A short sequence in the N-terminal region is required for the trimerization of type XIII collagen and is conserved in other collagenous transmembrane proteins

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The recombinant transmembrane protein type XIII collagen is shown to reside on the plasma membrane of insect cells in a 'type II' orientation. Expressions of deletion constructs showed that sequences important for the association of three α 1(XIII) chains reside in their N- rather than C-terminal portion. In particular, a deletion of residues 63–83 immediately adjacent to the transmembrane domain abolished the formation of disulfide-bonded trimers. The results imply that nucleation of the type XIII collagen triple helix occurs at the N-terminal region and that triple helix formation proceeds from the N- to the C-terminus, in opposite orientation to that of the fibrillar collagens. Interestingly, a sequence homologous to the deleted residues was found at the same plasma membrane-adjacent location in other collagenous transmembrane proteins, suggesting that it may be a conserved association domain. The type XIII collagen was secreted into insect cell medium in low amounts, but this secretion was markedly enhanced when the cytosolic portion was lacking. The cleavage occurred in the non-collagenous NC1 domain after four arginines and was inhibited by a furin protease inhibitor.

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Introduction

Two of the 19 collagens described in vertebrates are classified as transmembrane proteins, namely collagen types XIII and XVII (Pihlajaniemi and Rehn, 1995). These are not structurally homologous, except that both have been predicted to have a transmembrane domain near their N-terminus. The superfamily of collagens also includes transmembrane proteins that have short collagen-like domains, namely the type I and II macrophage scavenger receptors, C1q, a collagen-like subcomponent of the first component of complement C1, and a macrophage receptor with a collagenous structure (MARCO) (Elomaa *et al.*, 1995; Pihlajaniemi and Rehn, 1995). These membrane-associated collagenous proteins have no structural function, but they participate in host defence. The newest member of the group of transmembrane proteins with

collagen-like domains is the ectodysplasin-A (EDA) family of proteins (Srivastava *et al.*, 1997).

The type XIII collagen molecule is predicted to be composed of three collagenous domains (COL1–3) and four non-collagenous domains (NC1–4), two of them separating the collagenous domains and two located at the N- and C-terminal ends of the polypeptide (Pihlajaniemi *et al.*, 1987; Pihlajaniemi and Tamminen, 1990; Tikka *et al.*, 1991; Pihlajaniemi and Rehn, 1995; Hägg *et al.*, 1998; Kvist *et al.*, 1999). Type XIII collagen produced in insect cells forms homotrimers, and the three collagenous domains fold into a stable triple-helical conformation (Snellman *et al.*, 2000). The purified protein has been shown to interact with the integrin-type collagen receptor α 1 β 1 (Nykqvist *et al.*, 2000). The N-terminal NC1 domain of type XIII collagen contains a highly hydrophobic transmembrane domain, which appears to anchor type XIII collagen molecules to the plasma membrane of the cells (Hägg *et al.*, 1998). Interestingly, alternative splicing is predicted to affect the structures of the COL1, NC2 and COL3 domains of the human and mouse type XIII collagen chains (Pihlajaniemi and Tamminen, 1990; Tikka *et al.*, 1991; Juvonen and Pihlajaniemi, 1992; Juvonen *et al.*, 1992, 1993; Peltonen *et al.*, 1997). *In situ* hybridization data suggest that type XIII collagen mRNAs have a wide tissue distribution (Sandberg *et al.*, 1989; Juvonen *et al.*, 1992, 1993).

The N-terminal transmembrane domain raises the question of the mode of chain association and direction of triple-helix formation. In the case of fibril-forming procollagens, assembly is initiated by three α -chains associating via their C-propeptides, and subsequently the triple-helical domain nucleates at the C-terminal end and propagates in a C- to N-terminal direction (Bächinger *et al.*, 1980, 1981; Bruckner *et al.*, 1981; Doege and Fessler, 1986). McLaughlin and Bulleid (1998) have proposed that procollagen chain assembly occurs through a two-stage recognition event, i.e. association of the chains, driven by residues within the C-propeptide, followed by nucleation and alignment of the helix, driven mainly by sequences present at the C-terminal end of the triple-helical domain (Bulleid *et al.*, 1997; Lees *et al.*, 1997). Since membrane-associated collagenous polypeptides such as type XIII collagen polypeptides remain inserted in the rough endoplasmic reticulum membrane through their N-terminal anchorage throughout their biosynthetic phases, the folding of the triple helix may conceivably proceed from the N- to the C-terminus, and could be preceded by association of the α -chains through their N-terminal parts. In order to study this hypothesis, we expressed different type XIII deletion constructs in insect cells using the baculovirus expression system (Gruenwald and Heitz, 1993). This system was chosen since studies of type XIII collagen at the protein level have proved difficult

because of its low level of expression and the lack of suitable antibodies. First, immunofluorescence staining and electron microscopy of the insect cells expressing type XIII collagen were used to demonstrate that type XIII collagen molecules are anchored into the plasma membrane in a 'type II' orientation. We then showed that association of the disulfide-bonded trimers of type XIII collagen requires the N-terminal parts of this polypeptide, while the C-terminal parts are not necessary. Sequences of importance for $\alpha 1(\text{XIII})$ chain association were found to be conserved in several collagenous transmembrane proteins. Furthermore, type XIII collagen was found to be cleaved into the culture medium by a furin-like protease.

