Coiled Coils Ensure the Physiological Ectodomain Shedding of Collagen XVII*

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Background: α-Helical coiled coils represent potential oligomerization motifs in vital proteins. α-Helical coiled coils, frequent protein oligomerization motifs, are commonly observed in vital proteins. Here, using collagen XVII as an example, we provide evidence for a novel function of coiled coils in the regulation of ectodomain shedding. Transmembrane collagen XVII, an epithelial cell surface receptor, mediates dermal-epidermal adhesion in the skin, and its dysfunction is linked to human skin blistering diseases. The ectodomain of this collagen is constitutively shed from the cell surface by proteinases of a disintegrin and metalloprotease family; however, the mechanisms regulating shedding remain elusive. Here, we used site-specific mutagenesis to target the coiled-coil heptad repeats within the juxtamembranous, extracellular noncollagenous 16th A (NC16A) domain of collagen XVII. This resulted in a substantial increase of ectodomain shedding, which was not mediated by disintegrin and metalloproteases. Instead, conformational changes induced by the mutation(s) unmasked a furin recognition sequence that was used for cleavage. This study shows that apart from their functions in protein oligomerization, coiled coils can also act as regulators of ectodomain shedding depending on the biological context.

Results: Targeting coiled coils in the juxtamembranous linker region of transmembrane collagen XVII significantly increased intracellular cleavage by unmasking a furin recognition sequence.

Conclusion: Coiled coils in collagen XVII play a pivotal role to ensure its physiological ectodomain shedding.

Significance: This study revealed a novel role of coiled coils as stabilizer of a specific protease cleavage site.

α-Helical coiled coils represent the most frequent protein oligomerization motifs in nature, and they are often found in vital proteins (1–4). Indeed, the prediction is that 3–5% of all protein residues form coiled coils (5). These are generally characterized by heptad repeat sequences, αabcdefg, within which the first a and the fourth d positions are commonly occupied by hydrophobic amino acids (1–4). Particularly, leucine is typically found at d position, where it plays a role as a “leucine zipper” for oligomerization (6).

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 (7). Coiled coils are found in most, if not all, members of the collagen superfamily (8). In fibrillar collagens, they are usually located in the C-terminal propeptides and function to initiate trimerization and therefore triple-helix folding (8). In contrast, in cell surface-associated transmembrane collagens, the N-terminal noncollagenous domains harbor the coiled-coil sequences (8, 9), consistent with the fact that in these collagens the triple helix formation and ectodomain folding proceed from the N to C terminus (8, 9). 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 (10). Ectodomain shedding is mainly catalyzed by proteinases of the “a disintegrin and metalloprotease” (ADAM) family (11), and it is involved in a variety of essential functions mediated by TNF-α, amyloid precursor protein, Notch1, epidermal growth factor receptor ligands (11, 12), or transmembrane collagens (13, 14), for example.

Collagen XVII is an epithelial cell surface receptor in the skin. Its vital role in dermal-epidermal adhesion and cell migration is

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3The abbreviations used are: ADAM, a disintegrin and metalloprotease; M-β-CD, methyl-β-cyclodextrin; DHS, N-hydroxysuccinimide; Dec, decanoyl; CMK, chloromethyl ketone.
indirectly demonstrated by the fact that its dysfunction in genetic and acquired human diseases results in skin blistering (13, 14). Collagen XVII is a type II transmembrane protein with an intracytoplasmic N terminus and an extracellular collagenous C terminus (13, 14). The ectodomain can be proteolytically released (shed) from the cell surface both in vitro (15) and in vivo (16, 17) to yield a shorter collagenous triple-helical molecule. The cleavage occurs at different sites within the juxtamembranous NC16A domain (16, 18). Under physiological conditions, ADAM9, -10, and -17 appear to be the major sheddases (19), but involvement of neutrophil elastase and serine proteinases has been suggested in pathological settings such as bullous pemphigoid, an autoimmune blistering disease (16, 20, 21).

