Two Forms of Collagen XVII in Keratinocytes
A FULL-LENGTH TRANSMEMBRANE PROTEIN AND A SOLUBLE ECTODomain

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The cDNA sequence of human collagen XVII predicts an unusual type II transmembrane protein, but a biochemical characterization of this structure has not been accomplished yet. Using domain-specific antibodies against recombinant collagen XVII fragments, we identified two molecular forms of the collagen in human skin and epithelial cells. Full-length collagen XVII appeared as a homotrimeric transmembrane molecule of three 180-kDa α1(XVII) chains. The globular intracellular domain was disulfide-linked, and the N-glycosylated extra-cellular domain of three 120-kDa polypeptides was triple-helical at physiological temperatures. A second, soluble form of collagen XVII in keratinocyte culture media was recognized with antibodies to the ectodomain, but not the endodomain. The soluble form exhibited molecular properties of the collagen XVII ectodomain: a triple-helical, N-glycosylated molecule of three 120-kDa polypeptides. Northern blot analysis with probes spanning either the distal 5′ or the distal 3′ end of the collagen XVII cDNA revealed an identical 6-kb mRNA, suggesting that both the 180- and 120-kDa polypeptides were translated from the same mRNA, and that the 120-kDa polypeptide was generated post-translationally. In concert, keratinocytes harboring a homozygous nonsense mutation in the COL17A1 gene synthesized neither the 180-kDa α1(XVII) chain nor the 120-kDa polypeptide. Finally, treatment of normal keratinocytes with a synthetic inhibitor of furin protease convertases, decanoyl-RVKR-chloromethyl ketone, prevented the generation of the 120-kDa polypeptide. These data strongly suggest that the soluble 120-kDa polypeptide represents a specifically cleaved ectodomain of collagen XVII, generated through furin-mediated proteolytic processing. Thus, collagen XVII is not only an unusual type II transmembrane collagen, but the first collagen with a specifically processed, soluble triple-helical ectodomain.

Collagen XVII, also known as the 180-kDa bullous pemphigoid antigen or BP180, is a structural component of the hemidesmosomes in epithelial cells (1). The cDNA sequence predicts a type II integral transmembrane protein of 1497 amino acids, with an NH2-terminal intracellular domain of 466 amino acids, a transmembrane domain of 23 residues and a COOH-terminal extracellular domain of 1008 amino acid residues (2). Because of 15 collagenous subdomains characterized by -Gly-X-Y- repeat sequences within the ectodomain, the molecule was designated collagen XVII (3, 4). Traditionally, collagens are defined as triple-helical proteins with -Gly-X-Y- repeat sequences and with a function as a structural protein of the extracellular matrix (5). Among the more than 20 homo- and heterotrimeric collagens, types XVII and XIII represent the only putative transmembrane collagens (for review, see Ref. 6). However, probably as a result of their low level of expression in tissue and inaccessibility to standard biochemical analyses, the structures of these collagens were deduced from the cDNA sequences rather than from protein chemical data. Therefore, their molecular composition, folding, and assembly have remained a matter of conjecture. Nevertheless, Hirako et al. (7) studied collagen XVII from bovine cell lines with sucrose gradient centrifugation, rotary shadowing electron microscopy, and chemical cross-linking experiments, and suggested that it appeared as an asymmetric molecule with a globular head, central rod, and a flexible tail, with potential to trimer formation. In concert with these findings, recombinant extracellular fragments of human collagen XVII expressed in COS-1 cells showed a high molecular mass form with an elongated conformation (8). Immunoelectron microscopy demonstrated that antibodies against recombinant ectodomain fragments labeled structures outside the keratinocyte plasma membrane along the skin basement membrane (9).

The functions of collagen XVII are not known, but as a transmembrane component of the hemidesmosomes, it is likely to play a role in maintaining linkage between the intracellular and the extracellular structural elements and in anchoring the epithelia to the underlying basement membrane (10, 11). This concept is supported by pathological skin conditions. For example, in bullous autoimmune skin diseases, the presence of autoantibodies reactive with collagen XVII is associated with diminished epidermal-dermal cohesion (12–15), and in a mouse model passive transfer of collagen XVII antibodies resulted in skin blistering (16). Furthermore, heritable skin blistering disorders of the junctional epidermolysis bullosa group are associated with mutations in the gene for collagen XVII, COL17A1, and with absence or attenuated expression of collagen XVII (4, 17–28). Despite a growing number of COL17A1 mutations, the genotype-phenotype correlations and the molecular mechanisms underlying the phenotypes have remained elusive as a result of insufficient information on the structure and functions of normal human collagen XVII.