Results

Orientation of recombinant human type XIII collagen on the plasma membrane of insect cells

The N-terminal transmembrane domain is suggestive of a type II topography for type XIII collagen molecules,

Fig. 1. cDNAs encoding the human type XIII collagen α -chain variants. A schematic structure of the $\alpha 1(\text{XIII})$ collagen chain is shown at the top. The non-collagenous domains (NC1–4) are shown as dark grey boxes, the transmembrane domain (residues 39–61) as a black box and the collagenous domains (COL1–3) as light grey boxes. The locations of cysteine residues are indicated (C). The proteolytic cleavage site for furin (R_4) is indicated by an arrow. The deletion constructs used in the insect cell expressions are shown at the bottom. The abilities of the α -chain variants to form disulfide-bonded trimers are indicated as + or –.

in which the N-terminal portion is intracellular and the C-terminal portion extracellular. We have previously (Snellman *et al.*, 2000) generated recombinant baculoviruses that direct the synthesis of full-length (virus wthumanXIII) and N-terminally truncated type XIII collagen chains lacking the first 83 residues (del1–83) (Figure 1). Here we use the insect cell expression system to demonstrate the orientation of type XIII collagen molecules on the plasma membrane of the cells. High Five cells were infected with the virus wthumanXIII or the virus del1–83 together with the virus 4PH $\alpha\beta$ encoding the α - and β -subunits of human prolyl 4-hydroxylase. The enzyme is necessary in order to achieve sufficient 4-hydroxylation of prolines in the synthesized collagen chains (Lamberg *et al.*, 1996). As a control, cells were also infected with the virus 4PH $\alpha\beta$ alone. The cells were examined 48 h post-infection by western blotting, which demonstrated expression of type XIII collagen variants of the expected sizes (data not shown), and by immunofluorescence staining studies of non-permeabilized cells using the antibody XIII/NC3-1 (Hägg *et al.*, 1998), which recognizes the NC3 domain of human type XIII collagen. Cells expressing full-length type XIII collagen revealed a clear punctuate staining of the cell membrane (Figure 2A). This staining pattern indicates that type XIII collagen is oriented in the cell membrane so that its N-terminus is intracellular and its C-terminus extracellular (type II orientation). On the other hand, when the cells were producing N-terminally truncated type XIII collagen (del1–83), no cell surface staining was observed (Figure 2B). This implies that the del1–83 α -chain lacks the transmembrane domain that could anchor it to the plasma membrane. Cells infected only with the virus coding for both subunits of human prolyl 4-hydroxylase (4PH $\alpha\beta$) gave no surface staining (data not shown).

A similar punctuate staining pattern was also obtained in insect cells expressing full-length mouse type XIII collagen with a C-terminal His tag [moXIII(689)HIS],

Fig. 2. Immunofluorescence staining study of the orientation of type XIII collagen in the plasma membrane of insect cells. High Five cells were infected either with the wthumanXIII virus (A) or the del1–83 virus (B) together with a virus coding for both prolyl 4-hydroxylase subunits, virus 4PH $\alpha\beta$, and analysed 48 h post-infection. Non-permeabilized cells were immunostained with a rabbit polyclonal antibody against the NC3 domain of human type XIII collagen (antibody XIII/NC3-1). TRITC-conjugated swine antibody to rabbit IgG was used as a secondary antibody.

Fig. 3. Immunoelectron microscopy staining of insect cells expressing type XIII collagen. High Five cells were infected with the viruses moXIII(689)HIS and 4HP $\alpha\beta$ (A and B, magnification 32 000 $\times$ and 25 800 $\times$, respectively) or with virus 4HP $\alpha\beta$ alone (C, magnification 21 500 $\times$) and stained 48 h post-infection with antibody XIII/NC3-1 against the NC3 domain of the human type XIII collagen. The primary antibody was detected with a 10 nm protein A-gold conjugate. (D) An enlargement of a section of (B). Scale bars: 1 μ m.

which were fixed when using a monoclonal anti-histidine tag antibody to stain the non-permeabilized cells (data not shown).

Type XIII collagen was further characterized in terms of orientation and location by immunoelectron microscopy. Strong intracellular staining of insect cells expressing the moXIII(689)HIS collagen using the XIII/NC3-1 antibody was observed. A clear accumulation of gold particles was seen on the luminal side of the intracellular vesicle membranes, indicating that full-length type XIII collagen chains were inserted into the vesicle membrane with the C-terminus facing the lumen (Figure 3A, arrowheads). The prominent vesicle formation is a result of the virus infection and not the type XIII expression because the same phenomenon was also seen in control virus-infected cells but not in uninfected High Five cells (data not shown). These observed ultrastructural changes resemble cell necrosis, which has been reported to occur in Sf9 cells (Al-Rubeai and Singh, 1998). In addition to the vesicle membranes, the gold particles followed membranous

endoplasmic reticulum structures (Figure 3B and D, arrowheads). There is also staining on the extracellular side of the plasma membrane, which corresponds well with the immunofluorescence results (Figure 3B, arrows). This confirms the type II orientation of type XIII collagen.

