Type XIII Collagen and Other Transmembrane Collagens Contain Two Separate Coiled-coil Motifs, Which May Function as Independent Oligomerization Domains*

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Type XIII collagen is a homotrimeric transmembrane collagen composed of a short intracellular domain, a single membrane-spanning region, and an extracellular ectodomain with three collagenous domains (COL1–3) separated by short non-collagenous domains (NC1–4). Several collagenous transmembrane proteins have been found to harbor a conserved sequence next to their membrane-spanning regions, and in the case of type XIII collagen this sequence has been demonstrated to be important for association. We show here that this 21-residue sequence is necessary but not sufficient for NC1 association. Furthermore, the NC1 association region was predicted to form an α-helical coiled-coil structure, which may already begin at the membrane-spanning region, as is also predicted for the related collagen types XXIII and XXV. Interestingly, a second coiled-coil structure is predicted to be located in the NC3 domain of type XIII collagen and in the corresponding domains of types XXIII and XXV. It is found experimentally that the absence of the NC1 coiled-coil domain leads to a lack of disulfide-bonded trimers and misfolding of the membrane-proximal collagenous domain COL1, whereas the COL2 and COL3 domains are correctly folded. We suggest that the NC1 coiled-coil domain is important for association of the N-terminal part of the type XIII collagen α chains, whereas the NC3 coiled-coil domain is implicated in the association of the C-terminal part of the molecule. All in all, we propose that two widely separated coiled-coil domains of type XIII and related collagens function as independent oligomerization domains participating in the folding of distinct areas of the molecule.

Type XIII collagen is a type II transmembrane protein that is expressed in many tissues throughout development and adult life (1). It is located in focal adhesions of cultured fibroblasts and other cells and in adhesive structures of tissues such as the myotendinous junctions in muscle, intercalated discs in heart, and the cell basement membrane interphases (1, 2). The type XIII collagen ectodomain can bind to fibronectin, heparin, the basement membrane components nidogen-2 and perlecan, and the α1-subunit of integrin (3–5). Due to its location at the tissue and cell level and its binding properties, it has been postulated that type XIII collagen is involved in cellular adhesion and migration.

Type XIII collagen α chains produced in a recombinant insect cell culture system have been shown to form homotrimers (6). The primary structure is composed of three collagenous domains (COL11 to COL3), which are flanked and interrupted by non-collagenous domains (NC1 to NC4). The short cystolic domain and the transmembrane domain encompass about half of the NC1 domain, whereas the rest of the molecule forms the ectodomain, which is a rod of about 150 nm with two flexible hinges coinciding with the NC2 and NC3 domains (5). The primary structures of COL1, NC2, COL3, and NC4 can vary, on account of complex alternative splicing (7–10). It is been shown recently that extracellular sequences adjacent to the transmembrane domain are important for the association of type XIII collagen into trimeric molecules (4). It appears that triple helix formation proceeds in the opposite orientation than for the fibrillar collagens, i.e. from the N terminus to the C terminus. Because type XIII collagen does not contain a signal sequence, its translocation to the endoplasmic reticulum has been thought to be mediated by the transmembrane domain sequences, residues 37–59 in the mouse and 39–61 in man (11), as is known to occur with other type II transmembrane proteins (12).

By using homologous gene targeting, we have previously generated a mouse line, Col13a1N/N, expressing modified type XIII collagen that lacks the extreme 96 N-terminal residues, including the cystotic, transmembrane, and association domains, which are replaced by unique sequences not found in any other protein (13). Analysis of tissues and cultured cells derived from homozygous Col13a1N/N mice shows that the altered type XIII collagen molecules are transported to roughly the correct location despite the lack of a transmembrane domain. Expression of the N-terminally altered type XIII collagen molecules results in changes in muscle integrity in the genetically targeted mice, including abnormalities in the sarcolemma–basement membrane interphase. Immunoelectron microscopy has indicated that the mutant molecules are situated in the adjacent extracellular space, whereas wild-type type XIII collagen molecules are adherent to the plasma membrane. Moreover, cells extracted from the mutant mice showed decreased

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The abbreviations used are: COL, collagenous domain; NC, non-collagenous domain; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; 4PH, prolyl 4-hydroxylase; MSRs, macrophage scavenger receptors; MES, 4-morpholineethanesulfonic acid; SRCL, scavenger receptor with C-type lectin.
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adhesiveness to the basement membrane component type IV collagen.

