The Role of Disulfide Bonds and α-Helical Coiled-coils in the Biosynthesis of Type XIII Collagen and Other Collagenous Transmembrane Proteins

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Type XIII collagen is a type II transmembrane protein with three collagenous (COL1–3) and four noncollagenous domains (NC1–4). The human α1(XIII) chain contains altogether eight cysteine residues. We introduced point mutations to six of the most N-terminal cysteine residues, and we show here that the two cysteines 117 and 119 at the end of the N-terminal noncollagenous domain (NC1) are responsible for linking the three α1(XIII) chains together by means of interchain disulfide bonds. In addition, the intracellular and transmembrane domains have an impact on trimer formation, whereas the cysteines in the transmembrane domain and the COL1, the NC2, and the C-terminal NC4 domains do not affect trimer formation. We also suggest that the first three noncollagenous domains (NC1–3) harbor repeating heptad sequences typical of α-helical coiled-coils, whereas the conserved NC4 lacks a coiled-coil probability. Prevention of the coiled-coil conformation in the NC3 domain is shown here to result in labile type XIII collagen molecules. Furthermore, a new subgroup of collagenous transmembrane proteins, the Rattus norvegicus, Drosophila melanogaster, and Caenorhabditis elegans colmedins, is enlarged to contain also Homo sapiens collomin, and Pan troglodytes, Mus musculus, Tetraodon nigroviridis, and Danio rerio proteins. We suggest that there is a structurally varied group of collagenous transmembrane proteins whose biosynthesis is characterized by a coiled-coil motif following the transmembrane domain, and that these trimerization domains appear to be associated with each of the collagenous domains. In the case of type XIII collagen, the trimeric molecule has disulfide bonds at the junction of the NC1 and COL1 domains, and the type XIII collagen-like molecules (collagen types XXIII and XXV) and the colmedins are similar in that they all have a pair of cysteines in the same location. Moreover, furin cleavage at the NC1 domain can be expected in most of the proteins.

The collagen family of proteins contains a continuously expanding subgroup of collagenous transmembrane proteins. For a protein to join this subfamily, it needs to fulfill two crite-ria; it should be a type II transmembrane protein with at least one collagenous domain. The currently accepted members are as follows: the type XIII collagen-like proteins (collagen types XIII, XXIII, and XXV), type XVII collagen, the class A macrophage scavenger receptor-like proteins (MSRs (macrophage scavenger receptors), MARCO (macrophage receptor with collagenous structures), and SRCL (scavenger receptor with C-type lectin)), ectodysplasin-A, and the colmedins (1, 2).

We have studied the biosynthetic features of type XIII collagen by expressing it in insect cells using the baculovirus expression system, and we have demonstrated a disulfide-bonded homotrimeric molecule with three triple helical collagenous domains (3). Furthermore, the association of the three chains takes place in the N-terminal NC1 coiled-coil region, being followed by formation of the triple helix in an N- to C-terminal direction (4, 5). Further analysis revealed a second conserved α-helical coiled-coil domain in the NC3 domain, indicating that type XIII collagen harbors at least two independent oligomerization domains (5).

The biological function of type XIII collagen has been investigated by generating transgenic mice overexpressing mutant α1(XIII) chains with a long in-frame deletion of their COL2 sequences (6). The mutant CDNA construct also lacked the extreme 38 amino acids, including the whole NC4 domain. Expression of these mutant COL2del chains led to defects in placental formation or to cardiovascular defects in offspring from heterozygous matings, resulting in embryonic lethality. Disulfide-bonded trimeric COL2del molecules were found to be synthesized by cultured fibroblasts derived from the mutant mice.

The large olfactomedin-like (OLF) gene family includes seven evolutionarily and functionally distinct subfamilies (7). The most primitive clade in the OLF family, subfamily VI, is characterized as containing one or two collagenous domains preceding the OLF domain. So far nine members of the sub-group have been identified as follows: Homo sapiens BAD18742, Mus musculus CRG-L2, Gallus gallus XP_425097, Tetraodon nigroviridis CAF99838 and CAG05536, Drosophila melanogaster CG6867, Anopheles gambiae XP_315876, and Caenorhabditis elegans colmedin (cof-2) and unc-122. Four of these, namely Rattus norvegicus gliomedin (homologous to M.
**Amino Acid Sequence Analysis and Secondary Structure Prediction**—The residues essential for the coiled-coil structure in the NC3 domain of type XIII collagen were determined by changing individual residues to proline and running the COILS program (version 2.1) (10). Coiled-coil predictions for H. sapiens del1–38, E430P, A431P, and BAD18742; human constructs. The recombinant virus was then amplified twice, plaque-purified, and amplified three times. We also used the previously described recombinant baculovirus coding for both subunits of human prolyl 4-hydroxylase (4PHαβ) (16).