The full spectrum of biological functions of collagen XVII ectodomain shedding remains uncertain, but binding to laminin-332 and association with migration and differentiation of keratinocytes seem clear (13, 14). Consistent with this notion, we have shown that migrating keratinocytes constitutively shed and leave the collagenous ectodomain of collagen XVII in the extracellular matrix (22, 23). However, the regulation mechanisms of collagen XVII ectodomain shedding are still unclear.

The first clue 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 (8, 9, 24). 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 (16), 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 (25), 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.

**EXPERIMENTAL PROCEDURES**

**In Silico Prediction and Targeting Potential Coiled Coils on Collagen XVII**—The candidate regions for coiled coils on human collagen XVII (NM_000494) were assessed using the COILS (version 2.2) and Paircoil2 (26) programs. To determine essential residues for coiled-coil regions, each leucine was changed into a proline as described previously (9).

Human collagen XVII cDNA (a kind gift from Dr. L. Borra- dori) was introduced into the NotI site of pcDNA5/FRT (Invitrogen) (16). This construct was designated as COL17. For site-directed mutagenesis of the coiled-coil sequence, two different 1187-bp fragments spanning the EcoRV and Clal DNA restriction sites, which contain substitution mutations of either Leu495 or both Leu502 and Leu495 into Pro, were chemically produced and cloned into pUC57 (GenScript) (Fig. 1B). EcoRV- and Clal-digested DNA fragments were then inserted into the COL17-pcDNA5/FRT vector. These constructs were designated as L495P and L495P/L502P.

**Cell Cultures and Transfection**—The Flp-In-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 (16, 23).

**Recombinant Protein Expression**—Subconfluent stably transfected Flp-In-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 (15). Then the cells were lysed for 30 min on ice in a buffer containing 1% Nonidet P-40, 0.1 mM NaCl, 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 1 mM Pefabloc (15). The medium proteins were concentrated either with ethanol precipitation (15) or by Amicon ultrafiltration cassette (30 kDa, Millipore) before further processing. For characterization of responsible sheddases and transportation of cellular proteins, protease inhibitors, including 10 μM Marimastat (British Biotech), 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 α-antitrypsin Portland, or 50 ng/ml macrocytic lactone brefeldin A (Cell Signaling) were added into the medium as described previously (27). Isolation of proteins from the cell surface was carried out using EZ-Link Sulfo-NHS-SS-Biotin (21328, Thermo Scientific) as described elsewhere (15, 27).

**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 antibodies were reacted for 1 h at room temperature. The signals were visualized by ECL-Plus (GE Healthcare).

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 (20) were used in 1:50 (NC16A-1) and 1:2,000 dilutions (NC16A-3). For detection of the intracytoplasmic domain of collagen XVII, the polyclonal goat antibody N18 (Santa Cruz Biotechnology) was diluted 1:500. A new rabbit polyclonal antibody (Ab-SILP) was produced using the synthetic peptide NH2-SILPYGDS, as described previously (16, 23). Purification of the Ab-SILP was performed using SulfoLink coupling resin (20404, Thermo Scientific) according to the manufacturer’s instructions, and the final concentration of 1 μg/ml of the antibody...
was used. The β-tubulin antibody Ab6046 (Abcam) in a 1:2,000 dilution was employed as an internal loading control for immunoblotting.

**RT-PCR**—Total RNA was extracted from subconfluent transformed Flp-In-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′-tacattacgtcctgtgtag-3′) and B14 (5′-cgccgcctgcttcaccacct-3′) to yield a 374-bp COL17A1 cDNA fragment spanning nucleotides 4117–4491. The primers GAP-F (5′-tacctgctgccctctgtc-3′) and GAP-B (5′-gaggctctgctcaccacct-3′) were used for amplification of GAPDH cDNA (NM_002046.3) as a control.