Studies with the Col13a1ΔN mouse have revealed a role for the N terminus of type XIII collagen in anchoring muscle cells to the basement membrane. Nevertheless, the mutant molecules were secreted, and they may retain intact some of their functional properties. This prompted us to study the molecular properties of the N terminally altered type XIII collagen. The amount of type XIII collagen in tissues is very low, and thus insect cell expression was used to obtain sufficient protein for these studies. A series of N-terminal variants was tested for their ability to form stable disulfide-bonded type XIII collagen molecules. The data led us to search for other regions in addition to the NC1 domain that may be important for chain association and stability in type XIII collagen and other collagenous transmembrane and non-transmembrane proteins.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Generation of Recombinant Baculoviruses—Human type XIII collagen variant del1–38 and del1–83 viruses have been described previously (6). E-26 (14), a CDNA clone covering the coding sequence for type XIII collagen except for the beginning of the translation, was used as a template for generating human variant del1–61. Complementary oligonucleotides (nucleotides 660–692 in human type XIII collagen cDNA bearing a translation stop codon in position 678–680, under GenBank™ data base accession number AJ293824 (4)) were used as primers in a mutagenesis reaction performed using a site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene). The CDNA was transported into the insect cell expression vector pVL1392 (Invitrogen) by EcoRI digestion and ligation. Sequences coding for the altered N termini of mouse type XIII collagen were obtained by reverse transcription followed by amplification with PCR. Total RNA was isolated from the skeletal muscle of a mouse expressing wild-type N-terminally altered type XIII collagen and transcribed into single-stranded DNA using a type XIII collagen-specific reverse oligonucleotide primer complementary to nucleotides 1025–1039 (under GenBank™ data base accession number NM_007731 (11)) as described previously (13). The long altered N-terminal sequences were amplified using a sense oligonucleotide primer corresponding to nucleotides in the loxP sequence (5’-CAGGGGATCCAAATGCTATACGAAGTT-3’) as a template for generating a KpnI restriction enzyme recognition sequence was included in the 5’-end of both sense primers, and the antisense primer used in both amplifications was complementary to nucleotides 984–1003 in mouse type XIII collagen cDNA (under GenBank™ data base accession number NM_007731 (11)). The mouse type XIII collagen cDNA msOIII (689), lacking exons 15, 31, and 36 (4), was digested with KpnI and EcoRI digestion and ligation, and the short sequences using a primer corresponding to nucleotides 5818–5840 in the first intron (under GenBank™ data base accession number AF063666 (17)). KpnI and EcoRI restriction enzyme recognition sequences were included in the 5’-end of both sense primers, and the antisense primer used in both amplifications was complementary to nucleotides 984–1003 in mouse type XIII collagen cDNA (under GenBank™ data base accession number NM_007731 (11)). The mouse type XIII collagen cDNA msOIII (689), lacking exons 15, 31, and 36 (4), was digested with KpnI and EcoRI digestion and ligation, and the short sequences using a primer corresponding to nucleotides 5818–5840 in the first intron (under GenBank™ data base accession number AF063666 (17)). KpnI and EcoRI restriction enzyme recognition sequences were included in the 5’-end of both sense primers, and the antisense primer used in both amplifications was complementary to nucleotides 984–1003 in mouse type XIII collagen cDNA (under GenBank™ data base accession number NM_007731 (11)). The mouse type XIII collagen cDNA msOIII (689), lacking exons 15, 31, and 36 (4), was digested with KpnI and EcoRI digestion and ligation, and the short sequences using a primer corresponding to nucleotides 5818–5840 in the first intron (under GenBank™ data base accession number AF063666 (17)). KpnI and EcoRI restriction enzyme recognition sequences were included in the 5’-end of both sense primers, and the antisense primer used in both amplifications was complementary to nucleotides 984–1003 in mouse type XIII collagen cDNA (under GenBank™ data base accession number NM_007731 (11)).

Recombinant baculoviruses were generated by transfecting the construct DNAs together with modified Autographa californica nucleopolyhedrovirus DNA into Spodoptera frugiperda Sf9 insect cells using the BaculoGold transfection kit (Pharmingen). The recombinant viruses were plaque-purified and amplified as described previously (18).

Analysis of Recombinant Proteins Produced in Insect Cells by SDS-PAGE and Immunoblotting—High Five insect cells were cultured as monolayers in TNM-FH (Sigma) insect cell medium supplemented with 10% fetal bovine serum (Bioclear) and, when infected, in serum-free monolayers in TNM-FH (Sigma) insect cell medium supplemented with 0.1 M 4PH (Roche Applied Science) to prevent proteolytic cleavage. The cells were washed with PBS and collected as described previously. They were then homogenized in 67 mM Tris-HCl, pH 7.5, 267 mM NaCl, 0.2% Triton X-100 supplemented with Complete Protease Inhibitor Mixture (Roche Applied Science) for 30 min on ice. The cell lysate was centrifuged at 8000 × g for 10 min at 4 °C, the supernatant was recovered, and the precipitate was dissolved in 1% SDS. Samples of the different fractions were analyzed by denaturing SDS-PAGE under reducing or non-reducing conditions. The samples were subjected to Western blot analysis with anti-human type XIII polyclonal antibody XIII/NC3-1 (11), detected with enhanced chemiluminescence. The percentage of secreted protein was estimated using the Quantity One Quantitation software (Bio-Rad).

Pepsin Digestion of Recombinant Proteins—Cell lysate supernatants not supplemented with any protease inhibitor were digested with 0.1–0.15 mg/ml pepsin (Roche Applied Science) for 2–5 min at room temperature. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-human type XIII collagen antibodies XIII/NC3-55 (6), XIII/NC3-1 (11), and XIII/NC4-SO (6) and anti-mouse type XIII collagen antibody XIII/NC1-Q610 (2).

