Matrilin-4 is the most recently identified member of the matrilin family of von Willebrand factor A-like domain containing extracellular matrix adapter proteins. Full-length matrilin-4 was expressed in 293-EBNA cells, purified using affinity tags, and subjected to biochemical characterization. The largest oligomeric form of recombantly expressed full-length matrilin-4 is a trimer as shown by electron microscopy, SDS-polyacrylamide gel electrophoresis, and mass spectrometry. Proteolytically processed matrilin-4 species were also detected. The cleavage occurs in the short linker region between the second von Willebrand factor A-like domain and the coiled-coil domain leading to the release of large fragments and the formation of dimers and monomers of intact subunits still containing a trimeric coiled-coil. In immunoblots of calvaria extracts similar degradation products could be detected, indicating that a related proteolytic processing occurs in vivo. Matrilin-4 was first observed at day 7.5 post-coitum in mouse embryos. Affinity-purified antibodies detect a broad expression in dense and loose connective tissue, bone, cartilage, central and peripheral nervous systems and in association with basement membranes. In the matrix formed by cultured primary embryonic fibroblasts, matrilin-4 is found in a filamentous network connecting individual cells.

The matrilin family of oligomeric extracellular matrix proteins contains von Willebrand factor A (vWFA) like domains (1). In mouse, four matrilins exist with matrilin-1, previously referred to as cartilage matrix protein, being the prototype member. The expression of matrilin-1 and -3 is restricted to skeletal tissues, whereas the matrilin-2 and -4 have a broad tissue distribution. It has been suggested that matrilins act as adapter molecules connecting other proteins and proteoglycans in the extracellular matrix (2).

Mouse matrilin-4 has the modular structure typical for matrilins and consists of two vWFA-like domains connected by four epidermal growth factor (EGF)-like domains and a C-terminal α-helical coiled-coil domain predicted to mediate oligomerization (3). In addition, a unique splice variant, which does not contain the N-terminal vWFA-like domain, was identified in mouse (3). Matrilin-4, like matrilin-1, lacks the stretch of frequently positively charged amino acids found in other matrilins between the signal peptide cleavage site and the vWFA-like domain at the N-terminal end of the mature protein. The mouse matrilin-4 precursor consists of 624 amino acid residues, and after cleavage of the 21-amin acid signal peptide a mature protein with a predicted minimal mass of 66.4 kDa is formed (3). Human matrilin-4 is highly homologous to the mouse protein with an overall identity of 91% and a maximum identity of 97% in the second vWFA-like domain. Due to a mutation in the splice donor site of the third intron of the human gene, the exon, which corresponds to that specifying the first EGF-like domain in mouse, is not expressed in man. Instead of the four EGF-like domains present in mouse, the human matrilin-4 contains three, two, or one EGF-like domains, probably depending on a differential usage of splice acceptor sites in the exons coding for EGF-like domains (4). When matrilin-4 expression was studied in the mouse by Northern hybridization, mRNA could be detected in lung, sternum, brain, kidney, and heart (3). In the human, matrilin-4 expression was demonstrated by reverse transcriptase-polymerase chain reaction in lung and placenta, as well as in the embryonic kidney cell line 293-EBNA and in WI-26 fibroblasts (4). The broad tissue distribution is reminiscent of that of matrilin-2, and phylogenetic analyses show (1) that matrilin-4 and matrilin-2 descend from a common ancestor, further indicating a close relationship.

