Changes of Matrilin Forms during Endochondral Ossification

MOLECULAR BASIS OF OLIGOMERIC ASSEMBLY*

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To understand the molecular properties of matrilin-3, a newly discovered member of the novel extracellular matrix protein family, we cloned a MAT-3 cDNA from developing chicken sternum. Real time quantitative reverse-transcription polymerase chain reaction indicates that MAT-3 mRNA is mainly expressed in the proliferation zone of a growth plate. It is also expressed in the maturation zone, overlapping with that of the mature chondrocyte-abundant matrilin-1 mRNA. This suggests that matrilin-3 may self-assemble in the proliferation zone, in addition to its co-assembly with matrilin-1 during endochondral ossification. Transfection of a MAT-3 cDNA into COS-7 cells shows that MAT-3 predominantly forms a homotetramer but also a trimer and a dimer. Co-transfection of both MAT-3 and MAT-1 cDNAs results in three major matrilins as follows: (MAT-1), (MAT-3), and (MAT-1)(MAT-3). Thus matrilin-3 may assemble into both homotypic and heterotypic oligomers. Our analysis shows that the assembly of MAT-3 does not depend on the number of epidermal growth factor repeats within the molecule, but the presence of Cys412 and Cys414 within the coiled-coil domain, which form covalent disulfide linkage responsible for both homooligomerization of MAT-3 and heterooligomerization of MAT-3 and MAT-1. Our data suggest that the varying synthetic levels of matrilins in different zones of a growth plate may result in a change of matrilin oligomeric forms during endochondral ossification.

In a cartilaginous growth plate, extracellular matrix (ECM) molecules mediate cell-matrix and matrix-matrix interactions, thereby providing tissue integrity and a matrix permissible for chondrocyte differentiation and subsequent ossification. Some members of matrilins, a novel ECM protein family, have been shown to be expressed specifically in developing cartilage rudiments but not in adult articular cartilage (1, 2). The prototype of the matrilin family, cartilage matrix protein/matrulin-1, has been shown to interact with both collagens (3) and aggregans (4). Thus, matrilins may play an important role in the assembly of the ECM networks (5).

The matrilin family consists at least of four members (2).

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¶The abbreviations used are: ECM, extracellular matrix; MAT, matrilin; RT-PCR, reverse-transcription polymerase chain reaction; EGF, epidermal growth factor; WT, wild type; TAMRA, N,N',N'-tetramethyl-6-carboxyrhodamine; FAM, 6-carboxyfluorescein.

Whereas matrilin-2 and -4 are mainly expressed in non-cartilaginous tissues such as bone and lung, matrilin-1 is expressed specifically in the pre-hypertrophic mature zone of a growth plate (6). Matrilin-3, a novel member of the matrilin family, has also been found to express exclusively in developing cartilage (7). However, it is not known whether it is expressed by chondrocytes during a specific developmental stage, similar to matrilin-1. The identification of the co-assembly product, a heterotetramer (MAT-1)(MAT-3), from growth cartilage (1) suggests that the synthesis of the two matrilins may overlap. However, it is not clear whether their expression patterns are identical. Our study will determine the expression pattern of MAT-3 mRNA in a growth plate and compare it with that of MAT-1.

All the members of matrilin family contain von Willebrand factor A domains, EGF-like domains, and a heptad repeat-coiled-coil domain at the carboxyl terminus, which is responsible for the oligomerization of the molecule (8, 9). The structural differences among the members of matrilins are in two aspects. First, while matrilin-1, -2, and -4 contain two A domains (A1 and A2) separated by EGF-like domains, matrilin-3 lacks the A2 domain. We have shown that the absence of the A2 domain, which is adjacent to the coiled-coil domain, may modulate the oligomerization of matrilins, whereas the A1 domain is not involved in the oligomer formation process (5). For example, the deletion of the A2 domain from matrilin-1 converts a trimeric form, the major oligomeric form of matrilin-1, into a mixture of trimers, dimers, and monomers (5). In addition, the deletion of A2 domain from matrilin-1 abolishes its ability to form collagen-independent filaments (5). Thus, the A2 domain plays an important role in matrilin assembly. Matrilin-3 lacks the A2 domain (7). Its oligomeric forms from self-assembly remain unknown. It is also not clear whether there are other heterooligomeric forms, besides (MAT-1)(MAT-3), that result from co-assembly of matrilin-1 and -3. Our study will determine the homo- and heterooligomeric forms of matrilin-3.

