Molecular Structure and Tissue Distribution of Matrilin-3, a Filament-forming Extracellular Matrix Protein Expressed during Skeletal Development*

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Matrilin-3 is a recently identified member of the superfamily of proteins containing von Willebrand factor A-like domains and is able to form hetero-oligomers with matrilin-1 (cartilage matrix protein) via a C-terminal coiled-coil domain. Full-length matrilin-3 and a fragment lacking the assembly domain were expressed in 293-EBNA cells, purified, and subjected to biochemical characterization. Recombinantly expressed full-length matrilin-3 occurs as monomers, dimers, trimers, and tetramers, as detected by electron microscopy and SDS-polyacrylamide gel electrophoresis, whereas matrilin-3, purified from fetal calf cartilage, forms homotetramers as well as hetero-oligomers of variable stoichiometry with matrilin-1. In the matrix formed by cultured chondrosarcoma cells, matrilin-3 is found in a filamentous, collagen-dependent network connecting cells and in a collagen-independent pericellular network. Affinity-purified antibodies detect matrilin-3 expression in a variety of mouse cartilaginous tissues, such as sternum, articular, and epiphyseal cartilage, and in the cartilage anlage of developing bones. It is found both inside the lacunae and in the interterritorial matrix of the resting, proliferating, hypertrophic, and calcified cartilage zones, whereas the expression is lower in the superficial articular cartilage. In trachea and in costal cartilage of adult mice, an expression was seen in the perichondrium. Furthermore, matrilin-3 is found in bone, and its expression is, therefore, not restricted to chondroblasts and chondrocytes.

The matrils constitute a recently discovered family of non-collagenous proteins (1) belonging to the von Willebrand factor A (vWFA)like superfamily. To date, there are four matrilins known. Matrilin-2 (2, 3) and matrilin-4 (4, 5) have a broad tissue distribution, whereas the expression of matrilin-1 (also known as cartilage matrix protein) (6–8) and matrilin-3 (9–11) is more restricted to skeletal tissues. The division of the family into two subgroups can also be concluded from evolutionary studies (1). The descent from a common ancestor and the divergence through duplication of whole domains indicates the possibility of the different family members providing similar functions in different tissues. The at least partially coordinated expression of matrilin-1 and -3 gains further functional significance through the recent discovery of hetero-oligomers formed by matrilin-1 and -3 in epiphyseal cartilage of fetal calf femur (12).

Matrilin-3 has most features of the modular structure typical for matrilins and consists of an N-terminal vWFA-like domain, four EGF-like domains, and a C-terminal α-helical coiled-coil oligomerization domain (9–11), but it lacks the second vWFA-like domain that is present in all other matrilins. Similarly, a unique mouse matrilin-4 splice variant lacking the N-terminal vWFA-like domain was recently identified (4). In addition, matrilin-3 possesses a domain with a high content of positively charged amino acids between the N-terminal vWFA-like domain and the signal peptide cleavage site. The mouse matrilin-3 precursor consists of 481 amino acid residues and after cleavage of the 27-amino acid signal peptide a mature protein with a predicted minimum molecular mass of 48.9 kDa is formed (9). Matrilin-3 from human and chicken are highly homologous to the mouse protein and differ mainly in the N-terminal positively charged domain, which is longer in human and shorter in chicken. Additionally, all four EGF-like domains of human and mouse matrilin-3 contain an insertion of a single aspartic acid residue not found in the corresponding chicken sequences. Matrilin-3 expression was so far studied by Northern hybridization, in which the signal was more pronounced at the periphery than in the center of the cartilage (10).

We have recombiantly expressed the full-length mouse matrilin-3 as well as a truncated version lacking the coiled-coil domain in a mammalian expression system. The truncated matrilin-3 was used for production of a specific antisera that allowed immunohistochemical characterization of matrilin-3 expression and an analysis of assembly forms in the pericellular matrix formed by cultured chondrocytes. The full-length protein was used for structural studies by which the molecular dimensions and oligomeric state of recombinant matrilin-3 could be determined. Purification of native matrilin-3 from fetal calf cartilage allowed a comparison with the naturally occurring hetero-oligomers formed with matrilin-1.

