Matrilins constitute a family of four modular extracellular proteins that are involved in the development and homeostasis of cartilage and bone. To reveal their homo- and heterotypic oligomerization propensities, we analyzed the four human matrilin coiled-coil domains by biochemical and biophysical methods. These studies not only confirmed the homo- and heterotypic oligomerization states reported for the full-length proteins but revealed seven novel matrilin isoforms. Specific heterotrimeric interactions of variable chain stoichiometries were observed between matrilin-1 and matrilin-2, matrilin-1 and matrilin-4, and matrilin-2 and matrilin-4. In addition, matrilin-1 formed two different specific heterotramers with matrilin-3. Interestingly, a distinct heterotrimer consisting of three different chains was formed between matrilin-1, matrilin-2, and matrilin-4. No interactions, however, were observed between matrilin-2 and matrilin-3 or between matrilin-3 and matrilin-4. Both homo- and heterotypic oligomers folded into parallel disulfide-linked structures, although coiled-coil formation was not dependent on disulfide bridge formation. Our results indicate that the heterotypic preferences seen for the matrilin coiled-coil domains are the result of the packing of the hydrophobic core rather than ionic interactions. Mass spectrometry revealed that the concentrations of the individual chains statistically determined the stoichiometry of the heteromers, suggesting that formation of the different matrilin chain combinations is controlled by expression levels.

Matrilins are a family of four modular extracellular matrix proteins that show a similar structure consisting of one or two von Willebrand factor A(vWFA)1 domains, a varying number of EGF-like repeats, and a C-terminal coiled-coil domain (1). The functions of matrilins are poorly defined. Matrilins may play a role in stabilizing the extracellular matrix structure, since they can self-associate into supramolecular structures, resulting in the formation of filamentous networks (2–7). It has been shown that at least in the case of matrilin-1 and matrilin-3, these networks can either be associated with collagen fibrils or be collagen-independent. In the case of matrilin-1, it appears that the collagen-matrilin interaction is periodic, and it has been proposed that matrilin-1 may play a role in collagen fibril assembly (2, 4). Consistent with this hypothesis, ultrastructural studies of the cartilage of growth plates of matrilin-1 null mice revealed an abnormal type II collagen fibrillogenesis and fibril organization in the matrix of the zone of maturation (8). Furthermore, matrilin-1 binds to the aggrecan core protein (9) through an interaction that can become covalently stabilized (10), suggesting a role for matrilins in connecting different extracellular matrix components over a distance with their filaments to form an integrated network. In addition to binding to other matrix proteins, it has been proposed that matrilin-1 interacts with integrin α5β1 receptors and so may play a role in cell signaling (11). Recently, it has been demonstrated that mutations in the vWFA domain in matrilin-3 are associated with multiple epiphyseal dysplasia (12), confirming a role for the protein in the development and homeostasis of cartilage and bone.

Matrilin-2 (5, 13) and matrilin-4 (7, 14, 15) have a broad tissue distribution, whereas the expression of matrilin-1 (also known as cartilage matrix protein) (9, 16, 17) and matrilin-3 (6, 18–20) is more restricted to skeletal tissues. However, matrilin-1 expression appears to be less limited to cartilage during embryonal development (21). These complementary and in part overlapping expression patterns of matrilins gain additional potential functional significance through the recent discovery of hetero-oligomers formed by matrilin-1 and matrilin-3 in cartilage (6, 22, 23). It was shown that their assembly was not dependent on the number of EGF-like repeats but on the presence of the two cysteines within the coiled-coil domain, which form covalent disulfide bonds responsible for both homo- and hetero-oligomerization (24). These findings raise the question of whether hetero-oligomer formation can also exist between other matrilins. To address this issue, we investigated the homo- and hetero-oligomerization properties of the four human matrilins. To avoid difficulties associated with the supramolecular assembly properties of the proteins, these analyses were limited to the individual coiled-coil domains produced by recombinant expression in E. coli. Moreover, coiled-coil domains usually display the same oligomerization state as the full-length proteins. The structures and assembly products of the individual proteins and all possible chain combinations...
were assessed by SDS-PAGE, nondenaturing PAGE, mass spectrometry, CD spectroscopy, and analytical ultracentrifugation. These studies not only confirmed the known homotypic and heterotypic oligomerization forms reported for the full-length proteins but also revealed seven novel matrilin chain combinations. We demonstrate that the concentrations of the individual chains statistically determine the stoichiometry of the heteromers. Together with the thermodynamic results that revealed an increased thermal stability of the hetero-oligomers, this finding suggests that formation of the different matrilin chain combinations is controlled by expression levels.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids and Production of Recombinant Proteins—Synthetic genes encoding the amino acid sequences of the C-terminal coiled-coil domains of the four matrilin family members shown in Fig. 1B were prepared by PCR with optimal codon usage for Escherichia coli (25). The PCR products were ligated into the BamHI/EcoRI site of the bacterial expression vector pHis, a derivative of pET-15b (Novagen). Recombinant insert DNA was verified by Sanger dyeideoxy DNA sequencing.