In the present study, we used domain-specific antibodies and biochemical analyses to characterize collagen XVII from normal human skin and epidermal keratinocytes. We show that the collagen occurs in two triple-helical forms, as a full-length
transmembrane protein and as a soluble ectodomain, a specific proteolytic cleavage product of the full-length molecule.

**MATERIALS AND METHODS**

**Production of Recombinant Fragments and Domain-specific Antibodies to Collagen XVII**—Recombinant fusion proteins corresponding to two different fragments of the human collagen XVII were generated using the bacterial pQE expression system (Qiagen, Hilden, Germany). The fusion proteins contained an NH-terminal His tag for easy purification of the expression products from bacterial lysates. The fusion protein Col17-intra spanned amino acids 61–360 in the intracellular domain, whereas the fusion protein Col17-extra corresponded to amino acids 1292–1497 in the carboxyl terminus of the extracellular domain of collagen XVII (Fig. 1). The corresponding cDNAs were synthesized by First Strand cDNA Synthesis Kit (Amersham, Braunwegen, Germany) of mRNA from normal human keratinocytes isolated by the QuickPrep mRNA isolation kit (Pharmacia, Freiburg, Germany). The polymerase chain reaction amplification was performed with the sense primer 5′-ACGGATCCGCAGCGGCTACATAAACTC-3′ and the anti-sense primer 5′-GGGAAGCTTGGTGTTCTCCTCGTTT-3′ for the fusion protein Col17-intra, and with the sense primer 5′-CCGGATTCCGAGAGGCTTCTCCTCATTGCT-3′ and the anti-sense primer 5′-GGGAAGCTTGGTGTTCTCCTCGTTT-3′ for the fusion protein Col17-extra. To generate the expression clones, 5′ end primers contained a BamHI site and 3′ end primers contained a HindIII site for cloning into the vector pQE32 (Col17-intra) and pQE30 (Col17-extra). The correct orientation and ligation of both clones was verified by dyeoxyquinucleotide sequence analysis. The proteins were produced using the QIExpress Type IV kit (Qiagen) according to the manufacturer’s instructions and purified with affinity chromatography on Ni2+–NTA-agarose.

Immunization of rabbits with the fusion proteins was performed using standard procedures (Eurogentec, Ougrée, Belgium). Since no immune response was obtained for Col17-extra, chicken were immunized, and the antibodies were purified from egg yolks by filtration and precipitation with polyethylene glycol (29). Bullous pemphigoid patient sera (30) that was strongly reactive with the NC16a-domain (15), but not with other domains of collagen XVII, was also used for immunoblotting.

**Cell Cultures**—Normal human keratinocytes were obtained by trypsinization of skin biopsy samples, the human keratinocyte cell line HaCaT was a generous gift of Dr. N. Fuesnig, German Cancer Research Center (DKFZ), Heidelberg, Germany. All cells were cultured in serum-free, keratinocyte growth medium, supplemented with bovine pituitary extract and epidermal growth factor (KGM, Life Technologies, Inc.) as described previously (30). Prior to extraction and immunoblotting experiments, the cells were grown in the presence of 50 μg/ml 1-ascorbate for 48 h (26).

**Protein Extractions**—For analysis of collagen XVII from cell cultures, protocyl and the media were processed separately. The cell layers were extracted for 30 min on ice with 1 ml/75 cm2 of a buffer containing 1% Nonidet P-40, 0.1 M NaCl, 25 mM Tris-HCl, pH 7.4, and 10 mM EDTA, 1 mM Pefabloc (Merck, Darmstadt, Germany), and when appropriate, 14 μg/ml chymostatin, 7 μg/ml antipain, 7 μg/ml leupeptin, and 14 μg/ml pepstatin as proteinase inhibitors (31). The cell lysate was then scraped with a rubber policeman, and the extract was centrifuged at 14,000 × g at 4 °C. The supernatant was used for further analyses. In some experiments, the above extraction was preceded by incubation of the cells with 0.5 μM NaCl in 0.05 M Tris-HCl, pH 8.2, to release neutral salt soluble proteins. For analysis of medium proteins, proteinase inhibitors were added immediately after collecting the medium onto ice. After removing cellular debris by centrifugation 1000 rpm for 10 min, the proteins from 10 ml medium were precipitated with ammonium sulfate to 30% saturation for 4 h at 4 °C. After centrifugation at 15,000 × g for 60 min at 4 °C, the pellets were dissolved in 100 μl of a buffer containing 65 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM Pefabloc (Merck), and 1 mM EDTA (32). Fifty to 100 μl of the cell extracts or the medium concentrate were used for the enzyme digestions and 10–30 μl for immunoblotting.