**Analysis of Recombinant Proteins Produced in Insect Cells by SDS-PAGE and Immunoblotting**—Sf9 or High Five insect cells were cultured as monolayers in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) or in serum-free Express Five medium (Invitrogen) at 27 °C. Cells at a density of 6 × 10^5 cells/ml were infected with viruses coding for the human constructs. The recombinant virus was then amplified twice, plaque-purified, and amplified three times.
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were analyzed by denaturing SDS-PAGE under reducing or nonreducing conditions, followed by Western blot analysis with a polyclonal antibody against a bacterial fragment corresponding to the NC1 domain of mouse type XIII collagen, excluding the transmembrane domain (antibody XIII/NC1-Q610 (17)), part of the NC2 domain of human type XIII collagen (antibody XIII/NC2–55 (3)), the whole NC3 domain of human type XIII collagen (antibody XIII/NC3-1 (18)), or the C-terminal end of the COL3 domain and the whole NC4 domain of human type XIII collagen (antibody XIII/NC4-SO (3)) and detected with enhanced chemiluminescence.

RESULTS

Formation of Disulfide-bonded Trimers of Type XIII Collagen Variants—Full-length human α1(XIII) chains have four pairs of cysteine residues located in the NC1, COL1/NC2, and NC4 domains (Fig. 1A). We have shown in previous studies that some of the cysteines in the NC1 domain of human type XIII collagen, and possibly those at the junction of the COL1 and NC2 domains, are interchain-bonded, whereas the two cysteine residues in the NC4 domain are likely to form intrachain bonds (3). To actually pinpoint the cysteines responsible for the interchain linking of the trimeric type XIII collagen molecule, the previously indicated potential interchain-forming cysteines were mutated to serines as shown in Fig. 1A. In addition, the role of the C-terminal cysteines was tested by using a COL2del variant lacking the NC4 domain (Fig. 1B). The COL2del α1(XIII) chains also contain an in-frame deletion in the COL2 domain (Fig. 1B). The human del1–38 variant was used as a control in all the experiments, because it forms disulfide-bonded trimers with three triple helical domains, and its expression level is far higher than that of full-length mouse and human α1(XIII) chains. In all cases the insect cells were co-infected with viruses encoding recombinant α1(XIII) and prolyl 4-hydroxylase (4PHαβ) in order to produce hydroxylated type XIII collagen chains (19). The infected cells were harvested, homogenized, and centrifuged 48 h post-infection. The cell pellets were analyzed on SDS-PAGE under nonreducing conditions followed by Western blotting with the antibody XIII/NC3-1. As we have demonstrated before (3, 5), the deletion variant del1–38 assembled into trimeric disulfide-bonded molecules, whereas the deletion variant del1–61 did not (Fig. 2A, del1–38 and del1–61). In the case of the cysteine-mutated variants, the Cys-1S2S and Cys-5S6S chains were able to form disulfide-bonded trimers (Fig. 2A, Cys-1S2S and Cys-5S6S), whereas the absence of cysteines 3 and 4 (Cys-3S4S) abolished the formation of disulfide-bonded trimers (Fig. 2A, Cys-3S4S). For-
thermore, the formation of disulfide-bonded dimers was also prevented in the case of the Cys-3S4S variant. On the other hand, the Cys-3S and Cys-4S variants were able to form disulfide-bonded dimers, but the formation of disulfide-bonded trimers was diminished (Fig. 2A, Cys-3S and Cys-4S), indicating that both of these cysteine residues are necessary for the formation of a disulfide-bonded type XIII collagen trimer (Fig. 2B).

The COL2del variant assembled to trimeric disulfide-bonded molecules (Fig. 2A, COL2del) providing experimental evidence to our previous suggestion that these two cysteines in the NC4 domain form intrachain bonds. Deletion of the 83 amino acid residues from the interior of the COL2 domain was not detrimental to its capacity to form a triple helix.