We recombinantly expressed the full-length mouse matrilin-4 in a mammalian expression system. The recombinant protein was used for production of a specific antisemum, which allowed immunohistochemical characterization of matrilin-4 expression in the mouse and an analysis of supramolecular assembly forms in the extracellular matrix formed by cultured primary embryonic fibroblasts. The full-length protein was used for structural studies by which the molecular dimensions and oligomeric state of recombinant matrilin-4 could be determined. Structural analysis also revealed a unique, conserved cleavage site used for proteolytic processing of matrilin-4. Extraction of native matrilin-4 from mouse tissues allowed a characterization of the naturally occurring oligomers by SDS-PAGE and Western blotting.
terminal SpeI and a 3′-terminal Nof restriction site. The cDNA was inserted into the expression vector pCEP-Pu (5) downstream of the signal encoding sequence for the BM-40 signal peptide. The vector contained either an N-terminal His6-Myc tag downstream of the signal peptide sequence encoding the BM-40 signal peptide. The vector contained inserted into the expression vector pCEP-Pu (5) downstream of the C-terminal strepII-tagged matrilin-4 cDNA for purification. The recombinant plasmids were introduced into the human embryonic kidney 293-EBNA cell line (Invitrogen) by transfection with DACC-30™ (Eurogentec). The cells were selected with puromycin (1 μg/ml) and were transfected to serum-free medium for harvesting of the recombinant protein. After filtration and centrifugation (1 h, 10,000 g), cell pellets containing the N-terminally His6-Myc-tagged matrilin-4 was applied to a nickel-nitrilotriacetic acid column (10 ml, Qiagen). The bound fraction was eluted with 0.25 M imidazole in 2 M urea, 0.3 M NaCl, 50 mM NaH2PO4, pH 8.0. The matrilin-4-containing fractions were diluted with an equal volume 2 M urea, 50 mM Tris-HCl, pH 7.4, and concentrated on a SP HiTrap column (1 ml, Amersham Pharmacia Biotech). Elution was achieved with 1 x NaCl, 2 M urea, 50 mM Tris-HCl, pH 7.4. The cell culture supernatant containing the C-terminally strepII-tagged matrilin-4 was dialyzed against 1 mM EDTA, 0.1 M Tris-HCl, pH 8.0, applied to a streptactic column (3 ml, IBA), and eluted with 2.5 mM desthiobiotin, 1 mM EDTA, 0.1 M Tris-HCl, pH 8.0.

Preparation of Antibodies to Matrilin-4—The purified, N-terminally His6-Myc-tagged matrilin-4 was used to immunize New Zealand rabbits. Serum obtained was purified by affinity chromatography on a column containing the C-terminally strepII-tagged matrilin-4 coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech). The specific antibodies were eluted with 0.1 M glycine, pH 2.5, and the eluate was neutralized with 1 x Tris-HCl, pH 8.5.

Tissue Extraction—Mouse calvaria was extracted for 6 h at 4 °C with 10 volumes (ml/g wet tissue) of 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA and 2 mM N-ethylanilide, and mouse brain each consecutively with 5 volumes for 1 min at 4 °C with 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4 (TBS), TBS containing 10 mM EDTA, TBS containing 10 mM EDTA and 4 mM urea, and finally 0.1 M Tris-HCl, pH 7.4, containing 10 mM EDTA and 4 mM GdnHCl. All extraction buffers contained 2 mM phenylmethylsulfonyl fluoride.

SDS-Polyacrylamide Gel Electrophoresis, Immunoblotting, and Determination of N-terminal Sequences—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (8). For immunoblotting the proteins were transferred to nitrocellulose and incubated with the proteins were transferred to nitrocellulose and incubated with the antibodies obtained was purified by affinity chromatography on a column containing the C-terminally strepII-tagged matrilin-4 coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech). The specific antibodies were eluted with 0.1 M glycine, pH 2.5, and the eluate was neutralized with 1 x Tris-HCl, pH 8.5.

Mass Spectrometry—MALDI-TOF mass spectrometry was carried out in a Bruker Reflex II mass spectrometer equipped with a 24-sample SCOUT source and video system, a nitrogen UV laser (λmax = 337 nm), and a dual channel plate detector (Bruker Daltonik, Bremen, Germany). 1 μl of the sample solution was placed on the target, and 1 μl of a freshly prepared saturated solution of α-cyan-4-hydroxycinnamic acid (Alrich) in acetonitrile/H2O (2:1 with 0.1% trifluoroacetic acid) was added. For MALDI-TOF mass spectrometry of intact proteins, sinapinic acid (Alrich) was used as matrix. When required, the matrix was reduced with 0.01 M DTT 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 Reflex III. Calibration was based upon the M, of recombinant protein A (Repligen) of 44,610.3 and bovine serum albumin (BSA) (Sigma) of 66,433.5, respectively. Between 50 and 500 single laser shots were summed into an accumulated spectrum. External calibration was carried out using a mixture of six synthetic peptides with molecular masses between 1046 and 2466 Da as well as the protonated dimer of the matrix (379 Da).