**Immunoblotting**—For immunoblotting, proteins were separated on SDS-PAGE using gels with either 7% polyacrylamide or 3–15% polyacrylamide gradients under non-reducing or reducing (1 mM dithiothreitol) conditions. The incubations with the first antibodies were overnight, and with the alkaline phosphatase-linked anti-rabbit-, -chick,- and -human second antibodies for 2 h.

**Immunoprecipitation of Collagen XVII after Cell Surface Biotinylation**—Epidermal keratinocytes or HaCaT cells were incubated with 50 μg/ml ascorbic acid for 48 h, washed extensively and incubated with 3 μg/ml biotinylated-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) in 0.15 M NaCl, 0.05 mM sodium borate, pH 8.0, for 15 min. The reaction was stopped with 1 mM NH4Cl. After extensive washing, the cells were extracted with the Nonidet P-40-containing extraction buffer as described above, followed by immunoprecipitation with domain-specific collagen XVII antibodies.

Prior to immunoprecipitation, preclearing was achieved by incubating 50 μl of protein A-Sepharose (Pharmacia, Uppsala, Sweden) with 500 μl of biotinylated cell extract containing 0.1% SDS for 2 h at 4 °C. After centrifugation for 5 min at 300 × g, the supernatants were added to protein A-Sepharose/antibody complexes. These were prepared by adding 50 μl of rabbit antichicken IgG (Jackson, 1:4000) to 30 μl of protein A-Sepharose in 500 μl of the above Nonidet P-40 buffer with 0.1% SDS (Nonidet P-40 buffer, 0.1% SDS). Alternatively, 50 μl of chicken antibody Col17ecto-1 and 5 μl of rabbit-anti-chicken-IgG (Dianova, Hamburg, Germany) were added to 30 μl of protein A-Sepharose in 500 μl of Nonidet P-40 buffer, 0.1% SDS. The complexes were rotated for 2 h at 4 °C and centrifuged at 300 × g for 5 min, and the antibody/protein-A-Sepharose pellets were washed three times with Nonidet P-40 buffer, 0.1% SDS. The supernatants of preclarified cell extracts were added to these antibody complexes and rotated at 4 °C overnight. After extensive washing with Nonidet P-40 buffer, 0.1% SDS, the pellets were suspended in 0.8 μg/ml ascorbic acid, 2% SDS, 5% glycerol, 0.1 mM Tris, pH 6.8, heated for 5 min at 95 °C, and centrifuged for 5 min at 300 × g. The supernatants were loaded onto 7% SDS-PAGE and analyzed in blots using streptavidin-coupled alkaline phosphatase (Sigma, Deisenhofen, Germany) for detection.

**Enzyme Digests**—For assessment of the domain structure and stability of collagen XVII, cell and medium extracts were subjected to collagenase, pepsin, sequential pepsin/trypsin, or N-glycosidase F digestions. The incubation with 40 units/ml highly purified bacterial collagenase (Proteomen Biotech, Inc., Lyndebrook, NJ) was carried out in 50 μl of collagen cell extract containing 15 μM CaCl2 and 1 mM Pefabloc (Merck) for 4 h at 37 °C (34). For a limited pepsin digestion of collagen XVII, 100 μl of extracts were acidified by adding glacial acetic acid to a final concentration of 0.1%, and the samples were incubated with 1 μg/ml pepsin (Fluka, Deisenhofen, Germany) at 5°C for 2–24 h (34). After neutralization with unbuffered Tris, the samples were either directly precipitated with ethanol at −20 °C overnight or treated with 10 μg/ml trypsin (Sigma) at temperatures between 15°C and 47°C for 2 min (35, 36). The reaction was stopped by adding soy bean trypsin inhibitor (Sigma) to a final concentration of 10 μg/ml (36). For deglycosylation, 50 μl of the protein extract were treated with 10% β-mercaptoethanol for 10 min at 60°C prior to digestion with 10 units/ml N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) overnight at 37°C.

