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C-terminal Truncation Impairs Glycosylation of Transmembrane Collagen XVII and Leads to Intracellular Accumulation*

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Collagen XVII, a type II transmembrane protein in hemidesmosomes, is involved in the anchorage of stratified epithelia to the underlying mesenchyme. Its functions are regulated by ectodomain shedding, and its genetic defects lead to epidermal detachment in junctional epidermolysis bullosa (JEB), a heritable skin fragility syndrome, but the molecular disease mechanisms remain elusive. Here we used a spontaneously occurring homozygous *COL17A1* deletion mutant in JEB to discern glycosylation of collagen XVII. The mutation truncated the distal ectodomain and positioned the only *N*-glycosylation site 34 amino acids from the newly formed C terminus, which impaired efficient *N*-glycosylation. Immunofluorescence staining of authentic JEB keratinocytes and of COS-7 cells transfected with the mutant indicated intracellular accumulation of collagen XVII precursor molecules. Cell surface biotinylation and quantification of ectodomain shedding demonstrated that only about 15% of the truncated collagen XVII reached the cell surface. The cell surface-associated molecules were *N*-glycosylated in a normal manner, in contrast to the molecules retained within the cells, indicating that *N*-glycosylation of the ectodomain is required for targeting of collagen XVII to the plasma membrane and that reduced accessibility of the *N*-glycosylation site negatively regulates this process. Functional consequences of the strong reduction of collagen XVII on the cell surface included scattered deposition of cell adhesion molecule laminin 5 into the extracellular environment and, as a consequence of faulty collagen XVII-laminin ligand interactions, aberrant motility of the mutant cells.

Collagen XVII belongs to the family of collagenous transmembrane proteins, which function both as cell membrane receptors and as extracellular matrix components and are involved in a broad spectrum of biological events, ranging from cell adhesion to host defense (1). The group members typically are trimers of identical polypeptides, α-chains, which contain an N-terminal intracellular domain, a single transmembrane stretch, and a large extracellular C terminus with one or more collagenous subdomains, *i.e.* a structure characteristic of type II transmembrane proteins. The ectodomain is shed from the cell surface by metalloproteinases of the ADAM (a disintegrin and metalloprotease domain) family to yield a shorter form of the molecule, which remains stable in the extracellular space after the release (2). As a component of the hemidesmosomes, collagen XVII is involved in the anchorage of stratified epithelia in the skin, the mucous membranes, or the eye to the underlying mesenchyme.

Mutations in the collagen XVII gene, *COL17A,* cause JEB, a genodermatosis characterized by mechanically induced detachment of the epidermis from the dermis. To date, over 30 different *COL17A1* mutations have been reported (Human Gene Mutation Database, Cardiff University; Refs. 3 and 4). Most are null mutations resulting in premature termination codons and, subsequently, in nonsense-mediated mRNA decay, whereas some missense mutations and small deletions have been reported (5–10).

Bioinformatics predict four potential *N*-glycosylation sites within the amino acid sequence of collagen XVII, three in the endodomain and one in the ectodomain, 77 amino acids proximally from the C terminus. We have previously shown that this latter site, an Asn-Val-Thr sequon, is *N*-glycosylated (11). *N*-Glycosylation is catalyzed within the lumen of the endoplasmic reticulum (ER)[2] by the translocon-associated multisubunit enzyme complex oligosaccharyltransferase (OST) and involves the transfer of oligosaccharides to an Asn residue in the sequon Asn-*X*-(Thr/Ser) (12, 13). The oligosaccharides are needed for proper folding and stabilization of the protein, which is impor-

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[2] The abbreviations used are: ER, endoplasmic reticulum; JEB, junctional epidermolysis bullosa; KGM, keratinocyte growth medium; OST, oligosaccharyltransferase; PBS, phosphate-buffered saline; PNGase, *N*-glycosidase F; HaCaT, human adult low calcium high temperature keratinocytes.

tant for correct intracellular transport and targeting, such as the cell surface integration of type II transmembrane proteins (14).

Apart from the sequence in and around the sequon, the efficiency of *N*-glycosylation in the ER lumen is greatly influenced by the accessibility of the sequon to the lumenally orientated active site of OST. Studies utilizing *in vitro* transcription/translocation systems showed that *N*-glycosylation efficiency decreased significantly when the sequon was fewer than 60 residues from the C terminus (15, 16). Because the distance between the ribosome P-site and OST active site is estimated to be around 65 residues, inefficient *N*-glycosylation was presumed to be due to the sequon failing to reach the active site of OST prior to dissociation of the polypeptide from the ribosome (15, 16).

Here we provide molecular evidence that truncation of the distal C terminus of collagen XVII impairs its *N*-glycosylation. We describe a C-terminal deletion mutant of collagen XVII in JEB, in which the Asn-Val-Thr sequon is located only 34 amino acids from the newly formed C terminus. This leads to accumulation of most of the mutated collagen XVII within the cell and its proteasomal and/or lysosomal degradation. Only about 15% of the mutated collagen XVII reached the cell surface, which is not sufficient to ensure proper anchorage of keratinocytes to the basement membrane.

