachment as well as the dihydropyridine-sensitive calcium channel and the inter-α-trypsin inhibitor (reviewed in Refs. 8 and 9). The A domains of several proteins have been shown to bind extracellular matrix molecules such as collagen (10–15) and the glycosaminoglycans heparin and hyaluronic acid (16, 17). EGF-like domains are found in many classes of molecules, and some of them have been shown to bind calcium ions (18). The function of the EGF-like domain in CMP is not established. The carboxyl-terminal heptad repeat domain of CMP (7) has structural similarity to the coiled-coil domains of fibrinogen and members of the laminin, tenascin, and thrombospondin families (18–21). The function of the heptad repeat domains in these molecules has been shown to be the formation of hydrophobic interactions between three adjacent and similar coils, in effect forming homo- or heterotrimers (22).

CMP monomer contains 12 cysteine residues. Each of the two CMP-A domains has two cysteines, one at the amino-terminal end and one at the carboxyl-terminal end of each domain. The EGF-like domain has six cysteine residues. The final two cysteines are at the beginning of the carboxyl-terminal heptad repeats (4–6). In this communication we test the hypotheses that CMP-A domains become stabilized by intradomain disulfide bonds, that the cysteine residues in the carboxyl-terminal tail are involved in homotrimer formation (5, 23), and that the heptad repeats in the carboxyl-terminal tail are involved in the initiation of trimer formation (7). These hypotheses were tested by the creation of a variety of CMP mutants and the expression of these mutant CMP molecules in COS-7 cells that do not normally express CMP.

MATERIALS AND METHODS

Construction of CMP Mutants—The locations of the 12 cysteine residues, numbered C1 (at the amino terminus) through C12 (at the carboxyl terminus), in a CMP monomer are shown in Fig. 1. The wild-type chicken cDNA sequence was cloned into pcDNA1 vector.

* This work was supported in part by Grant HD-22016 from the National Institutes of Health, a grant from the Swiss National Science Foundation (to U. H.), and grants from the Arthritis Foundation (to M. M. T. and Q. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Genzyme Corporation, 1 Mountain Rd., Framingham, MA 01701.

§ Present address: Dept. of Pathology, Washington University Medical Center, St. Louis, MO 63110.

¶ Present address: Dept. of Pathology, Washington University Medical Center, St. Louis, MO 63110.

To whom correspondence should be addressed: Cutaneous Biology Research Center, Massachusetts General Hospital, MGH East, Bldg. 149, 13th St., Charlestown, MA 02129. Tel.: 617-726-4183; Fax: 617-726-4189; E-mail: pgoetinck@cbrc.mgh.harvard.edu.

† The abbreviations used are: CMP, cartilage matrix protein; DMEM, Dulbecco’s modified eagle medium; PCR, polymerase chain reaction; EGF, epidermal growth factor.
Construction of Mini-CMP—The model of CMP structure postulates that the carboxyl-terminal cysteines are involved in trimerization. To decrease the number of cysteine residues and thereby simplify the analysis of the mutagenesis, a mini-CMP was created that consists of the CMP-A2 domain and the heptad repeat-containing tail domain. This mini-CMP has only four cysteine residues, C9 and C10 in the CMP-A2 domain and C11 and C12 in the tail domain. The numbering of the cysteine residues of full-length CMP is also used in the numbering of the cysteine residues in the mini-CMP. The constructs were transiently expressed in COS-7 cells that normally do not express any CMP. Wild-type, full-length CMP expressed in this COS-7 system is indistinguishable on a Western blot from CMP isolated from chicken sterna (data not shown).

Mini-CMP Behaves like Full-length CMP—According to our model of CMP folding, C9 and C10 would form an intradomain disulfide bond, and C11 and C12 would be involved in the trimerization of mini-CMP. A Western blot of mini-CMP under reducing conditions shows that the molecule migrates as a single band with an apparent molecular mass of about 28 kDa (Fig. 3A, lane 1). This value is in agreement with the molecular mass predicted from chicken cDNA sequence analysis. A Western blot of mini-CMP under nonreducing conditions shows that the protein migrates as a single band of 85 kDa (Fig. 3A, lane 4). This electrophoretic pattern is consistent with the formation of a disulfide-bonded mini-CMP trimer. Full-length CMP has an apparent molecular mass of 54 kDa under reducing conditions (Fig. 3A, lane 2) and 144 kDa under nonreducing conditions (Fig. 3A, lane 5). The electrophoretic behavior of the mini-CMP is analogous to that of the expressed full-length CMP, in that both molecules are secreted exclusively as disulfide-bonded trimers.

