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Noncollagenous NC16 Subdomains of Collagen XVII Important for Triple Helix Folding Direction

Areida S. K.[1], Müller P. K.[2]

[1]Zoology Department, Faculty of Science, Mansoura University, Mansoura, Egypt

[2]Medizinische Universität zu Lübeck, Institut für Medizinische Molekularbiologie, D-23538 Lübeck, Germany

Abstract: ***α-Helical coiled coils represent the most frequent protein oligomerization in nature and they are often found in vital proteins. In this study, using collagenXVII as, a dermal-epidermal cell surface protein in the skin, we found evidence for a newly function of coiled coils in the triple helix folding in collagenXVII. Analysis of the primary sequence of collagen XVII with the COILS program, revealed two regions within the extracellular NC16a, b domains which are predicted to form coiled coil structures. One predicted coiled coil region with six heptad repeats conserved between human, mouse and chicken starts within the transmembrane (NC16b) region and extends into the extracellular NC16a domain. The amino acid residues predicted to be located within the membrane are LLLTWLLLLGLLFGL. The second predicted coiled coil region with two heptad repeats conserved between human, mouse and chicken is located within the NC16a domain close to the first collagenous domain C15 (EEVRKLKARVDELERI and RKKLMMEQENGNLR, respectivelly). After α1(XVII) chains synthesis, and are localized in the membrane as single chains. The coiled coil structure adjacent to the transmembrane domain mediates the chain selection before folding of the triple helix can take place. The shorter predicted coiled coil region helps to align the three α1(XVII) chains in the correct stagger. The folding of triple helical structures starts from the N-terminal and proceeds to the C-terminal end. Our data indicate that the short coiled-coil domain of amino acids plays a central role in both structural and functional regulation of collagen XVII, in addition to triple helix folding directions.***

Keywords: coiled coils, CollagenXVII, protein oligomerization, transmembrane domain
Corresponding Author: areida2002@yahoo.com

1. Introduction

α-Helical coiled coils represent the most frequent protein oligomerization motifs in nature, and they are often found in vital proteins (Kammerer, 1997; Burkhard et al., 2001; Mason and Arndt, 2004; Grigoryan and Keating, 2008). Indeed, the prediction is that 3–5% of all protein residues form coiled coils (Wolf et al., 1997). These are generally characterized by heptad repeat sequences, -abcdefg-, within which the first and the fourth d positions are commonly occupied by hydrophobic amino acids (Grigoryan and Keating, 2008). Particularly, leucine is typically found at d position, where it plays a role as a "leucine zipper" for oligomerization (Landschulz et al., 1988).

In extracellular matrix proteins, which guide cell functions in most tissues, mostly three-stranded coiled-coil structures mediate oligomerization and thus provide functional advantages such as multivalency, enhanced binding strength, or combined functions of different domains for the protein (Engel, 2004). Coiled coils are found in most, if not all, members of the collagen superfamily (McAlinden et al., 2003). In fibrillar collagens, they are usually located in the C-terminal propeptides and function to initiate trimerization and therefore triple-helix folding. In contrast, in cell surface-associated transmembrane collagens, the N-terminal noncollagenous domains harbor the coiled-coil sequences, consistent with the fact that in these collagens the triple helix formation and ectodomain folding proceed from the N to C terminus (Snellman et al., 2007). Thus far, the major, if not sole, function of coiled coils in collagens has been believed to be trimerization prior to triple helix formation. In this study, using collagen XVII as an example, we provide evidence for a novel function of coiled coils in transmembrane collagens, *i.e.* in the control of ectodomain shedding.