Second, while matrilin-1 contains only one EGF-like domain, matrilin-3 contains four EGF-like domains. Matrilin-2 and -4 also contain multiple EGF repeats (2). It is not known whether the variation of the number of EGF repeats will affect the assembly of matrilins. We will test the role of the EGF repeats in matrilin-3 assembly by examining the oligomeric formation of the matrilin-3 molecules containing 1–4 EGF repeats.

Among all the matrilins, the position and number of cysteines in every corresponding domain is conserved (2). Each A domain has two cysteines, one at the NH2 terminus and one at the COOH terminus. Each EGF-like domain contains six cysteines. There are two additional cysteines at the NH2 terminus of the coiled-coil domain. It has been shown that such two cysteines in matrilin-1 (Cys455 and Cys457) are responsible for forming intermolecular disulfide bonds linking MAT-1 sub-
units together (9). Matrilin-3 is composed of one A domain, four EGF repeats, and a coiled-coil domain. Thus it contains a total 28 cysteines. We hypothesize that the two cysteines in the coiled-coil domain of matrilin-3 (Cys\textsuperscript{455} and Cys\textsuperscript{457}) are responsible to form intermolecular disulfide bonds to link covalently matrilin subunits together, similar to the roles of those corresponding cysteines in matrilin-1. We will test this hypothesis in this study.

To achieve all of these aims, we cloned a MAT-3 cDNA from chick sternal cartilage. To examine the oligomerization of matrilin-3, MAT-3 peptides that consisted of both the EGF repeats and the coiled-coil domain were expressed by COS cells, similar to our approach to characterizing the oligomer formation of matrilin-1 (9). Furthermore, MAT-3 cDNAs were co-transfected with MAT-1 cDNAs to characterize the co-assembly of these two classes of matrilins. The function of the EGF repeats and the cysteine residues within the coiled-coil domain in the assembly of matrilin-3 oligomers were also determined.

MATERIALS AND METHODS

Cloning and Construction of Matrilin-3 cDNAs—Matrilin-3 cDNA was cloned from total RNA isolated from chick sternal cartilage using primers 2 and 4 (Fig. 1). Total RNA was isolated from 17-day embryonic chick sterna using RNaseasy kit (QIAGEN). RT-PCR of matrilin-3 mRNA was performed using Taq one-tube RT-PCR system (Roche Molecular Biochemicals) according to the manufacturer’s instruction. In brief, RNA (500 ng), dNTP (0.2 mM/each), dithiothreitol (5 mM), RNase inhibitor (5 units), primers (0.4 μM/each), reaction buffer (1×), and enzyme mix (1 μl) were added in one tube, and the volume was adjusted to 50 μl. The reverse transcriptions were performed at 50 °C for 30 min and then heated at 94 °C for 2 min. Two-step PCRs were used in the same tube under the following conditions: 94 °C 30 s, 50 °C 30 s, and 68 °C 1.5 min for 10 cycles, and then the annealing temperature was raised to 55 °C for another 20 cycles. The nucleotide sequence of a 654-base pair MAT-3 cDNA was determined by DNA sequencing and was found to be identical to that from GenBank\textsuperscript{TM} (accession number AJ000055), except the difference of seven nucleotides as follows: T755C, A961G, C1028T, T1073C, C1124T, and T1172C. Among these, only A961G causes an amino acid change from Lys\textsuperscript{313} to Arg\textsuperscript{313}. A matrilin-3 cDNA fragment encoding 217 amino acids (Ala\textsuperscript{235} to Val\textsuperscript{452}) was linked, by overlapping PCR, with a signal peptide cDNA sequence of matrilin-1 to ensure its secretion from cells (Fig. 1). This cDNA (MAT-3/4EGF) was cloned into an expression vector pCDNA3.1/V5-His (Invitrogen, Carlsbad, CA). Mat-3 EGF deletions and cysteine (Cys\textsuperscript{412} and Cys\textsuperscript{414}) point mutations were made by overlapping PCR and cloned to pCDNA3.1 in a similar fashion (Fig. 1). In addition, a wild-type matrilin-1 cDNA, a mini-MAT-1 cDNA, a MAT-1 cysteine (Cys\textsuperscript{455} and Cys\textsuperscript{457}) mutant cDNA, and a cDNA of the NC1 domain of type X collagen from previous studies (9, 10) were also cloned into pCDNA3.1V5-His vectors. The sequence of all the inserts was confirmed by DNA sequencing.