The recombinant proteins were expressed in E. coli JM109(DE3) host strain (Novagen). The His6-tagged proteins were purified by immobilized metal affinity chromatography on Ni2+-Sepharose (Novagen). Separation of the polypeptide chain fragments from their His6 tags by thrombin cleavage was carried out as described in the manufacturer’s instructions. The peptides contain two additional residues, Gly and Ser, at their N termini. They originate from the expression plasmids and are not part of the coding sequences. The recombinant polypeptide chain fragments were analyzed in 5 mM sodium phosphate buffer (pH 7.4) supplemented with 150 mM sodium chloride. The peptide concentrations were determined by tryptophan and tyrosine absorption in 6 M GuHCl (26).

Preparation of Disulfide-linked Homo- and Hetero-oligomers—The purified matrilin homo-oligomers were reduced with 10 mM DTT for 1 h at 37 °C, precipitated with 75% ammonium sulfate and redissolved in 200 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1 mM EDTA. For reoxidation of cysteines, oxidized and reduced glutathione were added to final concentrations of 9 and 0.9 mM, respectively (27). After 3 days, the peptides were dialyzed against 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl.

For the analysis of hetero-oligomers by electrospray mass spectrometry, all possible chain combinations involving two different proteins were mixed at molar ratios of 1:1, 2:1, and 1:2 in the presence of 10 mM DTT and 5 mM GuHCl, heated to 90 °C for 2 min, incubated at 37 °C, and dialyzed against 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl.

For isolation of specific hetero-oligomers, one His-tagged matrilin chain was mixed with an excess of another non-His-tagged matrilin (two in case of the heterotrimer comprising three different chains), heated to 90 °C for 2 min, and incubated at 37 °C for 1 h. For purification on Ni2+-Sepharose and removal of the His tag, heteromers were dialyzed against 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl.

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Gel Electrophoresis—Nondenaturing PAGE and Tricine/SDS-PAGE (28) were performed on 12 × 13-cm slab gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Apparent molecular masses were obtained by comparison with low molecular mass markers (Amersham Biosciences and Sigma).

Mass Spectral Analyses—For mass spectral analysis, the peptides were chromatographed on a 100-μm inner diameter column packed with Vydac C18 reverse-phase material (5-μm particle size). The proteins were eluted with a linear 20-min gradient from 0.1% trifluoroacetic acid to 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 50 μl/min. Fractions were analyzed on a linear ion trap mass spectrometer (29). Peptide masses were obtained by comparison with low molecular mass markers (Amersham Biosciences and Sigma).
Matrilin Isoforms

Table I

| Protein | $s_{20,w}$ | Molecular mass (kDa) | Calculated$^d$ (monomer) | Disulfide-linked | Reduced |
|---------|------------|----------------------|--------------------------|-----------------|---------|
| (ccMat1) | 1.6        | 16.2                 | 15.3                     | 5.1             | 2 (5.8 ± 0.2) | >90     |
| (ccMat2) | 2.1        | 15.5                 | 18.6                     | 6.2             | 55 (2.0 ± 0.2) | 47      |
| (ccMat3) | 1.9        | 22.1                 | 20.8                     | 5.2             | 75 (5.8 ± 0.2) | 58      |
| (ccMat4) | 1.5        | 17.5                 | 16.8                     | 5.6             | 40 (±0.2) | 65      |

$^a$ The recombinant proteins were analyzed at 20 °C in 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl.
$^b$ The chain number of the coiled-coils is indicated by subscripts numbers.
$^c$ Based on previous experimental data, the errors of the sedimentation velocity and sedimentation equilibrium values are within 5–10%.
$^d$ For simplicity, only the first decimal place is shown.