Ectodomain shedding, the release of extracellular domains of transmembrane proteins is one form of proteolytic maturation of functional proteins. Proteolytic processing of proteins is a common and crucial event in biology, underlined by the fact that more than 2% of mammalian genes encode proteases (Overall and Blobel, 2007). Ectodomain shedding is mainly catalyzed by proteinases of the "a disintegrin and metalloprotease" family (Reiss and Saftig, 2009), and it is involved in a variety of essential functions mediated by TNF-α, amyloid precursor protein, Notch1, epidermal growth factor receptor ligands (Huovila et al., 2005), or transmembrane collagens (Franzke et al., 2005). For example, Collagen XVII is an epithelial cell surface receptor in the skin. Its vital role in dermal-epidermal adhesion and cell migration is indirectly demonstrated by the fact that its dysfunction in genetic and acquired human diseases results in skin blistering (Franzke et al., 2003). Collagen XVII is a type II transmembrane protein with an intracytoplasmic N terminus and an extracellular collagenous C terminus. The ectodomain can be proteolytically released (shed) from the cell surface both *in vitro* (Franzke et al., 2002) and *in vivo* (Hirako et al., 2003) to yield a shorter collagenous triple-helical molecule. The cleavage occurs at different sites within the juxtamembranous NC16A domain (Nishie et al., 2010). Under physiological conditions, ADAM9, -10, and -17 appear to be the major sheddases (Franzke et al., 2009), but involvement of neutrophil elastase and serine proteinases has been suggested in pathological settings such as bullous pemphigoid, an autoimmune blistering disease (Lin et al., 2012). Migrating keratinocytes constitutively shed and leave the collagenous ectodomain of collagen XVII in the

extracellular matrix (Tasanen et al., 2004; Nishie et al., 2011). However, the regulation mechanisms of collagen XVII ectodomain shedding are still unclear.

The first cue of functional association of coiled coils and ectodomain shedding in transmembrane collagens comes from the fact that the coiled-coil heptad repeats are located within the juxtamembranous noncollagenous domains adjacent to the cell surface, which also harbor the sheddase recognition and cleavage sites (Balding et al., 1997). Interestingly, in collagen XVII, the physiological cleavage sites are located 8–11 amino acid residues C-terminally from the coiled coils within the NC16A domain (Nishie et al., 2010), indicating the coiled coils are not included in the shed ectodomain. In addition, it has been reported that the recombinant collagenous COL15 domain of collagen XVII can form a trimeric structure without the NC16A domain (Van den Bergh et al., 2006), suggesting that trimerization of collagen XVII may not always require coiled-coil repeats.

These findings led us to investigate the biological functions of coiled coils in the NC16A domain of collagen XVII. We targeted the coiled-coil heptad repeats in the NC16A domain using site-directed mutagenesis and uncovered a novel and essential role of the coiled coils within the NC16A domain in the regulation of collagen XVII ectodomain shedding.

2. Material and Methods

2.1 Prediction and Targeting Potential Coiled Coils on Collagen XVII

The candidate regions for coiled coils on human collagen XVII (NM000494) were assessed using the COILS (version 2.2)[McDonnell et al., 2006] programs. To determine essential residues for coiled-coil regions, each leucine was changed into a proline as described previously (Snellman et al., 2007).

2.2 Cell Cultures and Transfection

The 293 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mm l-glutamine, and 1 mm sodium pyruvate (all Invitrogen). To establish stably expressing cell lines, pcDNA5/FRT plasmid with wild type and mutant collagen XVII cDNA, as well as the empty vector as a control, were co-transfected with pOG44 into the Flp-In-293 host cells by Lipofectamine 2000 (Invitrogen). Stably expressing cells were selected under 200 µg/ml hygromycin B (Invitrogen), as described previously (Nishieet al., 2010).

2.3 Recombinant Protein Expression

Subconfluent stably transfected -293 cells were incubated in serum-free DMEM for 24 h prior to harvesting. Freshly prepared ascorbic acid was added to the culture medium in a final concentration of 50 µg/ ml to allow prolyl and lysyl hydroxylation of collagen and proper triple helix formation (Franzke et al., 2002). Then the cells were lysed for 30 min on ice in a buffer containing 1% Nonidet P-40, 0.1 m NaCl, 25 mm Tris-HCl, pH 7.4, 10 mm EDTA, and 1 mm Pefabloc. proteins, protease inhibitors, including 10 µm Marimastat, 1 mm Pefabloc SC (Roche Applied Science), 1:100 diluted protease inhibitor mixture P8340 (Sigma), 0.1 mm membrane permeable furin inhibitor Dec-RVKR-CMK (344930, Calbiochem), 8 µm of membrane impermeable furin inhibitor α1-antitrypsin Portland, or 50 ng/ml macrocyclic lactone brefeldin A (Cell Signaling) were added into the medium as described previously (Veit et al., 2007).