EXPERIMENTAL PROCEDURES

COL17A1 Mutation Detection—Following informed consent, EDTA-blood samples were obtained from the index patient and her parents. Genomic DNA was extracted using a QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The entire coding region of *COL17A1* (GenBank™ accession number M91669) and the exon/intron boundaries were amplified from genomic DNA by PCR, using primer pairs as reported by Gatalica *et al.* (17). Primer sequences for exon 54 and 55 were 5′-TTGATGTCCCAAGCTTCCAG-3′ and 5′-CTAATGAGCGTCAGCCTTGC-3′, giving rise to a 416-bp product. Direct sequencing of PCR products in both directions was performed using standard methods. Samples were submitted to automated sequencing in an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Darmstadt, Germany). DNA sequences were compared with the reference sequence from the NCBI Entrez Nucleotide data base.

Cell Cultures and Transient Transfections—Skin biopsies of the patient were obtained shortly after birth and at the age of 1.5 years. Keratinocytes were obtained by trypsinization of control and the JEB skin biopsies and cultured in serum-free, low calcium keratinocyte growth medium (KGM; Invitrogen, Karlsruhe, Germany) for 2–6 passages as described (18). 48 h before extraction or staining, 50 μg of L-ascorbate/ml were added to the cultures. HaCaT keratinocytes were cultured in serum-free KGM as described previously (11). COS-7 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum at 37 °C. COS-7 cells were seeded to 90% confluence on 6-well plates and on the next day, transiently transfected with 0.5 μg of wild-type or mutant collagen XVII cDNA/well and Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen). Prior to the measurements, the cells were incubated with 50 μg/ml L-ascorbate for 48 h to allow for full prolyl and lysyl hydroxylation of newly synthesized collagens. For analysis of glycosylation, HaCaT keratinocytes or transfected COS-7 cells were seeded in medium containing 25 or 100 nM tunicamycin and subsequently cultured for 48 h. After washing twice with PBS, the cells were further grown in serum-free keratinocyte basal medium or Dulbecco's modified Eagle's medium supplemented with the previously used tunicamycin concentrations for 5 h to determine the ectodomain release (shedding assay).

mRNA Analysis—mRNA was isolated from JEB and control keratinocytes with Oligotex Direct mRNA mini kit (Qiagen) as described by the manufacturer. For semiquantitative reverse transcription-PCR, the Titan One tube reverse transcription-PCR Kit (Roche Applied Science, Mannheim, Germany) was used with 30 ng of mRNA as a template and primer sets specific for human glyceraldehyde-3-phosphate dehydrogenase (sense primer, 5′-gga gcc aaa agg gtc atc atc tc-3′; antisense primer, 5′-gtc atg agt cct tcc acg ata cc-3′) and collagen XVII (sense primer, 5′-cag cgg gaa ggt ctt tac agc c-3′; antisense primer, 5′-cac ttc cac cag ctg cag ca-3′). Amplification was done using 30 cycles with denaturation at 94 °C for 30 s, annealing for 30 s at 60 °C, and extension at 68 °C for 45 s. The densities of the fractionated PCR products were analyzed using Gel-Pro Express V. 4.0 software (Media Cybernetics Inc., Gleichen, Germany).

Immunofluorescence—Immunofluorescence staining of human skin and keratinocytes was performed with standard techniques (2, 19, 20) using polyclonal antibodies against the NC16A domain and the distal C terminus of collagen XVII (2) and mouse monoclonal antibody BM165 against the human laminin α3 chain (a gift from Dr. R. E. Burgeson, MGH/Harvard Cutaneous Biology Research Center, Boston, MA). Transfected COS-7 cells were cultured on coverslips and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and stained with the NC16A antibody. The secondary antibodies were Cy3- and fluorescein isothiocyanate-labeled species-specific immunoglobulins (Dianova, Hamburg, Germany and Sigma, Deisenhofen, Germany, respectively). Fibrillar actin was stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma). Single stainings were observed with an Axiophot microscope equipped with an Axio Cam MRc digital camera. Double stainings were analyzed by laser scanning confocal microscopy (Leica, Heidelberg, Germany). Images of horizontal and vertical sections were captured with single channel excitation using the Leica software. PhotoShop (Adobe) was used for image superimposition.