Recombinant Protein Purification, N-terminal Sequencing, and Pepsin Digestions—High Five cells in suspension were cultured in Express Five medium in a Cerratom BS 4 shaker (B. Braun Biotech) with 130 °C non-constant agitation at 27 °C. The High Five cells were co-infected at a density of 1 × 10^6/ml with the virus encoding mouse XIII^N-short at m.o.i. 5 and with the virus 4PH at m.o.i. 1. Once they were infected, ascorbate phosphate (80 μg/ml) was added to the culture medium daily. 500 ml of cell culture medium was separated from the cells 48 h post-infection by centrifuging at 3400 × g for 10 min at room temperature, and 2 ml EDTA was added, after which the medium was further centrifuged at 40,000 × g for 45 min at 4 °C to remove the debris and viruses. The medium was then applied to a Resource S 6-ml column (Amersham Biosciences) and eluted using a gradient program on AKTA Explorer 10 (Amersham Biosciences). The fractions were analyzed by Western blotting using the antibody XIII/NC3-1, and those containing XIII^N-long protein were concentrated to 1 ml and further digested with 0.15 mg/ml pepsin (Roche Applied Science) for 2 min at room temperature. The digestion products were separated by SDS-PAGE and electroblotted onto a ProBlott™ membrane (Applied Biosystems), which was stained with Serva Blue R (Serva). The authenticity of the XIII^N-short protein was confirmed by N-terminal protein sequencing using a 492 Procise™ protein sequencer (Applied Biosystems).

For the sequencing of XIII^N-long proteins in the cell lysate, infected insect cells from a 100-ml suspension culture were harvested after 48 h of infection by centrifuging at 3400 × g for 10 min at room temperature. The digestion products were separated by SDS-PAGE and electroblotted onto a ProBlott™ membrane (Applied Biosystems) and sequenced using the ABI sequencer 492 Procise™ protein sequencer (Applied Biosystems).

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RESULTS

Secretion of Type XIII Collagen Molecules Expressed in Insect Cells—The type XIII collagen molecules synthesized by the Col13a1PNS mouse lacked the cytosolic, transmembrane, and association domains (the 96 extreme N-terminal residues encoded by exon 1) but retained the large collagenous ectodomain. Surprisingly, these molecules were located in association with adherence structures apparently secreted into the pericellular matrix, and they were correctly located in focal adhesions in cultured cells derived from these mice (13). To test the effect of the altered N terminus on association and folding of the mutant α1(XIII) chains, we prepared recombinant DNA expression constructs encoding identically altered mouse α1(XIII) chains (Fig. 1), the deleted N-terminal sequences in the Col13a1PNS mouse being replaced by either 65 or 11 residues of sequences unique to the mutant α1(XIII) chains depending on which of the two potential new translation initiation sites was used. Consequently, two constructs were prepared, namely XIII\(^{N\text{-long}}\) and XIII\(^{N\text{-short}}\), corresponding to full-length α1(XIII) chains from residue 97 onwards and preceded by the longer or shorter mutant N termini (Fig. 1A). The del1–38 variant lacks the cytosolic domain but corresponds to full-length human α1(XIII) chains in terms of chain association and folding, and this was used as a control on account of its superior expression levels relative to full-length human and mouse α1(XIII) chains (Fig. 1B (6)). In addition, constructs del1–61 and del1–83 were prepared, encoding human α1(XIII) chains lacking either the first 61 or 83 residues, respectively (Fig. 1B). In each case insect cells were infected with viruses encoding α1(XIII) and prolyl 4-hydroxylase (4PHαβ), the latter being necessary in order to obtain hydroxylated recombinant collagen chains when using insect cells as hosts (22).

Proteins were extracted from infected insect cells by homogenizing the cells in a buffer containing Triton X-100, and the remaining precipitates were solubilized in 1% SDS. The SDS fractions contained only minimal amounts of protein, these were excluded from the final pictures, which correspond to the same cell number, and the samples were fractionated on denaturing SDS-PAGE gels under reducing or non-reducing conditions and analyzed by Western blotting.

Because the SDS fractions contained only minimal amounts of protein, these were excluded from the final pictures, which contain only the Triton X-100 cellular fractions and medium samples for each variant (Fig. 2). It has been shown previously that about half of the del1–38 α1(XIII) chains are secreted into the medium through proteolytic cleavage by one or more furin-type proteases when cultured in medium supplemented with serum (4). A furin consensus sequence can be found at amino acid residues 105–108 in human type XIII collagen (under GenBank\(^{TM}\) data base accession number CAC00688 (4)) and at

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**Fig. 1.** Type XIII collagen cDNA variants used for recombinant protein production. A mutant mouse type XIII collagen polypeptide (α1(XIII)\(^{M}\)) is presented schematically in A, with the collagenous domains (COL1–COL3) indicated as white boxes, the non-collagenous domains (NC1–NC4) as black boxes, and the transmembrane domain as a light gray box. The altered N terminus is shown as a dark gray box, and the authentic mouse type XIII N terminus is illustrated separately. The N-terminal sequences of the mouse variants derive from a cDNA sequence in which there are two possible translation start codons (\(\text{M}_1\)). A full-length human type XIII collagen polypeptide is shown in B. The numbers of amino acid residues in the various domains and cysteine residues (C) are indicated, as is the protease-recognizing site (\(\text{R}_1\)). The mouse and human deletion variants used in this work are shown as full-length polypeptides.