Mini-CMP Can Trimerize with Full-length CMP—To test if mini-CMP can oligomerize with full-length CMP, mini-CMP and full-length CMP cDNAs were co-transfected into COS-7 cells. Western blot analysis of the medium of the co-transfected cells shows two bands (28 and 54 kDa) under reducing conditions (Fig. 3A, lane 3) that correspond to the monomers of mini-CMP and full-length CMP. Under nonreducing conditions there are four distinct bands (Fig. 3A, lane 6). The molecular weight of the band with the fastest electrophoretic mobility corresponds to a disulfide-bonded trimer consisting of three mini-CMP monomers. The molecular mass of the second band corresponds to that of a trimer consisting of two mini-CMP monomers and one full-length CMP monomer. The molecular mass of the third band corresponds to a trimer consisting of one mini-CMP monomer and two full-length CMP chains. The band with the slowest electrophoretic mobility is a trimer consisting of three full-length CMP monomers. Observations that only four bands are present under nonreducing conditions and that two of these bands correspond to mini-CMP trimer and full-length CMP trimer clearly prove the existence of CMP as trimers. The various forms observed on the gel are represented diagrammatically in Fig. 3B.

Cysteine Residues C11 and C12 Are Necessary for Interchain Disulfide Bond Formation in Mini-CMP—To test the roles of responding to the amino acids Phe<sup>200</sup> to Val<sup>204</sup> in the CMP-A2 domain cross-linked to keyhole limpet hemocyanin by means of N-succinimidyl 3-(pyridylthio)propionate through a cysteine residue introduced at the amino group of Phe<sup>200</sup> during the synthesis of the peptide, as described earlier (26). The antibodies were subsequently affinity purified on a column consisting of purified chicken CMP linked to Sepharose CL-4B (Pharmacia Biotech Inc.). Detection of antibody bound to proteins on the Immobilon-P membrane was done with peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) using ECL substrate (Amer sham Corp.) and exposure to x-ray film from Kodak or Fuji.
C11 and C12 in mini-CMP, a mini-CMP was created in which these two cysteines were converted to serine residues. Mutating C11 and C12 in mini-CMP results in a protein that migrates with an apparent molecular mass of 26 or 28 kDa under nonreducing or reducing conditions, respectively (Fig. 4, lanes 2 and 4). Therefore, no trimers or other oligomers formed from this mutant version of mini-CMP. This is in contrast with the mobility of the trimer form (85 kDa) of the mini-CMP under nonreducing conditions (Fig. 4, lane 1) and the monomer (28 kDa) under reducing conditions (Fig. 4, lane 3). The slight difference in the electrophoretic mobility of the mutated mini-CMP under nonreducing (Fig. 4, lane 2) compared with that under reducing conditions (Fig. 4, lane 4) reflects the presence of a single intrachain disulfide bond between C9 and C10. Thus, C11 and C12 are necessary for the formation of interchain disulfide bonds of the mini-CMP. The results also indicate that an intradomain disulfide bond involving C9 and C10 in the CMP-A2 domain can form in the absence of C11 and C12. Therefore, the formation of this intrachain disulfide bond is independent of interchain disulfide bonds.

Residues C11 and C12 Are Sufficient for the Formation of Disulfide Bonds in the Mini-CMP—To test directly the roles of C9 and C10 in mini-CMP, we expressed a mini-CMP in which both C9 and C10 had been altered by mutagenesis. This mini-CMP, which has only two cysteines, C11 and C12, migrates with a molecular mass of 94 or 26 kDa under nonreducing or reducing conditions, respectively (Fig. 4, lanes 5 and 6). The slightly slower electrophoretic mobility of the mutated mini-CMP under nonreducing (Fig. 4, lane 2) compared with that under reducing conditions (Fig. 4, lane 4) reflects the presence of a single intrachain disulfide bond between C9 and C10. Thus, C11 and C12 are necessary for the formation of the interchain disulfide bonds of the mini-CMP. The results also indicate that an intradomain disulfide bond involving C9 and C10 in the CMP-A2 domain can form in the absence of C11 and C12. Therefore, the formation of this intrachain disulfide bond is independent of interchain disulfide bonds.