Electrospray mass spectrometry was carried out with a Micromass Q-TOF II instrument (Micromass, Manchester, UK). The samples were introduced directly into the mass spectrometer without on-line ionization. Prior to the analysis the samples were desalted and concentrated using home-built microcolumns consisting of ~2.5 μl of C18 reversed phase material (ODS-AG, 120 Å, 50 μm, YMC Europe, Schermbeck, Germany) in a gel-loader tip (Biozym, Hessisch-Oldendorf, Germany). The adsorbed peptides were washed with 5% formic acid and subsequently eluted with 3 μl of 5% formic acid/methanol (1:1) directly into a metal-coated nanospray capillary (Protana AS, Odense, Denmark). The sample was introduced into the mass spectrometer using an electrospray ion source. The spray voltage was adjusted between 2700 and 3000 V, and the block temperature was set to 30 °C. For MS experiments collision energy was set to 12 V, whereas for MS/MS experiments this was raised to a value between 25 and 40 V depending on size and charge state of the precursor ion to obtain optimal fragment ion spectra. Calibration was carried out between m/z 400 and 2500 using 40 μm H3PO4.

RESULTS

Recombinant Expression and Purification of Matrilin-4—cDNAs encoding the sequence of mature mouse matrilin-4 were cloned into vectors carrying nucleotide sequences coding for

Structure and Expression of Matrilin-4

Deparaffinization ensued through incubation for 30 min in Rotihistol (Oldendorf, Germany). The adsorbed peptides were washed with 3 μl of 5% formic acid/methanol (1:1) directly into a metal-coated nanospray capillary (Protana AS, Odense, Denmark). The sample was introduced into the mass spectrometer using an electrospray ion source. The spray voltage was adjusted between 2700 and 3000 V, and the block temperature was set to 30 °C. For MS experiments collision energy was set to 12 V, whereas for MS/MS experiments this was raised to a value between 25 and 40 V depending on size and charge state of the precursor ion to obtain optimal fragment ion spectra. Calibration was carried out between m/z 400 and 2500 using 40 μm H3PO4.

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either an N-terminal His6-Myc tag (HM4) or a C-terminal strepII tag (M4S). The use of different tags at different positions allowed the evaluation of their potential effects on the properties of the recombinant protein. The constructs were inserted into the pCEP-Pu vector utilizing the secretion signal sequence of BM-40 (5). The recombinant plasmids were introduced into human embryonic kidney 293-EBNA cells and maintained in an episomal form. The secreted matrilin-4 protein constructs were purified from the cell culture medium by affinity chromatography on either a streptavidin or a Ni2⁺ column. The correct usage of the predicted signal peptide cleavage site was confirmed by N-terminal protein sequencing of the purified recombinant strepII-tagged matrilin-4.

**Recombinant Expression of Matrilin-4 Yields Trimers, Dimers, and Monomers—**SDS-PAGE analysis of both the N- and C-terminally tagged proteins showed the presence of trimers, dimers, and monomers, indicating that the tags do not influence the oligomerization (Fig. 1). The His6-Myc tag and the strepII tag contribute 5.5 and 1.3 kDa, respectively, to the mass of each subunit. Without reduction, His6-Myc-tagged matrilin-4 gave three strong bands with apparent molecular weights of 235 (t), 155 (d + cc), and 66 kDa (m – cc), respectively, and fainter bands at 87 (m + 2 cc) and 73 kDa (m). Due to incomplete closure of disulfide bonds minor amounts of uncleaved, free monomers are often seen in matrilin samples. The strepII-tagged matrilin-4 gave strong bands with apparent molecular weights of 210 (t), 150 (d + cc), and 95 kDa (m + 2cc), respectively, and fainter bands at lower molecular weights. The minor bands seen between 65 and 80 kDa most likely represent a mixture of intact monomers with proteolytically processed monomers. The band at 21 kDa is a disulfide-bonded complex of three coiled-coil region fragments (see also Fig. 4). Reduction of the strepII-tagged matrilin-4 yielded a strong band with an apparent molecular mass of 67 kDa (m) (Fig. 1). MALDI-TOF mass spectrometry of non-reduced, strepII-tagged matrilin-4 resulted in three single charged molecule ion peaks at 219 (t), 153 (d + cc), and 87 kDa (m + 2cc) (Fig. 2A). After complete reduction, one single molecule ion peak was detected at 72.9 kDa (m) (Fig. 2C). The theoretical molecular mass of the strepII-tagged, mature matrilin-4 is 67.6 kDa, indicating that 7% of the mass of the recombinantly expressed protein was contributed by post-translational modifications. The larger species observed without prior reduction presumably represent trimers and modified dimers and monomers of the 72.9-kDa subunit.