Molecular mass of the monomer based on its amino acid sequence.

The sediments and sedimentation experiments were carried out on a Beckman Optima XL-A analytical ultracentrifuge equipped with a 5-mm sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. Protein concentrations were adjusted to 0.2–1.2 mg/ml. Sedimentation velocity experiments were performed at a rotor speed of 45,000 rpm, and sedimentation was measured by the absorbance at 234 nm. Sedimentation coefficients were corrected to standard conditions (water, 20°C) as a function of temperature. A heating rate of 0.01 °C/min was used. The GuHCl concentrations were determined from the refractive index according to Pace (30). Data analysis was performed with the Sigma Plot (Jandel Scientific) software package.

Analytical Ultracentrifugation—Sedimentation equilibrium and sedimentation velocity experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments) equipped with a 12-mm Epon double-sector cells in an An-60 Ti rotor. The recombinant proteins were analyzed in 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. Protein concentrations were adjusted to 0.2–1.2 mg/ml. Sedimentation velocity runs were performed at a rotor speed of 54,000 rpm, and sedimentation data was analyzed by the absorbance at 234 nm. Sedimentation coefficients were corrected to standard conditions (water, 20 °C; Ref. 31). Sedimentation equilibrium measurements were carried out at 20 °C at rotor speeds from 22,000 to 28,000 rpm. Molecular masses were calculated in 15-mM Tricine, 1 mM NaCl solutions in a dual-sector experiments from ln A versus r² plots, where A represents the absorbance and r is the distance from the rotor center (31). A partial specific volume of 0.73 ml/g was used for all calculations.

Results

Design of the Matrilin Coiled-coil Proteins—The amino acid sequences of the four proteins termed ccMat1, ccMat2, ccMat3, and ccMat4 are displayed in Fig. 1B. They were designed as follows. 1) To obtain specific disulfide-linked oligomers, which can be characterized by nonreducing SDS-PAGE and mass spectroscopy, the conserved cysteine residues flanking the N terminus of the matrilin coiled-coil domains were included in the proteins. For chicken matrilin-1, it has been shown by NMR that the cysteines form a ring of intra-chain disulfide bridges that stabilize the coiled-coil structure (32). Together with analytical ultracentrifugation measurements, the cysteines can also be useful to probe the relative helix orientation of the coiled-coils. 2) To be able to distinguish them by nondenaturing PAGE, all proteins were negatively charged but differed in their net charge. For that purpose, two additional aspartate residues were introduced at the N terminus of ccMat2. Net charges of the monomers are −1 for ccMat1, −3 for ccMat2, −2 for ccMat3, and −4 for ccMat4. 3) To be able to quantitate the proteins by tyrosine or/and tryptophan absorption, an artificial tryptophan residue was included at the N terminus of ccMat1 and ccMat4.

The Matrilin Coiled-coil Domains Form Specific Homo-oligomers of High Thermal Stability—The four recombinant proteins were produced by expression in E. coli and purified as described under “Experimental Procedures.” The homogeneity of the recombinant proteins was assessed by Tricine/SDS-PAGE under reducing conditions and revealed single bands of the expected monomer molecular masses (Fig. 2A, lanes 1–4). After redox shuffling of the proteins, Tricine/SDS-PAGE under nonreducing conditions revealed the specific formation of covalently linked homotrimers for ccMat1, ccMat2, and ccMat4 and a covalently linked homotetramer for ccMat3 (Fig. 2A, lanes 5–8). Consistent results were obtained by electrospray mass spectrometry (Table I). Moreover, analytical ultracentrifugation sedimentation equilibrium studies confirmed the oligomerization states of the peptides and indicated that the peptides did not further assemble to higher aggregates (Table I).

This finding was also supported by nondenaturing PAGE analysis of the proteins (Fig. 2B). Nondenaturing gels separate on the basis of a combination of molecular mass, shape, and net charge of proteins. The relative migration of ccMat1, ccMat2, and ccMat4 homotrimers with net charges of −3, −9, and −12, respectively, can be expected as a result of their similar molecular masses and probably also similar shapes (Fig. 2B, lanes 5, 6, and 8). The faster electrophoretic mobility of the ccMat3 homotetramer with a net charge of −8 relative to that of ccMat2 homotrimer may be explained by the formation of a more compact structure, which is less resistant to migration in the electric field (lane 7). Notably, a very similar pattern was obtained in the presence of the reducing agent DTT (lanes 1–4), indicating that homo-oligomer formation of matrilins is not dependent on disulfide bridge formation.