**Northern Blotting**—For Northern bloting, 1.5 μg of mRNA isolated from cultured cells with OligoDirect mRNA Minikit (Qiagen, Hilden, Germany) was separated on a 0.8% agarose gel containing formaldehyde, transferred onto a positively charged nylon membrane (Boehringer Mannheim) overnight, and immobilized by baking at 120°C for 30 min. The membranes were pre-hybridized and then hybridized with digoxigenin (DIG)-labeled collagen XVII cDNA (4) at 50°C. The DIG labeling of the cDNA was performed with the DIG DNA labeling kit (Boehringer Mannheim, Mannheim, Germany) following the manufacturer’s instructions for randomly primed DNA labeling. After hybridization, the filters were washed to a final stringency of 0.1 × standard SSPE (0.1× standard saline citrate, 0.1% sodium dodecyl sulfate). The cDNA–mRNA hybrids were detected with alkaline phosphatase-labeled anti-DIG-antibodies and visualized by chemiluminescence using CDP-Star™ substrate (Boehringer Mannheim).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; DIG, digoxigenin; kb, kilobase(s).
The native conformation of collagen XVII was preserved typically procollagen deposition in the tissue. The detergent was necessary for solubilization of collagen XVII from the cells, since no collagen XVII was extractable with phosphate-buffered saline or 0.5 M NaCl, but not in dermis extracts (lane 2). SDS-PAGE with a 4.5–15% polyacrylamide gradient, B, immunoprecipitation of collagen XVII after cell surface biotinylation, with antibody Col17ecto-1 (lane 1) and antibody SA 3485 (lane 2). After separation on 7% SDS-PAGE, the blot was visualized with streptavidin-coupled alkaline phosphatase. Both antibodies precipitated a biotinylated 180-kDa polypeptide corresponding to the α1(XVII) chain. Molecular sizes, as determined by marker proteins of 200, 112, and 80 kDa, are indicated.

RESULTS

Domain-specific Collagen XVII Antibodies—Polyclonal antibodies raised against recombinant procaryotic fragments spanning the endodomain and the distal ectodomain of collagen XVII (Fig. 1) gave an intensive immune response in rabbits (antibody SA 3485) or in chicken (antibody Col17ecto-1). The antibodies showed no cross-reactivity or reaction with other hemidesmosomal components or basement membrane proteins, such as BP230, laminin 5, collagen IV, or collagen VII. In contrast, they specifically recognized collagen XVII extracted from skin or cultured cells in immunoblots and by immunoprecipitation. They did not work in immunofluorescence staining of skin cryosections or cultured cells.

Collagen XVII Extraction from the Epidermis and Cultured Epithelial Cells—Collagen XVII could be extracted from human epidermis, but not dermis (Fig. 2A) with a chaotropic buffer containing 8 M urea and 2% SDS as described previously (30). The native conformation of collagen XVII was preserved by extraction of cultured keratinocytes with a neutral buffer containing 1% Nonidet P-40 as a detergent (31). Notably, the α1(XVII) chains from both sources showed similar migration on SDS-PAGE (Fig. 2A, lanes 1 and 2), indicating that the tissue form of collagen XVII was similar to the cell form, and that no typical procollagen → collagen conversion (38) occurred prior to deposition in the tissue. The detergent was necessary for solubilization of collagen XVII from the cells, since no collagen XVII was extractable with phosphate-buffered saline or 0.5 M NaCl, 0.1 M Tris, pH 7.4, or 0.1 M acetic acid (data not shown).

The transmembrane location of collagen XVII was verified biochemically using keratinocyte cell surface biotinylation and subsequent immunoprecipitation with antibodies to the endodomain and ectodomains. Specifically, semi-confluent keratinocytes were biotinylated under conditions that preserved cell integrity to allow for labeling of the putative extracellular domain only. The cells were then extracted with a neutral buffer containing 1% Nonidet P-40, collagen XVII was immunoprecipitated from the extract, and the precipitates were visualized with streptavidin as a marker. Both antibodies SA 3485 and Col17ecto-1 precipitated a biotinylated 180-kDa band (Fig. 2B), demon-
Stratifying that the polypeptide recognized by them contained a biotinylated extracellular domain.