Real Time Quantitative RT-PCR—The transcripts of the genes of matrilin-1, -3, and type X collagen in different zones of a growth plate were quantified with quantitative real time RT-PCR (PerkinElmer Life Sciences), as described previously (11). Briefly, the tibiotarsal growth plate from 15-day chick embryos was cut into three zones, proliferation, maturation, and hypertrophy, under a dissection microscope, according to the method published previously (6). Total RNA was isolated from pooled cartilage pieces from the same zone by RNaseasy Kit (QIAGEN). Real time quantitative RT-PCR was performed by using AmpliTaq Gold polymerase (PerkinElmer Life Sciences ABI) with 20 ng of total RNA for each reaction.

Real time RT-PCR was performed with specific primers and probes corresponding to different genes. For each mRNA detection, a fluorogenic probe and two primers for PCR (forward and reverse) were synthesized (PerkinElmer Life Sciences ABI). The internal oligonucleotide probe was labeled with a fluorescent dye carboxyfluorescein (FAM) at the 5′ end and N\textsubscript{2}\textsubscript{−}N\textsubscript{2}\textsubscript{−}N\textsubscript{−}tetramethyl-6-carboxyfluorescein (TAMRA) at the 3′ end. The probe hybridized with the cDNA regions amplified by PCR. When both dyes were present in an intact probe, TAMRA acted as a quencher for FAM by absorbing at the FAM emission spectra. When the internally hybridized probe was degraded by the 5′-exonuclease activity of Taq polymerase during the course of PCR, these two dyes were separated in solution, resulting in a subsequent increase in the amount of fluorescence released during each amplification cycle was proportional to the amount of specific PCR products generated in that cycle.

For chick MAT-3 mRNA detection, the forward and reverse primers were 5′-CTTGTTGAGACGCTCAGTGTG-3′ and 5′-GGGTGGTTAATTGCTGCGGCACT-3′. The internal probe was 5′-FAM-5′-TGTCTTCTATGGGGCTCCTTA-3′. The probe hybridized with the cDNA regions amplified by PCR. When both dyes were present in an intact probe, TAMRA acted as a quencher for FAM by absorbing at the FAM emission wavelengths. When the internally hybridized probe was degraded by the 5′-exonuclease activity of Taq polymerase during the course of PCR, these two dyes were separated in solution, resulting in a subsequent increase in the amount of fluorescence released during each amplification cycle was proportional to the amount of specific PCR products generated in that cycle.

For chick MAT-1 mRNA, the forward and reverse primers were 5′-AGCTGCTGCTCAGTGTG-3′ and 5′-GGGTGGTTAATTGCTGCGGCACT-3′. The internal probe was 5′-FAM-5′-TGTCTTCTATGGGGCTCCTTA-3′.
Expression and Oligomerization of Matrilin-3

Real Time Quantitative RT-PCR—To examine the gene expression pattern of Mat-3, real-time quantitative RT-PCR was performed with mRNA isolated from the growth plate cartilage of three development stages as follows: proliferating, mature, and hypertrophic. Consistent with previously published results, obtained by using RT-hybridization and Western blot (Table I) showed that Mat-1 mRNA was mainly expressed in the maturation zone, and relatively little in the proliferation or hypertrophic zone, and that type X collagen mRNA was mainly expressed in the hypertrophic zone. This indicates that real time RT-PCR can be used to quantify accurately gene expression during development. Different from the mRNA distribution patterns of type X collagen and MAT-1, MAT-3 mRNA was predominantly expressed in the proliferation zone of a growth plate. It was also expressed in the maturation zone but very low in the hypertrophic zone. Thus, matrilin-3 is expressed at a much higher level than MAT-1 in the proliferation zone, whereas MAT-1 is expressed at a much higher level than MAT-3 in the maturation zone (Table I). These data suggest that matrilin-3 may self-assemble in the proliferation zone in addition to its co-assembly with matrilin-1.