**Proteolytic Processing of Recombinant Matrilin-4—**The consistent occurrence of matrilin-4 as a mixture of trimers, dimers, and monomers could be due either to an incomplete assembly of a triple coiled-coil or to cleavage of subunits close to the assembly domain, after formation of the trimer, yielding dimeric and monomeric fragments. Direct SDS-PAGE and Western blot analysis of non-purified culture media from 293-EBNA cells...
transfected with N- and C-terminally tagged matrilin-4 (Fig. 3) provided an indication of a C-terminal processing of matrilin-4. Bands that correspond to intact trimers as well as dimers and monomers derived from these trimers were observed (Fig. 3, lanes 1 and 2). Also truncated monomers lacking the coiled-coil domain of the N-terminally His<sub>6</sub>-Myc-tagged and C-terminally strepII-tagged matrilin-4 could be detected (Figs. 3 and 4). Furthermore, the C-terminal processing was confirmed by Western blot analysis of reduced samples from cell culture supernatant of 293-EBNA cells expressing N-terminally His<sub>6</sub>-Myc-tagged matrilin-4 (Fig. 3, lanes 3 and 4). Both the unprocessed and the C-terminally processed matrilin-4 subunits were detectable by the antisem against matrilin-4.

We used MALDI-TOF mass spectrometry to confirm the C-terminal processing by analysis of the fragments released upon cleavage. Partial reduction of the strepII-tagged matrilin-4 on the target yielded new peaks at 146 (d) and 80 kDa (m + cc) in addition to the 72.9 kDa (m) fully reduced monomer (Fig. 2B). This results from the loss of 7-kDa fragments from the 153-kDa (d + cc) and the 87-kDa (m + 2cc) molecule ions, respectively (Fig. 2A). After complete reduction, in addition to the fully reduced monomer of 72.9 kDa, a molecule ion of 6944.8 Da (cc) was detected (Fig. 2C, inset), presumably resulting from the release of a disulfide-bonded fragment. The mass of this fragment corresponds to the C-terminal end of the recombinant matrilin-4 starting with the amino acid residue Gly<sup>572</sup> (3) and lacking the very C-terminal lysine residue, with the theoretical mass of 6943.7 Da (Fig. 4). Matrilin-4 fragments without the coiled-coil domain could not be detected as they do not bind to the streptactin column.

SDS-PAGE of recombinant strepII-tagged matrilin-4 without prior reduction shows a band with an apparent molecular mass of ~21 kDa (3cc) that is lost upon reduction (Fig. 1). Isolation of this band followed by in gel trypsin digestion and MALDI-TOF analysis revealed the presence of a trypptic fragment of a mass of 2552.2 Da corresponding to the sequence Gly<sup>572</sup> to Arg<sup>594</sup>, indicating a cleavage between amino acid residues Glu<sup>571</sup> and Gly<sup>572</sup> in the region between the second vWFA-like domain and the coiled-coil domain (Fig. 4). Under reducing conditions a 7-kDa (cc) fragment of strepII-tagged matrilin-4 was isolated by SDS-PAGE. Subsequent in gel digestion with trypsin and endoprotease Glu-C followed by ESI-Q-TOF mass spectrometry yielded two sequences, SICPEEGIGATELSPECESLVEFQGRTLAGELSTQLNLARLTERLEENOLASRK<sup>574</sup>AAWSPHQPFEK<sup>*</sup> and SICPEEGIGATELSPECESLVEFQGRTLAGELSTQLNLARLTERLEENOLASRK<sup>565</sup>SPECESLVEFQGRTLAGELSTQLNLARLTERLEENOLASRK<sup>574</sup>AAWSPHQPFEK<sup>*</sup>, originating from the N-terminal end and the C-terminal strepII tag of the 7-kDa (cc) fragment, respectively (Fig. 4), supporting that the fragment results from a proteolytic cleavage between the second vWFA-like domain and the two cysteine residues stabilizing the coiled-coil. In addition, the localization of the cleavage site between residues Glu<sup>571</sup> and Gly<sup>572</sup> was unequivocally confirmed by Edman sequencing of the 21-kDa (3cc) band (Fig. 1) which gave GIGAHITELRS (Fig. 4). A cleavage at this point releases a matrilin-4 subunit, less its coiled-coil domain, which remains disulfide-bonded to the assembly domains from the other matrilin-4 chains (Fig. 4).