**Depletion of Plasma Membrane Cholesterol**—Subconfluent cells were washed twice with PBS and then treated with 5–20 mM methyl-β-cyclodextrin (M-β-CD, Sigma) in serum-free DMEM for 60 min (28). The medium proteins were concentrated as described above.

**Definition of the N Terminus of the Shed Ectodomain of Mutant Collagen XVII**—Precipitated medium proteins were boiled for 5 min with Laemmlis’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, as described (16). 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 (16).

**RESULTS**

**Collagen XVII Has Coiled Coils within the Juxtamembranous NC16A Domain**—In silico prediction by COILS (29) indicated that the stretch Val492–Ile505 in the juxtamembranous NC16A domain of collagen XVII contained two heptad repeats of potential coiled coils (Fig. 1A). The amino acid sequence of this region in collagen XVII is highly conserved among different species (Fig. 1A), suggesting that it has a vital function. When one or both leucines at the d position, Leu495 and/or Leu502, 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. 1, B and C). Accordingly, the calculated probability value for the prediction of coiled coils in this region by Paircoil2 was significant (p = 0.057) for wild type COL17, whereas those of the mutants L495P and L495P/L502P were both decreased (p = 0.33 and 0.68, respectively).

**Vital Role of Coiled Coils in the NC16A Domain for Trimerization 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. Isogenic expression of genes encoding these constructs was confirmed by RT-PCR (Fig. 2A). For recombinant protein expression, to ensure proper folding of collagen XVII, ascorbic acid was added to the cell culture medium. It is a cofactor for collagen hydroxylases and necessary for proper triple helix formation (15). Full-length collagen XVII is a homotrimer of three 180-kDa polypeptides, and the shedding generates an ectodomain consisting of three 120-kDa collagenous fragments (15, 17). On SDS-PAGE under mild denaturing conditions, the wild type trimeric ectodomain isolated from culture medium migrated at about 360 kDa; under denaturing conditions (boiling of the sample), it was dissociated into 120-kDa monomer forms (Fig. 2B). To further evaluate the stability of the triple-helical trimers, ascorbic acid was omitted from the protein expression system. This treatment did not have a significant effect on the stability of wild type COL17 ectodomain, but it led to increased generation of monomers in both mutants (Fig. 2B). These observations indicate that coiled coils in the NC16A domain play a role in trimerization and triple helix formation of the collagen XVII ectodomain.

**Targeted Mutations in Coiled Coils Resulted in Increased Ectodomain Shedding**—The ectodomain shedding of both collagen XVII mutants was substantially increased as compared with wild type COL17 (Fig. 2B). Analysis of the cytoplasmic domain of collagen XVII revealed a significantly reduced amount of full-length mutant in cell lysates (Fig. 2C). This observation suggests that increased ectodomain shedding of the mutants led to exhaustion of cellular collagen XVII and indicates that coiled coils in the NC16A domain are important for regulation of ectodomain shedding of the molecule.

**Ectodomain Shedding in Coiled-coil Mutants Was Not Mediated by Physiological Sheddase(s)**—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. Consistent with previous reports (28), ectodomain shedding of wild type COL17 was completely suppressed by Marimastat, a broad spectrum matrix metalloproteinase inhibitor (Fig. 3A). Unexpectedly, Marimastat did not suppress the ectodomain shedding of the mutants (Fig. 3A). Instead, an inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin, E-64, leupeptin, and pepstatin significantly suppressed ectodomain shedding of both mutants, although the shedding of wild type COL17 was not affected (Fig. 3A). Treatment with the general serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc) alone resulted in a very similar inhibition, arguing that serine proteases are mainly involved in the shedding of the mutants (Fig. 3A). These results indicate that the shedding of both mutant forms is associated with protease(s) other than the physiological sheddase(s) of collagen XVII. To further characterize the involved serine protease(s), two different furin-specific inhibitors were used. The membrane-permeable furin inhibitor Dec-RVKR-CMK significantly suppressed the shedding of both coiled-coil mutants, although the membrane-impermeable inhibitor α1-antitrypsin Portland showed no inhibitory activity (Fig. 3A), indicating that the increased shedding of the mutants is most likely driven by intracellular cleavage by furin, which is consistent with the fact that furin is mainly localized within the trans-Golgi network (30, 31).