2.4 Immunoblotting

Concentrated cell culture medium and cell lysate were mixed with Laemmli's sample buffer, and the samples, with or without boiling for 5 min, were separated on SDS-PAGE using 4–13% gradient or 7% uniform gels, followed by transfer onto a nitrocellulose membrane. After blocking the membranes with 2% skimmed milk in Tris-buffered saline and incubation with primary antibodies in the same buffer overnight at 4 °C, HRP-conjugated secondary antibody was reacted for 1 h at room temperature (Hofmann et al., 2010). The following antibodies were employed for immunoblotting: mouse monoclonal antibody NC16A-1 and NC16A-3 directed to distinct epitopes within the NC16A domain of collagen XVII.

2.5 RT-PCR

Total RNA was extracted from subconfluent transformed -293 cells with RNeasy Mini kit (Invitrogen), followed by RT-PCR using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Total cDNA was then amplified with primers F14′ (5 - tacaatgagctggctgtgag-3′) and B14 (5′-cggcttgacagcaatacttc-3′) to yield a 374-bp COL17A1 cDNA fragment spanning nucleotides 4117–4491. The primers GAP-F ′(5 - tcatctctgccccctctgct-3′) and GAP-B ′ (5 - cgacgcctgcttcaccacct-3′) were used for amplification of GAPDH cDNA.

2.6 N Terminus of the Ectodomain of Mutant Collagen XVII

Precipitated medium proteins were boiled for 5 min with Laemmli's sample buffer, followed by SDS-PAGE. To acetylate all free N termini, a fraction of the medium proteins was treated with 40 mm sulfo-acetate NHS (Thermo Fisher Scientific) before SDS-PAGE. After the SDS-PAGE, the Coomassie Blue-stained 120-kDa band corresponding to the monomer form of the ectodomain was cut out the gel and subjected to mass spectrometry (MS) analysis (Japan Bio Services), as reported previously (Zimina et al., 2005).

3. Results

3.1 Coiled coils in the transamembrane and NC16A domains of collagen XVII.

Prediction by COILS indicated that the transmembrane and NC16A domain of collagen XVII contained two heptad repeats of potential coiled coils (Fig-1A, *B*). The amino acid sequence of this region in collagen XVII is highly conserved among different species (Fig-4*A*), suggesting that it has a vital function. When one or both leucines at the d position,

Leu[495] and/or Leu[502], were changed into proline(s), the probability scores predicting a coiled-coil structure calculated by COILS were significantly reduced from 1.0 to 0.4 and 0, respectively (window; amino acids 14 (Fig-4, *A* and *B*). Accordingly, the calculated probability value for the prediction of coiled coils in this region was significant (p = 0.057) for wild type COL17.