**Electron Microscopy**—The purified strepII-tagged full-length matrilin-4 was submitted to electron microscopy after negative staining with uranyl formate (Fig. 5). The protein particles were heterogeneous in size, and a closer examination of single particles revealed that all species from monomer to trimer were...
sequential extraction with TBS alone (A), TBS with 10 mM EDTA on 4–8% polyacrylamide gels without prior reduction, transferred to B, were separated recombinant C-terminally strepII-tagged matrilin-4 (B) were separated from days 10.25 and 14.25 post-coitum. At day 10.25 post-coitum, matrilin-4 was found in the trabeculae of the heart (A) as well as in the cephalic menenchymal tissue (mt) (ne, neuroepithelium). At day 14.25 post-coitum (C), matrilin-4 is present in the cartilage primordia of the vertebra (vb), sternum (stn), and the hip (hp), in the anlage of cranial bones such as the bones of the nasal cavity (nc), Meckles cartilage (me), and basisphenoid bone (bb). Furthermore, it could be detected in the thyroid (td) and tracheal (tc) cartilages and around the terminal bronchioli of the lung (lg). Bar, 1.8 (C), 0.08 mm (A and B).
Fig. 8. Tissue distribution of matrilin-4. Immunohistochemistry was performed on paraffin-embedded (A–E and G–J) or frozen (F) tissue from newborn (A, C–E, and G–J) or 6-week-old (B and J) mice, or from a day 15.5 post-coitum mouse embryo (F), which were demineralized if needed (B). Tissues were incubated with an affinity-purified antiserum against matrilin-4 followed by either biotin-SP-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated streptavidin (A–D and G–J), or by Cy3-conjugated goat anti-rabbit IgG (E and F). In the knee of a newborn mouse (A), matrilin-4 was highly expressed throughout the cartilage including the developing articular surface (as), the perichondrium (pc), periost (po), and ligaments (lm). In the epiphyseal cartilage primordium of the distal femur (fm) and proximal tibia (tb), matrilin-4 was found in the zone of resting (rt), proliferating (pl), and hypertrophic (ht) cartilage. In the knee of a 6-week-old mouse (B), a strong signal could be detected...
detected in mouse epiphyseal and tracheal cartilage (14).

Sequential extraction of mouse brain indicated a differential solubility of the individual oligomeric forms of matrilin-4 (Fig. 6C). All forms could be solubilized in EDTA-containing buffer but not in TBS alone, which indicates a contribution to the anchorage by a divalent cation-dependent mechanism. Denaturation with urea released large additional amounts of trim-meric matrilin-4 and the 170-kDa form. Further denaturation with 4 M GdnHCl released only the trimeric form of matrilin-4, indicating that the unprocessed protein is most strongly bound to insoluble tissue elements.

Matrilin-4 Is Deposited in Loose and Dense Connective Tissue, below Epithelia, in Smooth Muscle and in Nervous Tissue—The tissue distribution of matrilin-4 was studied by immunohistochemistry using affinity-purified antibodies on cryostat and paraffin-embedded sections of embryonic, newborn, and 6-week-old mice. The earliest expression of matrilin-4 was detected by immunofluorescence in the ectoplacental cone at day 7.5 post-coitum (results not shown). At day 10.25 post-coitum matrilin-4 is present in the heart (Fig. 7A), cephalic mesenchymal tissue (Fig. 7B), and somites, while at day 14.5 post-coitum it was found in the primordial skeleton and cranial bones, in the larynx, and in the lung (Fig. 7C). A similar characterization of the expression of matrilin-1, -2, and -3 in mouse embryos has been performed earlier (15).