CD spectroscopy was used to test for secondary structures of the proteins. The far-ultraviolet CD spectra with minima near 208 and 222 nm (Fig. 3A) recorded from the four matrilin coiled-coil domains at 5 °C and total chain concentrations of 130 μM were characteristic for α-helical structures. Based on $\theta_{222}$ values of about −33,000 degrees cm² dmol⁻¹ for 100% α-helix (33), helical contents of >80% were calculated. The thermal stabilities of the four recombinant matrilin polypeptide chains were assayed by temperature-induced unfolding profiles recorded by CD at 221 nm (Fig. 3B; Table I). Due to their high thermal stabilities, GuHCl combined with increasing temperature was used to completely unfold the pro-
The four recombinant matrilin proteins fold into stable homotypic coiled-coil structures. A, far-ultraviolet CD spectra recorded from the proteins in 5 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl at 5 °C and total chain concentrations of 130 μM. Temperature-induced unfolding profiles of the proteins monitored by CD following the change of the mean molar residue ellipticity at 221 nm, \([\theta]_{221}\), under nonreducing (B) and reducing (10 mM DTT) (C) conditions are shown. The coiled-coil domains were analyzed under the same buffer and concentration conditions as in A except that the addition of GuHCl was necessary to completely unfold the proteins under nonreducing conditions.
T. Kiefhaber, unpublished data.

The chain number of the coiled-coils is indicated by subscript numbers.

For simplicity, only the first decimal place is shown. Values in parentheses represent the calculated masses of the proteins.

The addition of GuHCl was necessary to unfold the proteins. The final GuHCl concentrations were determined from the refractive index according to Pace (30) and are indicated in parentheses. ND, not determined.

The profiles exhibited the sigmoidal shapes typical for coiled-coil structures. The $T_m$ values obtained for the proteins are summarized in Table I. Only ccMat1 showed a biphasic transition, indicating cold denaturing. A similar thermal unfolding profile was observed for the chicken matrilin-1 coiled-coil domain and the pentameric coiled-coil domain of cartilage oligomeric matrix protein (ccCOMP) in the presence of high GuHCl concentrations (34). All thermal transitions were concentration-independent and reversible, with >90% of the starting signal regained on cooling (data not shown).

To test for the influence of disulfide bonds to their thermal stability, the four proteins were unfolded in the presence of the reducing agent DTT. Although all proteins still showed high thermal stabilities, significantly lower $T_m$ values and less sharp transition profiles were obtained under reducing conditions without GuHCl (Fig. 3C). While ccMat1 only partially melted, complete unfolding profiles were observed for ccMat2, ccMat3, and ccMat4. The $T_m$ values of the reduced hetero-oligomers are summarized in Table I.

Taken together, all four matrilin proteins folded into disulfide-linked coiled-coil structures of high thermal stabilities. Analytical ultracentrifugation together with SDS-PAGE under nonreducing conditions demonstrated that the helices of the four matrilin coiled-coils are arranged in a parallel manner. Formation of these specific homotypic structures was not dependent on disulfide bridge formation. The fact that consistent oligomerization states were reported for the full-length proteins demonstrates the validity of our approach.

The Matrilin Coiled-coil Domains Form Nine Distinct Hetero-oligomers—To investigate the properties of the four matrilin coiled-coil domains for heterotypic assembly, all possible chain combinations involving two different proteins were mixed at molar ratios of 1:1, 2:1, and 1:2. Furthermore, all possible combinations involving three different chains were mixed in equimolar amounts as described under “Experimental Procedures.”

To assess heteromer formation of the matrilin proteins and to determine their chain stoichiometries, electrospray mass spectrometry was used (Table II). With the exception of ccMat3, which interacted only with ccMat1, heteromer formation was observed for all other chain combinations. For all of these complexes, disulfide-linked heterotrimeric structures were found, except for ccMat1 and ccMat3, which folded into disulfide-linked tetramers. The concentration of the individual peptides statistically determined the stoichiometry of the heteromers. As a result, both possible stoichiometries were obtained for the heterotrimers containing two different chains, (ccMatX)1(ccMatY)2 and (ccMatX)2(ccMatY)1 (where X represents 1, 2, or 4, and Y is 1, 2, or 4). Two different stoichiometries were also seen for the heterotetramers, ccMat1,ccMat3, and ccMat1,ccMat4. In contrast, a heterotetramer containing one chain of ccMat3 and three chains of ccMat1 was never observed. Interestingly, mass spectrometry revealed a heterotrimer, consisting of three different chains of ccMat1, ccMat2, and ccMat4.