MATERIALS AND METHODS

Expression and Purification of Recombinant Matrilin-3—The full-length and the truncated matrilin-3 cDNA-construct, lacking the coiled-coil domain, were generated by polymerase chain reaction on a murine full-length clone (9). Suitable primers introduced a 5′ terminal SpeI and a 3′ terminal NotI restriction site. The digested cDNA-constructs were
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**Fig. 1.** SDS-PAGE analysis of recombinant matrilin-3 proteins and demonstration of the specificity of the matrilin-3 antiserum by immunoblot. Full-length matrilin-3 (M3) (lanes 1 and 3) and matrilin-3 lacking the coiled-coil domain (M3-) (lanes 2 and 4) were submitted to SDS-PAGE without (-SH) (lanes 1 and 2) and with (+SH) (lanes 3 and 4) prior reduction and stained with Coomassie Brilliant Blue. Asterisks mark the different oligomeric forms of non-reduced matrilin-3. Proteins extracted from sternum were submitted to SDS-PAGE, blotted to nitrocellulose, stained with Ponceau S (lane 5), and developed with the affinity-purified matrilin-3 antiserum (lane 6). All samples were separated on 4–15% polyacrylamide gels. Molecular mass of marker proteins (right) is given in kDa.

Phosphor imaging was performed as described by Laemmli (14). For immunoblots, the proteins were transferred to nitrocellulose and incubated with a dilution of the appropriate rabbit antiserum. Bound antibodies were detected using peroxidase-conjugated swine anti-rabbit IgG (Dakopats), 3-aminopthalhydrazide (1.25 mM), p-coumaric acid (225 mM), and 0.01% H$_2$O$_2$. Reference samples of protein from mouse sternum were extracted overnight with 0.1 M NaCl, 0.05 M Tris/HCl, pH 7.4, containing 0.01 M EDTA, 2 mM phenylmethylsulfonyl fluoride and 2 mM N-ethylmaleimide at 4°C. For sequencing, both recombinant proteins were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted to a polyvinylidine difluoride membrane (Immobilon P, Millipore). Protein bands were cut out, and their N-terminal amino acid sequences were determined in a Applied Biosystems 473A protein sequencer.

**In Situ Hybridization—**Limb sections from newborn mice were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4°C, washed overnight with phosphate-buffered saline (4°C), dehydrated, and embedded in paraffin. Sections of 7 μm were cut, mounted on 3-aminopropyltriethoxy-silane treated glass slides, dewaxed in xylene, and rehydrated. After washing in phosphate-buffered saline, they were digested with 10 μg/ml proteinase K, postfixed, and acetylated with 0.25% acetic anhydride. The sections were hybridized overnight at 55°C with digoxigenin-labeled riboprobes covering the first 827 nucleotides of the matrilin-3 cDNA (9). After hybridization, the sections were washed in 50% formamide, 2× SSC for 30 min at 52°C; digested with RNase A; and washed once with 2× SSC and twice with 0.2× SSC for 20 min at 52°C. The immunological detection of the digoxigenin-labeled sections was carried out according to the instructions of the manufacturer (Roche Molecular Biochemicals) with additional use of polystyrene alcohol in the detection solution (15).

**Immunohistochemistry—**Immunohistochemistry was performed on cryosections frozen on dry ice in Tissue-Tek® (Miles, Inc.) as well as on paraffin-embedded sections of fetal, newborn and 6-week-old mice. The cryosections were used either unfixed or fixed with 1% paraformaldehyde for 10 min., treated for 1 h with 0.04 units/ml chondroitinase ABC (Sigma), and incubated twice for 20 min in 1% H$_2$O$_2$. Immunolabeling was done by consecutive treatment of the sections for 1 h with affinity-purified antibodies to matrilin-3 and peroxidase conjugated swine anti-rabbit IgG. Both antibodies were diluted in 1% (w/v) bovine serum albumin in Tris-buffered saline and the slides developed with 3-amino-9-ethylcarbazole. The paraffin-embedded tissues were prefixed and demineralized after fixation in 0.6 M EDTA, Tris-buffered saline, pH 8.0, for 21 days. Deparaffinization ensued through incubation for 30 min in rothirol (Carl Roth GmbH, Karlsruhe, Germany) at 52°C. After rehydration, immunostaining was performed as described above, but with the incubation time with the first antibody increased to 18 h. For immunofluorescence detection, a Cy3-conjugated affinity-purified goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) was used. Nonspecific antibody binding was blocked by incubation for 1 h.
with 5% (w/v) normal goat serum in Tris-buffered saline.