The NC3 Coiled-coil Domain Point Mutant Proteins Are Labile—We have shown in previous studies that type XIII collagen contains potential coiled-coil domains in its NC1 and NC3 domains (5). This is illustrated here for del1–38 using the COILS program (Fig. 3A, del1–38). Proline residues are known to introduce a kink into a helix, leading to disruption of the coiled-coil structure (20, 21). Thus, to study the role of the NC3 coiled-coil domain in the stability of type XIII collagen, each residue in the NC3 29-residue coiled-coil domain of del1–38 was changed to a proline, and the effect was analyzed using the COILS program. This showed residues Glu-430 and Ala-431 to be important for the coiled-coil structure, as replacement of Glu-430 with proline reduced the probability of coiled-coil formation from nearly 100% to less than 30% (Fig. 3A, del1–38 and E430P), and replacement of Ala-431 almost prevented the pre-
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![Figure 4: 
A-Helical coiled-coil prediction for human collomin and alignment of coiled-coil sequences for human type XIII collagen and collomin. A, collomin protein sequence was studied for putative coiled-coil domains using the COILS program, as in Fig. 3. The abscissa shows the scale in amino acids (aa), and the y axis indicates coiled-coil probability. A schematic structure for the collomin chain is shown below the graph. The noncollagenous domains are shown as black boxes, the transmembrane domain as a gray box, the collagenous domains as white boxes, and an olfactomedin domain as a turquoise box. The potential furin cleavage site in collomin is marked by an arrow. B, alignment of the amino acid residues in the NC1 domain of type XIII collagen with the corresponding residues in collomin achieved using a BLOSUM matrix. The amino acid sequences are shown in one-letter codes. Identical amino acids are indicated by black boxes and similar ones by gray boxes. The conserved trimerization motif is indicated above the alignment with a bar.

dicted coiled-coil formation (Fig. 3A, A431P). Based on these predictions, the corresponding residues in the del1–38 variant were mutated to prolines using site-directed mutagenesis, and the recombinant viruses E430P and A431P were produced and used together with 4PHβ to infect insect cells. The del1–38 virus was used as a control. After harvesting, homogenization, and centrifugation, the supernatant samples were fractionated on SDS-PAGE under nonreducing or reducing conditions, followed by Western blotting with the antibody XIII/NC1-Q610, XIII/NC2–55, XIII/NC3-1, and XIII/NC4-SO (Fig. 1B). The potential furin cleavage site was predicted at amino acid position 94 at the N terminus, following the potential coiled-coil domain but preceding the collagenous domain (data not shown). The D. melanogaster protein contains one highly potential coiled-coil domain at its N terminus after the transmembrane domain, and two furin cleavage sites are proposed at positions 20 and 181, the latter situated between the coiled-coil and collagenous domain (data not shown). Furthermore, the C. elegans proteins were found to have multiple potential coiled-coil domains along the protein molecule, albeit with low probability (data not shown). As with the other colmedins, potential furin sites could be found in the N-terminal noncollagenous domain, at position 46 for cof-2 and position 67 for unc-122 (data not shown).

The collagenous and OLF domain-containing proteins H. sapiens BAD18742, M. musculus CRG-L2, G. gallus XP_425097, T. nigroviridis CAF99838 and CAG05536, and An. gambiae XP_315876 have been recently discovered (7). We searched these proteins for potential transmembrane domains, coiled-coils, and furin cleavage sites using the TMHMM Server version 2.0, the COILS program, and the ProP 1.0 Server, respectively. To begin with, the C-terminal end of the H. sapiens BAD18742 protein was shown to be identical to H. sapiens collomin (GenBank™ data base accession number NP_861454) but included further 5’ sequences representing the full-length H. sapiens collomin, so the latter name is used here (data not shown). The full-length H. sapiens collomin was predicted to possess a transmembrane domain at amino acids 13–35 with its N terminus inside the cell and C terminus outside, thus being a type II transmembrane protein (Fig. 4A). The COILS program gave multiple potential coiled-coil domains for H. sapiens collomin, and the one with the highest potential was that starting from the predicted transmembrane domain (Fig. 4A). A potential furin cleavage site was mapped at position 94 (Fig. 4A, arrow). Sequence comparisons indicated that a 22-residue stretch (residues 68–89) of the 40-residue NC1 coiled-coil domain of H. sapiens type XIII collagen is 41% identical to and 45% homologous with the corresponding sequences in H. sapiens collomin (Fig. 4B).