The specificity of matrilin heteromer formation was demonstrated when selected hetero-oligomers from each chain combination (Fig. 4, lanes 5–11) were compared with the homooligomeric species (Fig. 4, lanes 1–4) on nondenaturing gels. All hetero-oligomers migrated at positions intermediate to their component chains, indicating that the complexes adopt a compact structure with an intermediate net charge. Furthermore, a very similar pattern was obtained in the presence of DTT (Fig. 4, lanes 12–18), indicating that heterotypic interactions are mediated by noncovalent interactions and are not dependent on disulfide bridge formation. Fig. 4 shows no evidence for association of the hetero-oligomers to higher aggregates, a result that is supported by analytical ultracentrifugation of the purified (ccMat1)1(ccMat2)1(ccMat4)1 complex. A molecular mass of 19.6 kDa and no higher aggregates were observed, which is consistent with formation of a trimeric structure.

The purified matrilin heteromers were further characterized by CD spectroscopy. As expected, the far-ultraviolet CD spectra recorded from the heterotypic coiled-coil domains were characteristic for a-helical structures (data not shown). As judged from the CD signal at 221 nm, all hetero-oligomers were >80% helical (data not shown).

Like those of the homo-oligomers, the thermal stabilities of the heterotypic matrilin complexes required the addition of GuHCl for the recording of temperature-induced unfolding profiles. The profiles were recorded at total chain concentrations ranging from 20 to 100 μM. They were >90% reversible (data not shown) and, as expected for covalently linked oligomers, concentration-independent (Fig. 5; Table II).

The thermal stability of heterotetramer ccMat1,ccMat2,ccMat3,ccMat4 could be directly compared with those of the component homooligomers, because all three species were measured in the same GuHCl concentration of 5.8 M. The value obtained for the heterotetramer is about 15°C higher than the measured value of 72°C for (ccMat1)1(ccMat2)1, which is consistent with formation of a trimeric structure.

The purified matrilin heteromers were further characterized by CD spectroscopy. As expected, the far-ultraviolet CD spectra recorded from the heterotypic coiled-coil domains were characteristic for a-helical structures (data not shown). As judged from the CD signal at 221 nm, all hetero-oligomers were >80% helical (data not shown).

Like those of the homo-oligomers, the thermal stabilities of the heterotypic matrilin complexes required the addition of GuHCl for the recording of temperature-induced unfolding profiles. The profiles were recorded at total chain concentrations ranging from 20 to 100 μM. They were >90% reversible (data not shown) and, as expected for covalently linked oligomers, concentration-independent (Fig. 5; Table II).

The thermal stability of heterotetramer ccMat1,ccMat2,ccMat3,ccMat4 could be directly compared with those of the component homooligomers, because all three species were measured in the same GuHCl concentration of 5.8 M. The value obtained for the heterotetramer is about 15°C higher than the measured value of 72°C for (ccMat1)1(ccMat2)1. This value is about 8°C lower than the measured $T_m$ obtained for the heterotrimer (Table II). For the other heterotrimers, the same tendency was observed. For (ccMat1)2(ccMat2)1(ccMat4)1, (ccMat1)2(ccMat3)1, and (ccMat1)2(ccMat4)1, corrected $T_m$ values of 72, 55, and 48°C, respectively, were calculated, which are 6, 3, and 27°C, respectively, lower than the measured values. These findings indicate that matrilin hetero-oligomers are thermodynamically favored over homo-oligomers.

Taken together, our results demonstrate that the four ma-

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Y. Guo, R. A. Kammerer, and J. Engel, unpublished data.

