Matrilin-2, a Large, Oligomeric Matrix Protein, Is Expressed by a Great Variety of Cells and Forms Fibrillar Networks*

Dorothea Piecha‡, Selen Muratoglu§, Matthias Mör gelin‡, Nik Hauser‡, Daniel Studer‡, Ibolya Kiss§, Mats Paulsson‡, and Ferenc Deák‡‡

From the ‡Institute for Biochemistry, Medical Faculty, University of Cologne, D-50931 Cologne, Germany, the §Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, H-6701 Szeged, Hungary, the ¶Department of Cell and Molecular Biology, Lund University, S-22100 Lund, Sweden, and the ‡‡M. E. Müller-Institute for Biomechanics, CH-3010 Bern, Switzerland

Matrilin-2 is a member of the protein superfamily with von Willebrand factor type A-like modules. Mouse matrilin-2 cDNA fragments were expressed in 293-EBNA cells, and the protein was purified, characterized, and used to immunize rabbits. The affinity-purified antiserum detects matrilin-2 in dense and loose connective tissue structures, subepithelial connective tissue of the skin and digestive tract, specialized cartilages, and blood vessel walls. In situ hybridization of 32P-labeled riboprobes localizes the matrilin-2 mRNA to fibroblasts of dermis, tendon, ligaments, perichondrium, and periosteum; connective tissue elements in the heart; smooth muscle cells; and epithelia and loose connective tissue cells of the alimentary canal and respiratory tract. RNA blot hybridization and immunoblotting revealed both matrilin-2 mRNA and protein in cultures of a variety of cell types, confirming the tissue distribution. Alternative splicing affects a module unique for matrilin-2 in all of the above RNA sources. SDS-polyacrylamide gel electrophoresis and electron microscopy reveals matrilin-2 from tissue extracts and cell line cultures as a mixture of mono-, di-, tri-, and tetramers. Matrilin-2 is substituted with N-linked oligosaccharides but not with glycosaminoglycans. Because of other, yet unidentified, cell-type dependent posttranslational modifications, the monomer is heterogeneous in size. Immunofluorescence showed that matrilin-2 functions by forming an extracellular, filamentous network.

Extracellular matrix provides physical support to the cells, delineates pathways for cell migration during differentiation and tissue regeneration, and provides the necessary milieu for the normal cell metabolism and development. Collagen fibers and proteoglycan aggregates provide the structural basis for matrix architecture. Noncollagenous proteins modulate the organization of these elements, form collagen-associated or independent networks, and are parts of cell migratory pathways.

The matrix molecules share homologous modules, protein domains of common evolutionary origin, but a great functional variability of the homologous modules in different proteins has been observed. The recently discovered matrilins (for a review, see Ref. 1) are typical modular proteins belonging to the superfamily with von Willebrand factor type A-like (vWFA) modules. Members of the matrilin family are found in a wide variety of extracellular matrices. Matrilin-1, formerly called cartilage matrix protein, and matrilin-3 (2, 3) are abundant in cartilage, while matrilin-2 (4) and matrilin-4 (5) show a broader tissue distribution. Thus, all forms of connective tissue appear to contain at least one form of matrilin, indicating a general and important function for this protein family.

Matrilin-2 was found to contain the same protein modules in the same order as matrilin-1 (4). The precursor protein in mouse is 956 amino acids long and consists of a putative signal peptide, two vWFA domains connected by 10 epidermal growth factor-like modules, a potential oligomerization domain, and a unique segment. The ability of the 38 C-terminal amino acid moieties to form an α-helical coiled-coil was shown by Pan and Beck (6). Matrilin-2 mRNA was detected by filter hybridization in a variety of mouse organs including calvaria, uterus, heart, and brain as well as fibroblast and osteoblast cell lines. A group of 120–150-kDa bands was, after reduction, recognized specifically with an antiserum against the matrilin-2-glutathione S-transferase fusion protein in media of the matrilin-2-expressing cell lines. Immunolocalization of matrilin-2 in developing skeletal elements showed reactivity in the perichondrium and the osteoblast layer of trabecular bone.

In order to gain a better understanding of the potential function of matrilin-2, we have determined the spatial expression of the gene by radioactive in situ hybridization. A new antiserum with a higher titer to the native matrilin-2 was raised using, as an antigen, matrilin-2 expressed in a eukaryotic cell line, and the protein was immunolocalized in mouse tissues. Furthermore, matrilin-2 was purified from media of cells overexpressing the full-length protein and visualized by electron microscopy to provide information on the molecular dimensions and oligomeric structure of the protein. The structural information was extended by SDS-PAGE analysis of the intact protein and the reduced subunits. Posttranslational modification of the protein and alternative splicing of the mRNA were also characterized. Finally, formation of an extracellular network in cultures of cells expressing matrilin-2 was demonstrated by indirect immunofluorescence. The potential

* This work was supported by joint grants from the Volkswagen-Stiftung (I/71 654) and the Bilateral German-Hungarian Cooperation Program (D-10/96/WTZ), Hungarian National Scientific Research Foundation Grants OTKA T023803 and T029157, Deutsche Forschungsgemeinschaft Grant Ki 558/10–3, and a grant from the Köln Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary. Tel.: 36-62-432-2232; Fax: 36-62-433-506; E-mail: matrix@nucleus.szbk.u-szeged.hu.