Analysis of the Novel Collagenous Transmembrane Proteins—To extend our understanding of the conserved primary structure features of collagenous transmembrane proteins, the new members of this subfamily, namely the colmedins R. norvegicus gliomedin, D. melanogaster CG6867, and C. elegans cof-2 and unc-122 (2, 8), were analyzed for potential coiled-coils and furin cleavage sites using the COILS program and the ProP 1.0 Server, respectively. The R. norvegicus gliomedin was found to possess at least one potential coiled-coil domain at its N-terminal end, extending from the transmembrane domain, and one...
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The α-helical coiled-coil domains that are known to work as specific oligomerization domains, determining the association state and chain composition of the relevant molecule, are emerging as essential for the assembly of most collagens (5, 22). The transmembrane collagens are integrated into the plasma membrane in a type II orientation, and the collagenous ectodomain is folded in an N- to C-terminal direction. A closer look at type XVII collagen reveals that most, if not all, of its noncollagenous domains have potential coiled-coil features, except for the extreme C-terminal NC16 domain (Fig. 5) (5). Type XIII collagen also contains coiled-coil features in all of its noncollagenous domains, except again for the extreme C-terminal NC4 domain (Fig. 3A, del1–38, and Fig. 5). This suggests that every collagenous domain “needs” a nearby coiled-coil region in its N terminus in order to associate and fold correctly. Thus, in the case of type XIII collagen, the conserved NC4 domain can be considered completely “free” for other purposes.

Computational predictions of the NC3 domain of type XIII collagen suggested that its coiled-coil conformation is important for the correct folding of the corresponding molecule, and this prediction was found to be correct in in vitro mutagenesis studies. In nonreduced SDS-PAGE analysis the mutant α1(XIII) chains remained monomeric, suggesting that the formation of stabilizing interchain disulfide bonds in the NC1 domain was compromised. One explanation for the absence of interchain disulfide bonds could be that their formation is hindered when the prolyl 4-hydroxylase enzyme associates with abnormal procollagen molecules (23). Moreover, the mutant α1(XIII) chains were found to be labile, with very few full-length α1(XIII) chains detectable in reduced SDS-PAGE analysis. Furthermore, folding of the COL3 domain seemed to be most severely affected, because this domain was absent among the degradation fragments.

We have previously shown that absence of the NC1 coiled-coil domain leads to lack of disulfide-bonded trimers and misfolding of the membrane-proximal collagenous domain COL1, whereas the COL2 and COL3 domains are correctly folded (5). Taken together with the present data, it seems that the two main coiled-coil regions of type XIII collagen, in the NC1 and NC3 domains, act as independent nucleation sites for the formation of the following trimeric collagenous domain, and because the NC2 domain is also predicted to harbor a coiled-coil conformation, although at a low probability, it may act in a similar manner and contribute to the stabilization of the COL2 domain. Altogether the data are consistent with the hypothesis that every collagenous domain needs its own nearby N-terminal coiled-coil region for proper folding and stability.

It should be noted that the structure of the NC2 domain can vary depending on alternative splicing of the precursor RNA (24). Interestingly, different compositions of the alternatively spliced exons 14 and 15 encoding the NC2 domain gave different low probabilities for a coiled-coil conformation using the COILS program, i.e. a 0.012% probability for +exon14/-exon15, a 0.002% probability for +exon14/+exon15, and 0% probabilities for −exon14/+exon15 and −exon14/−exon15. On the other hand, it has been shown for matrilins, which are also known to assemble via a coiled-coil domain, that the concentrations of the individual chains statistically determined the stoichiometry of the heteromers, indicating that expression levels of the different matrilin chains determine the resulting chain combination of the oligomer (25, 26). It
may thus be that the tissue-specific isoforms are formed partly on the basis of the concentration of individual chains and partly on the basis of their coiled-coil conformation. This could also be a practical way of changing the protein response in tissues, by simply controlling it through alteration in the expression levels of particular chains.

Coiled-coil domains in extracellular proteins have been shown to be frequently flanked by pairs of closely spaced cysteine residues, which form interchain disulfide bonds (27). As we show here, the type XIII collagen trimers are subsequently stabilized through interchain disulfide bonds involving Cys-117 and Cys-119 at the end of the NC1 domain (Fig. 1A, C3 and C4). Like type XIII collagen, the cartilage matrix protein, i.e. matrilin-1, contains two closely spaced cysteines in its heptad repeat-containing tail domain, which have been shown to be both necessary and sufficient for interchain disulfide bond formation resulting in a stable trimer. If, however, the heptad repeats of the tail domain were deleted, trimers failed to form even if the cysteines were present (28). Furthermore, in addition to collagen type XIII, collagen types XXIII and XXV also harbor closely spaced cysteines (CXCXGPPP) just in front of the first collagenous domain (Fig. 5) (29, 30), suggesting that this pair of cysteines also have an interchain-type stabilizing effect in these closely related collagenous transmembrane collagens. Type XVII collagen is devoid of cysteines in its ectodomain region, however, highlighting its differences relative to the other transmembrane collagens (31). This is consistent with the finding that the conserved nucleation site for triple helix folding in other collagenous transmembrane proteins is not necessary for the formation of a stable triple helical collagen XVII trimer (4, 32). On the other hand, type XVII collagen contains two other potential coiled-coil regions following the previously described conserved nucleation site (5). The first of these seems to be essential for chain association because the del528–547 construct lacking this coiled-coil region was abnormally folded, preventing shedding of these mutant α1(XVII) chains (32).