Cloning and Construction of Matrilin cDNAs—To characterize the assembly of Mat-3, a MAT-3 cDNA was cloned by RT-PCR from mRNA isolated from embryonic chicken sternal cartilage (see “Material and Methods”). Since it has been established that the oligomeric assembly of matrilins is determined by the COOH-terminal coiled-coil domain (8) and modulated by the domain adjacent the coiled-coil (5), we constructed a MAT-3 cDNA that contains the coiled-coil domain and the neighboring four EGF repeats (Fig. 2, MAT-3/4EGF). A V5 tag and a His tag were attached to the COOH terminus of the molecule for identification of the recombinant protein with antibodies against these tags. A signal peptide sequence from MAT-1 was attached to the NH₂ terminus of the molecule to ensure the secretion of the recombinant protein.

Two groups of MAT-3 cDNA mutants were made. To determine whether the multiple EGF domains of MAT-3 were involved in its assembly, three EGF deletion mutants were created from deleting the EGF domain one at a time from WT-MAT-3 (Fig. 2, MAT-3/3EGF, 2EGF, and 1EGF). To determine whether the 27th and 28th cysteine residues (Cys₁¹² and Cys¹¹⁴), located at the beginning of the heptad repeat coiled-coil, were involved in covalently linking MAT-3 subunits, these two cysteines were mutated into serines (Fig. 3). Upon reduction, all of these bands were reduced to 34 kDa (Fig. 3), consistent with that of a MAT-3 trimer (Table II); a weak band at 102 kDa (Fig. 3), consistent with the calculated molecular weight of a MAT-3 tetramer (Table II); a weak band at 102 kDa (Fig. 3), consistent with that of a MAT-3 trimer (Table II); and a very weak band at 68 kDa (Fig. 3), consistent with that of a MAT-3 dimer (Table II). In addition, higher order multimeric forms of MAT-3 could also be seen from the Western blot (Fig. 3). Upon reduction, all of these bands were reduced to 34

### Table I

| Matrilin-3 | Matrilin-1 | Collagen X |
|------------|------------|------------|
| Proliferation zone | Maturation zone | Hypertrophic zone |
| 157,358 ± 23,190 | 34,091 ± 3,858 | 4,124 ± 329 |
| 86,673 ± 3,071 | 363,175 ± 90,333 | 2,183 ± 0 |
| 1,778 ± 894 | 15,828 ± 1,186 | 23,573 ± 1,478 |

**RESULTS**

**Real Time Quantitative RT-PCR**—To examine the gene expression pattern of MAT-3, real-time quantitative RT-PCR was performed with mRNA isolated from the growth plate cartilage of three development stages as follows: proliferating, mature, and hypertrophic. Consistent with previously published results, obtained by using RT-hybridization and Western blot (Table I) showed that MAT-1 mRNA was mainly expressed in the maturation zone, and relatively little in the proliferation or hypertrophic zone, and that type X collagen mRNA was mainly expressed in the hypertrophic zone. This indicates that real time RT-PCR can be used to quantify accurately gene expression during development. Different from the mRNA distribution patterns of type X collagen and MAT-1, MAT-3 mRNA was predominantly expressed in the proliferation zone of a growth plate. It was also expressed in the maturation zone but very low in the hypertrophic zone. Thus, matrilin-3 is expressed at a much higher level than MAT-1 in the proliferation zone, whereas MAT-1 is expressed at a much higher level than MAT-3 in the maturation zone (Table I). These data suggest that matrilin-3 may self-assemble in the proliferation zone in addition to its co-assembly with matrilin-1.

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Expression and Oligomerization of Matrilin-3

FIG. 2. Constructs of matrilin-3 and matrilin-1 cDNAs. Full-length MAT-3 consists of a signal peptide, A1 domain, four EGF repeats, and a coiled-coil domain. Full-length MAT-1 consists of a signal peptide, two A domains (A1 and A2) separated by an EGF domain, and a coiled-coil. All the cysteines in MAT-3 and MAT-1 are numbered. All constructs derived from the full-length MAT-3 and MAT-1 are indicated.