T. Kiefhaber, unpublished data.

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**Table II**

| Protein<sup>a</sup> | Molecular mass<sup>b</sup> Tm<sup>c</sup> | mass spectroscopy (calculated) | °C |
|---------------------|--------------------------------|-------------------------------|-----|
| (ccMat1)<sub>1</sub>(ccMat2)<sub>1</sub> | 16.4 (16.4) | 80 (4 M) | 32 |
| (ccMat1)<sub>1</sub>(ccMat2)<sub>2</sub> | 17.5 (17.5) | ND | 40 |
| (ccMat1)<sub>1</sub>(ccMat3)<sub>2</sub> | 20.7 (20.7) | ND | 45 |
| (ccMat1)<sub>1</sub>(ccMat4)<sub>2</sub> | 20.6 (20.6) | 81 (5.8 °C) | 48 |
| (ccMat1)<sub>1</sub>(ccMat4)<sub>4</sub> | 16.3 (16.3) | ND | 45 |
| (ccMat1)<sub>1</sub>(ccMat4)<sub>4</sub> | 15.8 (15.8) | 78 (5 °C) | 40 |
| (ccMat2)<sub>2</sub>(ccMat4)<sub>1</sub> | 17.4 (17.4) | 58 (3 °C) | 40 |
| (ccMat2)<sub>2</sub>(ccMat4)<sub>2</sub> | 18.0 (18.0) | ND | 45 |
| (ccMat3)<sub>1</sub>(ccMat2)<sub>2</sub>(ccMat4)<sub>1</sub> | 16.9 (16.9) | 75 (4 °C) | 40 |

<sup>a</sup> The chain number of the coiled-coils is indicated by subscript numbers.

<sup>b</sup> For simplicity, only the first decimal place is shown. Values in parentheses represent the calculated masses of the proteins.

<sup>c</sup> The addition of GuHCl was necessary to unfold the proteins. The final GuHCl concentrations were determined from the refractive index according to Pace (30) and are indicated in parentheses. ND, not determined.
trilin coiled-coil domains can fold into nine disulfide-linked parallel hetero-oligomers. Notably, the formation of these distinct isoforms is not dependent on disulfide bonds. Two of these chain combinations have recently been reported for the full-length matrilin-1 and matrilin-3 proteins (6, 22–24). Together with the fact that coiled-coil domains usually display the same oligomerization properties as the full-length proteins, our findings strongly suggest that the other seven matrilin isoforms also exist in nature. Although our results shed light on the structural organization of matrilins, the functional significance of heteromer formation remains to be elucidated.

**DISCUSSION**

As a step toward our goal to understand matrilin structure and function, we have characterized the four human matrilin coiled-coil domains and all possible chain combinations thereof by biochemical and biophysical methods. Coiled-coil domains usually display the same oligomerization state as the full-length proteins but offer the advantage of avoiding difficulties associated with the full-length proteins, such as supramolecular assembly. We found that ccMat1, ccMat2, and ccMat4 all formed disulfide-linked, three-stranded, parallel homotrimers, whereas ccMat3 folded into a disulfide-linked, four-stranded, parallel coiled-coil structure (Fig. 6). Consistent oligomerization states have previously been reported for the full-length proteins (5–7, 35), synthetic peptides, and recombinant proteins comprising the coiled-coil domains from matrilin-1 and matrilin-2 (32, 36–38). Based on the coiled-coil structures, we were able to identify nine different matrilin isoforms, seven of which have not been described previously. The possible matrilin chain combinations are shown in Fig. 6. An unexpected feature of the matrilin hetero-oligomers consisting of two different polypeptide chains is that they can exist in different stoichiometries. The most plausible explanation for this observation is that the thermodynamic stabilities of the heterotypic coiled-coils with the same chain composition are very similar. Our studies not only confirmed two heterotetramers of different chain stoichiometries reported for biochemically isolated matrilin-1 and matrilin-3 (6, 23) but also demonstrated that these interactions occur via the proteins’ coiled-coil domain. These findings emphasize the validity of our approach and demonstrate that the characterization of the coiled-coil domains from a protein family can be used to predict their oligomerization properties.

The α-helical coiled coil is the most widespread subunit oligomerization motif found in proteins (39–42). It is a type of protein structure consisting of 2–5 amphipathic α-helices that “coil” around each other in a slight supertwist (39–42). The sequences of left-handed coiled coils are characterized by a heptad repeat of seven residues denoted a–g with a 3,4-hydrophobic repeat of mostly apolar amino acids at positions a and d (43, 44). Interactions between the core residues a and d and the two flanking positions e and g determine the number of strands, the parallel or antiparallel orientation of α-helices, and the homo- or heterotypic association of subunits into a coiled coil (for a review, see Refs. 39 and 42).