Isolation of the Non-collagenous and Collagenous Domains—

For characterization of the domain structure, native collagen XVII was subjected to limited proteolytic digestions. Treatment with highly purified bacterial collagenase yielded a band of approximately 65 kDa on SDS-PAGE under reducing conditions (Fig. 3A), corresponding to the fragment predicted from the cDNA sequence (2). Under non-reducing conditions, the collagenase-resistant fragment migrated with an apparent molecular mass of 170 kDa (Fig. 3, lane 2); digestion for 6 h with additional 90-kDa fragment (lane 3), and digestion for 24 h with a single pepsin-resistant fragment of about 90 kDa (lane 4, arrow). Tryptic digestion under conditions in which globular sequences are preserved yielded several intermediate products. After 2 h of digestion, 140-, 120-, and 105-kDa fragments (lane 3); after 4 h, 120- and 105-kDa fragments (lane 4), and after 24 h, one pepsin-resistant fragment of 90 kDa (lane 5) were identified with the antibody IF 77/95. Note that the migration of the pepsin fragments was identical under reducing and non-reducing conditions. Molecular sizes, as determined by marker proteins of 200, 112, 80, and 50 kDa, are indicated.

Limited pepsin and/or trypsin digestion was used to isolate the collagenous domain, since triple-helical structures resist proteolysis under conditions in which globular sequences are digested (35). Incubation of collagen XVII with 1 μg/ml pepsin at 5 °C for 2 h was sufficient to digest the endodomain. During an extended incubation, the digestion proceeded in a stepwise manner, yielding several intermediate products with apparent sizes of 140, 120, and 105 kDa, and a final pepsin-resistant fragment of about 90 kDa, both under reducing and non-reducing conditions (Fig. 3, B and C), demonstrating that the collagenous domain did not contain disulfide bonds. Limited trypsin digestion also resulted in a 90-kDa fragment (Fig. 3B, lane 5), indicating that the collagenous domain was triple-helical. The NH₂-terminus of the pepsin/trypsin-resistant fragments extended to the NC16a domain, since the antibody IF 77/95 recognized all intermediate fragments. In contrast, antibody Col17ecto-1 raised against the 205 most COOH-terminal amino acid residues of collagen XVII reacted only with the 140-, 120-, and 105-kDa fragments, but not with the 90-kDa fragment (Fig. 4, lane 9), indicating that an extended pepsin incubation or trypsin treatment eliminated the distal ectodomain (compare with Fig. 1).

The Ectodomain Contains N-Linked Oligosaccharides—

Deglycosylation with N-glycosidase F resulted in faster migration of the 180-kDa a1(XVII) chain on SDS-PAGE (Fig. 4, lanes 1 and 2). When collagenase digestion preceded deglycosylation, no difference in the molecular mass of the endodomain was noted (Fig. 4, lanes 3 and 4). In contrast, differences emerged when the extracellular domain was deglycosylated: shifts in migration of the 140- and 120-kDa, but not of the 105- and 90-kDa pepsin fragments were observed (Fig. 4, lanes 5–8). These findings are consistent with N-glycosylation of the -N-V-T- site in the distal COOH terminus (Asn-1421, numbered according to Giudice et al.; Ref. 2) of collagen XVII. This site lies within the segment recognized by the antibody Col17ecto-1, which is eliminated during extended pepsin digestion (Fig. 4, lane 9).

Stability of the Collagenous Ectodomain—

The thermal stability of the extracellular domain of collagen XVII was assessed with trypsin or sequential pepsin/trypsin digestions as probes for the triple-helical conformation (35, 36). Native collagen XVII in the cell extracts was first subjected to pepsin treatment at 5 °C for 2 h to remove the globular domain, followed by a trypsin probing for 2 min at temperatures between 15 and 47 °C. Alternatively, the pepsin step was omitted. The resulting 90-kDa digestion product remained stable between 15 and 37 °C, but melted between 38 and 44 °C, as assessed by immunoblotting with the antibody IF 77/95 (Fig. 5). Quantitation of the scanned immune signals determined that the extracellular alignment was lost to about 50% at 41.5 °C at neutral pH under the buffer conditions used.