Residues C11 and C12 Are Also Necessary for Interchain

**Fig. 2. Construct production and cysteine mutations.** The relative locations of the primers used to produce the various CMP constructs are shown underneath the schematic model of CMP in A. Cysteines were mutated to the amino acids indicated by single letters. The introduced stop codons are shown as stars. B shows the sequence of all primers used in 5' to 3' orientation. The primers are numbered as in A. Lower case letters indicate mismatches with the template sequence. The mutated codons are listed in bold, and underlining indicates the stop codons introduced for the heptad-less constructs. Nonadjacent areas of overlap with template, used to connect the signal sequence to the CMP-A2 domain in the recombinant PCR, are separated by a tilde (—). The primer pairs, templates, and introduced mutations for the single or recombinant PCRs are listed in C. The delta symbol (Δ) indicates deletion of the corresponding domains. N/A, not applicable.

**Fig. 3. Mini-CMP behaves as full-length CMP.** Conditioned medium of COS cells transfected with mini-CMP (lanes 1 and 4), full-length CMP (lanes 2 and 5), or co-transfected with the cDNAs of both constructs (lanes 3 and 6) was separated on a 8% gel, blotted to a polyvinylidene difluoride membrane, and incubated with antiserum D2/1476 against the CMP-A2 domain. Bound antibodies were detected with a peroxidase-coupled secondary antibody and a chemiluminescence detection kit. Lanes 1–3 were run under reducing conditions (+), and lanes 4–6 were run under nonreducing conditions (−). Molecular weights of the protein standards are shown on the left × 10^3. Schematic drawings of the obtained mixed multimers between mini- and full-length CMP are shown in B. The total number of CMP-A domains/multimer is indicated in parentheses and corresponds to the numbers shown on the right side of A.
Cysteines and Heptad Repeats in CMP Trimer Formation

Disulfide Bond Formation in Full-length CMP—To test if C11 and C12 are responsible for the formation of interchain disulfide bonds in full-length CMP as they are in mini-CMP, both C11 and C12 were mutated in full-length CMP. Mutating both C11 and C12 of full-length CMP results in a protein that migrates with an apparent molecular mass of 45 kDa under nonreducing conditions (Fig. 5, lanes 5 and 6) were run under nonreducing (−) and reducing (+) conditions on a 11% gel and analyzed by Western blotting as described in the legend of Fig. 3. Molecular weights of the protein standards are shown on the left × 10⁻². Above each lane in the blot is a figure representing the mini-CMP construct used. The open box represents the CMP-A2 domain, and the solid line represents the COOH-terminal tail domain. The cysteines (C) and the mutated cysteines (X) are shown. The (−) and (+) symbols indicate the absence or presence of reducing agent during electrophoresis. Since full-length CMP with mutated cysteines C11 and C12 (lanes 3 and 4) were run under nonreducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions on a 11% gel and analyzed by Western blotting as described in the legend of Fig. 3. Molecular weights of the protein standards are shown on the left × 10⁻². Above each lane in the blot is a figure representing the CMP construct used. The open box demonstrates the CMP-A1 and -A2 domains, the black box represents the EGF-like domain, and the solid line indicates the COOH-terminal tail. The cysteines (C) and the mutated cysteines (X) are shown. The (−) and (+) symbols indicate the absence or presence of reducing agents during electrophoresis.

DISCUSSION

An important aspect of the macromolecular organization of the extracellular matrix is that some of its components spontaneously assemble into homotypic or heterotypic oligomers. The formation of triple helices of collagens and the assembly of these trimers in supramolecular structures are examples of such assemblies, as are the formation of laminins, thrombospondin, and tenasin (18–22).

The organization of CMP into a functional trimer requires that a number of co- or post-translational modifications occur both within and between monomers. We have postulated that intramonomeric changes include the formation of disulfide bridges within the A domain and the EGF-like domain (5, 23). The intermonomeric changes include the initiation of the trimer formation through the establishment of coiled-coil α-helices followed by the stabilization of the trimer through disulfide bonds involving the two cysteines in the heptad repeat-containing tail. Although reducing agents are necessary to obtain CMP monomers in denaturing gels, the interchain disulfide bonds are not necessary for the maintenance of the trimeric structure under otherwise non-denaturing conditions (7).