N-terminal sequences are needed for formation of disulfide-bonded trimers

The full-length and four deletion constructs were used to study the mechanism by which the α 1(XIII) chains associate into trimeric molecules (Figure 1). The deletion constructs included del1–83, and another previously described recombinant baculovirus, namely the del1–38 variant that directs the synthesis of N-terminally deleted chains lacking the intracellular portion (Snellman *et al.*, 2000). Furthermore, two new recombinant baculoviruses were produced: del63–83, encoding α 1(XIII) chains with an in-frame internal deletion encompassing a stretch of 21 amino acids after the transmembrane domain, and del442–668, directing synthesis of chains lacking the

Fig. 4. Reducing SDS-PAGE analysis of the type XIII collagen α -chain variants. Sf9 cells were infected with viruses coding for various type XIII collagen α -chain variants together with a virus coding for both prolyl 4-hydroxylase subunits, harvested 48 h post-infection, homogenized and centrifuged. The cell pellets were resuspended in 1% SDS. Aliquots of the cell pellets were electrophoresed by 8% SDS-PAGE under reducing conditions and analysed by western blotting with antibody XIII/NC3-1. Lane 1, the type XIII collagen virus used for infection was wthumanXIII; lane 2, del1-83; lane 3, del1-38; lane 4, del63-83, lane 5, del442-668. Two lower molecular mass forms of the del63-83 monomer were also seen in the western blot experiment. The middle-sized form recognized by antibody XIII/NC3-1 is unspecific, since it is also present with the same intensity in cells expressing only prolyl 4-hydroxylase (data not shown). The unspecific band is seen only in lane 4 because more protein was loaded into this lane than the others.

C-terminal one-third of the molecule (Figure 1). Thus, five viruses coding for different type XIII collagen α -chain variants, namely wthumanXIII, del1-83, del1-38, del63-83 and del442-668, were used separately to infect Sf9 cells, together with the virus 4PH $\alpha\beta$. The cells were collected 48 h post-infection, homogenized and centrifuged. The cell supernatants were then removed, the cell pellets resuspended in 1% SDS, and aliquots of the supernatants and pellets analysed by SDS-PAGE under reducing or non-reducing conditions, followed by western blotting with antibody XIII/NC3-1. The calculated molecular masses of the wthumanXIII, del1-83, del1-38, del63-83 and del442-668 α -chains are 65, 56.5, 61.4, 62.5 and 43.6 kDa, respectively, while the observed ones were 89, 79, 83, 86 and 57 kDa, respectively, thus showing the expected differences (Figure 4, lanes 1-5). These size differences between the calculated and observed molecular masses are probably due to the fact that collagens have anomalous electrophoretic behaviour relative to globular proteins, presumably due to their high imino acid content (Furthmayr and Timpl, 1971).

The abilities of the different type XIII collagen α -chain variants to form disulfide-bonded trimers were studied by analysing cell supernatants under non-reducing conditions. Wild-type type XIII collagen α -chains were found to associate into disulfide-linked trimeric molecules, while the N-terminally truncated type XIII collagen del1-83 α -chains failed to do so [Figure 5, lanes 1 and 2; trimers (T) indicated by an arrow]. As the del1-83 α -chains are devoid of most of the N-terminal NC1 domain, including the transmembrane domain, the role of the N-terminal sequences in the formation of disulfide-bonded trimers of

Fig. 5. Non-reducing SDS-PAGE analysis of trimer formation by the type XIII collagen α -chain variants. The viruses encoding various type XIII collagen α -chain variants together with a virus coding for both prolyl 4-hydroxylase subunits were used to infect Sf9 cells, which were harvested 48 h post-infection, homogenized and centrifuged. Aliquots of the cell supernatants were electrophoresed by 5% SDS-PAGE under non-reducing conditions and analysed by western blotting with antibody XIII/NC3-1. Lane 1, the type XIII collagen virus used for infection was wthumanXIII; lane 2, del1-83; lane 3, del1-38; lane 4, del63-83, lane 5, del442-668. Trimers (T) of type XIII collagen α -chain variant molecules in lanes 1-4 are indicated by an arrow. Monomers (M) of the corresponding molecules are also indicated. A trimer (T) of the del442-668 molecule in lane 5 is shown by an arrowhead.

type XIII collagen was studied further. To test whether the intracellular sequences were necessary for trimer formation, the N-terminally truncated del1-38 α -chains lacking the cytosolic part of the NC1 domain were expressed in insect cells. The del1-38 α -chains were observed to form disulfide-bonded trimeric molecules effectively, however [Figure 5, lane 3; trimers (T) indicated by an arrow], so that the cytosolic part of type XIII collagen appears not to be obligatory for trimer association and the formation of stabilizing interchain disulfide bonds. Interestingly, del63-83 α -chains lacking 21 amino acid residues in the ectodomain adjacent to the transmembrane domain failed to associate into disulfide-bonded trimers (Figure 5, lane 4). On the other hand, del442-668 α -chains lacking the COL3 and NC4 domains were able to do so effectively [Figure 5, lane 5; trimers (T) are indicated by an arrowhead]. The additional fragments in lanes 1, 3 and 5 in Figure 5 are thought to represent dimers of corresponding α -chains, and the band in between the trimer and the dimer in lane 1 is likely to represent a degradation product of the trimer.