**Fig. 2.** Western blotting analysis of expressed recombinant proteins in cell and medium fractions under reducing or non-reducing conditions. Cells were infected with viruses containing Triton X-100. Proteins analyzed by 10% SDS-PAGE under reducing conditions are shown in A and by 5% SDS-PAGE under non-reducing conditions are shown in B. The samples for both panels were derived from cells infected with viruses coding for del1–38 (lanes 1 and 2), XIII\(^{N\text{-long}}\) (lanes 3 and 4), XIII\(^{N\text{-long}}\) (lanes 5 and 6), del1–61 (lanes 7 and 8), and del1–83 (lanes 9 and 10) and extracted with a buffer containing Triton X-100 (lanes 1, 3, 5, 7, and 9), or else the medium proportioned to the cell number (lanes 2, 4, 6, 8, and 10). The antibody used was XIII/NC3-1. Trimmers (T), dimers (D), and monomers (M) are shown by arrows.

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residues 103–106 in the mouse protein (under GenBank™
data base accession number NP_031757 (11)). Here we used
serum-free medium, and only about 10% of the del1–38 protein
was secreted (Fig. 2B, lanes 1 and 2), possibly because of a
decrease in protease activators in the medium. Similar or
slightly higher portions of the protein, namely about 50% for
XIII
N
short, 15% for XIII
N
long, 40% for del1–61, and 15% for
del1–83, were found to be secreted (Fig. 2B, lanes 3–10).

Mutant XIII
N
short and XIII
N
long α1(XIII) Chains Are
Processed at a Furin Site—Western blotting of XIII
N
long cell
fractions revealed two bands with molecular masses of 90 and 80
kDa (Fig. 2A, lane 5), whereas the predicted molecular mass for
XIII
N
long is 64 kDa. This difference is known to reflect the high
imino acid content of collagens in addition to post-translational
modification of the polypeptides (23). Purification and se-
quencing of the cell fraction proteins indicated that the 90-kDa
chain had the N-terminal MLYEVIRSLLE predicted for intact
XIII
N
long (13), whereas the 80-kDa band corresponded to
polypeptides with the N-terminal 147GQPGEKGAPG located at
amino acid residue 147 in the mouse protein, and thus the
latter represented a degradation product of the full-length
XIII
N
long α chain. Sequence analysis of the two bands observed
in the medium indicated that the upper band with the N-
terminal MLYEVIRSLLE represented full-length XIII
N
long and the lower band had an N terminus of 107EAPKMSPGCN
(residue 107 of the mouse α1(XIII) chains). Thus the lower band
lacked the 75 extreme N-terminal residues (65 residues of
mutant sequences and residues 97–106 of the mouse α1(XIII)
chains) preceding the predicted furin cleavage site, indicating
that the XIII
N
long chains were cleaved at the predicted furin
site in the manner described previously for the human del1–38 α chains (4). The observed molecular mass for secreted
XIII
N
short was 80 kDa (Fig. 2A, lane 4) and the calculated mass
for full-length was 58 kDa. Only one major band was observed
in the medium of cells synthesizing the XIII
N
short α chains, and
identification of its N-terminal sequence, 107EAPKMSPGCN,
indicated that it lacked the 21 extreme N-terminal residues (11
chains) preceding the predicted furin cleavage site, indicating
that the XIII
N
short chains were cleaved at the predicted furin
site in the manner described previously for the human del1–38 α chains (4), and some part of the full-length XIII
N
long α chains were secreted without proteolytic processing.

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, as depicted in Fig. 1B. We have shown previously that
interchain disulfide bonds are found in the NC1 domain of
human type XIII collagen and possibly in the COL1 and NC2
domains, whereas the cysteine residues occurring in the NC4
domain form intrachain bonds (6). Full-length mouse α1(XIII)
chains contain nine cysteine residues, differing from the hu-
man equivalents in possessing an extra pair of cysteines in the
transmembrane domain and lacking a cysteine residue in the
NC2 region (Fig. 1A). Deletion variant del1–38 contains all
eight cysteine residues, and it has been shown that this variant
forms disulfide-bonded homotrimers in a similar manner to
full-length human α chains (4). Thus we compared the ability of
the other recombinant α1(XIII) chains to form disulfide-bonded
trimers with del1–38. Western blot analysis of non-reduced
samples of the del1–38 variant revealed disulfide-bonded tri-
mers both in the cell supernatant and in the medium, and it is
notable that monomers are not present in the medium (Fig. 2B,
lanes 1 and 2). The slightly faster mobility of the trimer protein
in the medium is due to the furin cleavage, as shown previously
(4). Full-length mouse α1(XIII) chains form disulfide-bonded
homotrimers in the same manner (data not shown). Due to the
sequence alteration in the N terminus, XIII
N
short and XIII
N
long
α1(XIII) chains lack the NC1 association domain and four of
the extreme N-terminal cysteine residues, whereas instead
XIII
N
long contains two new cysteine residues in its unique
sequences (Fig. 1A). The XIII
N
long samples also contain trace
amounts of trimers in the cell extracts, but the ratio of trimers
to monomers is extremely low, so that only monomers could be
visualized in the medium (Fig. 2B, lanes 5 and 6). In the case of
the XIII
N
short α1(XIII) chains no disulfide-bonded trimers
could be detected in the cells or medium (Fig. 2B, lanes 3 and 4).
Thus it can be concluded that the N-terminally mutant
α1(XIII) chains are deficient in terms of their capacity to form
disulfide-bonded trimers. It should be noted that in some of the
samples a band between the trimers and the monomers was
observed representing dimers of α1(XIII) chains (Fig. 2B).