Time-lapse Videomicroscopy and Image Analysis—Keratinocytes were seeded in the center of wells (10-μl drop/well, 24-well plates, Costar) in KGM supplemented with laminin 5 (2.5 μg/ml) to allow rapid cell adhesion. After 4 h, the cells were washed with PBS, and the wells were filled with fresh KGM. 12 h later, cell movement was recorded in a thermally controlled chamber (37 °C, 5% CO_2) placed on an inverted microscope (Axiovert S100TV, Zeiss) equipped with a digital CCD camera (Xillix MicroImager, Richmond, British Columbia, Canada). Phase contrast photographs were automatically captured every 5 min for 800 min and stored with the Openlab software system

(Improvision, Coventry, UK.). The sequences of images were converted to QuickTime movies, and migration tracks were analyzed using the Dynamic Image Analysis System software (Solltech Inc., Oakdale, IA). Extracted migration parameters included cell velocity (speed in μm/min) and processive index defined by the ratio between the linear and the absolute distances covered by a cell during the time of recording.

Immunodetection of Proteins—For immunoblotting, keratinocytes were extracted with a buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and proteinase inhibitors (6, 11), and the medium proteins were precipitated with chloroform/methanol (2, 11). The protein content was determined with micro Lowry assay (DC protein detection kit, Bio-Rad, Germany) using bovine serum albumin as standard. Normalized quantities of the proteins were subjected to immunoblotting with the following polyclonal antibodies: Ecto-5, recognizing epitopes within the 50 most C-terminal amino acids of collagen XVII, and the antibody NC16A (8, 11, 19).

Recombinant C-terminally Deleted Collagen XVII—The C-terminal deletion construct with 18 novel amino acids followed by a stop codon was generated using the full-length cDNA for human collagen XVII cloned into the NotI site of pcDNA3 (Invitrogen, San Diego, CA) and the GeneTailor site-directed mutagenesis system (Invitrogen Europe, Leek, The Netherlands). In the first mutagenesis reaction, the original amino acids ^{1450}GPKGDR^{1455} were replaced with SRTQR-STOP using the primers 5′-GGAGAGATGGGCACTCCATTCCAGGACCCAAAGGTGAGGCCCTGCTGGG-3′ and 5′-TGGAGTGCCCATCTCTCCTTTTTGCCCAGG-3′ (the codons of the novel amino acids underlined). Using the first mutated construct as a template, the amino acids ^{1444}GEMGTP^{1449} were replaced with KRRDGH using the primers 5′-GGACCCCCTGGGCAAAAAAAAAGGAGAGATGGGCACTCCAGGACCCAA-3′ and 5′-TTTTTGCCCAGGGGGGTCCTTGAATGGCTCC-3′. In the last mutagenesis reaction, the second mutated construct was used as a template to replace the amino acids ^{1437}QGPPGQK^{1443} with HSRTPWA using the primers 5′-CAAACTTATGGAGCCATTCATTCAAGGACCCCCTGGGCAAAAAGGAGAGAT-3′ and 5′-AATGGCTCCATAAGTTTGGAAGAAGTCCAT-3′. The PCR products were purified using QIAquick gel extraction kit (Qiagen, Hildesheim, Germany). The resulting deletions and all amplicons were confirmed by DNA sequencing. The *N*-linked glycosylation consensus site in collagen XVII was disrupted by site-directed mutagenesis. Therefore, the asparagine in ^{1421}NVT^{1423} was converted into threonine in ^{1421}TVT^{1423} using the QuikChange II site-directed mutagenesis kit (Stratagene, Germany). For transfection, the COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Semiconfluent cells were transfected with the Lipofectamine 2000 (Invitrogen) using 10 μg of DNA/75-cm^2 culture flask. Transfected cells were grown in serum-free medium containing 50 μg/ml L-ascorbate, which was added every 24 h to allow hydroxylation of collagen and proper triple-helix formation. The media were collected 48 h after transfection and processed, in parallel with the cell layers, as described (11).

Surface Biotinylation—Transiently transfected COS-7 cells were cultured with 50 μg/ml of L-ascorbate for 48 h, washed extensively, and surface-biotinylated with D-biotinoyl-ϵ-aminocaproic acid *N*-hydroxysuccinimide ester according to the manufacturer's recommendations (Roche Applied Science). After extensive washes with PBS for five times, the cells were extracted with a buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and proteinase inhibitors (6, 11). The extracts were immunoprecipitated with collagen XVII antibody Endo-2 (2), and the precipitates were immunoblotted both with the NC16A antibody, to detect total collagen XVII, and with a streptavidin-coupled alkaline phosphatase (Sigma) to detect biotinylated collagen XVII. The software TotalLab1D V1.00 from Phoetix (Biostep GmbH Jahnsdorf, Germany) was used for semiquantitative analysis of the signals.

Deglycosylation—For removal of *N*-linked carbohydrate residues, samples of cell lysate or concentrated cell medium containing 50 μg of protein were treated with 10% β-mercaptoethanol for 10 min at 100 °C prior to digestion with 10 units/ml *N*-glycosidase F (PNGase F) or 5 units/ml endoglycosidase H overnight at 37 °C (New England Biolabs).