** The abbreviations used are: vWFA, von Willebrand factor type A-like; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis.

This paper is available on line at http://www.jbc.org

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function and suggested molecular architecture of the protein is discussed.

**MATERIALS AND METHODS**

**Mouse Strains and Cell Cultures**

BALB/c or NMRI mouse strains were used for RNA or protein analysis, respectively. The mouse fibroblastic cell lines WEHI 164 and NIH 3T3, the rat osteogenic sarcoma UMR-106, and the small intestine epithelial IEC-6 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The mouse C2T7 cells with skeletal muscle characteristics (7), the smooth muscle-like cell 9E11G (8), the keratinocyte carcinoma PVD/Al (9), the rat Schwann cell line RN22 (10), and the Swarm rat chondrosarcoma cell line (RSJC) (11) were obtained from the laboratories of origin. The mouse immortalized endothelial cells m1END, derived from mesenteric lymph nodes (12), and the SV40-transformed lymphoid vascular endothelial cell SVEC (13) were provided by L. Sorokin and R. Hallmann (Erlangen); smooth muscle cells (SMC) from rat aorta were cultured by F. Michaelisen (Cologne), using standard methods (14). Unless recommended otherwise by the supplier, the cell lines were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), and utilized for RNA and protein analyses.

**RNA Preparation and Analysis**

Total RNA was prepared from guanidinium thiocyanate extracts of various cell lines using the RNA isolation kit of Stratagene. For RNA blot analysis, 7-μg aliquots were electrophoresed, blotted to Hybond N filter (Amersham Pharmacia Biotech) and hybridized consecutively with pCRP12 cDNA (4) and chicken 27 S rRNA gene fragment. For the study of RNA alternative splicing, total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies) using oligo(dT) primer. Nested polymerase chain reactions were performed using first the distal primers 5′-GGACGGGCTCCGAGTGA-3′ and 5′-CTGTATCTCAGGGTATTTTC-3′ and then the proximal primer pair 5′-CATGGACAGACCTCTCCTCT-3′ and 5′-TTTGTTGTAGACCGTGAAAG-3′ flanking the unique region of mouse matrilin-2.

**In Situ Hybridization**

**Preparation of Tissue Sections**—For paraffin embedding, tissue specimens were fixed overnight in 95% ethanol, 1% acetic acid; dehydrated in ethanol; cleared in xylol; and embedded in low melting point paraffin (Paraplast, Sigma). Sections of 7–9 μm were cut and mounted on poly-L-lysine-coated slides. For cryostat sections, specimens were fixed overnight in 4% paraformaldehyde, 8 mM NaHPO4, 0.15 M NaCl, pH 7.4; treated with RNase T1 (1 unit/ml); and washed again at 53 °C in 50% formamide, 0.2 M dithiothreitol; and then hybridization was performed with riboprobes at a final activity of 1–4 × 107 cpm/ml, 3-μm sections were cut.

**Hybridization**—For pretreatment, in situ hybridization, and washing, the protocol outlined by Hofstetter et al. (15) was used. Briefly, sections were deparaffinized and rehydrated, if necessary, and then digested with proteinase K (1 μg/ml), postfixed, and acetylated in 0.25% acetic anhydride. Riboprobe were labeled with [35S]CTP and hydrolyzed to 150-nucleotide average length. The sections were hybridized for 12–16 h at 53 °C with riboprobes at a final activity of 1–4 × 107 cpm/ml, depending on their length. After hybridization, the tissue sections were washed at 53 °C in 50% formamide, 2 × SSC, 1 μM EDTA, 10 μM dithiothreitol; treated with RNase T1 (1 unit/ml); and washed again at 53 °C in 50% formamide, 0.2 × SSC, 1 μM EDTA. The slides were dehydrated and dipped in LM1 photoemulsion (Amersham Pharmacia Biotech). Autoradiography was performed for 5–10 days, and sections were counterstained in Mayer’s hematoxylin (Merck).