After birth, the protein is abundant in dense connective tissue, including perichondrium, periostea, tendon, and ligaments (Fig. 8A). Furthermore, matrilin-4 is highly expressed in cartilage (Fig. 8, A–D and I). In the knee of a newborn mouse (Fig. 8A), matrilin-4 is present at the joint surface, as well as in the resting, proliferating, and hypertrophic cartilage, while at 6 weeks (Fig. 8B) it was found in the epiphyseal growth plate and in the cartilage remnants in the metaphysis but not in the articular cartilage. In loose connective tissue, it is present in the epiphyseal growth plate (gp) and in the calcified area (ca) of newly synthesized bone. In a transverse section through the head (C), matrilin-4 was found in the developing occipital bones (ob), bones of the nasal cavity (nc), the nasal septum (ns), the inner and outer mesenchymal layer of hair follicles (hf), and in the primordium of the upper incisors (ic). In the thorax (D), matrilin-4 is present in the trachea (tc), in costal cartilage (cc), in vertebral bodies (cb) and their transverse processes (tp), in the manubrium sternum (ms) and processes xiphoideus (px), in the clavicula (cv), and in the spinal cord (sc) (os, esophagus). In the skin (E), matrilin-4 was found in the fibrocollagenous network of the papillary layer (pl) and associated with the dermal-epidermal basement membrane (bmm). Further signals were detected in the smooth muscle (sm) layer of the intestine (F) (lm, lumen) and in the interalveolar walls (iw) of the lung (G). In the eye (H), matrilin-4 is present in the cornea (cn) and sclera (sr) and strongly in the limbus (lb). In the heart, matrilin-4 was found in the fibrous skeleton (arrow, D) and in the valves (vv, I). Matrilin-4 is broadly distributed in the brain (C and J), where it is present in the neocortex (nc), striatum (st), pallidum (pd), thalamus (tm), hippocampus (hc), cerebellum (cb), pons (ps), and medulla oblongata (mo). Bar, 2.8 (C), 2.3 (J), 1.6 (D), 0.7 (H), 0.6 (I), 0.5 (A and B), 0.3 (G) and 0.075 mm (E and F).
under stratified squamous epithelia (Fig. 9B'), in the papillary
layer of the dermis (Fig. 8E), in the smooth muscle layer of the
intestine (Fig. 8F), and in the interalveolar walls of the lung
(Fig. 8G). In the esophagus (Fig. 9B') and the dermis (Fig. 8E)
matrilin-4 was found associated with the basement membrane.
In the eye (Fig. 8H) it is expressed in the cornea and sclera and
strongly in the limbus. Furthermore, matrilin-4 is present in
the fibrous skeleton and in the valves of the heart (Fig. 8, D and
I), in the primordium of the incisors (Fig. 8C), and at lower
levels in the adrenal gland (not shown).

Matrilin-4 is also widely distributed in the central nervous
system and in peripheral nerves. In the adult brain (Fig. 8J),
matrilin-4 was observed in the neocortex, striatum, pallidum,
thalamus, cerebellum, pons, medulla oblongata, and in the
chordoid plexus (results not shown). It was further present in
the spinal cord (Fig. 8D) and in the sympathetic ganglion
(results not shown).

The Matrilin-4 Gene Is Transcribed in Fibroblasts, Chondro-
blasts, Osteoblasts, Epithelial, Muscle, and Neuronal Cells—
The cellular origins of matrilin-4 were revealed by in situ
hybridization and the results compared with the information
on the deposition of the protein derived from immunohisto-
chemistry. In sections through a humeroradial joint (Fig. 9, A
and A'), the highest mRNA levels could be detected in the
perichondrium and at the joint surface, in good agreement with
to the staining pattern obtained by immunohistochemistry.
In the esophagus (Fig. 9, B and B'), epithelial cells show clear
hybridization signals. Matrilin-4 mRNA was also detected in
Purkinje cells in the cerebellum (Fig. 9, C and C'). Skeletal
muscle cells and osteoblasts showed a weak but significant
level of gene expression (results not shown).

Matrilin-4 Forms an Extracellular Filamentous Network in
Cell Culture—In order to study the extracellular assembly
forms of matrilin-4, mouse primary embryonic fibroblasts were
cultured in the presence of ascorbate and analyzed by immu-
nofluorescence. The filamentous network (Fig. 10) seen was
reminiscent of that detected by antibodies to matrilin-1 (16), -2
(11), and -3 (10) in other cell culture systems.