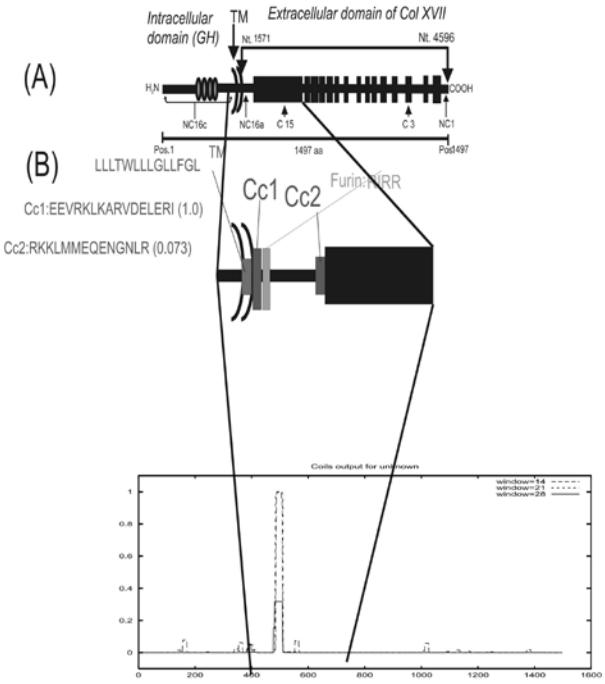

Figure 1: Coiled coils in the NC16A domain of collagen XVII. [A] Schematic illustration of collagenXVII, a type II transmembrane protein. [B] Prediction by COILS indicated high probability for a coiled-coil oligomerization domain.

3.2 Mutations in the coiled coils increased ectodomain shedding of collagen XVII.

To address the role of coiled coils within the NC16A domain, stably transformed Flp-In-293 cells expressing wild type (COL17) and mutant (L495P/L502P or L495P) collagen XVII were produced. Wild type collagen XVII cDNA (COL17) and the mutants were stably transfected into -293 cells. Isogenic expression of these constructs was confirmed by RT-PCR (Fig-2A). Immunoblotting of medium proteins were detected by antibody NC16A in the presence and absence of ascorbic acid (Fig-2B). The trimeric shed ectodomain of collagen XVII was migrated around 360 kDa under mildly denaturing conditions (Fig-2B). When the samples were boiled before SDS-PAGE, the ectodomain migrated as a 120-kDa monomer form. In the presence of ascorbic acid in the culture medium, the wild type COL17 ectodomain was completely trimeric. In contrast, both mutants contained a relatively large fraction of monomeric polypeptides in the absence of ascorbic acid. The amount of the shed ectodomain of both mutants was significantly increased, as compared with wild type COL17 (Fig-2B). Immunoblotting of the cell lysates was analsized with antibodies to the intracytoplasmic domain, which recognized the membrane-bound full-length form of wild type COL17 (a trimer of about 540 kDa and a monomer of about 180 kDa, *arrows*) (Fig-2C). This was significantly reduced in cells expressing the mutants (Fig-2C), suggesting that increased ectodomain shedding had consumed cellular storages of collagen XVII.

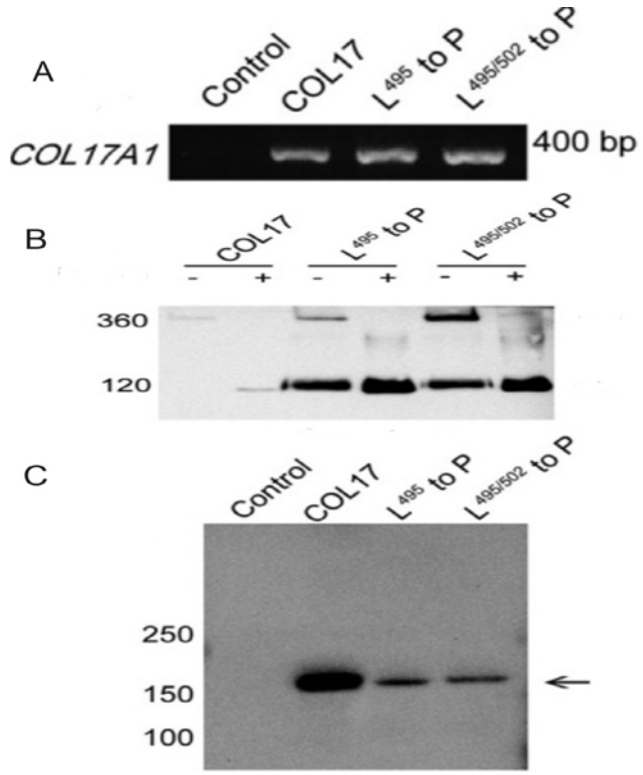

Figure 2: Mutations in the coiled coils domain increased ectodomain shedding of collagen XVII. [A] Wild type collagen XVII cDNA (COL17) and the mutants were stably transfected into 293 cells. [B] Immunoblotting of medium proteins under boiling conditions. [C] Immunoblotting of the cell lysates under boiling conditions