FIG. 3. Matrilin-3 forms a series of homo-oligomers. Conditioned medium of COS cells transfected with MAT-3/4EGF was separated on a 10% gel, blotted to a membrane, and incubated with antisera against the V5 tag. Bound antibodies were detected with a peroxidase-coupled secondary antibody and a chemiluminescence detection kit. Lanes 1–3 represents three independent experiments with transfection efficiencies from low (lane 1), medium (lane 2), to high (lane 3). All yield similar results. Reducing conditions are indicated and molecular mass is shown on the right.

FIG. 4. EGF domain deletion mutants behave as MAT-3/4EGF. Conditioned medium of cells expressing MAT-3/1EGF (lane 1), MAT-3/2EGF (lane 2), MAT-3/3EGF (lane 3), and MAT-3/4EGF (lane 4) were run under non-reducing and reducing conditions as indicated, on a 4–10% gradient gel and analyzed by Western bloting as described in the legend of Fig. 2. Molecular mass is shown on the left. BSA, bovine serum albumin.

| Table II | Calculated molecular weight of matrilin-1 and matrilin-3 homo- and hetero-oligomers |
|----------|--------------------------------------------------------------------------------------|
|          | Monomer | Dimer | Trimer | Tetramer |
| Mat-3/4EGF | 34  | 68  | 102  | 136  |
| Mat-3/3EGF | 27  | 54  | 81   | 108  |
| Mat-3/2EGF | 20  | 40  | 60   | 80   |
| Mat-3/1EGF | 13  | 28  | 39   | 52   |
| WT-MAT-1  | 54  | 200 |      |      |
| Mini-MAT-1 | 30  | 90  |      |      |
| (Mat-1/2/Mat-3)2 | 178 |      |      |      |
| (Mini-Mat-1/2/Mat-3)2 | 128 |      |      |      |

*Apparent molecular weight from electrophoresis.

kDa (Fig. 3, Reducing), the predicted molecular mass of a MAT-3 monomer (Table II). This indicated that matrilin-3 could self-assemble into a tetramer, a trimer, a dimer, and other multimeric forms. The predominant form of the matrilin-3 oligomer was a homotetramer. Furthermore, the oligomers were linked covalently by disulfide bonds.

Transfection of Mat-3 EGF Deletion Mutants—To determine whether EGF repeats were involved in the oligomeric formation of MAT-3, a series of MAT-3 EGF deletion mutants were transfected. Under non-reducing conditions, transfection of the MAT-3/1EGF resulted in a major product of 52 kDa (Fig. 4, Non-reducing, lane 1), which was consistent with the predicted molecular mass of MAT-3/1EGF (16). The second product of 136 kDa was also a product from MAT-1 single transfection (Fig. 5A, Non-reducing, lane 1), which was established previously in this study as a MAT-1 tetramer (Figs. 3 and 4). One co-assembly product was 178 kDa was seen (Fig. 5A, Non-reducing, lane 1), which was absent from single transfections of either MAT-1 or MAT-3 alone (Fig. 5A, Non-reducing, lane 3). The apparent molecular weight of this co-assembly product was identical to the predicted molecular weight of (MAT-3/1EGF)2 (MAT-3/1EGF)2 (Fig. 5A, Non-reducing, D2, lane 1).

To verify further the co-assembly products, the MAT-3/4EGF...
was co-transfected with a Mini-MAT-1, which contained only the A2 domain and the coiled-coil of MAT-1. A previous study has shown that the mini-MAT-1 behaves exactly like wild-type MAT-1 during oligomer formation (9). Co-transfection resulted in two products that contained MAT-1 (Fig. 6B, Non-reducing, V5 and D2) as follows: a mini-MAT-1 homotrimer (90 kDa) and a mini-MAT-1 and MAT-3 hetero-oligomer (128 kDa), whose molecular weight was identical to that of (mini-MAT-1)₂(MAT-3)₂ (Table III). Thus, MAT-3 assembled with MAT-1 in the molecular form of (MAT-1)₂(MAT-3)₂.

Site-directed Mutagenesis of Cys⁴¹² and Cys⁴¹⁴ of Mat-3—To determine whether Cys⁴¹² and Cys⁴¹⁴ (the 27th and 28th cysteines) were the sites that covalently link the matrilin-3 subunits, we performed transfection of the MAT-3 mutant (Mat-3/C₂⁷,₂⁸) (Table III). Thus, Cys⁴¹² and Cys⁴¹⁴ were responsible for covalently linking MAT-3 subunits.