Cell Culture of Chondrosarcoma Cells—The Swarm rat chondrosarcoma cell line (16) was obtained from Dr. J. Kimura (Henry Ford Hospital, Detroit, MI). The immortalized cells were cultured on plastic chamber slides in Ham’s F-12 nutrient mixture supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 units/ml streptomycin, and, if desired, 50 μg/ml l-ascorbic acid.

Electron Microscopy—Electron microscopy was performed as described previously (3, 17) using matrilin samples with typical concentrations of 5–10 μg/ml in Tris-buffered saline that were stained with 0.75% uranyl formate.

Mass Spectrometry—Sample preparation for MALDI-TOF mass spectrometry was performed as described (18). As a matrix, α-cyano-4-hydroxycinnamic acid (Sigma) was used. When desired, the protein was reduced with 0.01 m dithiothreitol on the target for 1 h at 37 °C. Cations were detected and analyzed utilizing the high mass detector in the linear mode of a Bruker Biflex III. Calibration of the time of flight analyzer was based upon the Mr of recombinant protein A (Repligen) of 44,810.3 and the Mr of bovine serum albumin dimer (Sigma) of 132,859.0.

RESULTS

Recombinant Expression of Matrilin-3 Constructs—A cDNA fragment encoding the sequence of mature mouse matrilin-3 and a fragment lacking the oligomerization domain were inserted into the pCEP-Pu vector utilizing the secretion signal sequence of BM-40 (13). The recombinant plasmids were introduced into 293-EBNA cells and stably maintained in an episomal form. The secreted matrilin-3 proteins were purified from the medium (Fig. 1), and their identities were confirmed by N-terminal sequencing. Surprisingly, the first eight amino acid residues encoded by the cDNA-constructs were missing, presumably due to the cleavage at the potential furin protease cleavage site Arg-Leu-Ala-Arg (amino acids 31–34). The resulting N-terminal sequence of the mature protein starts with Ala55-Ser-Val. The purified protein lacking the oligomerization domain was used to immunize rabbits. After affinity purification, the antisera reacted specifically with matrilin-3 (Fig. 1).

Recombinantly Expressed Matrilin-3 Forms Oligomers—Purified recombinant full-length matrilin-3 was analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 1). Without reduction four bands representing the tetra-, tri-, di-, and monomeric forms of matrilin-3 were detected. The purified full-length matrilin-3 was submitted to electron microscopy after negative staining with uranyl formate (Fig. 2A). The stained particles were heterogeneous in size, and a closer examination of single particles revealed that all species from monomer to tetramer were present in the sample. At high magnification, it was seen that in oligomers all subunits are joined at a single point in a manner reminiscent of the bouquet-like structure known from matrilin-1 (19) and matrilin-2 (3), but different in that in matrilin-3 a stalk made up from the four EGF-repeats is clearly resolved. Monomers have either a stretched tadpole-like or a collapsed shape.