3.3 Shedding of coiled-coil mutants were independent of physiological sheddases.

To disclose the mechanisms of increased ectodomain shedding of collagen XVII in the coiled-coil mutants, protease inhibitors were employed to determine the proteinase classes involved. Ectodomain shedding of wild type COL17 was completely suppressed by the matrix metalloproteinase inhibitor Marimastat but not by other proteinase inhibitors (Fig-3). In contrast, shedding of both mutants was considerably inhibited by a mixture of serine and cysteine proteinase inhibitors and by Pefabloc, indicating that increased shedding of the mutants was mediated by proteases distinct from the physiologically relevant ADAMs (Fig-3A). Furthermore, the membrane-permeable furin inhibitor Dec-RVKR-CMK significantly suppressed the shedding of the coiled-coil mutants, although the membrane-impermeable inhibitor α1-antitrypsin Portland showed no inhibitory activity, indicating that the increased shedding of the mutants is most likely driven by intracellular cleavage by furin (Fig-3A). M-β-CD, a reagent that disrupts lipid rafts by depletion of cholesterol content, increased ectodomain shedding of both wild typeCOL17 and the mutants, indicating that lipid rafts and the plasma membrane microenvironment did not contribute to increased shedding of the mutants (Fig-3B). Western blot analysis in *A* and *B* was performed using antibody NC16A. Mapping of

cleavage sites using defined antibody epitopes. The black bars show epitopes of antibodies, NC16A. The arrow heads indicate physiological sheddase cleavage sites Fig-3C). Antibody NC16A-3 reacted weakly with the shed ectodomain of wild type COL17 but strongly with the mutants (Fig-3). Antibody NC16A needed extensive exposure time to detect shed ectodomain of wild type COL17.

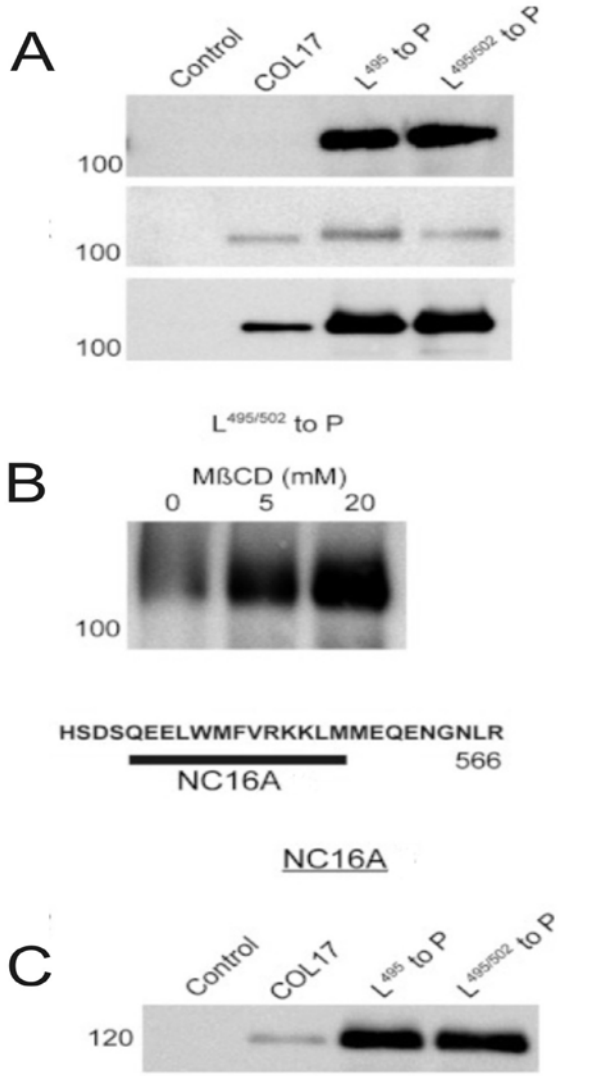

Figure 3: Shedding of coiled-coil mutants was independent of physiological sheddases. [A] Ectodomain shedding of wild type COL17. [B] Increased ectodomain shedding of both wild types COL17 and the mutants. [C] Mapping of cleavage sites using defined antibody epitopes.