The Transmembrane Domain Is Implicated in Efficient Chain
Association—To test whether inclusion of the previously
described association domain (4) would restore the capacity of
the α1(XIII) chains to form disulfide-bonded trimers, the
del1–61 variant lacking only the cytosolic and transmembrane
domains was tested. Only minute amounts of trimers were
detected in the cell extract; however, the trimer/monomer ratio
was very low compared with del1–38, lacking only the cytosolic
domain (Fig. 2B, lanes 7 and 1, respectively), and only mono-
mers were detected in the medium (Fig. 2B, lane 8). Further-
more, the del1–83 α chains, which practically correspond to the
XIII
N
long and XIII
N
short α chains in terms of their type XIII
collagen sequences but lack the 11 or 65 residues of mutant
N-terminal sequences, did not form disulfide-bonded trimers
(Fig. 2B, lanes 9 and 10). Del1–83 contains the pairs of cysteine
residues near the end of the NC1, at the end of the COL1, and
at the beginning of the NC2 domain, but at least in this variant,
these cysteines appear not to be utilized for interchain bonds.
All in all, the data suggest that the previously identified trans-
membrane-proximal chain association region of about 20 resi-
dues is not sufficient to ensure proper association and disulfide
bonding of the α1(XIII) chains, as the transmembrane domain
is also required.

The COL1 Domains of the N-terminally Truncated α1(XIII)
Chain Variants Are Pepsin-sensitive—Although the N-termin-
ally truncated α chains are lacking the sequence shown to be
important for α1(XIII) chain association, these modified α
chains may also associate and trimerize through other se-
quences. Thus we considered it possible that they could form
triple-helical trimers that lack interchain disulfide bonds and
thus were not detected in the non-reduced Western blot (Fig.
2B). To study this possibility, High Five insect cells were co-
fected with the viruses del1–61, del1–83, XIII
N
short, or
XIII
N
long together with 4Phoβ, and the ability of the modified
chains to form stable triple-helical domains was assessed en-
zymatically. The results were compared with High Five cells
infected with the virus del1–38 together with 4Phoβ, because
insect cell expression of del1–38 α chains has been shown
previously (6) to result in the formation of homotrimers with
three triple-helical collagenous domains.

The Triton X-100-soluble proteins in the cell supernatants
were digested with pepsin, which is unable to digest triple-
helical collagen sequences, and thus any pepsin-resistant frag-
ments are likely to be derived from triple-helical molecules.
Samples of the pepsin-digested cell supernatants were ana-
lyzed by SDS-PAGE under reducing conditions, followed by
Western blotting with various antibodies. Digestion of the
del1–38 sample resulted in four pepsin-resistant fragments
subjected to Western blotting with antibodies XIII/NC3-1 (A) 12% SDS-PAGE under reducing conditions, and analyzed by Western blotting (homogenized, and centrifuged. Aliquots of the cell supernatants were digested with pepsin for 2–5 min at room temperature, electrophoresed by 12% SDS-PAGE under reducing conditions, and analyzed by Western blotting (lanes 1–3), XIII/NC2-55 (lanes 4–6), XIII/NC3-1 (lanes 7–9), and XIII/NC4-SO (lanes 10–12). Lanes 1, 4, 7, and 10 refer to del1–38 samples; lanes 2, 5, 8, and 11 to del1–61 samples; and lanes 3, 6, 9, and 12 to del1–83 samples, all digested with pepsin. B provides a schematic presentation of the pepsin-resistant fragments of the variants del1–38, del1–61, and del1–83 and the antibodies used, and the location of the cysteine residues (C). C, the pepisin-digested cell supernatant samples XIII/NC1-Q610 (lanes 2 and 5) and XIII/NC2-55 (lanes 3 and 6) together with del1–38 (lanes 1 and 4) were subjected to Western blotting with antibodies XIII/NC3-1 (lanes 1–3) and XIII/NC4-SO (lanes 4–6). The XIII/NC1-Q610 protein partly purified from the conditional medium (lane 7) was digested with pepsin and analyzed on 12% SDS-PAGE gel under reduced conditions followed by transfer to nitrocellulose filter and Coomassie Blue staining. A schematic presentation of the pepsin-resistant fragments of the mouse N-terminally altered variants XIII/NC1-Q610 and XIII/NC2-55 is given in D, in which the cysteine residues (C) and antibodies used are also indicated.

when detected using the antibody XIII/NC1-Q610, which reacts with NC1 domain sequences (Fig. 3A, lane 1). Their observed molecular masses were 19, 22, 26, and 29 kDa. On the other hand, faint 19- and 22-kDa bands could be detected during digestion of the del1–61 and del1–83 samples (Fig. 3A, lanes 2 and 3), but once the digestion was complete, no bands representing the COL1 domain could be detected.