Matrilin-3 differs from the other matrilins in terms of its tetrameric oligomerization state. It is well known that the residues at the a and d positions can exert a major influence on oligomer selection. Crucial roles in oligomer specification are played by leucine and the β-branched residue isoleucine (45). Tetramers are known to be favored by sequences enriched by leucine residues at the α sites and isoleucine at the d sites, whereas trimers display a more even distribution of hydrophobic residues at these positions. The preferences for specific amino acids at the core positions of coiled-coils can be explained by differences in packing geometry in these structures. Since only matrilin-3 contains a single isoleucine residue at a d position of the third heptad repeat, it is tempting to speculate that this residue specifies the four-stranded oligomerization state of the protein. Considering the importance of isoleucine for specifying the oligomerization state, chicken matrilin-3 may possibly even form a trimer because this residue is not conserved in the avian species. In addition, the d position of the fourth heptad repeat of all known matrilin-3 species is occupied by residues that are not frequently found at these positions (46). It should be noted that residues at the b, c, ε, and g positions of the tetramer also contribute to the hydrophobic core (45). Thus, the amino acid sequence at these positions can also influence the oligomerization state. Accordingly, Beck et al. (37) observed a switch in the oligomerization state of the coiled-coil domain of matrilin-1 after substitution of a single residue. After replacing arginine 487, which forms a potential interchain ionic interaction with a glutamate 492, with glutamine, the peptide folded into a homotetramer at neutral pH. Interestingly, the mutant peptide folded into a homotrimer at acidic and basic pH. It should be noted, however, that the interchain salt bridge is an unlikely determinant in specifying the four-stranded oligomerization state, because the interaction is conserved among the human and mouse matrilins.

Our findings raise the question about the molecular determinants that specify that matrilin-3 interacts with matrilin-1 but not with matrilin-2 and matrilin-4. In line with our results, it has been established by phylogenetic analysis that the coiled-coil domain of ccMat3 is most closely related to ccMat1 (1). Interestingly, all coiled-coil structures containing matrilin-3 are four-stranded. It is well established that hetero-oligomerization can occur as a “relief” of repulsive electrostatic interactions between residues in the g position with residues in the e′ position of a neighboring chain in a homo-oligomer (for a review, see Ref. 47). Electrostatic interactions between residue i of chain 1 in g and residue i′ + 5 in e of chain 2 are prominent because of the proximity of these residues (45). Repulsive in-
teractions disrupt the complementary packing at the interface of the coiled-coil, thus accounting for the instability of the homodimers. However, interchain $g$ and $e'$ interactions do not explain hetero-oligomer formation of matrilins. There is only one potential attractive interaction between residues of the last two heptads that is conserved in all four coiled-coils (Arg or Lys in $g$ to Glu in $e'$; see Fig. 1B). Tetramers also exhibit two types of ion pairs that are rarely found in trimers. These are $g$ to $b'$ and $c$ to $e'$ salt bridges. Like the $e$ and $g$ residues, however, these salt bridges are unlikely to account for the hetero-oligomerization specificity of matrilins. Furthermore, hetero-oligomer formation of matrilins is not the result of homo-oligomer instability, because all four homotypic matrilin coiled-coils fold into very stable structures. Because electrostatic interactions do not provide an explanation, we therefore suggest that the heterotypic preferences seen for the matrilin coiled-coil domains are the result of the packing of the hydrophobic core. Our hypothesis is consistent with a recent study of Keating et al. (48), who addressed the role of hydrophobic residues at the $a$ and $d$ positions in determining heterotypic interaction specificity. These authors showed that computational modeling of coiled-coil structures can be used to predict interaction energy differences that agree quantitatively with experimental results positions in determining interaction specificity. The calculations could be used to predict coiled-coil partnering preferences that arise from core packing. Accordingly, residues that are known to favor trimer or tetramer formation over dimerization (such as Ile or Val at the $d$ position) were found to destabilize the heterodimers.