3.4 Disruption of coiled coils in the NC16A domain and hypothetical model of triple helix formation of collagen XVII based on the results presented.

Analysis of the primary sequence of collagen XVII with the COILS program (Lupas et al., 1991), revealed two regions within the extracellular NC16a domain which are predicted to form coiled coil structures. One predicted coiled coil region with six heptad repeats conserved between human, mouse and chicken starts within the transmembrane region and extends into the extracellular NC16a domain (Fig-4A) (Fig-1B). The amino acid residues predicted to be located within the membrane are LLLTWLLLLGLLFGL (Fig-4A). The second predicted coiled coil region with two heptad repeats conserved between human, mouse and chicken is located within the NC16a domain close to the first collagenous domain C15(EEVRKLKARVDELERI AND RKKLMMEQENGNLR, respectivelly) (Fig-4B) (Fig-1B). After α1(XVII) chains synthesis, and are localized in the membrane as single chains (Fig-4C). It is hypothesized that the coiled coil structure adjacent to the transmembrane domain mediates the chain selection before folding of the triple helix can take place (Fig-4D). The shorter predicted coiled coil region could help to align the three α1(XVII) chains in the correct stagger (Fig-4E). Folding of the triple helical structures starts from the N-terminal and proceeds to the C-terminal end (Fig-4F). The above data indicated that site-targeted mutation of coiled coils in the NC16A domain resulted in increased ectodomain shedding that is independent of the physiological sheddases and is most likely driven by furin. To address the precise cleavage mechanism, MS was employed to elucidate the cleavage sites.

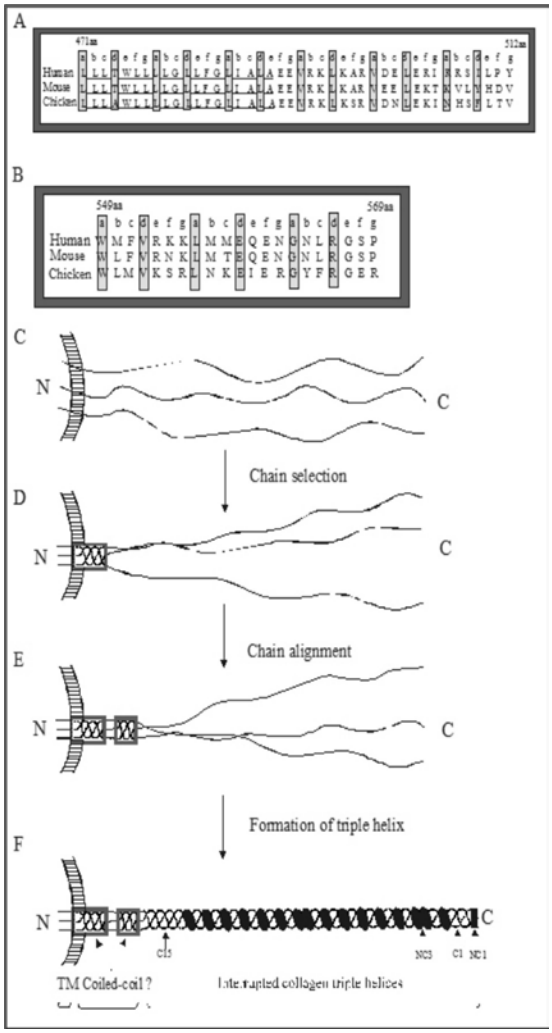

Figure 4: Regions of predicted coiled coil structures [A, B] and hypothetical model of triple helix formation and stability [C-F] of collagen XVII based on the results presented. Analysis of the primary sequence of collagen XVII with the COILS program.