RESULTS

Mutation Analysis Reveals a C-terminal Deletion of Collagen XVII Ectodomain—After diagnostic antigen mapping had suggested JEB and collagen XVII abnormalities in a newborn female with mild trauma-induced acral skin blistering and slight involvement of the nails (Fig. 1*A*), *COL17A1* mutation analysis was performed. Sequencing of all 56 *COL17A1* exons revealed a homozygous duplication of four nucleotides in positions 4305–4308 in exon 54 (Fig. 1*B*), designated as c.4305_4308dupCATT (nucleotide +1 is the A of the ATG-translation initiation codon). The duplication resulted in a frameshift, starting with codon 1437, and a premature termination codon 18 codons downstream. These changes generated a new sequence of 18 amino acids, which replaced the 61 most C-terminal amino acids of the ectodomain in the truncated protein (Fig. 1*C*). In contrast to many mutations leading to premature termination codons, this one did not cause significant nonsense-mediated mRNA degradation. Reverse transcription-PCR demonstrated the presence of collagen XVII mRNA, nearly at the same level as in the controls (not shown).

Expression of Truncated Collagen XVII in JEB Keratinocytes—The presence of truncated collagen XVII was shown first by immunofluorescence staining of the skin of the patient. Antibodies to the juxtamembranous NC16A domain of collagen XVII showed a positive, yet moderately weaker, staining of the basement membrane than in normal skin (Fig. 2*A*, *upper panels*). In contrast, Ecto-5, an antibody directed against the distal C-terminal ectodomain, yielded no signal in JEB skin, indicating that a deleted molecule was present *in situ* (Fig. 2*A*, *lower panels*). To obtain additional evidence for synthesis of truncated collagen XVII, keratinocytes were isolated from the skin of the patient and cultivated. Immunoblot analysis of cell extracts and media was used to analyze collagen XVII expression (Fig. 2*B*). Antibodies to the NC16A domain recognized the