**Expression and Purification of Recombinant Matrilin-2**

Partially overlapping cDNA fragments in the mouse matrilin-2 clones pCRP207, pCRP190, and pCRP12 (4) were combined to full-length cDNA using suitable restriction enzymes. One AflI site was inserted immediately upstream of the first AUG codon by polymerase chain reaction. After digestion with AflI and NcoI, a 3.3-kilobase pair cDNA fragment was inserted into the expression vector pCEP-Pu (16). One 12–16 h at 53 °C with riboprobes at a final activity of 1–4 × 107 cpm/ml, depending on their length. After hybridization, the tissue sections were washed at 53 °C in 50% formamide, 2 × SSC, 1 μM EDTA, 10 μM dithiothreitol; treated with RNase T1 (1 unit/ml); and washed again at 53 °C in 50% formamide, 0.2 × SSC, 1 μM EDTA. The slides were dehydrated and dipped in LM1 photoemulsion (Amersham Pharmacia Biotech). Autoradiography was performed for 5–10 days, and sections were counterstained in Mayer’s hematoxylin (Merck).

**Immunoblotting of Cell Culture Media and Mouse Organ Extracts**

Cell cultures were grown to confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The cell layers were washed and cultured for 48 h without serum, and the medium was harvested. Several mouse organs were homogenized using a Polytron homogenizer and extracted with 0.25 M NaCl, 50 mM Tris-HCl, pH 7.4, containing as protease inhibitors 10 mM EDTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride. After centrifugation, aliquots of the supernatant were added to SDS-polyacrylamide gel electrophoresis according to the protocol of Laemmli (17), using gradient gels of 4–15% polyacrylamide. Proteins were transferred electrophoretically to a nitrocellulose filter and developed with affinity-purified antiserum to matrilin-2, followed by peroxidase-conjugated swine anti-rabbit IgG (DAKO) and the ECL chemiluminescence procedure (Amersham Pharmacia Biotech) as suggested by the suppliers.

**Immunohistochemistry and Immunofluorescence of Cell Layers**

Immunohistochemistry was performed as described previously (18), using the affinity-purified anti-matrilin-2 antiserum together with a swine anti-rabbit IgG-peroxidase complex and 3-amino-9-ethylcarbazole as substrate on unfixed cryosections from adult and newborn mouse. For immunofluorescence of cell cultures, cells were plated onto poly-L-lysine–coated slides, and slides were fixed in 2% paraformaldehyde in phosphate-buffered saline for 10 min. In some experiments, cells were permeabilized by treatment with 10% Nonidet P-40 in phosphate-buffered saline for 10 min. Nonspecific antibody binding was blocked by incubation with 1% (w/v) bovine serum albumin in phosphate-buffered saline for 1 h. The cells were treated with the affinity-purified antibody to matrilin-2 for 1 h followed by Cy3-conjugated affinity-pure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Pictures were taken with a Zeiss Axiophot microscope equipped with a fluorescence source.

**Analysis of Posttranslational Modifications**

The potential substitution with sulfated glycosaminoglycans was determined by metabolic labeling. 283-EBNA cells transfected with pCEP-Mtr2 were grown in serum- and sulfate-free minimal essential medium containing 50 μM [35S]Sulfate (Amersham Pharmacia Biotech). After 48-h labeling period, the media were harvested and precipitated with trichloroacetic acid (final concentration 12%). The radiolabeled proteins were separated by SDS-PAGE, and radioactive bands were visualized by fluorography after treatment with 1 M sodium salicylate.

**Chondroitinase ABC and Heparitinase Digestions—**Cell culture media from 283-EBNA cells transfected with pCEP-Mtr2 were incubated with 0.7 milliunits/μl heparitinase I (Sigma; from Flavobacterium heparinum) and 1.7 milliunits/μl chondroitinase ABC (Sigma) for 7 h at 37 °C. Aliquots of the digested and control media were analyzed after SDS-PAGE and immunoblotting with specific antisera to matrilin-2.

To test for the presence of N-glycosidically linked oligosaccharides, 283-EBNA cells transfected with pCEP-Mtr2 were grown in serum-free medium.
Structure and Expression of Matrilin-2

Dulbecco's modified Eagle's medium for 48 h in the presence of tunicamycin (Sigma) at 0.5 mg/ml. Media from tunicamycin-treated and control cells were analyzed after SDS-PAGE and immunoblotting with specific antisera to matrilin-2. Parallel blots were developed with antibodies to nidogen, which is endogenously produced by the 293-EBNA cells and served as a positive control of better known glycosylated nidogen (not shown).

N-Glycosidase F Digestion—Purified matrilin-2 in incubation buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM sodium chloride, 0.5% Nonidet P-40, and 0.1% SDS was denatured by heating at 100 °C for 2 min. After denaturation, the protein was incubated with 0.3 units of N-glycosidase F (Roche Molecular Biochemicals) per mg of protein for 20 h at 37 °C. The control sample was treated similarly without adding N-glycosidase F. The digested and control samples were analyzed by 4–15% SDS-PAGE and stained with silver nitrate.