The plasma membrane-adjacent sequence necessary for type XIII collagen chain association is conserved in several collagenous transmembrane proteins

Since the 21-residue sequence appeared to be important for $\alpha 1(\text{XIII})$ chain association, we investigated whether any of the other collagenous transmembrane proteins had a similar sequence. This led to the identification of a homologous region in several of the other molecules (Figure 6). More specifically, 13 residues of the 21-residue type XIII collagen sequence are 70, 46 and 61% identical and 70, 54 and 69% homologous to a corresponding

XIII 62 HFRTAE LQARVLRLEAERGEQQ
 XVII 498 AEEVRK LKARVDELERIRRSIL
 MARCO 70 LQARLRVLEMYFLNDT
 EDA-A1 61 YLELR SELRRERGAES
 consensus LqarV eLererg dt

Fig. 6. Multiple alignment of collagenous proteins containing transmembrane domains and a derived consensus sequence. First row, human type XIII collagen; second row, human type XVII collagen; third row, human MARCO (a macrophage receptor with a collagenous structure); and fourth row, human EDA-A1 (ectodysplasin-A1). The amino acid sequence is shown in one-letter codes. Alignment was achieved using a Blosom62 comparison matrix. The identical amino acids are indicated by black boxes and similar ones by grey boxes.

sequence in type XVII collagen, MARCO and EDA, respectively (Li *et al.*, 1993; Bayes *et al.*, 1998; Elomaa *et al.*, 1998). In all of these proteins the conserved sequence is located immediately adjacent to the transmembrane domains of the respective proteins (Figure 6). Otherwise their N-terminal non-collagenous domains did not reveal significant homologies. Furthermore, the conserved sequence was not clearly detectable in the macrophage scavenger receptors or the A, B and C chains of C1q.

Secretion of full-length and N- and C-terminally truncated type XIII collagen molecules expressed in insect cells

To find out whether type XIII collagen molecules are secreted and proteolytically processed, viruses directing synthesis of full-length chains, chains lacking the intracellular portion (del1–38) and C-terminally truncated chains (del442–668) were used together with the virus 4PH $\alpha\beta$ to infect High Five cells (Figure 1). The cells were grown in serum-free medium for 48 h, after which they were collected along with the culture media, homogenized in phosphate-buffered saline (PBS) with protease inhibitors and centrifuged. The supernatant and the pellet were separated, and the pellet was extracted with 0.3 M NaCl, 0.2% Triton and 0.07 M Tris buffer pH 7.4 with protease inhibitors for 30 min at 4°C. The extracted pellet was then centrifuged and the Triton-extracted supernatant was collected. Aliquots of the cell supernatant, the Triton-extracted supernatant and the culture medium were analysed by SDS-PAGE under reducing conditions, followed by western blotting with the antibody XIII/NC3-1. In the case of full-length type XIII collagen, a major band was observed in the Triton-extracted supernatant fraction (Figure 7, lane 2), while only very faint bands were seen in the cell supernatant and medium fractions (Figure 7, lanes 1 and 3, the bands are visible only in the original autoradiographs). The N-terminally truncated type XIII collagen was observed in both the PBS-soluble and Triton-extracted cell fractions, and also in significant amounts in the medium (Figure 7, lanes 4–6). The C-terminally truncated type XIII collagen was most strongly observed in the Triton-extracted supernatant fraction, and bands could also be seen in the PBS-soluble cell fraction (Figure 7, lanes 7 and 8).

Interestingly, there was a difference in the observed molecular mass of the del1–38 protein when comparing the cell fractions with the medium one, as the medium fraction showed two slightly lower molecular mass bands

Fig. 7. SDS-PAGE analysis of recombinant type XIII collagen expressed in insect cells. The viruses with human XIII (lanes 1–3), del1–38 (lanes 4–6) and del442–668 (lanes 7–9) together with a virus coding for both prolyl 4-hydroxylase subunits were used to infect High Five cells. The infected cells were harvested 48 h post-infection, homogenized in PBS with protease inhibitors, centrifuged and the cell supernatant was collected. The remaining cell pellet was extracted with Triton, centrifuged, and the resulting Triton-extracted cell supernatant was collected. The culture medium was also collected. Aliquots of the PBS-extracted cell supernatant (lanes 1, 4 and 7), the Triton-extracted cell pellet (lanes 2, 5 and 8) and the culture medium (lanes 3, 5 and 9) were electrophoresed by 10% SDS-PAGE under reducing conditions and analysed by western blotting with antibody XIII/NC3-1. The bands below the full-length polypeptides of each variant in lanes 4, 5, 7 and 8 are not visible in cells infected only with the prolyl 4-hydroxylase virus (not shown) and thus they represent degradation products.

(Figure 7, lane 6). About 50% of the del1–38 α -chains were observed to be secreted, and the full-length type XIII collagen and C-terminally truncated type XIII collagen were also secreted from the insect cells, although in lesser amounts, i.e. <10% of the total. The secreted proteins in these two cases likewise had a slightly lower molecular mass than the respective cellular fractions (data not shown).

Determination of the N-terminal sequences of the secreted and membrane-bound recombinant type XIII collagen forms

The secreted form of the N-terminally truncated type XIII collagen (del1–38) was thought to be derived from the membrane-bound form through cleavage of the ectodomain. To study this further, the N-termini of the secreted and membrane-bound del1–38 α -chains were sequenced in order to identify the possible cleavage site. High Five cells were infected for 48 h, after which the culture medium and the cells were collected, partially purified, fractionated by SDS-PAGE under reducing conditions, and blotted onto a membrane. Bands corresponding to the membrane-bound and secreted del1–38 α -chains were cut out and their N-terminal sequences were determined.