**Coiled-coil Mutations in the NC16A Domain Did Not Affect Plasma Membrane Microlocalization of Collagen XVII**—Ectodomain shedding of collagen XVII can be significantly influenced by the plasma membrane microenvironment, in particular by lipid rafts (28). M-β-CD is a known disruptor of lipid rafts by depletion of the membranous cholesterol levels, which
causes increased ectodomain shedding of collagen XVII (28). Therefore, M-β-CD was added to cells expressing either wild type COL17 or the coiled-coil mutants. This resulted in a dose-dependent increase in ectodomain shedding in the mutants (Fig. 3B), suggesting that the mutated coiled-coil structure did not influence targeting of collagen XVII into the lipid rafts. Thus, the mechanism that leads to increased shedding does not depend on the plasma membrane environment.

Consequences of Targeting Coiled Coils of Collagen XVII on Distinct Epitopes—The NC16A domain of collagen XVII is highly antigenic, and autoimmunity to this region results in the blistering disease bullous pemphigoid (32, 33). Therefore, it was of interest to assess whether mutations within the coiled coils induced epitope changes. To this end, we used two well-characterized antibodies with known epitope sequences to map their reactivity with wild type COL17 and the mutants. The
Role of Coiled Coils in Ectodomain Shedding

FIGURE 2. Targeted mutations in the coiled coils significantly increased ectodomain shedding of collagen XVII. A, wild type collagen XVII cDNA (COL17) and the mutants L495P (L<sup>495</sup> to P) and L495P/L502P (L<sup>495</sup>P to P and L<sup>502</sup>P to P) were stably transfected into Flp-In-293 cells. Isogenic expression of these constructs was confirmed by RT-PCR. B, immunoblotting of medium proteins by antibody NC16A-3 in the presence (left panel) and absence (right panel) of ascorbic acid. The trimeric shed ectodomain of collagen XVII migrated around 360 kDa (arrows) under mildly denaturing conditions, i.e. without boiling of the samples. When the samples were boiled before SDS-PAGE, the ectodomain migrated as a 120-kDa monomer form (arrowheads). In the presence of ascorbic acid in the culture medium, the wild type COL17 ectodomain was completely trimeric, although both mutants contained small amounts of a lower molecular mass band around 240 kDa, which most likely represents the dimerized form (stars). 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. C, immunoblotting of the cell lysates 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). This was significantly reduced in cells expressing the mutants, suggesting that increased ectodomain shedding had consumed cellular storages of collagen XVII. This underlines the importance of the coiled coils in the NC16A domain for correct regulation of ectodomain shedding of collagen XVII.

monoclonal antibody NC16A-3, which recognizes the sequence Gln<sup>545</sup>–Met<sup>557</sup> (20), reacted with the shed ectodomain of wild type COL17, but it was clearly stronger with the mutants L495P and L495P/L502P (Fig. 3C). The monoclonal antibody NC16A-1, which recognizes the sequence Ser<sup>515</sup>–Arg<sup>523</sup> (20), reacted very faintly, if at all, with the shed ectodomain of wild type COL17 but robustly with the shed ectodomain of the mutants L495P and L495P/L502P. Because most of the physiological sheddase cleavage sites are located C-terminal to the recognition sequence of NC16A-1 (16), this suggested that mutation of coiled coils may have yielded additional ectopic cleavage site(s) closer to the cell surface (Fig. 3C). To address this further, we generated the new antibody Ab-SILP, which recognizes the sequence region Ser<sup>508</sup>–Ser<sup>515</sup>, the adjacent C-terminal sequence stretch from the putative furin cleavage site. Interestingly, this antibody specifically detected the COL17 ectodomain that was cleaved on the furin recognition motif, but it showed no reactivity against full-length COL17 (data not shown). As a result, Ab-SILP only reacted with the cleaved ectodomain of the mutants but not wild type COL17 (Fig. 3C). These results confirm that both mutants were cleaved in an ADAM-independent manner, most likely by furin.