Electron Microscopy

Purified matrilin-2 (10 μg/ml) was adsorbed to a 400-mesh carbon-coated copper grid, which was rendered hydrophilic by glow discharge at low pressure in air. The grid was immediatelyblotted, washed with two drops of water, and stained with 0.75% uranyl formate for 15 s. Samples were observed in a Jeol 1200 EX transmission electron microscope operated at 60-kV accelerating voltage and ×75,000 magnification. Images were recorded on Kodak ESTAR Thick Base 4898 plates without preirradiation at a dose of typically 2000 electrons/nm². Evaluation of the data from electron micrographs was done as described previously (19).

RESULTS

Matrilin-2 Is Deposited in All Forms of Connective Tissue, Some Types of Smooth Muscle, and a Few Epithelia—A cDNA fragment encoding the EGF-like modules and the second vWFA domain of mouse matrilin-2 was inserted into the pCEP-Pu vector, utilizing the secretion signal sequence of BM40 (16). The recombinant plasmid was introduced into the 293-EBNA cell line, where it was stably maintained in episomal form. The matrilin-2 fragment, secreted into the tissue culture medium, was purified and used to immunize rabbits. The antisera, after affinity purification, specifically reacts with matrilin-2 and does not show any cross-reactivity (see Fig. 5A).

Matrilin-2 was localized immunohistochemically in cryostat sections of adult and newborn mice (Fig. 1). The protein is most abundant in dense connective tissue, including tendon, ligaments, perichondrium, periosteum, dura mater, epineurium (Fig. 1, G–I); perimysium of skeletal heart, and smooth muscle (Fig. 1, B, C, E, and G); submucosa of alimentary canal (Fig. 1, E and F); the reticular layer of dermis (Fig. 1, A and G); spleen capsule; and the annulus fibrosus, chordae tendineae, and valves of heart (not shown). In loose connective tissue, local concentration of the protein is not as high. It is most abundant in the papillary layer of dermis (Fig. 1A) and spleen trabeculae (not shown). It is less abundant, but detectable, in the lamina propria of alimentary canal and the tunica adventitia of blood vessels and respiratory tract (not shown in detail). Matrilin-2 is also detectable to variable extents in specialized connective tissue, including the zones of proliferation and hypertrophy in epiphysyeal cartilage (Fig. 1G), elastic cartilage of the ear (not shown), fibrocartilage in the annulus fibrosus of the intervertebral disc (Fig. 1, H–I), and bone, where it lines the marrow cavities. The protein was abundant in the myometrium (not shown) and was also detectable between muscularis mucosae and muscularis externa of the alimentary canal, possibly associated with the nervous plexus (Fig. 1E). The amount of the protein was also above the detectability threshold in a few specialized epithelia, e.g., the sublingual gland in the newborn head and the lens epithelium or underlying basement membrane of day 16.5 embryos (not shown). In nervous tissue, matrilin-2 was observed in the dura and pia mater of brain and spinal cord as well as the perineurium of peripheral nerves (Fig. 1H).

Matrilin-2 Gene Is Transcribed in Fibroblasts, Osteoblasts, Smooth Muscle Cells, and Some Epithelial Cells—In order to reveal where the matrilin-2 mRNA is produced, eventually leading to extracellular deposition of the protein, we performed in situ hybridization. Three antisense riboprobes, complementary to nonoverlapping regions of the matrilin-2 mRNA were hybridized to cryostat and paraffin sections of 5–10-week-old mouse. The hybridization of the radioactive riboprobe was detected by autoradiography and the silver grains were visualized in dark field. Bright field photomicrographs of the same fields helped to identify the hybridizing tissues in the sections (Fig. 2). Parallel sections were hybridized with sense riboprobes and verified the specificity of hybridization (not shown).

The results of in situ hybridization confirmed and extended the data obtained by immunohistochemistry. Connective tissue cells are clearly positive in dense and loose as well as specialized connective tissue. Dense connective tissue fibroblasts show characteristic accumulation of grains in tendon, ligaments, perichondrium, periosteum (Fig. 2, A and B); cells in the reticular layer of dermis and at the base of hair papillae (Fig. 2A); and annulus fibrosus of heart, atriocentric valve, and chordae tendineae (Fig. 2D). Loose connective tissue cells also gave hybridization signals in the adventitia of trachea (Fig. 2, E and G) and the mesentery cells (Fig. 2C). Matrilin-2 gene expression was observed in epiphyseal cartilage, in the zones of proliferation and early hypertrophy (Fig. 2B), as well as in osteoblasts of the calvaria (not shown).