Co-transfection of Cys Mutants of Mat-1 and Mat-3—We hypothesized that Cys⁴¹² and Cys⁴¹⁴ (the 27th and 28th cysteines) of matrilin-3 also formed disulfide bonds with Cys⁴⁵⁵ and Cys⁴⁵⁷ (the 11th and 12th cysteines) of matrilin-1, which have been shown before to link covalently MAT-1 subunits (9). Thus disulfide bonds among these four residues form covalent linkages between MAT-1 and MAT-3. To test this hypothesis, a series of co-transfections were performed between cysteine mutants of MAT-1 and MAT-3. First, when MAT-1/C₁₁,₁₂ was co-transfected with MAT-3/4EGF (Fig. 6B, Non-reducing, lane 3), no hetero-oligomers between MAT-1 and MAT-3 were formed, although homo-oligomers of MAT-3 (tetramer, trimer, and to a less extent, dimer) still formed (compare Non-reducing lane 3 to lane 2). Thus, Cys⁴⁵⁵ and Cys⁴⁵⁷ of MAT-1 were responsible for linking MAT-1 to MAT-3. Second, when MAT-3/C₂⁷,₂⁸ was co-transfected with WT-MAT-1, it failed to form any hetero-oligomers (Fig. 6B, Non-reducing, lane 4), although MAT-1 homo-oligomers (trimer and to a less extent dimer) were formed (compare Non-reducing lane 4 to lane 1). Thus, Cys⁴¹² and Cys⁴¹⁴ of MAT-3 were responsible for covalently linking MAT-3 subunits to MAT-1. When MAT-1/C₁₁,₁₂ was co-transfected with MAT-3/C₂⁷,₂⁸ (Fig. 6B, Non-reducing, lane 5), nei-

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**Table III**

Amino acid residues at the a and d positions of the heptad repeat of the coiled-coil domain of chick matrilin-1 and -3

| Matrilin-3 | 1 | 2 | 3 | 4 | 5 | 6 |
|------------|---|---|---|---|---|---|
| a          | Q | L | L | L | Q |
| d          | I(β) | V(β) | L | V(β) | Y | V(β) |

| Matrilin-1 | a | d |
|------------|---|---|
|             | V(β) | I(β) | L | V(β) | I(β) | I(β) |
ther the homo-oligomers of MAT-1 and MAT-3 nor the hetero-oligomers between MAT-1 and MAT-3 (see Fig. 5A) were covalently linked. Thus, disulfide bonds among the two cysteines (Cys455 and Cys457) of MAT-1 and the two cysteines (Cys412 and Cys414) of MAT-3 were responsible for covalently linking the subunits of hetero-oligomers of MAT-1 and MAT-3 together.

**DISCUSSION**

ECM in growth plate cartilage is sequentially modified by differentiating chondrocytes and matured during endochondral ossification. The spontaneous assembly of the ECM oligomeric molecules during this maturation process depends on at least two factors as follows: (a) gene expression patterns of chondrocytes, and (b) oligomeric properties of the molecule. In this study, the expression and oligomerization of matrilin-3 are characterized. We have shown that, although both matrilin-1 and -3 are expressed specifically in growth plate cartilage, the expression pattern of MAT-3 is different from that of MAT-1. Mat-3 mRNA is expressed predominantly in the proliferation zone, where much less MAT-1 mRNA is expressed. In the maturation zone, much more MAT-1 mRNA is expressed than MAT-3 mRNA. Because the chondrocytes from the proliferation zone precede those from the maturation zone temporally during development, our data suggest that matrilin-3 may self-assemble in the proliferation zone, in addition to its co-assembly with matrilin-1.

The self-assembly of matrilin-3 was studied by transfection of a MAT-3 cDNA in COS-7 cells that do not synthesize endogenous MAT-3. Matrilin-3 forms a tetramer predominantly, but also a trimer and a dimer in small quantities. Thus, unlike matrilin-1 and -2, which form mainly trimers (9, 17), matrilin-3 forms tetramers predominantly. Analysis of the amino acid sequence of the coiled-coil domain of MAT-3 (Table III) suggests that, unlike the MAT-1 sequence that favors a triple helix, the MAT-3 sequence strongly favors the formation of a tetrameric helix. For example, the presence of β-branched residues at both “a” and “d” positions favors a tetrameric helix, and the presence of β residues at a positions also favors a tetrameric helix (18). Mat-1 contains β residues at both positions, and MAT-3 does not contain any β residues at a positions (Table III). Thus, MAT-1 favors trimer formation and MAT-3 favors tetramer formation. By using this rule to analyze all the members of the matrilin family, it can be predicted that the major oligomeric forms of matrilin-1, -2, and -4 are trimers and that of matrilin-3 is a tetramer. Although it has been shown that matrilin-1 and -2 form trimers (9, 17), and matrilin-3 forms tetramer, the oligomeric form of matrilin-4 remains to be determined.