Second Form of Collagen XVII: A Soluble Ectodomain—

To test for the presence of collagen XVII in cell culture media, concentrated keratinocyte medium was immunoblotted with collagen XVII antibodies. The antibodies Col17ecto-1 and IF 77/95 both showed reactivity with a 120-kDa polypeptide (Fig. 6), but no signal was obtained with the antibody SA 3485. For further characterization, the immunoreactive medium mate-
keratinocyte medium concentrate used for immunoblotting (Fig. 6) was derived from 10 ml of medium in a 50-cm² monolayer culture. In comparison, the maximal amount of epidermis extract that could be loaded onto SDS-PAGE corresponded to 0.04 cm² of skin surface. Presumably as a result of this quantitative difference, the 120-kDa polypeptide was not detected in standard epidermis extracts.

Collagen XVII Nullizygote Cells Lack the Soluble Form—Mutant keratinocytes from a patient with generalized atrophic benign epidermolysis bullosa were investigated for the presence of the 180- and 120-kDa polypeptides. The patient carried a homozygous deletion 522delAG in the COL17A1 gene, which led to a frameshift and a premature termination codon. The mutation caused nonsense-mediated mRNA decay and absence of collagen XVII in skin and keratinocytes in vitro (28). In contrast to normal controls, the immunoreactive 120-kDa polypeptide was not found in the medium of the mutant keratinocytes (Fig. 6A, lane 4). These findings are compatible with the prediction that the 120-kDa polypeptide is derived from the α1(XVII) chain.

Collagen XVII mRNA Expression in Keratinocytes—Northern blot analysis of mRNA from normal keratinocytes or HaCaT cells with collagen XVII cDNA revealed a 6-kb mRNA. An identical mRNA band hybridized with cDNA probes corresponding to the distal 5′ or to the distal 3′ end of the collagen XVII cDNA (Fig. 7), suggesting that the 180- and 120-kDa polypeptides were translated from the same mRNA transcript.

A Synthetic Furin Inhibitor Prevents Generation of Soluble Ectodomain—To investigate the possibility of specific proteolytic processing of collagen XVII ectodomain, normal keratinocytes were treated with proteinase inhibitors in vitro. The results obtained with 1–5 mM EGTA and 0.1–1 mM Pefabloc were ambiguous as a result of cytotoxic effects of the chemicals. However, a significant effect was noted with a synthetic inhibitor of proprotein convertases of the furin/PACE family (for review, see Ref. 39), decanoyl-RVKR-chloromethyl ketone (37). Because of its lipophilic structure, the compound can act both intra- and extracellularly and thus inhibit enzymes in both locations. Incubation of cultured normal keratinocytes with 100 μM decanoyl-RVKR-chloromethyl ketone for 20 h prevented the generation of the 120-kDa polypeptide (Fig. 8). This observation strongly suggested that the production of the 120-kDa polypeptides was, directly or indirectly, dependent on furin-mediated proteolytic processing.

DISCUSSION

Here, we show that human collagen XVII from epidermal keratinocytes is a homotrimeric transmembrane protein that occurs in two forms, as a full-length protein and as a soluble ectodomain. Biochemical and immunochemical analyses with
domain-specific antibodies demonstrated that the globular intracellular domain of the full-length protein is disulfide-linked, and that the collagenous extracellular domain is triple-helical and N-glycosylated. Previous investigations on bovine collagen XVII or recombinant fragments produced under culture conditions omitting ascorbic acid had provided indications for a longitudinal trimeric structure for collagen XVII in vitro (7, 8). The present experiments with collagen XVII synthesized in the presence of ascorbic acid to allow adequate prolyl and lysyl hydroxylation of the α1(XVII)-chains (5) demonstrated that the triple-helical ectodomain remains in a stable alignment and is resistant to proteolysis at temperatures above 40 °C, clearly higher than the physiological skin temperature of about 32 °C. Thus, the interrupted collagenous ectodomain is able to maintain flexibility of the protein for efficient ligand interactions at the skin basement membrane zone.