Muscle cells showed a detectable level of gene expression, albeit not as high as that in fibroblasts. A strong in situ hybridization signal was observed in the organs where previous Northern hybridization (4) showed a high steady state level of matrilin-2 mRNA. The uterus gave an overall strong hybridization signal (Fig. 2C). Heart was also strongly positive, but with a gradient toward the regions richer in connective tissue cells, e.g., the atria, auricle, valves, and chordae tendineae (Fig. 2D).

In addition to connective tissue cells and myoblasts, some epithelia also showed clearly positive hybridization signals. In paraffin sections, the secretory epithelium of esophagus, the mucosa, and serosa of colon as well as the seromucous glands of trachea showed strong hybridization (Fig. 2, E–H).

Relative Abundance of Matrilin-2 mRNA in Established Cell Lines—In some cases it was difficult to determine with certainty the cell types where the gene expression was observed by in situ hybridization. For example, smooth muscle cells are in close association with fibroblasts, and epithelial cells form thin layers in close proximity to the underlying connective tissue. Therefore, we examined matrilin-2 mRNA and protein production in homogeneous cultures of permanent cell lines. Total RNA samples were isolated from cultured cells, and the relative amount of matrilin-2 mRNA was estimated by Northern hybridization (Fig. 3). In all of the cell lines examined, expression of the gene was observed. We previously demonstrated that the fibroelastic cell lines L929, WEHI 164, NIH 3T3, and the rat osteogenic sarcoma UMR-106 expressed the gene (4). In the present experiment, the mRNA level in NIH 3T3 cells (Fig. 3, lane 1) was compared with other cell lines. In two samples, isolated from the rat chondrosarcoma cell line and the 9E11G smooth muscle-like cells, the matrilin-2 mRNA level was higher than in NIH3T3 cells. The other smooth muscle cell line, SMC, isolated from rat aorta, and the differentiated skeletal muscle myotube C2/7 contained less, but significant, amounts of matrilin-2 mRNA, confirming that the gene can be expressed in cells with myoblast characteristics. The intestinal epithelial cell line IEC6 and the Schwannoma cell line RN22 also expressed the gene at a detectable level. The least amount
Fig. 1. Immunohistochemistry of mouse tissue. Sections from newborn (A, F, G, H, I, and J) and adult (B–E) animals were made, and matrilin-2 (A–I) or matrilin-1 (J) was detected with affinity-purified antisera. A, in skin from the leg, strong immunostaining was observed in the papillary layer (pl), and somewhat weaker staining was seen in the reticular layer (rl) of dermis and at the base of hair papillae (hp). ep, epidermis. B, in the heart, the auricle (au) is stained more intensely than the atrium wall (aw). C, in the ventricles, the connective tissue surrounding some capillaries (ca) are positive, and staining of the basement membrane around myoblasts is weak. The interstitial connective tissue (ct) is strongly reactive. D, in kidney, the arcuate arteries (aa) show strong staining. E, the esophagus epithelium (ep) is not stained, but the underlying basement membrane and mucosa show strong immunoreaction. In the muscularis externa, connective tissue (ct) between the circular and longitudinal smooth muscle cell layers shows strong immunostaining, possibly associated with the nervous plexus. F, in the oral cavity of a newborn mouse, fibers of periodontal membrane around the developing incisor (i) show strong staining as well as the submucosa of hard and soft palate and the lamina propria of the tongue (to). There is no staining in the epithelial layers (ep). G, in the ossifying skeleton of the leg, the perichondrium (pc),
of matrilin-2 mRNA was found in the keratinocyte carcinoma PVD(AI). In a separate experiment, matrilin-2 mRNA was detected in the SVEC endothelial cell line (not shown). In summary, not only the connective tissue cell types contained matrilin-2 mRNA, but gene expression was also observed in myoblasts and in the epithelial and endothelial cell lines tested.

Previous analysis indicated that the sequence variability within the unique region may be a consequence of alternative splicing. Therefore, we performed reverse transcription-polimerase chain reaction analysis to determine if there is further mRNA heterogeneity within the translated region. In the SVEC endothelial and the rat chondrosarcoma cell lines, alternative splicing affected only the middle third of the unique module but not the region encoding the coiled coil, the vWFA2, or EGF-like modules (data not shown). Systematic comparison of the RNA samples showed that a 57-nucleotide-long region is alternatively retained or spliced out in all of the 10 cell lines studied (Fig. 4).