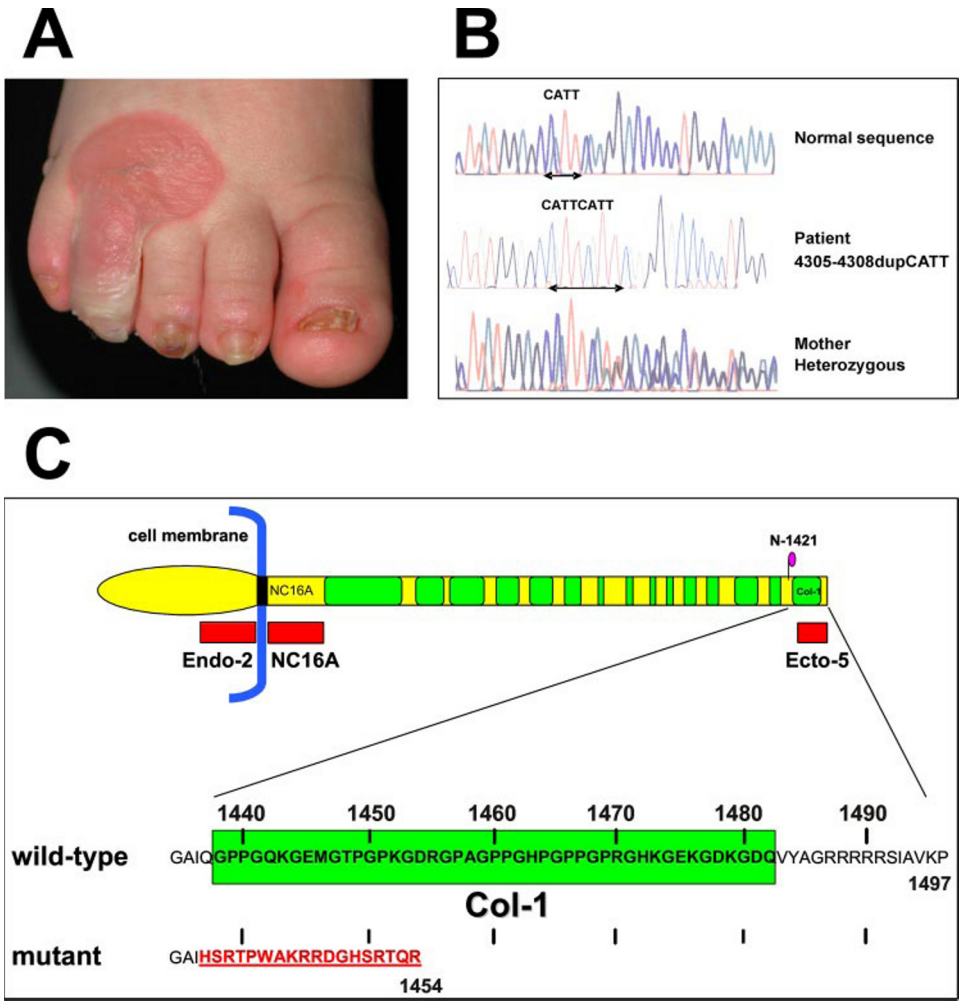

FIGURE 1. **A homozygous duplication in the *COL17A1* gene caused C-terminal truncation of collagen XVII.** *A*, clinical phenotype of the JEB patient. The female proband, offspring of consanguineous healthy parents, showed postnatal skin blistering. At the age of 18 months, blisters occurred on mechanically stressed regions, especially on hands and feet; also, toenail dystrophy was present. *B*, sequence analysis of the DNA of the patient disclosed in exon 54 of the *COL17A1* gene a homozygous duplication of four nucleotides (CATT), starting at nucleotide position 4305. It resulted in a frameshift and a premature termination codon, 18 codons downstream. Thus, the 61 most C-terminal amino acids of the collagen XVII ectodomain were deleted. *C*, schematic representation of transmembrane collagen XVII, with a globular endodomain, a short transmembrane stretch, and a large rod-like ectodomain, which contains 15 collagenous subdomains (*green*) separated by short non-collagenous sequences (*yellow*). An Asn-Val-Thr sequon for *N*-glycosylation is found at amino acid position 1421. The *red boxes* represent the epitopes of the domain-specific antibodies Endo-2, NC16A, and Ecto-5. Ecto-5 was raised against the 50 most C-terminal amino acids. In the *lower panel*, the distal part of collagen XVII ectodomain, including the NC 1 and Col-1 domain, is shown as an amino acid sequence; the Col-1 domain is highlighted in *green*. The sequence of the truncated collagen XVII is displayed below, and the sequence of the 18 amino acids generated by the frameshift is shown as *underlined red characters*.

180-kDa full-length transmembrane molecule and the shed 120-kDa form in control keratinocytes. In JEB keratinocytes, truncated collagen XVII was found, with an ~10-kDa reduction in molecular mass, *i.e.* 170 kDa for the transmembrane form and 110 kDa for the shed ectodomain (Fig. 2*B*, *upper panel*). The truncated polypeptides were recognized with the NC16A antibody, but not with Ecto-5, indicating that the distal C terminus of collagen XVII was deleted. In addition to the reduction in molecular mass, the amount of the shed form of collagen XVII was significantly reduced in JEB cells (Fig. 2*B*, *upper right blot*). Scanning densitometry of the blots revealed that in JEB cells, the quantity of shed collagen XVII was ~15% of the controls (Fig. 2*B*, *upper right panel*).

C-terminally Truncated Collagen XVII Accumulates within the Cell—Immunofluorescence staining of collagen XVII with the NC16A antibody showed a perinuclear/ER signal in control keratinocytes, whereas in JEB cells, the signal was distributed in the entire intracellular space (Fig. 2*C*). Moreover, a fraction of collagen XVII was distinctly co-localized with cortical actin in controls (Fig. 2*C*, *arrows*), indicating cell membrane targeting of collagen XVII. This was not the case in JEB cells (Fig. 2*C*). Together, these observations suggested that C-terminal deletion of collagen XVII leads to its intracellular accumulation. To verify this hypothesis, COS-7 cells were transiently transfected with a cDNA encoding wild-type human collagen XVII or the C-terminally truncated mutant. Subsequently, the surface of the transfected cells was biotinylated with a non-permeable reagent, sulfo-*N*-hydroxysuccinimide-biotin. This experiment revealed that only about 10–15% of mutant collagen XVII was present on the cell surface and that most of the mutant collagen XVII was not accessible to the biotinylation agent (Fig. 3*A*). Consistently with these findings, collagen XVII-overexpressing COS-7 cells contained the wild-type protein mainly in the perinuclear/ER area in immunofluorescence staining, whereas the C-terminally truncated collagen XVII accumulated within the cells, producing a patchy staining pattern (Fig. 3*B*).

C-terminal Truncation of Collagen XVII Impairs Co-translational N-Glycosylation—Collagen XVII has one actively used *N*-glycosylation site at position 1421, close to the distal C terminus (11). In the present mutant, the C-terminal deletion did not remove the 1421 Asn-Val-Thr sequon but reduced its distance from the C terminus from 77 to 34 amino acids (Fig. 4*A*). Since C-terminal truncation can alter co-translational *N*-glycosylation efficiency when the sequon is fewer than 60 residues from the C terminus (15, 16, 21), we determined whether intracellular accumulation and decreased cell surface targeting of mutant collagen XVII depended on its glycosylation state. Extracts of COS-7 cells transfected with wild-type or mutant collagen XVII were treated with PNGase F and subjected to immunoblot analysis. The 180-kDa wild-type col-