Disruption of Coiled Coils in the NC16A Domain Unmasked the Furin Recognition Motif of Collagen XVII—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 site(s).

Definition of the cleavage-generated new N termini of the mutant L495P/L502P was pursued, and MS analysis of Coo massie Blue-stained bands (Fig. 4A) detected distinct peptide fragments, including R↓SILPYGDSMDR↓I and R↓SILPYGDSMDRIEK↓D (↓ means end of the fragments). Because the samples were trypsinized prior to MS analysis, most N termini at R↓S sites may be artificial. To reveal genuine cleavage site(s), the purified protein samples were treated with a blocking reagent, sulfo-acetate NHS, before MS analysis to acetylate all free amines (16, 34, 35). MS of the acetylated samples disclosed N-terminally acetylated acetyl-SILPYGDSMDR and acetyl-SILPYGDSMDRIEK peptides, suggesting that Ser<sup>508</sup> represents the new N terminus of shed mutant collagen XVII ectodomain. Intriguingly, Ser<sup>508</sup> is located adjacent to the furin-recognition motif -RIRR- (Figs. 1A, 3C, and 4B), indicating involvement of furin in the shedding of the mutated protein.

Coiled-coil Mutants Were Predominantly Cleaved Intracellularly—The data obtained from collagen XVII shedding experiments using the membrane-permeable and -impermeable furin inhibitors Dec-RVKR-CMK and a1-antitrypsin Portland suggested that the intracellular cleavage by furin occurred intracellularly. To assess detailed information about the cleaving mechanism, we performed immunoblotting using sulfo-NHS biotin-labeled cell surface proteins. This resulted in reduced amounts of the shed ectodomain released into culture medium from coiled-coil mutants compared with that of wild type COL17 (Fig. 4C), suggesting the cleavage of the mutants occurred intracellularly. Finally, we used a macrolonic lactone brefeldin A, which inhibits translocation of proteins from the endoplasmic reticulum to the Golgi apparatus and consequently their secretion via secretory vesicles (36). Treatment with brefeldin A significantly reduced the amount of shed ectodomain of the mutants compared to the wild type controls (Fig. 4D). Taken together, these results let us conclude that cleavage of coiled-coil mutants by furin occurred predominantly intracellularly but not on the cell surface.
DISCUSSION

In this study, we uncovered a novel role for the coiled-coil motif in the juxtamembranous 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 (8, 9), the coiled-coil domains also regulate ectodomain shedding, a prominent post-translational modification of many transmembrane proteins (13, 14).

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 Val492–Ile505 and contains two heptad repeats. The substitution of only one amino acid, Leu495 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 L495P and L495P/L502P exhibited clearly reduced trimer stability of the ectodomain.

Unexpectedly, the mutation of the coiled-coil domain Val492–Ile505 also led to substantially enhanced ectodomain shedding. The structural change induced cleavage C-terminally from a furin recognition site, which is not utilized by physiologically relevant ADAMs. Furthermore, the membrane-permeable furin inhibitor Dec-RVKR-CMK also inhibited the shedding of wild type COL17, extended exposure was needed to obtain signals. Western blot analysis in A and B was performed using antibody NC16A-3. M, C, mapping of cleavage sites using defined antibody epitopes. The black bars show epitopes of antibodies Ab-SILP, NC16A-1, and NC16A-3. The red arrowheads indicate physiological sheddase cleavage sites. Antibody NC16A-3 reacted weakly with the shed ectodomain of wild type COL17 but strongly with the mutants. Antibody NC16A-1 needed extensive exposure time to detect shed ectodomain of wild type COL17. However, it reacted robustly with shed forms of mutant L495P/L502P and L495P, suggesting that proteolysis had occurred N-terminally of the physiological cleavage sites. Ab-SILP reacted selectively with the mutant ectodomain, suggesting ADAM-independent ectopic cleavage of the mutants adjacent to the furin-recognition motif. Red box, predicted coiled coils. Blue box, furin recognition motif. L495P (L495 to P) and L495P/L502P (L495 to P and L502 to P). α1-PDX, α1-antitrypsin Portland.