DISCUSSION

Recombinant Matrilin-4 Forms Homotrimers—SDS-PAGE
analysis, MALDI-TOF mass spectrometry, and electron micros-
copy showed the production of matrilin-4 homotrimers in 293-
EBNA cells transfected with matrilin-4 cDNA. In electron mi-
croscopy (Fig. 5) the trimeric form shows similarities to the
bouquet-like shape observed for other matrilins (2, 10, 11) with
a compact center from which stalk-like structures with globu-
lar ends extend. Self-interactions of the vWFA-like domains, as
proposed to occur between the A1 and A2 domains in matrilin-1
and -2 (2, 11), were not visible.

Zhang and Chen (17) used rules based on the nature of the
hydrophobic residues in positions “a” and “d” of the heptad
repeat of coiled-coils in GCN4 leucine zipper mutants (18) to
predict that based on sequence matrilin-4 would form trimers.
Our experimental work shows that this prediction holds true,
as is also the case for matrilin-1 and -3 that according to the
same rules should form trimers and tetramers, respectively.
However, the largest assembly form of matrilin-2 is a tetramer
(11) and not a trimer as predicted on the basis of the rules of
Harbury et al. (18). The trimers seen in matrilin-2 preparations
are, in analogy with matrilin-4, likely to be degradation prod-
ucts. In contrast to the tetrameric assembly form of full-length
matrilin-2, the isolated matrilin-2 coiled-coil domain assemblies
preferentially into a trimer (19). It appears that prediction of
the oligomeric state of matrilin coiled-coil domains (17) leaves
uncertainties and that experimental analysis is required.

Previous studies showed that the coiled-coil domain of ma-
trilin-1 folds autonomously (20), even though the second
vWFA-like domain possibly also plays a role (21). Matrilin-3,
which lacks a second vWFA-like domain, and matrilin-2, which
carries a unique segment adjacent to the coiled-coil, form tet-
ramers, whereas matrilin-1 and -4 form trimers. In contrast,
hetero-oligomers of matrilin-1 and -3 occur as both trimers and
tetramers (11, 13, 17). Therefore, the oligomerization may also
be influenced by the distance between the second vWFA-like
domain and the coiled-coil domain.

Matrilin-4 Dimers and Monomers Are Formed by Cleavage of
Trimers—Supernatants of matrilin-4 transfected 293-EBNA
cells contain a complex mixture of different oligomeric forms.
Such heterogeneity was also seen for matrilin-2 and -3, both
when expressed recombinantly and when extracted from tis-
sues (10, 11, 17), although matrilin-1 appears more homoge-
nous in tissues (Fig. 6) (22). It has been suggested that the
heterogeneity could result either from a proteolytic processing
(10, 11) or from an imperfect oligomerization (10, 17). Proteo-
lytic degradation products of matrilin-4 could be demonstrated
by mass spectrometry, Edman sequencing, and SDS-PAGE
analysis. A cleavage site could be identified that is located
between the second vWFA-like domain and the coiled-coil do-
main, leading to the formation of truncated forms that are
trimeric with respect to their coiled-coil domains but lack the
more N-terminal parts of the subunit. Only dimers and mono-
mers containing the intact trimeric coiled-coil were detected by
MALDI-TOF analysis (Fig. 3), which excludes imperfect oli-
gomerization as the cause for heterogeneity. The demonstra-
tion of N-terminally truncated but trimeric matrilin-4 directly
shows that the oligomerization is achieved by the C-terminal
coiled-coil domain. Further studies will be needed to determine
when and where the processing occurs in vivo and the nature of
the protease involved.

Matrilin-4 Extracted from Tissues Shows a Degradation Pat-
tern Reminiscent of Recombinant Matrilin-4—The pattern of
degradation products present in recombinant matrilin-4 shows
similarities to that seen for matrilin-4 extracted from tissues
(Fig. 6B), indicating that proteolytic processing occurs also in
vivo. The two glutamic acid residues N-terminal to the cleavage
site of matrilin-4 are conserved in all matrilins, indicating that
this cleavage site may be used also in other matrilins. For
matrilin-2 it was shown that the heterogeneous band pattern
seen in SDS-PAGE is not due to differential substitution with
glycosaminoglycans or N-linked oligosaccharides (11).