**The Matrilin-2-specific Antiserum Reacts with Multiple Protein Bands upon Electrophoresis of Unreduced Samples**—In order to gain information about the relative amount and presumed oligomeric structure of matrilin-2, culture media from cell lines and extracts from tissues were compared by SDS-
bands with apparent $M_r$ values between 100,000 and 130,000. The size heterogeneity was even more apparent when nonreduced samples from media of a variety of cell lines as well as extracts of skin and uterus were analyzed by immunoblotting (Fig. 5C). All the cell lines tested secreted detectable amounts of matrilin-2, with the exception of the epithelial cell line IEC6. Because the IEC6 cells showed production of matrilin-2 mRNA, we need to assume that the mRNA is translated and/or the protein is secreted with a very low efficiency in that cell line. While the 293-EBNA cells that had been transfected with pCEP-Mtr2 produced four groups of bands that may represent monomers, dimers, trimers, and tetramers, most cell lines secreted mainly the smallest and largest components. Extracts of skin and uterus showed, in addition, a relative abundance of potential trimers. The analysis was, however, complicated by the presence of discrete differences in the electrophoretic mobility of corresponding matrilin-2 bands between sources (Fig. 5C).

**Analysis of Posttranslational Modifications in Matrilin-2**—While the presence of matrilin-2 oligomers of variable sizes can be explained by source-specific differences in assembly, the multiple size of monomers indicated that, in addition to this, posttranslational processing occurs, possibly in a tissue-specific manner. A set of experiments was designed to explore this possibility (Fig. 6). The most obvious cause for the heterogeneity would be proteolysis after secretion into the culture medium. Samples of medium were, therefore, harvested at different periods of time show great similarities. With the exception of degradation fragments, which are clearly smaller than monomers, all components could be observed already after 8 h, indicating that proteolysis in the medium or in the intercellular compartment of tissues is not the major cause of heterogeneity. The size differences could also be due to a variable substitution with glycosaminoglycans or oligosaccharides. To test for the presence of sulfated glycosaminoglycans, cultures of wild type and matrilin-2-transfected 293-EBNA cells were, therefore, labeled with $^{35}$S]sulfate, and the media were analyzed by SDS-PAGE and, in part, immunoblotting to the matrilin-2 expressed within the unique region by reverse transcription-polymerase chain reaction. The analysis was performed as described under "Materials and Methods." Template RNA was isolated from rat chondrosarcoma tissue (RCS) or the mouse and rat cell lines indicated at the top. C, amplification of the longer segment using pCRP12 cDNA clone (4) as template. $M_r$ values ranging from 70,000 to 500,000. On the basis of the calculated $M_r \approx 104,300$ for the nonmodified matrilin-2 monomer, faster migrating bands must represent degradation products, and the slower ones may correspond to oligomeric forms. We can conclude that the secretory signal peptide of matrilin-2 was functionally active in 293-EBNA cells and that matrilin-2 can form oligomers stable enough to resist denaturing electrophoresis if the sample is not reduced. The medium from nontransfected 293-EBNA cells did not show any reactivity with the antiserum, demonstrating the specificity of this reagent.

The relative amounts of the different forms of matrilin-2, resolved by electrophoresis, differ somewhat between the crude media of transfected cells and preparations chromatographically purified from this source (Fig. 5B). The reason for this difference is that during purification we removed degradation fragments and enriched slightly for the oligomeric forms. Reduction of the purified material yielded several closely spaced PAGE and, after reduction, we obtained bands with apparent $M_r$ values between 100,000 and 130,000. The size heterogeneity was even more apparent when nonreduced samples from media of a variety of cell lines as well as extracts of skin and uterus were analyzed by immunoblotting (Fig. 5C). All the cell lines tested secreted detectable amounts of matrilin-2, with the exception of the epithelial cell line IEC6. Because the IEC6 cells showed production of matrilin-2 mRNA, we need to assume that the mRNA is translated and/or the protein is secreted with a very low efficiency in that cell line. While the 293-EBNA cells that had been transfected with pCEP-Mtr2 produced four groups of bands that may represent monomers, dimers, trimers, and tetramers, most cell lines secreted mainly the smallest and largest components. Extracts of skin and uterus showed, in addition, a relative abundance of potential trimers. The analysis was, however, complicated by the presence of discrete differences in the electrophoretic mobility of corresponding matrilin-2 bands between sources (Fig. 5C).

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cells either transfected with pCEP-Mtr2 (lane 2) or without transfection (lane 3) were cultured in the presence of 50 μCi/ml [35S]sulfate for 48 h, and aliquots of the media were applied to a 4–15% SDS-PAGE without prior reduction.

Matrilin-2 was detected by immunoblotting using an affinity-purified antiserum. B, electrophoretic analysis of sulfate-labeled products secreted into the medium. 293-EBNA cells either transfected with pCEP-Mtr2 (lane 2) or without transfection (lane 3) were cultured in the presence of 50 μCi/ml [35S]sulfate for 48 h, and aliquots of the media were applied to a 4–15% SDS-PAGE without prior reduction. The radioactive macromolecules were detected by autoradiography. Treatment of purified matrilin-2 with heparitinase ABC (lane 3) was negatively stained with uranyl for-

ticate to produce high resolution images by electron microscopy. Fields of stained molecules showed particles of heterogenous size (Fig. 7, top), and close examination of single particles revealed that all species from monomers to tetramers were present in the sample. At high magnification it was seen that, irrespective of the number of subunits within a molecule, all subunits are joined at a single point (Fig. 7, bottom), which presumably represents the coiled-coil α-helix assembled from the C-terminal domains. In most cases, the subunit is seen as a looped structure with a more heavily stained hole in the middle and frequently, but not always, carrying most mass in the periphery. The average diameter of the loops was 8 nm. Occasionally particles were seen where the loop was opened into a flexible rod, better representing the tandem array of domains making up the subunits.