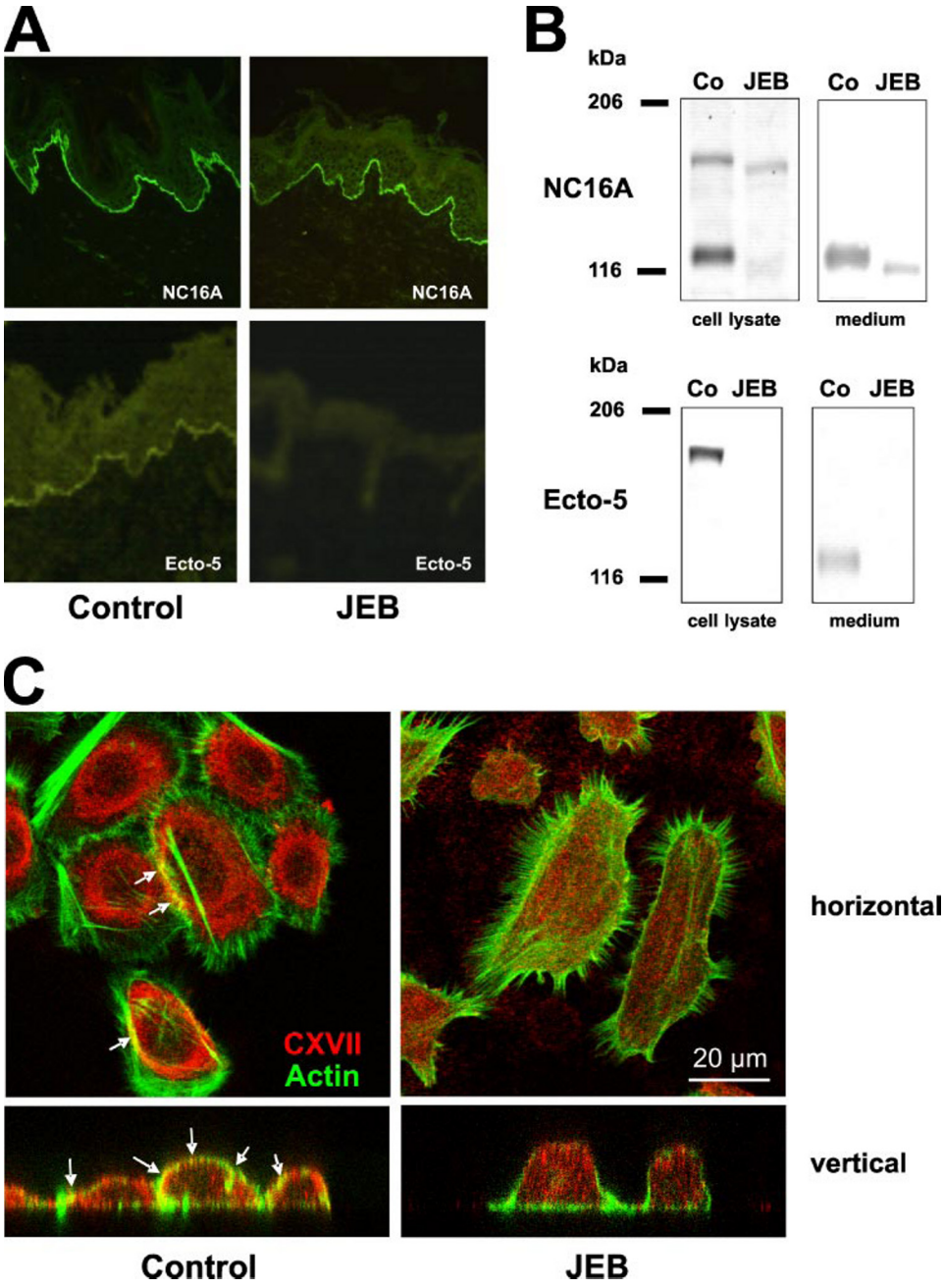

FIGURE 2. **C-terminally truncated collagen XVII (*CXVII*) in JEB skin and keratinocytes.** *A*, immunofluorescence staining of skin cryosections with domain-specific antibodies to collagen XVII. Staining of control skin with the antibody NC16A (*upper left panel*) and Ecto-5 (*lower left panel*) resulted in a strong signal along the dermal-epidermal junction. Staining of JEB skin revealed a positive, albeit weaker, signal with NC16A (*upper right panel*) and no staining with Ecto-5 (*lower right panel*), indicating that deleted collagen XVII was present *in situ*. *B*, immunoblot analysis of collagen XVII in cultured keratinocytes with antibodies NC16A (*upper panel*) and Ecto-5 (*lower panel*). Control keratinocytes (*Co*) contained both physiological forms of collagen XVII, the 180- kDa transmembrane and the 120-kDa shed ectodomain, in the cell lysate, and the 120-kDa shed ectodomain in the medium. In contrast, JEB keratinocytes contained only mutant collagen XVII, with the transmembrane form of ~170 kDa and the shed ectodomain of about 110 kDa (*upper panel*). Densitometric analysis showed that the intensity of the JEB ectodomain band was ~15% that of the control (*upper panel*). With Ecto-5 antibody, no signals were detected in JEB cell lysate or medium (*lower panel*). *C*, cultivated cells were double-labeled for fibrillar actin (*green*) and collagen XVII (*red*), and the stainings were analyzed by horizontal (*upper panels*) and vertical (*lower panels*) confocal sections. In control cells, the precursor products of collagen XVII have a restricted cytoplasmic localization, and a fraction co-localizes with cortical actin (*yellow*; *arrows*). In contrast, JEB cells accumulated the precursor products in the intracellular space, and there was no co-localization with cortical actin.