Western blotting of the pepsin-digested del1–38 sample with the NC2-specific antibody XIII/NC2-55 detected four pepsin-resistant fragments of sizes 26, 29, 30, and 32 kDa (Fig. 3A, lane 4). The bands recognized by both XIII/NC1-Q610 and XIII/NC2-55 antibodies are considered to represent pepsin-resistant fragments originating from the COL1 domain flanked by non-collagenous sequences, whereas the 30- and 32-kDa fragments represent the COL2 domain, as they can also be detected with an antibody XIII/NC3-1 raised against the NC3 domain of type XIII collagen (Fig. 3A, lane 7). When the pepsin-digested del1–61 and del1–83 samples were Western-blotted with antibody XIII/NC2-55, three fragments of size 30, 31, and 33 kDa, representing the COL2 domain (see below), could be observed (Fig. 3A, lanes 5 and 6). No bands representing the COL1 domain could be detected with this antibody (see below). Thus both antibodies against the NC1 and NC2 domains demonstrate that the COL1 domains of the del1–61 and del1–83 chains are pepsin-sensitive.

Western blotting of the pepsin-digested del1–61 and del1–83 α chains with the antibody XIII/NC3-1 detected pepsin-resistant fragments of 31 and 33 kDa (Fig. 3A, lanes 8 and 9), which were also detectable with antibody XIII/NC2-55 (Fig. 3A, lanes 5 and 6). In contrast to the COL1 domain, the COL2 domain fragments of the del1–61 and del1–83 chains can be detected at equal intensity to the COL2 domain fragments of the del1–38 chains. It should be noted that the mobility of the COL2 fragments recognized by the antibodies against the NC2 and NC3 domains is slower in the del1–61 and del1–83 samples than in del1–38. It is conceivable that the del1–61 and del1–83 chains are subject to a higher extent of post-translational modifications, due to delays in chain association and triple helix formation, and that this causes the slight difference in their electrophoretic mobility compared with del1–38.

Pepsin digestion of the del1–38, del1–61, and del1–83 α chains and subsequent Western blotting with antibody XIII/NC4-SO, produced against a synthetic peptide corresponding to the C-terminal end of the COL3 domain of human type XIII collagen and the whole NC4 domain, revealed one 36-kDa pepsin-resistant fragment of equal intensity in each case (Fig. 3A, lanes 10–12). This fragment thus represents the COL3 and NC4 domains. The pepsin digestion results with regard to the human variants are summarized in Fig. 3B.

The COL1 Domains of the N-terminally Altered Type XIII Collagen α Chain Variants Are Pepsin-sensitive—The del1–38 variant was used as a control for the mouse N-terminally altered XIII/NC1-Q610 and XIII/NC2-55 α1(XIII) chains as well. Pepsin digestions were performed, and Western blottings were prepared as described for the human variants above. The antibodies XIII/NC1-Q610 and XIII/NC2-55 were not used with mouse type XIII collagen, because the mouse XIII/NC1-Q610 and XIII/NC2-55 variants lacked most of the XIII/NC1-Q610 epitope, and the mouse NC2 sequences differed substantially from the human corresponding sequences. Western blotting with the XIII/
NC3-1 antibody revealed 30- and 32-kDa bands in the pepsin-digested sample of del1–38 and a 32-kDa band in pepsindigested samples of XII\textsuperscript{N-short} and XII\textsubscript{N-long} (Fig. 3C, lanes 1–3). Western blotting with the antibody XIII/NC4-50 identified a single band in all samples, of size 36 kDa in del1–38 (Fig. 3C, lane 4) and 38 kDa in the N-terminally altered samples (Fig. 3C, lanes 5 and 6).

To study the properties of all of the collagens, insect cells in suspension culture were infected with the variant XIII\textsuperscript{N-short} virus, and the corresponding protein was partly purified from the conditional medium, pepsin-digested, and analyzed by SDS-PAGE under reducing conditions, followed by transfer onto a ProBlott\textsuperscript{TM} membrane and Coomassie Blue staining. This revealed three pepsin-resistant fragments of sizes 30, 32, and 38 kDa (Fig. 3C, lane 7). The bands were cut from the membrane and subjected to N-terminal sequencing. The 32-kDa band was found to represent the fragment containing the COL2 domain and a portion of the NC2 domain, as the 32-kDa band from the membrane and subjected to N-terminal sequencing. The 32-kDa band was found to represent the fragment containing the COL2 domain and a portion of the NC2 domain, as the 32-kDa band from the membrane and subjected to N-terminal sequencing.

Moreover, the N-terminally altered protein molecules found in the insect cell culture media in the case of XII\textsubscript{N-long} and XII\textsuperscript{N-short} raised the question of additional association and folding elements to those present in the NC1 of type XIII collagen. Interestingly, the COILS program predicted that type XIII collagen would have a second coiled-coil sequence toward its C-terminal part, namely at residues 437–465 (Fig. 4, 29 residues in length). Similarly, strong coiled-coil predictions were given for type XXIII collagen, at residues 391–410 (Fig. 4, 20 residues), and type XXV collagen, at residues 422–450 (Fig. 4, 29 residues). In all of these collagens the second coiled-coil domain encompasses the NC3 domain.