The sequencing showed that the membrane-bound del1–38 α -chains maintained their correct N-terminus starting with the amino acid sequence MLPSPGSCGL, where the LPSPGSCGL sequence represents the start of the predicted transmembrane domain sequence of type XIII collagen at residue 39. The corresponding cDNA construct was created by deleting the cDNA sequence coding for the first 38 amino acids of human type XIII collagen and creating a new ATG translation start codon just before the cDNA sequence coding for the transmembrane domain. N-terminal sequencing of the two medium bands yielded the same sequence, i.e. EAPKTSPGCN, which corres-

ponds to the human type XIII collagen sequence (Hägg *et al.*, 1998) beginning at residue 109. These results suggest that the two secreted forms of the del1–38 α -chains were formed after proteolytic cleavage of the membrane-bound del1–38 protein, which removed part of the N-terminal NC1 domain from them, including the transmembrane domain. Since western blotting with antibody XIII/NC4-SO, which detects the C-terminal end of the type XIII collagen molecules, resulted in visualization of both secreted forms (data not shown), these may result from differences in glycosylation, for example, rather than differences in proteolytic processing.

Effect of decanoyl-RVKR-chloromethyl ketone on secretion of type XIII collagen

The cleavage site of the secreted type XIII collagen is preceded by the amino acid sequence RRRR, which implies that one or more furin-like proteinases are responsible for the cleavage. A family of seven mammalian proteinases has been identified recently, comprising the proprotein convertases PC1/PC3, PC2, furin/PACE, PC4, PACE4, PC5/PC6 and PC7/SPC7/LPC/PC8 (Nakayama, 1997; Seidah and Chretien, 1997). The selected cleavage sites of these proteinases occur in one or more exposed segments of the precursor, which generally contains the motif (R/K)-X_n-(R/K)↓, where $n = 0, 2, 4$ or 6 (amino acid single letter code in which X can be any amino acid except cysteine). In order to study whether a furin-like proteinase could be responsible for generating secreted type XIII collagen forms in insect cells, a furin-specific inhibitor, decanoyl-RVKR-chloromethyl ketone (Garten *et al.*, 1994), was tested. High Five cells were co-infected with the viruses del1–38 and 4PH $\alpha\beta$, and 100 μ M decanoyl-RVKR-chloromethyl ketone or a corresponding volume of methanol was added to the culture medium 24 h post-infection or else the infected cells were left untreated. The cells and culture media were then collected 48 h post-infection, the cells were homogenized and centrifuged, and the cell supernatants and media were analysed by SDS-PAGE under reducing conditions followed by western blotting with antibody XIII/NC3-1. Decanoyl-RVKR-chloromethyl ketone did not have any cytotoxic effects on the infected insect cells, nor was the expression of the membrane-bound del1–38 α -chains altered (Figure 8, compare lanes 1 and 2). The decanoyl-RVKR-chloromethyl ketone treatment did markedly reduce the formation of the secreted form of del1–38 α -chains, however (Figure 8, lane 4), whereas the methanol-treated (Figure 8, lane 3) and untreated infected cells (data not shown) generated the secreted form of del1–38 α -chains.

Discussion

Type XIII collagen molecules were shown to reside on the plasma membrane of insect cells in a 'type II' orientation, with the N-terminus intracellular and the C-terminus extracellular. The data reported here suggest that the N-terminal portion of the α 1(XIII) chains contains sequences that are important for chain recognition and association. C-terminally truncated type XIII collagen α -chains lacking the last 227 residues were able to form disulfide-bonded trimers as effectively as the wild-type

Fig. 8. Analysis of the effect of decanoyl-RVKR-chloromethyl ketone on secretion of type XIII collagen. The virus del1–38 together with a virus coding for both prolyl 4-hydroxylase subunits was used to infect High Five cells. Methanol (lanes 1 and 3) or decanoyl-RVKR-chloromethyl ketone (lanes 2 and 4) was added to the cells 24 h post-infection, and they were harvested 48 h post-infection, homogenized and centrifuged. The culture medium was also collected. Aliquots of the cell supernatant (lanes 1 and 2) and the culture medium (lanes 3 and 4) were electrophoresed by 10% SDS-PAGE under reducing conditions and analysed by western blotting with antibody XIII/NC3-1. The bands below the full-length polypeptides in lanes 1 and 2 are not visible in cells infected only with the prolyl 4-hydroxylase virus (not shown) and thus represent degradation products.

full-length type XIII collagen α -chains. On the other hand, deletion of most of the N-terminal NC1 domain of type XIII collagen, namely the extreme 83 residues, abolished the formation of disulfide-bonded trimers. Further deletion studies indicated that the first 38 residues preceding the transmembrane domain in the NC1 domain were not necessary for trimer formation, since the proportion of disulfide-bonded trimers was the same as with full-length α -chains. Furthermore, an internal in-frame deletion of 21 amino acids, residues 63–83, immediately adjacent to the type XIII collagen transmembrane domain, again abolished the formation of disulfide-bonded trimers. The lack of disulfide-bonded trimers could therefore suggest that this 21-residue ectodomain sequence is necessary for correct association of the three α 1(XIII) chains. Another possibility is that deletion of the 21-residue sequence influences proper folding of adjacent sequences that might form an interacting domain. It should be noted that alternative splicing does not affect any of the NC1 domain sequences (Pihlajaniemi and Tamminen, 1990; Tikka *et al.*, 1991; Juvonen and Pihlajaniemi, 1992; Juvonen *et al.*, 1992, 1993; Peltonen *et al.*, 1997).