lagen XVII band was completely sensitive to digestion with PNGase F (Fig. 4*B*). A single band of about 175 kDa was apparent after removal of *N*-linked oligosaccharide side chains. In contrast, the double band at 170 kDa representing mutant collagen XVII yielded in a single band after incubation with PNGase, indicating that C-terminally truncated collagen XVII was partially glycosylated (Fig. 4*B*). In contrast, the ectodomain of collagen XVII released into the medium, both wild-type and mutant, was completely sensitive to digestion with PNGase F, as shown by a mobility shift of ~5 kDa (Fig. 4*B*). Both ectodomains were insensitive to endoglycosidase H digestion, indicating complex carbohydrate structures derived from Golgi transition (data not shown; Ref. 22).

Tunicamycin is an antibiotic and inhibitor of the initial *N*-glycosylation of proteins during the passage through the ER lumen (23, 24). The treatment was used to verify whether lack of glycosylation of collagen XVII is mainly responsible for the intracellular accumulation and diminished targeting to the cell surface. For this purpose, COS-7 cells transfected with wild-type collagen XVII were treated with 0.1 and 0.5 μM tunicamycin. The treatment strongly inhibited glycosylation of collagen XVII, as shown in Fig. 4*C*, *left panel*, for the transmembrane form. Concomitantly, the quantity of the shed ectodomain in the medium of tunicamycin-treated cells was significantly reduced (Fig. 4*C*, *right panel*). The same results have been observed with tunicamycin-treated HaCaT cells (not shown).

Since tunicamycin treatment also influences the glycosylation of other proteins that may have an effect on collagen XVII targeting, we generated a glycosylation-deficient collagen XVII mutant by substituting the critical Asn residue in amino acid position 1421 by a Thr (N1421T). Transfection of COS-7 cells with N1421T cDNA resulted in exclusive expression and intracellular accumulation of unglycosylated collagen XVII and no production of the shed ectodomain, as determined by Western blot analysis of the cell lysate and concentrated medium (Fig. 4*D*).