It should be noted that the del1–61 variant lacking the intracellular and transmembrane domains and the cysteines 45 and 53 (Fig. 1A, C1 and C2) of type XIII collagen failed to form trimers despite the presence of cysteines 117 and 119. Although Cys-117 and Cys-119 at the end of the NC1 domain are absolutely required for the stabilization of type XIII collagen trimers (Fig. 1A, C3 and C4), the NC1 coiled-coil and these cysteines alone are not sufficient for trimer formation. Thus the intracellular and transmembrane domains have an important impact on trimer formation and stabilization. On the other hand, deletion of the 83 amino acid residues from the interior of the COL2 domain and lack of the C-terminal cysteine residues were not detrimental to its capacity to form a triple helix. In the case of type I collagen it has been suggested that the C-terminal globular domain, not the coiled-coil region, is crucial in the initiation of intramolecular assembly and heterotrimer selectivity (33). Studies with some other proteins suggest that heptad repeat-containing amino acid sequences may contain distinct coiled-coil trigger sites that are necessary to mediate coiled-coil formation (34–36). Hence, in addition to a coiled-coil domain, other sequences might be involved and important in trimerization acting either independently or together with the coiled-coil region.

Interestingly, most collagenous transmembrane proteins, namely collagen types XIII, XVII, XXIII, and XXV, and ectodysplasin-A1, harbor a potential furin cleavage site in their N-terminal NC1 domain, next to the transmembrane domain (Fig. 5) (1). On the other hand, the subfamily of class A macrophage scavenger receptor-like molecules, MSRs, MARCO, and SRCL, are devoid of any furin cleavage site. Furthermore, our analysis of the novel collagenous transmembrane protein subfamily consisting of the colmedins showed them to harbor a potential furin cleavage site in their N-terminal noncollagenous domain between the transmembrane domain and the (first) collagenous domain. This coincides well with the suggestion that unc-122 has a transmembrane and a secreted form (8). As a consequence of their potential shedding, it can be envisaged that the collagenous transmembrane proteins possess multiple functions both at the plasma membrane and in the extracellular matrix.

A closer look at the colmedin named *H. sapiens* collomin shows it to have features in common with the other collagenous transmembrane proteins, in particular a coiled-coil region extending directly from the transmembrane domain. This potential oligomerization domain for collomin was shown to be homologous with the corresponding domain in the type XIII collagen molecule. Furthermore, the type XIII collagen-like molecules (collagen types XIII, XXIII, and XXV) and the colmedins are similar in that they all harbor two closely located cysteines just preceding the (first) collagenous domain (Fig. 5).

A universal molecular determinant for the trimeric formation of short coiled-coils has recently been identified (37). The sequence motif RhXXhE (h indicates hydrophobic residue and X indicates any amino acid residue) is functionally conserved in many diverse protein families harboring short, three-stranded coiled-coil oligomerization domains, i.e. it is found in 86% of all short, autonomous, parallel three-stranded coiled-coils, including some of the collagenous transmembrane proteins, namely the type XIII collagen-like proteins, type XVII collagen, and MARCO. In these proteins the trimerization motif is included in the conserved NC1 association domain (4, 5). In the case of type XIII collagen, this motif is in the “acceptable” form RVLRLK, whereas in collomin it is surprisingly found as ALRALE (Fig. 4B). According to Kammerer *et al.* (37), replacement of the arginine residues by alanine should lead to the formation of a tetrameric structure, but this is unlikely to be the case for collomin, because it contains collagenous sequences that can be expected to form a trimeric structure.

Altogether, a picture is emerging that suggests that there is a structurally varied group of collagenous transmembrane proteins whose biosynthesis is characterized by a coiled-coil motif following the transmembrane domain, and that these trimerization domains appear to be associated with each of the collagenous domains. In the case of type XIII collagen, the trimeric molecule has disulfide bonds at the junction of the NC1 and COL1 domains, and several of the other collagenous transmembrane proteins have a pair of cysteines in the same loca-
tion. Moreover, furin cleavage at the NC1 domain can be expected in most of the proteins.

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