Purification of Native Matrilin-1/Matrilin-3 Hetero-oligomers from Fetal Calf Cartilage—Preliminary immunoblot results revealed that expression and/or solubility of matrilin-3 is much higher in fetal than in adult tissues (not shown). Therefore, fetal calf cartilage was extracted to obtain the tissue form of matrilin-3. Extraction under nondenaturing conditions, followed by chromatographic purification, yielded preparations highly enriched in matrilin-3 but also containing considerable amounts of matrilin-1. SDS-PAGE and immunoblot showed the presence of different matrilin-1/matrilin-3 hetero-oligomers as well as of homotrimeric matrilin-1 (Fig. 3). Reduction of the oligomeric forms resulted in two bands with apparent masses of 66 and 57 kDa (Fig. 3). These were by immunoblotting identified as matrilin-3 and matrilin-1, respectively (Fig. 3), in agreement with the recent assignment of Wu and Eyre (12) by N-terminal protein sequencing. MALDI-TOF mass spectrometry gave masses of 53.0 and 49.3 kDa for the subunits of matrilin-1 and -3, respectively (results not shown). Apparently the matrilin subunits show an anomalous behavior in SDS-PAGE, as earlier noted for matrilin-1 (20). In nonreducing SDS-PAGE, four bands of differing intensity could be distinguished in the range of 170–220 kDa. In addition, two weak bands were present at around 160 kDa, of which the upper band reacted with antibodies to both matrilin-1 and -3 and the lower only with antibodies to matrilin-1 (Fig. 3). These bands probably represent heterotrimers matrilin-1/matrilin-3 and homotrimeric matrilin-1. A sample that had been chromatographically enriched for the three largest oligomers, presumably tetramers, was further investigated with two-dimensional SDS-PAGE using nonreducing conditions in the first dimension and reducing in the second (Fig. 4). The oligomer with the highest apparent Mr was found to be composed only of matrilin-3 subunits, whereas the two forms of higher mobility contain both matrilin-3 and matrilin-1. The complex band pattern detected in SDS-PAGE under nonreducing conditions (Fig. 3) is in contrast to the recently published results (12) that point to the existence of only heterotetramers with a two and two stoichiometry.

MALDI-TOF mass spectroscopy of an nonreduced sample of the same preparation as shown in Fig. 3 yielded two broad
molecule ion peaks at around 156 and 202 kDa, indicating the presence of both trimeric and tetrameric populations (results not shown). A resolution of the different species within those peaks could not be achieved. In electron microscopy, tetrameric and, less often, trimeric molecules were seen that frequently displayed the bouquet-like shape typical for matrilins (Fig. 2B).

In addition to compact trimeric particles, presumably representing matrilin-1 homotrimers, and tetrameric particles with clearly visible stalks, closely resembling the recombinant matrilin-3 homo-oligomers, particles were seen that, judging from their morphology, could represent matrilin-1/matrilin-3 hetero-oligomers of varying stochiometries. Furthermore, trimeric and tetrameric particles were seen in which the globular domains show an almost linear arrangement.

Matrilin-3 Forms Extracellular Filamentous Networks in Cell Culture—In order to study the extracellular assembly forms of matrilin-3, the matrix produced by cultured Swarm rat chondrosarcoma cells was analyzed by immunofluorescence microscopy. When ascorbate was present in the cell culture medium, matrilin-3 was detected in an extensive, fibrillar network connecting cells over a distance of several cell diameters (Fig. 5A). These extended fibrils could not be detected in the absence of ascorbate (Fig. 5B). Such collagen-independent fibrils were formed at cell contacts that develop immediately after mitosis (Fig. 5C).