The principal function of coiled-coil domains is subunit oligomerization of multisubunit proteins. Oligomerization generates multivalency, provides high local concentrations of functional sites, and allows clustered domains to function in a concerted manner. Heteromers could further increase the structural and functional diversity of matrilins. Candidates are the vWFA domains, which are arranged in different ways in different isoforms (for a schematic view, see Figs. 1A and 6). These domains are present in a large number of other extracellular proteins, including collagens, complement factors, integrins, and von Willebrand factor (49, 50) and mediate self-interaction and ligand-binding activities (49). Thus, the ligand-binding affinity of vWFA domains could be modulated by the expression of matrilins as homo- and hetero-oligomers. Such a mechanism has also been proposed for the heparin-binding properties of thrombospondins (51), where thrombospondin-2 has a lower affinity than thrombospondin-1. Thus, the ligand-binding affinity could be varied by the formation of thrombospondin homo- and heterotrimers. Coiled-coil domains also play an important role in the formation of laminins for which isoform-specific functions are well established (52, 53). Laminins comprise a family of at least 14 heterotrimers that are assembled through a coiled-coil domain from at least 11 different polypeptide chains. Laminins function as structural components and are essential for morphogenesis but in addition interact with cell surface receptors such as integrins and $a$-dystroglycan. The many interactions of laminins are mediated by binding sites, often contributed by single domains, which may differ between different forms of laminin. One important function of many laminins is their ability to self-assemble into independent networks (54). Since self-assembly is mediated by the N-terminal domains, these interactions differ among isoforms.

The vWFA domain is the most likely candidate for the self-interaction seen for matrilins, leading to the formation of supramolecular structures, which may play a role in stabilizing the extracellular matrix structure. Also in this case, heterooligomerization could provide a means to increase the structural diversity of these complexes and generate assemblies with particular properties. This hypothesis is supported by a recent study on collagen V by Chanut-Delalande et al. (55).
These authors investigated the role of collagen V in homotypic and heterotypic fibril formation. They found that both collagen V heterotrimer and homotrimer formed thin fibrils. When mixed with collagen I, however, heterotrimer and homotrimer exerted different effects in heterotypic fibril formation. Unlike the heterotrimer, which was buried in the fibril interior, the homotrimer was localized as thin filamentous structures at the surface of wide collagen I fibrils and did not regulate fibril assembly. Its localization at the fibril surface suggests that the homotrimer can act as a molecular linker between collagen fibrils and/or macromolecules in the extracellular matrix. Interestingly, like our findings on matrilin heteromer formation, the control of collagen V homo- and heterotrimers seems to be determined by chain stoichiometry.

Furthermore, coiled-coil domains can also harbor binding sites for globular proteins. For example, the N-terminal domain of agrin binds to the coiled-coil domain of laminins (56). This interaction is important for the localization of agrin to the synaptic basal lamina and other basement membranes. In the hyperthermophilic archaeabacterium *Staphylothermus marinus*, a subtilisin-like protease interacts with the four-stranded right-handed coiled coil of the surface layer protein tetrabrachion (57, 58). Likewise, particular matrilin isoforms may contain binding sites for extracellular proteins that are not present in the homo-oligomers. Thus, depending on their respective distribution in tissues, the different matrilin isoforms might fulfill specific biological functions. This conclusion is also supported by a study of Chapman et al. (12), who demonstrated that mutations in the vWFA domain in matrilin-3 are associated with episcleral dysplasia.

The tissue distribution of matrilins indicates that some of the chain combinations identified in this study are more prominent than others. Matrilin-2 (5, 13) and matrilin-4 (7, 14, 15) have a broad tissue distribution, whereas the expression of matrilin-1 (also known as cartilage matrix protein) (9, 16, 17) and matrilin-3 (6, 18–20) is more restricted to skeletal tissues. Our results on the identification of distinct matrilin chain combinations strongly suggest that these isoforms also exist in nature. The recombinant proteins may be used to raise isoform-specific antibodies to identify and monitor particular chain combinations in tissue.

Due to its small size the heterotrimeric coiled coil consisting of matrilin-1, matrilin-2, and matrilin-4 should also be of interest as a hetero-oligomerization tool. Homotypic and heterodimeric coiled coils are frequently used to artificially cluster domains and peptides of interest. Due to the high local concentrations, in many cases clustering results in a significantly increased activity of the domains and peptides.

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**FIG. 6. Possible matrilin homo- and hetero-oligomers.** 1, matrilin-1; 2, matrilin-2; 3, matrilin-3; 4, matrilin-4. The same color code as in Fig. 1A is used.
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Characterization of the Matrilin Coiled-coil Domains Reveals Seven Novel Isoforms
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