Sequence comparisons indicated that a 21-residue stretch (residues 60–80) of the 40-residue NC1 coiled-coil domain of type XIII collagen is 48 and 38% identical and 52 and 57% homologous to the corresponding sequences in collagen types XXIII and XXV, respectively (Fig. 5A). In the case of the NC3 coiled-coil domain, 24 residues (residues 437–460) out of the 29-residue type XIII collagen sequence are 21 and 54% identical and 38 and 71% homologous to the corresponding sequences in collagen types XXIII and XXV, respectively (Fig. 5B).

**Coiled-coil Regions of Type XIII Collagen**—While extending the search for potential internal coiled-coil domains to encompass non-transmembrane collagens as well, we found the recently described type XXVI collagen (32) to have two potential coiled-coil domains (Fig. 6A), an N-terminal one in the NC1 domain, encompassing residues 129–159 (31 residues), and a C-terminal one in the NC3 domain, covering residues 345–388 (41 residues). Comparison of the NC1 and NC3 coiled-coil sequences of type XIII collagen with the whole sequence of type XXVI collagen revealed sequence homology between the respective NC1 coiled-coil domains (Fig. 6B) but not between the NC3 domains. More specifically, a 21-residue portion of the 40-residue type XIII collagen NC1 coiled-coil domain is 48% identical and 57% homologous to the corresponding sequence in type XXVI collagen (Fig. 6B).

**DISCUSSION**

Authentic type XIII collagen molecules lack an N-terminal signal sequence, and instead the transmembrane domain is presumed to be important for their endoplasmic reticulum translocation and subsequent transmembrane anchorage. Thus it is surprising that the mouse N-terminal mutant \(\alpha(XIII)
\) chains lacking the transmembrane and adjacent chain association domain were found to be correctly deposited in adherent structures in tissues and in focal adhesions in cultured cells (13). The data presented here, obtained using recombinant protein expression in insect cells, indicate that the N-terminally altered molecules are translocated into the endoplasmic reticulum and secreted out of the cell in a manner comparable with \(\alpha(XIII)
\) chains equipped with the transmembrane domain. This is evident from the similar or slightly higher amounts of type XIII collagen found in the insect cell culture media in the case of XII\textsubscript{N-long} and XII\textsuperscript{N-short} \(\alpha\) chains by comparison with membrane-anchored \(\alpha(XIII)
\) chains. Moreover, the N-terminally altered protein molecules found in
**Fig. 4. α-Helical coiled-coil prediction for collagenous transmembrane proteins.** The human protein sequences indicated were analyzed for putative coiled-coil domains using the COILS program (version 2.1 (21)). The scoring matrix MTIDK, derived from myosins, paramyosins, tropomyosins, intermediate filaments of types I–V, desmosomal protein, and kinesins, was used, with 2.5-fold weighting of residues in heptad positions a and d. Sliding window sizes of 14, 21, and 28 residues were used. The abscissa shows the scale in amino acids (aa), and the y axis indicates coiled-coil probability. Schematic structures for the corresponding molecules are shown below the graphs. The non-collagenous domains are shown as black boxes, the transmembrane domains as transparent gray boxes, the coiled-coil domains as striped red boxes, the collagenous domains as white boxes, and a tumor necrosis factor motif as a green box.
the media were proteolytically processed at a furin cleavage site. Membrane-anchored type XIII collagen molecules have been found previously (4) to be cleaved at the same site, and this cleavage can be inhibited by a furin inhibitor. Furin pro-proteases are predominantly situated in the trans-Golgi network, but they are also located in the plasma membrane (33). Thus lack of the 96 extreme N-terminal residues of authentic type XIII collagen does not impair proteolytic processing, and this cleavage is indicative of utilization of the same secretory pathway as in authentic molecules.

Human N-terminally truncated a1(XIII) chains lacking the first 83 residues have been shown to occur in part in the cytosolic compartment, whereas about half of the protein is translocated to the lumen of the endoplasmic reticulum and secreted into the medium (6). In the case of the XIII\textsuperscript{N-long} protein, we observed two bands in the cell fraction, one representing full-length a1(XIII)\textsuperscript{N-long} chains and the other lacking 115 amino acids of the altered sequence at the N terminus, the start of localization in mouse protein being at amino acid 147. We consider it likely that this truncated protein is a degradation product of the full-length a1(XIII)\textsuperscript{N-long} chains. Moreover, analysis of the a1(XIII)\textsuperscript{N-long} chains in the cell culture medium indicated that the more truncated ones were not secreted. Thus it appears that the sequences following residue 147 do not include information on translocation to the lumen of endoplasmic reticulum, but such information may be included in the sequence between residues 96 and 147 in the mouse type XIII collagen sequence.