**The Protein Forms an Extracellular Filamentous Network in Cell Culture**—In order to study the extracellular assembly forms of matrilin-2, the pericellular matrix of cultured primary smooth muscle cells from rat aorta was analyzed in immunofluorescence microscopy with specific antibodies against matrilin-2 (Fig. 8). Matrilin-2 was detected in an extensive, branched fibrillar network. The network may be connected to the cell surface, but in experiments where the cells were permeabilized (Fig. 8B), and thereby better outlined through the staining of the intracellular pool of immunoactive matrilin-2, it was clear that the fibrils were not limited to the cell surface but extended over and beyond cells.

**DISCUSSION**

**The Matrilin-2 Gene Is Transcribed in Fibroblasts, Myocytes, and Epithelial Cells, but the Protein Is Transported to Connective Tissue Structures**—During our previous work, matrilin-2 mRNA was found in skin of the tail, calvaria, heart, uterus, and brain (4). From the wide distribution of the transcript, expression of the gene in cell types common to many organs was presumed. In situ hybridization and Northern blotting data represented here confirmed gene expression in connective tissue cells but extended the expression pattern to muscle and epithelial cells. Most of the expressor cell types are of mesodermal origin, but the epithelia of trachea, esophagus, and intestine, which showed strong in situ hybridization signals, develop from endoderm. Expression of the matrilin-2 gene in cells of
various developmental lineages predicts a complex mechanism for regulation of gene expression.

In some cases, synthesis of the matrilin-2 mRNA was found in epithelial cells, but deposition of the protein was observed only in subepithelial connective tissue. It is likely that the matrilin-2 mRNA is translated in epithelial cells and that the protein is specifically transported to the subepithelial regions. It is known that some components of basement membranes are made by epithelial cells; others are contributions from the underlying connective tissue fibroblasts. Nidogen is exclusively made and the α1 and α2 chains of collagen IV are predominantly produced by dermal fibroblasts, but all three chains of laminin-1 can be expressed by keratinocytes, especially at the beginning of coculturing (20). In the case of matrilin-2, the possibility cannot be excluded that epithelial cells synthesize mRNA, but translation or protein transport is blocked. The absence of matrilin-2 from media of the IEC6 intestinal epithelium has functional consequences. Studies of matrilin-1 derived from tissue sources show that homooligomerization of the naturally occurring protein leads to the formation of trimers as the single predominant species (18). Recent work has, however, demonstrated restrictions in the stringency of coiled-coil formation among matrilins. In showing that a single point mutation in the coiled-coil domain of matrilin-1 may cause it to form tetramers instead of trimers (22) and that matrilin-1 occurs in vivo not only as a homotrimer but also in a heterotetramer together with matrilin-3 subunits (23). In the case of matrilin-2, it could be that this lack of stringency leads to the protein occurring in vivo as a mixed set of monomers and oligomers. This heterogeneity may have functional consequences, since ligand binding sites will occur in variable copy number within a single molecule, and differences in oligomerization state may, because of cooperativity, lead to differences in affinity for macromolecular ligands that may bind to more than one subunit within a single matrilin-2 molecule. The possibility cannot be excluded that the oligomers detected by immunoblot are heterooligomers formed between matrilin-2 and another matrilin. A candidate would be matrilin-4, which is expressed outside cartilage as shown by Northern blots that give signals in lung, liver, brain, sternum, kidney, and heart (5).