4. Discussion

α-Helical coiled coils represent the most frequent protein oligomerization motifs in nature and they are often found in vital proteins. In this study, we uncovered a novel role for the coiled-coil motif in the transmembranous NC16A domain of the collagen XVII molecule. Targeted mutation of critical amino acids within the heptad repeats induced ectodomain shedding by mechanisms unrelated to the physiological cleavage by ADAMs. Our results suggest that apart from the function as important oligomerization motifs in vital extracellular matrix proteins like thrombospondins and collagens (McAlinden et al., 2003; Snellman et al., 2007), the coiled-coil domains also regulate ectodomain shedding, a prominent post-translational modification of many transmembrane proteins (Franzke et al., ; Franzke etal., 2005). *In silico* prediction showed a striking probability of about 1.0 for coiled coils within the highly conserved NC16A domain of collagen XVII. The coiled-coil sequence spans amino acids Val (492) Ile (505) and contains two heptad repeats. The substitution of only one amino acid, Leu (495)in a–d- position of the first heptad, with proline reduced the propensity score to form coiled coils to one-half, and substitution of both leucines in the d position practically eliminated the probability of coiled coils. The importance of this coiled-coil sequence as an oligomerization domain in collagen XVII is underlined by the fact that the recombinantly expressed mutants exhibited clearly reduced trimer stability of the ectodomain.

Unexpectedly, the mutation of the coiled-coil domain led to substantially enhanced ectodomain shedding. The structural change induced cleavage C-terminally from a furin recognition site, which is not utilized by physiological sheddases for wild type collagen XVII (Nishie et al., 2010). It is well known that conformational changes of substrate proteins can influence the susceptibility to sheddases. However, the mechanisms are not yet fully understood. For example, in the case of collagen XVII, deletion of the stretch outside the physiological cleavage sites abrogates ectodomain shedding (Franzke et al., 2004), arguing for the importance of structural motifs as determinants for ADAM-dependent cleavage. Also, intramembranous cleavage of Notch1 by γ-secretase requires prior cleavage of its ectodomain within the juxtamembranous region by ADAM10 (McDonald et al., 2005; Steiner et al., 2008). Furthermore, it has been demonstrated that a mutation within the juxtamembrane stalk region of angiotensin I-converting enzyme increases its ectodomain shedding, which was not blocked by hydroxamate-based metalloproteinase inhibitors but serine protease inhibitors (McDonald et al., 2004; Alfalah et al., 2001) as observed in this study. Our current observations add coiled-coil domains to the spectrum of structural motifs that regulate conformation-dependent susceptibility of substrate proteins to sheddases.

It is of great interest that shedding of the mutant collagen XVII ectodomain occurred at Ser (504), adjacent to the furin-recognition sequence -RIRR-. This site differs from those used under physiological conditions (Hirako et al., 2003). Because collagen XVII possesses a furin-recognition motif, furin was initially believed to be a responsible sheddase (Schäcke et al., 1998; Thomas et al., 2002). However, detailed biochemical studies using a combination of proteinase inhibitors and stimulators and collagen XVII mutants disclosed as a major physiological protease (Franzke et al., 2009). In line with this, our previous investigations have defined several N termini of the shed ectodomain, including Asp, Leu, Gln, and Gly, all of which are distinct from the furin-recognition motif (Bosshart et al., 1994). Therefore, the role of furin seemed to be in the activation of the ADAMs.

The present observations support the conclusion that cleavage of collagen XVII ectodomain by different proteinases depends on the biological context. Because the cleavage of mutant collagen XVII by furin was mainly driven intracellularly, it can be speculated that the existence of the furin cleavage site is used as “quality control” during synthesis of the molecule (Giudice et al., 1993). Although no evidence exists so far that coiled-coil regions of collagen XVII are related to pathological settings, ectopic cleavage by the serine proteinase plasmin at unknown sites of the molecule has been observed in bullous pemphigoid (Nebenführ et al., 2002). Thus, it is likely that different enzymes can be involved in the cleavage of collagen XVII in a context-dependent manner.

In conclusion, we show here that the short coiled-coil domain of collagenXVII plays a central role in both structural and functional regulation of collagen XVII, in addition to triple helix folding directions.

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