The second interesting aspect of our finding is that the formation of MAT-3 oligomers is not exclusively tetrameric. Matrilin-3 also forms trimers, dimers, and even other multimeric forms in small quantities. Examination of the MAT-3 sequence (Table III, Matrilin-3) reveals that there is indeed one pair of Leu residues at the a and d positions, favoring trimer formation (18), although the majority of the a residues are Leu, and the d residues are β-branched, thus favoring tetramer formation (18). Therefore, this sequence may dictate that the majority of the MAT-3 oligomers are tetramers and the minority are trimers. Similar mixed sequence information, which contains both a majority component and a minority component, exists for all the matrils. For example, although the majority of the MAT-1 sequence (β-branched residues at both a and d positions) favors trimer formation, the minority of the sequence (β residues at the a position and Leu at the d position) may make the formation of a dimer possible as well (Table III, Matrilin-1). Indeed, a minority of matrilin-1 exists as dimers and other multimeric forms (1). Consistent with this hypothesis, it has been reported that, although the majority of matrilin-2 exists as trimers (19), tetramers and dimers exist as well (20). Thus, each matrilin may exist in a major oligomeric form and minor oligomeric forms as well. The heterogeneity of the matrilin oligomeric forms may contribute to the complexity of the extracellular matrix network.

The co-assembly of matrilin-3 and -1 was studied by co-transfection of their cDNAs. The major co-assembly product is (MAT-1)2(MAT-3)2, identical to the one identified from growth plate cartilage in vivo (1). From our data, it can be concluded that (MAT-1)2(MAT-3)2 is the major, if not the only, co-assembly product between matrilin-1 and -3. It is interesting to note that, although MAT-1 favors trimer formation and MAT-3 favors tetramer formation, the hetero-oligomeric form of these two adopt a tetrameric configuration. Thus, the tetrameric configuration of MAT-3 acts in a dominant fashion during the co-assembly process.

Members of the matrilin family contain different numbers of the EGF-like domains. Mat-1 contains 1; MAT-2 contains 10, and both MAT-3 and -4 contain 4. Our analysis has shown that the variation of the EGF repeats has little effect on the oligomeric properties of MAT-3, even though the EGF repeats abut the coiled-coil domain in the molecule. This suggests that the EGF-like domains in a matrilin do not influence the oligomeric formation process. In contrast, the pair of cysteines at the NH2 terminus of the coiled-coil domain is conserved among all the matrils. Our analysis has shown that this pair of cysteines in MAT-1 and MAT-3 is necessary and sufficient for the formation of intermolecular disulfide bonds connecting matrilin subunits. This includes the formation of both homo- and hetero-oligomeric matrils.

In summary, we have determined the gene expression pattern of matrilin-3 in a growth plate, and we identified several novel forms of matrilin-3. These data may have implications for understanding the matrix maturation process during development. In the proliferation zone of a growth plate, chondrocytes are dividing and very close to each other, thus there is very little matrix space between neighboring cells. In contrast, mature chondrocytes synthesize and deposit a large amount of matrix, thereby creating a large interstitial matrix region. It has been shown that the mature cartilage-specific matrilin-1 could form a filamentous network to connect collagen fibrils and aggrecans (5). Matrilin-3, which lacks the A2 domain, may not form filaments by itself (5). Our data suggest that there is a change of the molecular forms of matrils in a growth plate, from the proliferation zone to the maturation zone. In the proliferation zone, matrilin-3 is the major matrilin form, whereas matrilin-1 is the major form of matrils in the maturation zone. This synthetic change may convert matrilin forms from the ones that are incapable of forming long range matrix networks into those that can, thereby contributing to the matrix maturation process during endochondral ossification.

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