**Evidence for Self-interaction of vWFA Domains of Matrilin-2 and Its Potential Role in Fibrillar Network Formation in Cell Culture**—In electron microscopy using negative stain, the matrilin-2 subunits are most often seen as loop structures with a diameter of 8 nm, but in a smaller fraction of the particles they are seen to open into a flexible rod (Fig. 7). Based on x-ray crystallographic data, we may assume a diameter of 3.6 nm for vWFA domains and 2.1 nm for EGF-like domains. In a tandem array, the two A domains together with the 10 EGF-like domains would have a length of about 28 nm. A circle with a diameter of 8 nm has a circumference of 25 nm, and the measurements are, therefore, compatible with a model of the matrilin-2 subunit where a loop is formed through interactions between the two A domains. In earlier studies of matrilin-1, we similarly observed a compact structure of the subunits (18). Domain interactions within the subunit had to be assumed to species from monomers to tetramers are seen in electron microscopy of samples purified under nondenaturing conditions (Fig. 7). This proves that the occurrence of multiple bands in SDS-PAGE is not due to incomplete closure of interchain disulfide bridges followed by dissociation of the coiled-coil upon treatment with SDS, but either that coiled-coil α-helices with varying numbers of protein strands are formed or that matrilin-2 subunits are specifically proteolytically cleaved at a site close to the coiled-coil region before or around secretion. Pan and Beck (6) recently investigated the oligomerization of a synthetic peptide corresponding to the coiled-coil domain of matrilin-2. By means of chemical cross-linking, they found that a trimer was the preferred species, but even at high cross-linking reagent concentrations, at which a corresponding matrilin-1-related peptide exclusively shows a single band corresponding to a trimeric state (21), the matrilin-2 peptide showed nearly equal amounts of material running in the position of monomers, dimers, and trimers (6). Under these conditions, higher molecular mass bands, although of lower concentrations, which can be interpreted to represent tetramers and pentamers, were also found. The multiplicity of oligomers could not be abolished by increasing the ionic strength as it was found for the matrilin-1 peptide (21). Although these authors argue that the monomeric and dimeric states observed upon SDS-PAGE might be due to the limitations of the cross-linking approach, they could not rule out that indeed different oligomerization states for the matrilin-2 related peptide are possible. Studies of matrilin-1 derived from tissue sources show that homooligomerization of the naturally occurring protein leads to the formation of trimers as the single predominant species (18). Recent work has, however, demonstrated restrictions in the stringency of coiled-coil formation among matrilins in showing that a single point mutation in the coiled-coil domain of matrilin-1 may cause it to form tetramers instead of trimers (22) and that matrilin-1 occurs in vivo not only as a homotrimer but also in a heterotetramer together with matrilin-3 subunits (23). In the case of matrilin-2, it could be that this lack of stringency leads to the protein occurring in vivo as a mixed set of monomers and oligomers. This heterogeneity may have functional consequences, since ligand binding sites will occur in variable copy number within a single molecule, and differences in oligomerization state may, because of cooperativity, lead to differences in affinity for macromolecular ligands that may bind to more than one subunit within a single matrilin-2 molecule. The possibility cannot be excluded that the oligomers detected by immunoblot are heterooligomers formed between matrilin-2 and another matrilin. A candidate would be matrilin-4, which is expressed outside cartilage as shown by Northern blots that give signals in lung, liver, brain, sternum, kidney, and heart (5).
explain why the length determined from electron micrographs was considerably smaller than that expected from the dimensions of the domains in tandem array. Since the A-domains in matrilin-1 are connected by a single EGF-like domain, substructures within the subunit could not be resolved, while in the matrilin-2 subunit, where the A-domains are connected by 10 EGF-like domains, a loop around a heavily stained hole could be seen. For sterical reasons, it is most likely that the loop is formed by the EGF-like domains, which are held together through an interaction between the two A-domains. Indeed, structures indicating self-interactions between A-domains have been observed by electron microscopy of von Willebrand factor (24) and of the N-terminal globule of the domains have been observed. For sterical reasons, it is most likely that the loop is formed by the EGF-like domains, which are held together through an interaction between the two A-domains. Indeed, structures indicating self-interactions between A-domains have been observed by electron microscopy of von Willebrand factor (24) and of the N-terminal globule of the α3 chain of type VI collagen (25). Further, by x-ray crystallography of von Willebrand factor A1-domain a contact surface was detected between A1-domain pairs, suggesting a hypothetical mechanism for the regulation of protein assembly and heterologous ligand binding mediated by homophilic interactions of type A-domains (26). vWFA domains in those proteins have also been shown to be involved in other interactions (25, 27).

By immunofluorescence microscopy of the matrix formed by cultured smooth muscle cells (Fig. 8), we could show that matrilin-2 molecules assemble into an extracellular fibrillar network, where each fibril may have a length of several cell diameters and often divides into smaller branches. In similar studies, matrilin-1 was found in chondrocyte cultures in close association with collagen II fibers (28), and a filamentous network, independent of collagen fibers, was also observed when matrilin-1 was overexpressed using a retroviral system (29). Matrilin-1 constructs, in which the vWFA1 domain had been deleted, assembled into trimers but could not form filamentous structures, thereby implicating the vWFA1 domain as being involved in the polymerization reaction leading to fibril formation. In analogy, it is likely that the vWFA domains of matrilin-2 may interact with each other and that filaments may be formed by this interaction. We do not know at present the exact molecular composition of the matrilin-2-positive extracellular filaments, but we are pursuing the study of such interactions of matrilin-2 with itself and with other extracellular macromolecules that may form the basis for fibril formation.