To test this model, we engineered a mini-CMP in which the CMP-A1 and the EGF domains were deleted, thus reducing the number of cysteine residues in the mini-CMP from 12 to 4. Two of the cysteines are in the CMP-A2 domain and two are in the heptad repeat-containing tail domain. When the length of the tail domain of the mini-CMP was intact, we showed clearly that C11 and C12 are both necessary and sufficient for interchain disulfide bond formation that results in a stable trimer. If, then, the interchain disulfide bonds in full-length CMP can be explained in terms of multimers of the CMP constructs made that consist of wild-type CMP with a stop codon introduced between the C12 residue and the heptad repeats. A similar construct was made that had C11 and C12 mutated. Neither of these constructs formed disulfide-bonded trimers (Fig. 6, lanes 1 and 2). The proteins encoded by these constructs migrated as two close bands that cannot be explained in terms of multimers of the CMP construct. We interpret these two bands to reflect different intramonomeric disulfide bonds. Upon reduction both samples migrate as a single band with identical electrophoretic mobilities (Fig. 6, lanes 3 and 4). These results indicate that the heptad repeats are critical for the initiation of CMP trimer formation. Such trimers cannot form when the heptad repeats are not present, even when residues C11 and C12 are intact. Thus, residues C11 and C12 stabilize the trimer by forming interchain disulfide bonds.

The Heptad Repeats Are Essential for the Initiation of Disulfide-bonded Trimerization—It has been shown that CMP maintains its trimeric state under reducing non-denaturing conditions, and it has been suggested that the heptad repeats are responsible for the maintenance of the trimeric state (7). To test whether the heptad repeats have an effect on the formation of trimers in CMP, constructs were made that consist of wild-type CMP with a stop codon introduced between the C12 residue and the heptad repeats. A similar construct was made that had C11 and C12 mutated. Neither of these constructs formed disulfide-bonded trimers (Fig. 6, lanes 1 and 2). The proteins encoded by these constructs migrated as two close bands that cannot be explained in terms of multimers of the CMP construct. We interpret these two bands to reflect different intramonomeric disulfide bonds. Upon reduction both samples migrate as a single band with identical electrophoretic mobilities (Fig. 6, lanes 3 and 4). These results indicate that the heptad repeats are critical for the initiation of CMP trimer formation. Such trimers cannot form when the heptad repeats are not present, even when residues C11 and C12 are intact. Thus, residues C11 and C12 stabilize the trimer by forming interchain disulfide bonds.

The intermonomeric changes include the initiation of the trimer formation through the establishment of coiled-coil α-helices followed by the stabilization of the trimer through disulfide bonds involving the two cysteines in the heptad repeat-containing tail. Although reducing agents are necessary to obtain CMP monomers in denaturing gels, the interchain disulfide bonds are not necessary for the maintenance of the trimeric structure under otherwise non-denaturing conditions (7).
Cysteines and Heptad Repeats in CMP Trimer Formation

however, the heptad repeats of the tail domain are deleted, trimers will not form even if C11 and C12 are present. Therefore, the heptad repeats act as nucleation sites in the formation of the CMP trimer, and the trimers are subsequently stabilized through disulfide bonds involving C11 and C12. The establishment of trimers through the interaction of heptad repeats have been proposed for the formation of heterotrimers of laminin and homotrimers of tenasin, fibrinogen, and thrombospondin. As in CMP, closely spaced cysteine residues are at the amino and carboxyl end of the heptad repeats of these proteins (19–22). We also present evidence that intradomain disulfide bonds form between residues C9 and C10 within CMP-A2, suggesting that a similar bridge may exist between C1 and C2 in the CMP-A1 domain. Based on the presence of two intra-A domain disulfide bridges, along with the three disulfide bridges in the EGF-like domain, a very compact and globular CMP structure can be predicted. This is consistent with electron microscopic observations that native CMP trimer isolated from cartilage consists of three compact ellipsoids connected at one end (8, 7).

Not all A domains of other proteins have cysteine residues at their amino and carboxyl ends as seen in CMP. Some A domains have no cysteines and others have one, two, three, or five cysteines (8). When there are two cysteine residues in an A domain, they are most often situated at the amino and carboxyl ends of the domain. In those A domains, it is possible that the two cysteine residues form intradomain disulfide bonds as we have shown to exist in the CMP-A2 domain. However, the crystal structure of the A domain of an α-chain of the CR3 integrin suggests that the ends of an A domain may be juxtaposed even in the absence of an intradomain disulfide bridge (9).

Fig. 6. The heptad repeats are needed for the initiation of disulfide-bonded trimerization. Conditioned medium of cells expressing heptad-less CMP (lanes 1 and 3) and heptad-less CMP with mutated cysteines C11 and C12 (lanes 2 and 4) were run under nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions on a 11% gel and analyzed by Western blotting as described in the legend of Fig. 3. Molecular weights of the protein standards are shown on the left \( \times 10^{-3} \). Above each lane in the blot is a figure representing the CMP construct used. The open boxes indicate the absence or presence of reducing agents during electrophoresis.

REFERENCES