The importance of collagenous ectodomain sequences for ligand binding was also noted for other members of the family of membrane-associated collagenous proteins (40–42). In addition to collagen XVII, the group includes the macrophage scavenger receptors type I and type II, the B-chain of C1q complex and collagen XIII (for review, see Ref. 6). All of these have a carboxyl-terminal ectodomain with one or more triple-helical stretches. Since the macrophage scavenger receptors and C1q possess only a short triple-helix of 72–81 amino acid residues and do not exert structural functions, they have not been regarded as proper collagens. In contrast, collagen XIII is a structural protein with interesting analogies to collagen XVII. Both are epidermal collagens with a long collagenous ectodomain, and both colocalize with integrins, collagen XIII with α3β1 in focal contacts and collagen XVII with α6β4 in hemidesmosomes (10, 11). These features imply specific functions in epithelial cell adhesion for these molecules and suggest putative ligand interactions between the transmembrane collagens and the integrins in the basal keratinocytes.

Strikingly, keratinocytes and HaCaT cells secreted a shorter soluble triple-helical form of collagen XVII, in addition to maintaining the full-length transmembrane protein. Several lines of evidence support the hypothesis that the soluble form represents the ectodomain of collagen XVII. 1) Its apparent size of 120-kDa corresponds to the size of the ectodomain obtained by limited pepsin digestion of the full-length molecule; 2) it reacted with both the antibody Col17ecto-1 generated against the 205 most carboxyl-terminal amino acids of collagen XVII and the antibody IP 77/95 recognizing the NC16a-domain adjacent to the transmembrane domain; 3) it was not reactive with antibody SA 3485 to the intracellular domain of collagen XVII; 4) its sensitivity to collagenase, pepsin and N-glycosidase F was comparable to that of the extracellular domain of collagen XVII, both under reducing and non-reducing conditions; 5) it was not present in COL17A1 nullizygote keratinocyte cultures devoid of the α1(XVII) chain, suggesting that the production of the 120-kDa polypeptide depended on the synthesis of the 180-kDa chain; 6) Northern blots with cDNA probes spanning the distal 5’ or 3’ ends of collagen XVII cDNA revealed an identical 6-kb mRNA, suggesting that both polypeptides were translated from the same mRNA transcript. In summary, these data argue for the existence of the soluble ectodomain of collagen XVII and are compatible with the prediction that it is proteolytically released from the cell surface.

There is a growing body of evidence about soluble forms of type I or type II integral transmembrane proteins, including cell adhesion proteins, growth factor and cytokine receptors, or receptor ligands. The secreted forms are derived by selective post-translational proteolysis from the cell surface (for review, see Ref. 43). The cleavage generally occurs close to the extracellular face of the membrane, releasing a physiologically active protein, and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. The enzymes have been only partially characterized, but many can be grouped as metallo- and/or serine proteinases (43). They are localized at the cell surface, or are themselves integral membrane proteins which after activation can act close to the cell membrane in the extracellular space. The details of the activation mechanisms remain elusive, but phosphol esters or proteolytic cleavage, e.g. by the ubiquitous proprotein convertases of the furin/PACE-family (for review, see Ref. 39) are known to activate cell surface associated metalloproteinases (44). In concert with this, nearly all cell types appear to express furin both intracellularly and at the cell surface (45).

Based on these considerations, we tested the effect of a synthetic furin inhibitor, decanoyl-RVKR-chloromethyl ketone (37), on the generation of the soluble form of collagen XVII. Because of its lipophilic nature, the compound acts both intracellularly and extracellularly and inhibits furin in both locations. It showed no cytotoxic effects on keratinocytes and had no influence on the synthesis of the full-length α1(XVII) chain. Instead, it prevented the generation of the 120-kDa polypeptide, suggesting that a furin-mediated proteolytic process was required for the release of the collagen XVII ectodomain. Two kinds of putative mechanisms seem feasible in this context. First, furin activates the genuine collagen XVII converting proteinase at the cell surface, or second, furin processes collagen XVII directly. For the genuine convertase, a spectrum of matrix metalloproteinases present as candidates. At least one such enzyme with a furin activation site, the membrane type matrix metalloproteinase 1 (MT1-MMP or MMP-14) is expressed in the skin (44). Another enzyme expressed in keratinocytes, the 92-kDa gelatinase-B (MMP-9) was able to degrade a recombinant NC16a-COL14 fragment of collagen XVII (46). The alternative that furin or another member of the enzyme family is the genuine convertase appears attractive since collagen XVII contains a tribasic furin/PACE cleavage motif, -R-I-R-R-, 14 residues from the N-terminal end of the mature protein, and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. The enzymes have been only partially characterized, but many can be grouped as metallo- and/or serine proteinases (43). They are localized at the cell surface, or are themselves integral membrane proteins which after activation can act close to the cell membrane in the extracellular space. The details of the activation mechanisms remain elusive, but phosphol esters or proteolytic cleavage, e.g. by the ubiquitous proprotein convertases of the furin/PACE-family (for review, see Ref. 39) are known to activate cell surface associated metalloproteinases (44). In concert with this, nearly all cell types appear to express furin both intracellularly and at the cell surface (45).