Matrilin-3 Is Expressed in Dense Connective Tissue during Growth and Remodeling—Immunohistochemistry was performed on sections of mouse embryos and of tissues from newborn and 6-week-old mice. The earliest expression of matrilin-3 could be detected in a day 12.5 postcoitum mouse embryo in the cartilage anlage of the developing bones (not shown). At day 14.5 postcoitum, the primordial skeleton (e.g. rib cartilage, vertebral bodies, and the cartilage primordium of the legs) showed strong staining for matrilin-3 (Fig. 6A). At birth, matrilin-3 is present in the developing occipital bones and in the bones of the nasal cavity, whereas no expression was detected in maxilla and mandible (Fig. 6B). Matrilin-3 was also detected in the manubrium and corpus of sternum (Fig. 6C), as well as in the cartilage plates of trachea (Fig. 6D). In the tail of newborn mice, the cartilage primordium of the vertebral bodies showed a strong expression, in contrast to a weaker one in the annulus fibrosus and none in the nucleus pulposus (Fig. 6E). In long bones of newborn mice, e.g. the tibia, matrilin-3 is broadly expressed in the epiphysis and could be detected in both the territorial and interterritorial matrix of reserve, proliferating and hypertrophic cartilage (Fig. 6F). In the hypertrophic cartilage, the signal is weaker and in part missing in the lower
zone. Matrilin-3 was also detected in the ossified portion of the epiphysis, whereas no signal was seen in the articular cartilage. The strong expression in the epiphyseal growth plate persisted in long bones of 6-week-old mice (Fig. 6I). At this stage, matrilin-3 was also detected in peripheral and deeper portions of the articular cartilage and in the interior of the lateral meniscus. In addition to cartilaginous tissues, matrilin-3 was present in the osteoid around osteoblasts attached to bone trabeculae in the subchondral bone (Fig. 6H) and around osteocytes inside the cancellous bone (Fig. 6J). In the still growing distal portion of the radius, matrilin-3 was more abundant than in the ossified proximal end, where an epiphysis is no longer present (not shown). In spongy cartilage plates, matrilin-3 was present in filamentous structures that start and end in the chondroblasts and are arranged in parallel orientation relative to the ossification front (Fig. 6J). In cartilaginous tissue, it was deposited in the perichondrium around fibroblast-like cells and in structures spanning the distance from perichondrium to perichondrium (Fig. 6K). A similar, mainly perichondral expression was seen in the trachea (Fig. 6L). At no stage of development was matrilin-3 detected in extraskeletal tissues.

Matrilin-3 mRNA Is Transcribed in Chondrocytes and Osteoblasts—In situ hybridization was performed on paraffin sections of newborn mice. In limbs, the matrilin-3 gene was transcribed by all chondrocytes with the exception of those at the articular surface and the lower hypertrophic zone (Fig. 7A). A gradient in transcription of the matrilin-3 mRNA could be seen, with the strongest signals in the peripheral areas of the proliferative zone, weaker signals in the central part of the resting cartilage, and the weakest signals or none in the hypertrophic zone, where, however, most of the intracellular material is lost during the hybridization procedure. In the rare cases in which RNA was retained, weak hybridization signals appeared (Fig. 7A). Matrilin-3 is also transcribed by cells, presumably osteoblasts, that are located as a monolayer on the endosteal surface and on bone trabeculae, showing that the matrilin-3 detected in bone by immunohistochemistry is not only a remainder of the resorbed cartilage but also newly synthesized there (Fig. 7B). Furthermore, immunoblot analysis of U2-OS cell cultures (22) showed that this osteoblast-like cell line secretes matrilin-3 into the medium (results not shown).