FIGURE 3. Shedding of coiled-coil mutants was independent of physiological sheddases and lipid rafts. A, ectodomain shedding of wild type COL17 was completely suppressed by the matrix metalloproteinase inhibitor Marimastat but not by other proteinase inhibitors. 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. 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. Because Dec-RVKR-CMK also inhibited the shedding of wild type COL17, extended exposure was needed to obtain signals. B, M-β-CD, a reagent that disrupts lipid rafts by depletion of cholesterol content, increased ectodomain shedding of both wild type COL17 and the mutants, indicating that lipid rafts and the plasma membrane microenvironment did not contribute to increased shedding of the mutants. Western blot analysis in A and B was performed using antibody NC16A-3. C, mapping of cleavage sites using defined antibody epitopes. The black bars show epitopes of antibodies Ab-SILP, NC16A-1, and NC16A-3. The red arrowheads indicate physiological sheddase cleavage sites. Antibody NC16A-3 reacted weakly with the shed ectodomain of wild type COL17 but strongly with the mutants. Antibody NC16A-1 needed extensive exposure time to detect shed ectodomain of wild type COL17. However, it reacted robustly with shed forms of mutant L495P/L502P and L495P, suggesting that proteolysis had occurred N-terminally of the physiological cleavage sites. Ab-SILP reacted selectively with the mutant ectodomain, suggesting ADAM-independent ectopic cleavage of the mutants adjacent to the furin-recognition motif. Red box, predicted coiled coils. Blue box, furin recognition motif. L495P (L495 to P) and L495P/L502P (L495 to P and L502 to P). α1-PDX, α1-antitrypsin Portland.
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 (39) 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 Ser508, adjacent to the furin-recognition motif -RIRR- (blue box), suggesting involvement of furin or furin-like protease(s) as sheddases of the coiled-coil mutants. For comparison, the physiological cleavage sites in wild type collagen XVII are indicated by small red arrowheads. C, immunoblotting of sulfo-NHS-biotin-labeled cell surface proteins by NC16A-3 revealed strongly reduced amounts of the shed ectodomain in the supernatant of the coiled-coil mutants. D, treatment with brefeldin A significantly reduced the shed ectodomain of the mutants in the culture medium, as detected by NC16A-3. These results indicate that the cleavage of coiled-coil mutants by furin occurred intracellularly and not on the cell surface. L495P (L495 to P) and L495P/L502P (L495/502 to P)
ognition sequence -RIRR-. This site differs from those used under physiological conditions (16, 18). Because collagen XVII possesses a furin-recognition motif, furin was initially believed to be a responsible sheddase (40). However, detailed biochemical studies using a combination of protease inhibitors and stimulators and collagen XVII mutants disclosed ADAM9/10/17 as a major physiological protease (19). In line with this, our previous investigations have defined several N termini of the shed ectodomain, including Asp514, Leu524, Gln525, and Gly526, all of which are distinct from the furin-recognition motif (16). 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 proteases 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. Although no evidence exists so far that coiled-coil regions of collagen XVII are related to pathological settings, ectopic cleavage by the serine protease plasmin at unknown sites of the molecule has been observed in bullous pemphigoid (16, 20). Thus, it is likely that different enzymes can be involved in the cleavage of collagen XVII in a context-dependent manner.

In summary, we show here that the furin-recognition motif can be unmasked by conformational changes within the NC16A domain, and we demonstrate that the short coiled-coil domain of 14 amino acids plays a central role in both structural and functional regulation of collagen XVII.

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