Based on these considerations, we tested the effect of a synthetic furin inhibitor, decanoyl-RVKR-chloromethyl ketone (37), on the generation of the soluble form of collagen XVII. Because of its lipophilic nature, the compound acts both intracellularly and extracellularly and inhibits furin in both locations. It showed no cytotoxic effects on keratinocytes and had no influence on the synthesis of the full-length α1(XVII) chain. Instead, it prevented the generation of the 120-kDa polypeptide, suggesting that a furin-mediated proteolytic process was required for the release of the collagen XVII ectodomain. Two kinds of putative mechanisms seem feasible in this context. First, furin activates the genuine collagen XVII converting proteinase at the cell surface, or second, furin processes collagen XVII directly. For the genuine convertase, a spectrum of matrix metalloproteinases present as candidates. At least one such enzyme with a furin activation site, the membrane type matrix metalloproteinase 1 (MT1-MMP or MMP-14) is expressed in the skin (44). Another enzyme expressed in keratinocytes, the 92-kDa gelatinase-B (MMP-9) was able to degrade a recombinant NC16a-COL14 fragment of collagen XVII (46). The alternative that furin or another member of the enzyme family is the genuine convertase appears attractive since collagen XVII contains a tribasic furin/PACE cleavage motif, -R-I-R-R-, 14 residues from the N-terminal end of the mature protein, and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. The enzymes have been only partially characterized, but many can be grouped as metallo- and/or serine proteinases (43). They are localized at the cell surface, or are themselves integral membrane proteins which after activation can act close to the cell membrane in the extracellular space. The details of the activation mechanisms remain elusive, but phosphol esters or proteolytic cleavage, e.g. by the ubiquitous proprotein convertases of the furin/PACE-family (for review, see Ref. 39) are known to activate cell surface associated metalloproteinases (44). In concert with this, nearly all cell types appear to express furin both intracellularly and at the cell surface (45).
membrane proteins, the generation of a soluble ectodomain may be a process for rapidly down-regulating the protein from the cell surface. Alternatively, generation of a soluble form of the protein that has properties either identical with, or subtly different from those of the membrane bound form may be a way to fine-regulate signal transduction and/or cell attachment to the basement membrane during proliferation and differentiation of the epidermis. The soluble form was not detected in the epidermis with the present immunoblotting experiments. This is not, however, an indication of its lack from the dermo-epidermal junction, but the absence rather has quantitative reasons; large amounts of protein-rich epidermal extracts cannot be loaded onto SDS-PAGE, in contrast to medium concentrates with a low protein content. Future experiments with purification of the soluble ectodomain from large epidermal sheets will demonstrate ist presence in the skin in vivo.

Possibly, the soluble ectodomain of collagen XVII also has clinical relevance in human autoimmune blistering diseases. Using autoantisera from patients with bullous pemphigoid and linear IgA dermatosis, previous studies identified 97- and 120–125-kDa basement membrane proteins as autoantigens in skin and keratinocytes (47–49). A 120-kDa secreted keratinocyte protein, coined LAD-1, was defined using linear IgA dermatosis sera and a monoclonal antibody (47, 50). However, lack of systematic cross-reactivity of the human autoantisera or the monoclonal antibodies with the 97- and 120-kDa autoantigens and with collagen XVII have impeded the characterization of the proteins in question. Interestingly, a very recent study reported that the 97-kDa linear IgA bullous disease antigen isolated from the epidermis showed partial amino acid sequence identity with the extracellular domain of collagen XVII (51). Future investigations must reveal the molecular proper-