DISCUSSION

Matrilin-3 Is Able to Form Homo- and Hetero-oligomers—Matrilin-3 is able to form homo-oligomers, as detected by both SDS-PAGE (Fig. 1) and electron microscopy (Fig. 2A) of recombinant protein from the supernatants of 293-EBNA cells transfected with matrilin-3 cDNA. The oligomerization occurs through the coiled-coil domain, as the construct that lacks this domain only yields monomers (Fig. 1). In electron microscopy (Fig. 2A), the oligomeric forms show similarities to the bouquet-like shape of matrilin-1 and -2 (19, 3). However, the center of the particle accumulates more stain, as the single A-domains in matrilin-3 are connected to the coiled-coil domain by a stalk formed by four EGF domains. As each subunit contains only a single A-domain, self-interactions within the subunit cannot occur, and the arms of matrilin-3 are accordingly more extended than those of matrilin-1 and -2 (19, 3). The presence of all oligomeric forms from monomers to tetramers is surprising, but it has also been seen with matrilin-2, both when expressed recombinantly and when extracted from tissues (3). Matrilin-1 occurs in mature cartilage mainly as homotrimers (20), but the formation of homotetramers was described for a matrilin-1 peptide, covering the coiled-coil domain, where a single arginine residue was replaced with a glutamine (23). As dimers and monomers of matrilin-3 are seen not only under the denaturing conditions of SDS-PAGE (Fig. 1A) but also in electron microscopy (Fig. 2A), they are not due to deficient closure of disulfide bridges. In contrast, in native preparations from fetal bovine rib cartilage, even though small amounts of matrilin-1 dimers and monomers could be detected in SDS-PAGE, only intact, trimeric particles were seen by electron microscopy (19). Chen et al. (24) recently reported that deletion of the A2 domain in chicken matrilin-1 led to an incomplete assembly when this protein was recombinantly expressed in chick fibroblasts. They proposed that the A2 domain facilitates the formation of the directly adjacent coiled-coil during the biosynthesis. This is in agreement with our observation that matrilin-3 subunits that naturally lack the A2 domain occur in monomeric and dimeric forms as well as in higher oligomers. In matrilin-2, which also assembles incompletely, the A2 domain is present, but it is separated from the coiled-coil by a unique stretch of 75 amino acids in an unknown fold (2, 3). Possibly, the A2 domain may promote coiled-coil formation only when directly adjacent to the assembly domain. The incomplete oligomerization of matrilin-3 may, however, equally well reflect a low stability of the matrilin-3 trimeric and tetrameric coiled-coils, caused by the high proportion of imperfect heptad repeats in this matrilin, and it is also possible that subunits are lost from completely assem-
FIG. 6. Tissue distribution of matrilin-3. Frozen (A–E, L) and paraffin-embedded (F–K) sections from a day 14.5 postcoitum mouse embryo (A), newborn (B–F) and 6-week-old (G–L) animals were incubated with affinity-purified antibodies against matrilin-3 followed either by peroxidase-conjugated swine anti-rabbit IgG (A–F) or a Cy3-conjugated goat anti-rabbit IgG (G–L). In a day 14.5 postcoitum mouse fetus, immunostaining was detected in the cartilage primordia of the vertebral bodies (vb), the dorsal part of ribs (ri), and the heads of the humerus (hu) and the femur (fe) (A). In the head, the cartilage primordia of the supraoccipital (so), basioccipital (bo), basisphenoid (bs), turbinate (tu) and hyoid (hy), bones and petrous part of temporal bone (te) were stained (B). In the cartilage plates (cp) of corpus sternum and in costal cartilage (co), strong signals were present (C). Weaker signals could be detected in the ossification cores (os) between the cartilage plates. The cricotracheal cartilage of the trachea (tr) was also positive (D). Strong signals could be detected in the cartilage primordia of the vertebral bodies (cb). In the intervertebral disc, a weak signal could be found in the annulus fibrosus (af) but not in the nucleus pulposus (np) (E). The distal end of theibia shows a matrilin-3 expression both in the territorial and interterritorial matrix surrounding resting (rc), proliferating (pc) and hypertrophic (hc) chondrocytes, weaker signals in the trabecular calcified bone (cb), and none in the articular cartilage (ac) (F). Strong expression could be observed in the epiphyseal growth plate (gp) and the newly formed trabecular bone (tr) of tibia. Matrilin-3 is also present in the trabecular meshwork of the secondary ossification center (so), in the lateral meniscus (lm), in the subchondral bone of femur (sb), and at a low level in the peripheral articular cartilage (pa) (G). The higher magnification of the metaphyseal side of the growth plate of tibia shows immunostaining around osteoblasts (ob) attached to bone trabeculae (H). On the epiphyseal side of the growth plate, matrilin-3 was found on trabecular surfaces (ts) and around osteocytes (oc) inside the cancellous bone (I). In adult sternum, matrilin-3 was observed in filamentous structures (fl) between the lacunae, which are arranged in a parallel orientation to the ossification front (J). Strong immunostaining was found in the costal perichondrium (pe) around fibroblast-like shaped cells and inside the costal cartilage in structures with transverse orientation (arrows) (K). In adult trachea, strong immunostaining was found in the perichondrium...
of newborn mice.

In the mineralized part of tibia, mRNA was detected in cartilage (detected in cells of the superficial and central parts of the articular zone). No mRNA could be found in central areas of the lateral meniscus (material during the hybridization procedure. Weak signals were also seen in hypertrophic cartilage (hc) and in the center of the resting (A) zone, weak or no signals were detected, due in part to a loss of cellular material during the hybridization procedure. Weak signals were also found in central areas of the lateral meniscus (lm). No mRNA could be detected in cells of the superficial and central parts of the articular cartilage (A). In the mineralized part of tibia, mRNA was detected in cells lining bone trabeculae and in endosteal cells (eo) (B). Bar, 150 μm.