We have shown previously (6) that interchain disulfide bonds are formed by cysteines located in the NC1 domain or by the cysteines at the junction of the COL1 and NC2 domains, whereas the extreme C-terminal pair of cysteine residues in type XIII collagen molecules form intrachain disulfide bonds. The human deletion variants del1–61 and del1–83 and the mouse variants XIII\textsuperscript{N-short} and XIII\textsuperscript{N-long} lack the cysteine residues of the transmembrane domain but retain the pair of cysteines at the NC1/COL1 junction. Interestingly, none of the N-terminally mutant a1(XIII) chains was able to associate efficiently into disulfide-bonded trimers. This is the case even with the del1–61 a1(XIII) chains, which contain the entire NC1 association domain, conserved with other membrane-spanning collagenous proteins.

We have recently shown by means of pepsin and trypsin/chymotrypsin digestions that the del1–38 \alpha chains form correctly aligned disulfide-bonded trimers with all three collagenous domains in triple-helical conformation, and that interchain disulfide bonds were associated with the COL1 domain (6). We considered it possible that the \alpha chains translocated to the lumen of the endoplasmic reticulum and lacking the N-terminal residues could form triple-helical molecules even though they lacked interchain disulfide bonds. Interestingly, the use of pepsin resistance to map triple-helical domains indicated that despite lack of the transmembrane domain and the NC1 association domain, it is possible for the modified \alpha chains to form trimeric molecules, where the COL2 and COL3 domains are in a pepsin-resistant triple-helical conformation. Nevertheless, the COL1 domains of trimeric type XIII collagen molecules composed of modified \alpha chains are sensitive to pepsin. The results imply that the transmembrane and association domain sequences are needed for correct folding of the COL1 domain. These sequences are not included in the proteolytically processed ectodomain, which nevertheless has a stable triple-helical COL1 domain (5). Thus it appears that the triple-helical conformation remains stable once it has been formed.

Coiled-coil domains are thought to have an important role as oligomerization domains. We show here that it can be predicted that the NC1 association domain will form a coiled-coil structure. Furthermore, the results imply that chains lacking the NC1 coiled-coil region can associate through sequences residing in other parts of the molecule, but the ensuing trimers are incorrectly folded with respect to the COL1 domain. Studies with the various N-terminal variants indicated that type XIII collagen seems to have another domain responsible for association and folding of the molecule, from COL2 to NC4. This is presumably the NC3 domain, in view of the potential coiled-coil structure encompassing this domain and the conservation of this structure among type XIII collagen-like molecules. The importance of the NC1 and NC3 domains is further highlighted by the fact that neither the NC1 nor the NC3 domain of type XIII collagen is affected by alternative splicing, whereas the rest of the domains except for the COL2 domain can be alternatively spliced (7–10, 14, 34).

Mice expressing the N-terminally altered type XIII collagen (Col13a1\textsuperscript{p60N}) are viable and fertile but still cannot be considered completely normal. Expression of altered molecules affects mainly skeletal muscle integrity and can be detected as an abnormal plasma membrane-basement membrane interphase (13). Our data obtained with recombinantly produced, identically altered a1(XIII) chains indicate that the modified type XIII collagen molecules are correctly folded with respect to the C-terminal two-thirds of their structure. Consequently these mutant molecules may retain some aspects of the function of normal type XIII collagen. The findings recorded with the Col13a1\textsuperscript{p60N} mouse line and the properties of the mutant molecules also imply that the cytosolic and transmembrane domains of type XIII collagen are of functional significance but not necessarily for all aspects of type XIII collagen function. Moreover, this N-terminally altered type XIII collagen causes a considerably milder phenotype than with another a1(XIII) mutation in which a 90-residue in-frame deletion of COL2 domain...
leads to fetal lethality (35). In this case the mutant molecules remain attached to the plasma membrane, folding of the affected domain is likely to be impaired, and the mutant molecules may produce incorrect signals in the cell.

The family of collagenous transmembrane proteins currently has 9 members, i.e. collagen types XIII, XVII, XXIII, and XXV, the macrophage scavenger receptors (MSRs), a macrophage receptor with collagenous structures (MARCO), a scavenger receptor with C-type lectin (SRCL), the complement component C1q, and ectodysplasin-A (15, 29, 30, 36). Of these, human type XVII collagen, bovine MSRs, mouse MARCO, and human SRCL have been shown previously (15, 16, 24, 25) to possess coiled-coil structures, the coiled-coil region in type XVII collagen and MARCO extending directly from the transmembrane region, whereas that in the MSRs and SRCL is separated from the transmembrane domain by a spacer domain of 32 and 56 amino acid residues, respectively. Furthermore, the coiled-coil region is much shorter in type XVII collagen and MARCO than in MSRs and SRCL, 39 and 38 residues in the NC1 of human type XIII collagen (2) as white boxes. The signal sequence (SS) of type XXVI collagen is marked by a bar. Alignment of the amino acid residues in the NC1 of human type XIII collagen (1st row) with the corresponding residues in mouse type XXVI collagen (2nd row) is shown in B. Alignment was achieved using a BLOSUM matrix. The amino acid sequences are shown in one-letter codes. Gaps (--) were introduced for maximal alignment of the polypeptides. Identical amino acids are indicated by black boxes and similar ones by gray boxes.

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