There are four cysteine residues in the type XIII collagen NC1 domain, namely two in the transmembrane portion and two at the junction of NC1 and COL1 (Hägg *et al.*, 1998). We have previously shown that some of these NC1 domain cysteine residues participate in the formation of interchain disulfide bonds (Snellman *et al.*, 2000). Furthermore, additional cysteine residues occurring in the COL1 and NC2 domains may also participate in interchain disulfide bond formation while the cysteine residues occurring in the NC4 domain form intrachain bonds (Snellman *et al.*, 2000). Since we have tested only for the ability to form disulfide-linked trimers we cannot exclude the possibility that the del1–83 and del63–83 α -chains could form triple-helical trimers, which, however, are lacking interchain disulfide bonds and thus were not detected in the non-reduced western blot.

Nevertheless, del1–83 is only lacking the cysteines included in the transmembrane domain and the sequence deleted in del63–83 α -chains does not contain cysteines. Thus, at least the short deletion is not implicated in directly abolishing interchain disulfide bonds.

Interestingly, homologous sequences encompassing 13 residues of the 21-residue sequence were found adjacent to the transmembrane domains of type XVII collagen, MARCO and EDA, suggesting that this conserved ectodomain sequence may serve as a self-association sequence in several of the collagenous transmembrane proteins. Collagen chain association has been studied most extensively for fibrillar collagens, where chain association occurs through their highly homologous non-collagenous C-propeptides (McLaughlin and Bulleid, 1998). Recent work on the fibrillar collagens has focused on mechanisms involved in selective chain association and has resulted in delineation of a short discontinuous recognition sequence which is involved in selective association of the highly homologous pro α -chains of types I, III and V procollagens (Lees *et al.*, 1997). It should be noted that with the exception of the short conserved putative association sequence, the N-terminal non-collagenous domains of the collagenous transmembrane proteins are not homologous, and thus ample possibilities should exist for these chains to discriminate between each other, ensuring that each collagenous molecule has the correct α -chains in cells synthesizing more than one of the collagenous transmembrane proteins.

Taken together, the present findings show that sequences that are important for chain recognition and the association of type XIII collagen α -chains reside in the N-terminal portion of these α -chains rather than in the C-terminal part as is the case with the fibril-forming collagens (Doege and Fessler, 1986; Lees *et al.*, 1997). The results also imply that nucleation of the type XIII collagen triple helix occurs in the N-terminal region and that triple helix formation proceeds from the N- to the C-terminus, in the opposite orientation to that known to occur for the fibrillar collagens (Bächinger *et al.*, 1980, 1981; Bruckner *et al.*, 1981). Furthermore, it can be hypothesized that the assembly of other collagenous proteins with transmembrane domains, all having a type II orientation, occurs in the same fashion, i.e. association of the N-terminal parts of the three α -chains (as is suggested in this paper for type XIII collagen) followed by formation of the triple helix in an N- to C-terminus direction. In this context, the finding of a conserved sequence in several of the collagenous transmembrane proteins points to common features in their chain association.

We found that the recombinant type XIII collagen synthesized in insect cells could be released into the medium by ectodomain cleavage. The N-terminus of the secreted type XIII collagen began with amino acid residue 109 of the full-length type XIII collagen (Tikka *et al.*, 1991) and appeared to be cleaved by a furin-type protease. Previous studies have demonstrated that insect cells contain a furin-like protease (Kuroda *et al.*, 1986; Wells and Compans, 1990; Park *et al.*, 1995) and a *Spodoptera frugiperda* analogue of the proprotein convertase furin has been identified (Cieplik *et al.*, 1998). Furthermore, this insect furin was shown to have an identical substrate specificity and inhibitor profile to the mammalian furin,

and thus its activity could also be inhibited with the furin-specific inhibitor decanoyl-RVGR-chloromethyl ketone (Cieplik *et al.*, 1998). Previous work with cultured human keratinocytes endogenously expressing type XIII collagen has indicated that some of the molecules are released into the culture medium (Peltonen *et al.*, 1999). The medium form has almost identical mobility to the type XIII collagen obtained from the keratinocytes, suggesting that cleavage occurs close to the plasma membrane. Thus, the proteolytic processing observed for type XIII collagen expressed in insect cells may mimic the processing observed in cells naturally synthesizing this collagen (Peltonen *et al.*, 1999). There are at least two potential mammalian proprotein convertases, namely furin/PACE and PC7/SPC7/LPC/PC8, that could cleave type XIII collagen, for the following reasons: they are expressed in a broad range of tissues and cell lines, they prefer to cleave precursors at the general (R/K)-X-(R)-X-(R/K)-R↓ motif, where at least two of the residues in parentheses are present, and they are both membrane proteins mainly localized in the *trans*-Golgi network (TGN) but are also found at the cell surface (Seidah and Chretien, 1997).