Fig. 7. In situ hybridization of matrilin-3 mRNA in the skeleton of newborn mice. Antisense riboprobes labeled with digoxigenin were hybridized to paraffin-embedded sections of a knee (A) and tibia (B) of newborn mice. In tibia (left) and femur (right), mRNA was detected in most of the cartilaginous tissues (A). In the periphery of the resting and the proliferating (pc) cartilage, strong signals were present, whereas in hypertrophic cartilage (hc) and in the center of the resting zone, weak or no signals were detected, due in part to a loss of cellular material during the hybridization procedure. Weak signals were also found in central areas of the lateral meniscus (lm). No mRNA could be detected in cells of the superficial and central parts of the articular cartilage (A). In the mineralized part of tibia, mRNA was detected in cells lining bone trabeculae and in endosteal cells (eo) (B). Bar, 150 μm.

Evidence for a Role of Matrilin-3 in the Formation of Extracellular Filamentous Networks—Immunofluorescence microscopy of the matrix formed by cultured Swarm rat chondrosarcoma cells shows matrilin-3 in an extended filamentous network. The filaments have a variable thickness, often form branches and connect cells over a distance of several cell diameters (Fig. 5A). The formation of this network depends on the presence of ascorbate in the culture medium, implying that it contains also collagenous proteins. In the absence of ascorbate a matrilin-3-containing network is seen in close association with cells (Fig. 5B). This network focally connects cells and sometimes covers the whole chondrocyte (Fig. 5C). Interestingly, the filaments often connect two neighboring cells that appear to have recently undergone mitosis. Such connections, if they occur also in situ, could possibly provide chondrocytes with spatial information.

Similar results were obtained for the distribution of matrilin-1 in cultures of chicken chondrocytes, in which a more extended collagen-dependent network (21, 25) and a pericellular, collagen-independent network were also described (21). Recent studies revealed functional differences between the two A domains of matrilin-1. Deletion of the A2 domain leads to an impaired oligomerization, whereas the lack of either A domain results in an inability to form collagen-independent filaments. Furthermore, double point mutations of Asp-22 in the A1 and Asp-255 in the A2 domain disrupt matrilin-1 network formation (24). As these aspartic acid residues are part of the metal-ion-dependent adhesion site motif, an adhesion mechanism mediated by this site is very likely. Matrilin-2 also forms a fibrillar network when expressed by cultured smooth muscle cells (3), showing this feature to be common to several matrilins.

The matrilin-3 containing filamentous structures found in caudal sternal cartilage plates (Fig. 6J) could represent an in vivo counterpart of the fibrils seen in cell culture. These structures are oriented along the lateral growth axis of the sternum plate. Matrilin-3 positive, filamentous structures were detected also in costal and tracheal cartilage, in which, in both cases, the direction of the fibrils is perpendicular to the perichondrium (Fig. 6K).

Matrilin-3 Is Specific for Dense Connective Tissue and Expressed in Zones of Growth or Remodeling—Matrilin-3 has a tissue distribution similar to that of matrilin-1. Both proteins are found almost exclusively in skeletal tissues and apparently sometimes function together in the form of hetero-oligomers. Matrilin-3 is strongly expressed in growing skeletal tissues, e.g. the epiphyseal growth plate, or in bone undergoing growth and remodeling. In bone, it is not only a remnant found in not yet resorbed calcified cartilage, but it is actively synthesized by osteoblasts and osteocytes. In bovine trachea, the matrilin-3 expression is decreased upon maturation (12), and in 6-week-old mice, the residual expression is more restricted to the perichondrium (Fig. 6L). In contrast, matrilin-1 accumulates in bovine trachea, and the highest amounts are present in adult animals (26).

Even though matrilin-1 and matrilin-3 may occur in the same tissues, their relative amounts differ during the life span of the animal, and even though their functions may be related, they may in part fulfill those at different timepoints in development and maturation. Further work will be directed at determining the structure, composition, and function of matrilins.
containing filaments and the interactions by which they are formed.

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REFERENCES

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