Oligomeric forms of matrilins will be multivalent and able to
interact with and connect multiple other extracellular matrix
molecules. A proteolytic cleavage of one or two subunits would
release interaction partners from the supramolecular complex
formed and could modulate the overall binding properties of
matrilins. This is supported by the fact that the larger forms of
matrilin-4 require more strongly denaturing conditions for
their extraction from tissues (Fig. 6). Still a proportion of ma-
trilin-4 molecules could be extracted with EDTA-containing
buffer alone, indicating a cation-dependent anchorage in the
extracellular matrix. A similar behavior has been described for
matrilin-1 (2). The vWFA-like domains of matrilins contain the
metal ion-dependent adhesion site sequence motif, and the fact
that at least some interactions of matrilin-1 and -4 can be
stabilized by divalent cations indicates that this motif is indeed
functional.

Proteolytic cleavage of extracellular matrix molecules is a
common mechanism for the regulation of the matrix archite-
cture during remodeling, and perturbations in the balance of
proteolysis and de novo protein synthesis may form a basis for
pathological changes in tissue structure and function. Further-
more, degradation products may have a function different from
that of the unprocessed protein, e.g., endostatin, a fragment of the NC1 domain of collagen type XVIII, acts as an angiogenesis inhibitor (23). It remains to be investigated if fragments released by degradation of matrilins may have such independent new functions.

Matrilin-4 Is the Most Widespread Member of the Matrilin Family—Matrilin-4 is present in highest amounts in tissues derived from mesenchymal cells, but additionally in situ hybridization showed transcription in epithelial and neuronal cells, which have developed from endoderm and neuroectoderm, respectively. In contrast to matrilin-2 and matrilin-1 and -3 that have a complementary tissue distribution, matrilin-4 is found in most locations where one of the other matrilins is expressed. Similar to matrilin-2, it is present in a variety of non-skeletal tissues, but in contrast matrilin-4 is broadly expressed in cartilage where the matrilin-2 expression is limited to the hypertrophic zone of the growth plate. Furthermore, matrilin-4 is present in the surface layer of the developing articular cartilage, where no other matrilin has been detected. The partial overlap in the spatial expression of matrilins may allow a functional redundancy that could explain the lack of an overt phenotype seen when the matrilin-1 gene is interrupted (14, 24).

Matrilin-4 Forms an Extended Extracellular Network—Immunofluorescence microscopy of the matrix formed by cultured primary embryonic fibroblasts shows matrilin-4 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. 10). Similar results were obtained for matrilin-1 in cultures of chicken chondrocytes, where a more extended collagen-dependent (16, 25) and a pericellular, collagen-independent network was described (16). Matrilin-2 and -3 also form fibrillar networks when expressed by cultured rat aorta smooth muscle (11) or rat chondrosarcoma cells (10), indicating mechanisms of supramolecular assembly common to all matrilins.

The Matrilin Family Consists of Four Members—Matrilin-4 is in all probability the final member of the matrilin family. No further members have been detected in the sequence data bases including those provided by the human genome project. This is supported by phylogenetic studies showing that each of the four matrilin genes is located in a gene cluster together with members of other tetralog protein families such as the syndecans (26). Each member of the matrilin family has in the meantime been characterized with regard to structure and distribution in embryonal and mature tissues, which allows some conclusions about the overall role of the matrilin family. The widespread distribution of matrilins with at least one family member being present in nearly every tissue and at very different time points of development indicates an important role in the structure and function of an extracellular matrix. A common feature of the matrilins is the ability to participate in the formation of a filamentous network but probably also to bind ligands adjacent to or within those filaments. This is facilitated by the oligomeric structure, which enables the simultaneous interaction with multiple ligands. Ligand binding affinity could be modified by altering cooperativity through proteolytic processing, an event that may not be restricted to matrilin-4. The broad tissue distribution of matrilin-4, which nearly covers the expression domains of all other matrilins, makes it likely that matrilin-4 is the most versatile member of the family. Therefore, our further work will focus on matrilin-4 in the hope that detailed studies of this matrilin will enhance the general understanding of matrilin structure and function.

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