Surprisingly, secretion of the del1–38 protein, i.e. type XIII collagen lacking the cytosolic domain, from the insect cells was found to be very much more effective than secretion of the full-length type XIII collagen, suggesting that the absence of the cytosolic domain from the type XIII collagen molecule somehow exposes a proteinase cleavage site. Thus, the cytosolic part of type XIII collagen appears to be important for its membrane attachment. This domain contains one putative protein kinase C phosphorylation site, at residues 5–8 (Kennelly and Krebs, 1991). Whether the phosphorylation state of this residue regulates the membrane attachment and secretion of type XIII collagen remains to be seen. We have postulated that type XIII collagen may have a role as a cell adhesion protein that connects cells to their surrounding extracellular matrix or as a receptor for soluble ligands (Hägg *et al.*, 1998). The purpose of generating a soluble isoform of type XIII collagen could be to desensitize the cells to the cognate ligand, thus leading to a rapid loss of cell adhesion or receptor stimulation.

Materials and methods

Construction of recombinant baculoviruses

PCR was used to generate the cDNA del63–83 by deleting nucleotides 197–259 (Tikka *et al.*, 1991) from the cDNA huXIII described previously (Snellman *et al.*, 2000). The cDNA del63–83 was digested with *NotI*–*EcoRI* restriction enzymes, ligated into pVL1392, and the resulting construct was termed pVLdel63–83. The construct pVLdel442–668 was produced by using PCR to delete all the nucleotides after nucleotide 1196 (Pihlajaniemi and Tamminen, 1990) from the cDNA huXIII, followed by digestion with *NotI*–*EcoRI* restriction enzymes and ligation into pVL1392. Both pVL constructs were separately co-transfected with modified *Autographa californica* nuclear polyhedrosis virus DNA into *S.frugiperda* Sf9 insect cells using the BaculoGold transfection Kit (PharMingen). The recombinant viruses were collected, amplified, plaque purified and amplified again (Gruenewald and Heitz, 1993). The viruses were named del63–83 and del442–668. These and the previously generated viruses with humanXIII, del1–38 and del1–83 (Snellman *et al.*, 2000) are illustrated in Figure 1.

The cDNA moXIII(689) consists of clone 689 encoding the α 1-chain of mouse type XIII collagen (Peltonen *et al.*, 1997) with 5' nucleotides 466–857 and 3' nucleotides 2350–2926 generated by PCR using mouse cDNA clones as templates and ligated to the *BbsI* and *SnaI* sites of

clone 689. Next, the cDNA moXIII(689) was used to generate the pBacmoXIII(689)HIS construct by cloning it into the *EcoRI*–*NotI* site of vector pFastBac1 (Gibco-BRL, Life Technologies) with an extra nucleotide sequence coding for six histidine residues just before the stop codon. The recombinant bacmid DNA was produced and transfected into *S.frugiperda* Sf9 insect cells according to the BAC-TO-BAC Baculovirus Expression System Instruction Manual (Gibco-BRL, Life Technologies). The ensuing recombinant virus moXIII(689)HIS was collected and amplified (Gruenwald and Heitz, 1993).

Analysis of recombinant proteins in insect cell cultures

Monolayer cultures of insect cells (Sf9 or High Five; Invitrogen) were grown at 28°C in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) or in a serum-free HyQ CCM3 medium (HyClone). Cells at a density of 6×10^5 /ml were infected with different XIII collagen viruses (Figure 1) either with or without a virus coding for both the α - and β -subunits of human prolyl 4-hydroxylase (4PH $\alpha\beta$; Nokelainen *et al.*, 1998). The type XIII coding viruses were used in 5- to 10-fold excess over the prolyl 4-hydroxylase encoding virus. Ascorbate, when present, was added to the culture medium at a dose of 80 μ g/ml daily. The cells and culture medium were collected 48–72 h after infection and the cells washed with PBS before being homogenized in either homogenization buffer I (0.5 M NaCl, 0.1% Triton, 1 mM EDTA- Na_2 , 1 mM EGTA, 1 mM *N*-ethylmaleimide, 0.01% phenylmethylsulfonyl fluoride and 0.05 mM Tris buffer pH 8.0), homogenization buffer II (0.3 M NaCl, 0.2% Triton, 1 mM EDTA- Na_2 , 1 mM EGTA, 1 mM *N*-ethylmaleimide, 0.01% phenylmethylsulfonyl fluoride and 0.07 M Tris buffer pH 7.4) or homogenization buffer III [PBS with Complete™ protease inhibitor (Boehringer Mannheim)] and centrifuged at 8000 *g* for 10 min. The supernatant and pellet were separated, and the pellet was suspended in 1% SDS or extracted with 0.3 M NaCl, 0.2% Triton and 0.07 M Tris buffer pH 7.4 with Complete™ protease inhibitor for 30 min at 4°C. The extracted pellet was again centrifuged at 8000 *g* for 10 min and the supernatant was collected. The supernatants, pellets and media were analysed by denaturing SDS–PAGE under reducing or non-reducing conditions, followed by western blotting with a polyclonal antibody against the whole NC3 domain of human type XIII collagen (antibody XIII/NC3-1; Hägg *et al.*, 1998) or the C-terminal end of the COL3 domain and the whole NC4 domain of human type XIII collagen (antibody XIII/NC4-SO; Snellman *et al.*, 2000).

Immunofluorescence staining of insect cells expressing type XIII collagen

High Five insect cells were plated on culture dishes containing poly-L-lysine-coated coverslips (4/10 cm plate) and infected with the virus with human XIII or with the virus del1–83 together with the virus 4PH $\alpha\beta$. Infection with the virus 4PH $\alpha\beta$ alone was also performed as a control. The infected cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum for 48 h, after which the coverslips were removed for immunostaining and the remaining cells on the culture dishes were used for the lysate preparations. The non-permeabilized cells were immunostained for type XIII collagen as follows. The coverslips were rinsed extensively with PBS followed by fixation with 3% paraformaldehyde for 5 min at room temperature; the cells were rinsed 3×5 min with PBS, followed by incubation with 0.1 M glycine–PBS and blocking with 0.2% bovine serum albumin–PBS. Subsequently, the cells were incubated for 1 h with antibody XIII/NC3-1 using a dilution of 1:200. The cells were extensively rinsed with PBS and incubated with a secondary antibody, tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit antibody (Dako), at a 1:100 dilution for 1 h. After several PBS rinses, the coverslips were mounted using Immumount medium (Shandon). The immunostaining was examined with a Leitz Aristoplan microscope and photographed on Kodak Ektachrome Elite II 400 ASA colour slide film.

Immunoelectron microscopy of insect cells expressing type XIII collagen

Insect cells infected with the virus moXIII(689)HIS together with 4PH $\alpha\beta$ or with the virus 4PH $\alpha\beta$ alone were cultured on plastic cell culture dishes as described above. Forty-eight hours after infection, they were washed with PBS and collected from the plates with vigorous washing using 8% paraformaldehyde–0.2 M HEPES buffer pH 7.4, centrifuged at 6000 *g* for 4 min into a pellet and allowed to fix for a total of 30 min at room temperature. After fixation, the pellet was freeze-protected in 2.3 M sucrose–PBS for 20–30 min, divided into 1 mm² specimens and subsequently frozen in liquid nitrogen. The cell specimens were cut into 60 nm sections using a Leiz Ultracut cryomicrotome and placed on

Formvar-coated nickel grids. The sections were blocked with 10% fetal calf serum (FCS)–20 mM glycine–PBS for 10 min on an ice bath and incubated for 45 min with antibody XIII/NC3-1 diluted 1:200 with 5% FCS–20 mM glycine–PBS at room temperature. After extensive washing with 20 mM glycine–PBS, sections were incubated for 20 min with 10 nm AuroProbe protein A–gold conjugate (Amersham), which was diluted appropriately with 5% FCS–20 mM glycine–PBS. The sections were washed extensively with 20 mM glycine–PBS and post-fixed in 2.5% glutaraldehyde–PBS pH 7.4 for 2 min. After washing with distilled water, the specimens were counterstained and dry-protected with 0.3% uranyl acetate–2% methylcellulose for 10 min on an ice bath. The sections were viewed and photographed with a Philips 410LS electron microscope using an acceleration voltage of 60 or 80 kV.

N-terminal sequence analysis of secreted and membrane-bound type XIII collagen

The virus del1–38 together with the virus 4PH $\alpha\beta$ were used to infect High Five cells for 48 h, after which the culture media were harvested and centrifuged at 40 000 *g* for 45 min to remove the viruses. The supernatant was then batch-adsorbed to a pre-equilibrated P11 cation exchange matrix (Whatman) at pH 7.0 for 2 h. After the washing and elution steps, the fraction containing type XIII collagen was concentrated with Ultrafree Protein Concentrator (Millipore). At the same time, the corresponding cells were homogenized in PBS containing Complete™ Proteinase Inhibitor and then centrifuged at 280 *g* for 10 min. The pellet was harvested and treated with 500 mM Na_2CO_3 on ice for 2 h, and centrifuged at 40 000 *g* for 1.5 h. The pellet was collected and extracted with PBS containing 0.1% Triton, 10% glycerol and 2 M urea. After centrifugation at 12 000 *g* for 20 min, the supernatant was concentrated by the same method as above, and the resulting samples underwent SDS–PAGE and then were electroblotted onto a ProBlott membrane (PE Biosystems). The bands visualized by Coomassie Blue staining, which matched the antibody detection with antibody XIII/NC3-1, were cut away and loaded directly onto a 477A Protein Sequencer (Applied Biosystems Inc.).

Effect of decanoyl-RVKR-chloromethyl ketone on secretion of type XIII collagen

High Five cells were infected with the virus del1–38 together with the virus 4PH $\alpha\beta$. The infected cells were cultured in HyQ CCM3 medium and 100 μ M decanoyl-RVKR-chloromethyl ketone (Garten *et al.*, 1994; Bachem, Basel, Switzerland) or a corresponding volume of methanol was added to the culture medium 24 h post-infection. Untreated control cells were also prepared. The infections were allowed to continue until 48 h post-infection, at which time the cells and culture media were collected, and the cells washed with PBS and homogenized in 0.3 M NaCl, 0.2% Triton and 0.07 M Tris buffer pH 7.4 with Complete™ protease inhibitor and centrifuged. The cell supernatants, pellets and media were analysed by SDS–PAGE under reducing conditions, followed by western blotting with antibody XIII/NC3-1.

Sequence analysis of several collagenous transmembrane proteins

Sequence alignment of type XIII and XVII collagens, MARCO, EDA, the macrophage scavenger receptor and the A, B and C chains of C1q was performed with GCG's PILEUP program using default values (Version 10.0, 1999). The alignment was compiled into a figure using BOXSHADE. Further analyses were carried out using ANTHEPROT v.4.9 (Geourjon and Delage, 1995).

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