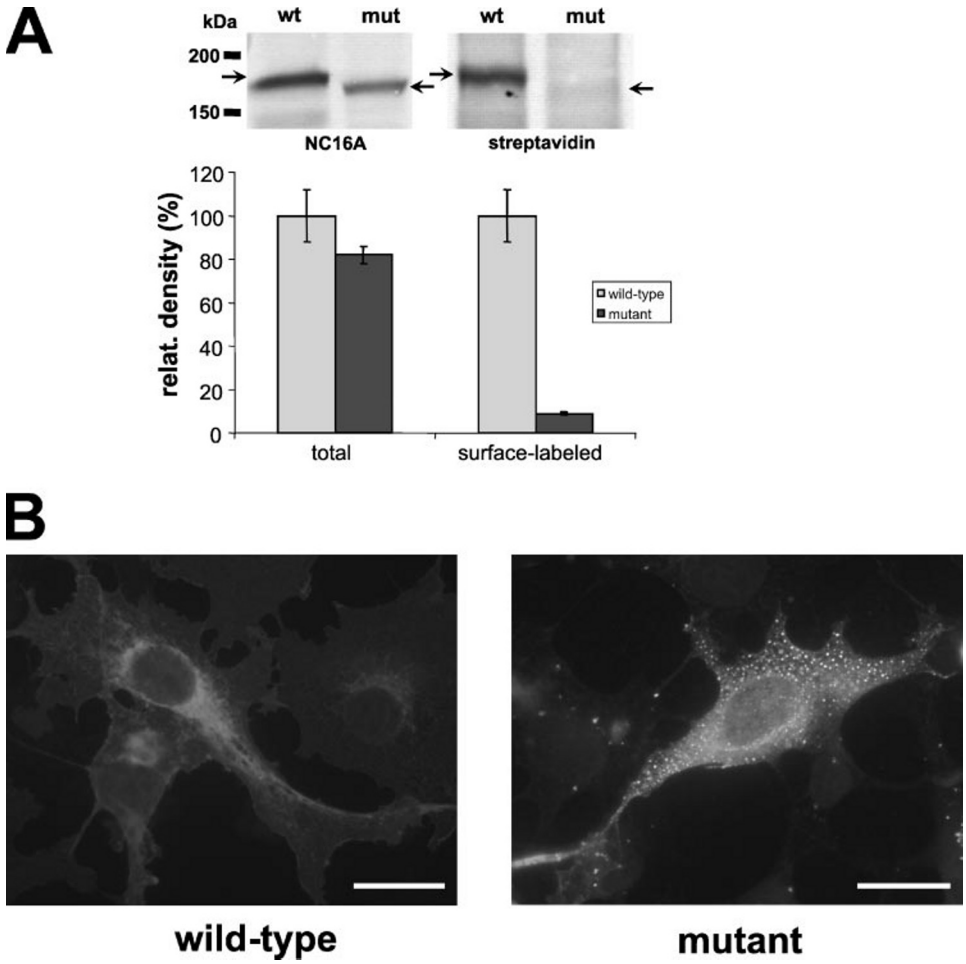

FIGURE 3. **Membrane integration and subcellular localization of collagen XVII in transfected COS-7 cells.** *A*, the relative (*relat.*) surface pools of wild-type (*wt*) and mutant (*mut*) collagen XVII expressed in COS-7 cells were determined by cell surface protein biotinylation. Transiently transfected COS-7 cells were cultured for 48 h, surface-biotinylated, and extracted, as described under "Experimental Procedures." After immunoprecipitation of the extracts with the antibody Endo-2, equal aliquots were immunoblotted with the antibody NC16A to determine total collagen XVII and with streptavidin-coupled alkaline phosphatase to detect biotinylated collagen XVII at the cell surface. Densitometric analysis demonstrated that the total amount of collagen XVII was only slightly less in the cells transfected with the mutant. However, significant differences were seen with the biotin-labeled fraction. Cells transfected with the mutant exhibited a significantly lower fraction of biotin-labeled collagen XVII. *B*, immunofluorescence staining of COS-7 cells transfected with wild-type (*left panel*) or mutant collagen XVII construct (*right panel*) with the NC16A antibody and fluorescein isothiocyanate-coupled secondary antibody. Fluorescence microscopy confirmed strong accumulation of the C-terminally truncated mutant collagen XVII precursor in the cells.

Abnormal Matrix Deposition and Motility of JEB Keratinocytes—To investigate whether lack of glycosylation and intracellular accumulation of truncated collagen XVII have functional consequences, we examined the motility of JEB keratinocytes and the deposition of laminin 5 and collagen XVII ectodomain in the extracellular milieu. Immunofluorescence staining showed that in cultures of control keratinocytes, laminin 5 and the shed ectodomain of collagen XVII were deposited in a concentric pattern in well circumscribed areas close to the cell bodies (Fig. 5*A*). In contrast, both proteins were deposited in a scattered manner, farther away from the cell bodies in cultures of JEB cells (Fig. 5*A*). Processing of laminin 5, which is needed for its integration in the basement membrane (25–27) was not altered in JEB keratinocytes, excluding the possibility that the scattered deposition of laminin 5 was caused by its deficient cleavage (not shown).

Analysis of cell motility by time-lapse videomicroscopy demonstrated that single cell tracks of control keratinocytes were rather linear, whereas JEB keratinocytes frequently changed direction of translocation (Fig. 5*B*, *colored trajectories* in *upper panels*). This was reflected by a significantly different ($p < 0.01$) mean processive index of 0.38 ± 0.17 ($n = 17$ cells) for the controls and of 0.23 ± 0.17 ($n = 18$ cells) for JEB keratinocytes. For an easier comparison, the net cell trajectories of wild-type and mutant keratinocytes were displayed as wind rose plots (Fig. 5*B*, *lower panels*), indicating that JEB cells characteristically moved with random directions, unlike the controls, which moved in a directed manner. The velocity of JEB and control keratinocytes were not significantly different (0.91 ± 0.23 and 0.95 ± 0.19 μm/min, respectively). Thus, lack of normal collagen XVII on the cell surface leads to loss of directed cell motility and abnormal deposition of extracellular adhesion molecules.

DISCUSSION

As a type II transmembrane protein, collagen XVII connects the intracellular keratinocyte intermediate filament network with the extracellular basement membrane zone. Its absence as a consequence of *COL17A1* null mutations leads to severe epidermal dysadhesion, whereas missense mutations often cause unfolding of the triple-helix, susceptibility to degradation by tissue proteinases, and functional insufficiency (8, 10). In this study, we analyzed a novel deletion, which was associated with mild skin blistering. A homozygous duplication in *COL17A1* resulted in replacement of the 61 most C-terminal amino acids of collagen XVII by a new sequence of 18 amino acids, eliminating the NC1 and Col-1 domains. Despite the premature termination codon caused by the nucleotide duplication, it did not lead to nonsense-mediated mRNA decay but allowed synthesis of truncated collagen XVII α-chains. Thus, the mutant was useful for assessing the functional role of the distal C terminus.

Bioinformatic predictions disclosed one potential *N*-glycosylation site at amino acid position 1421 in the C terminus of the molecule (11). The deletion reduces the distance of the site from the C terminus from 77 to 34 amino acids and concomitantly leads to a decrease in *N*-glycosylation. A similar effect has been described for the non-anchored prion

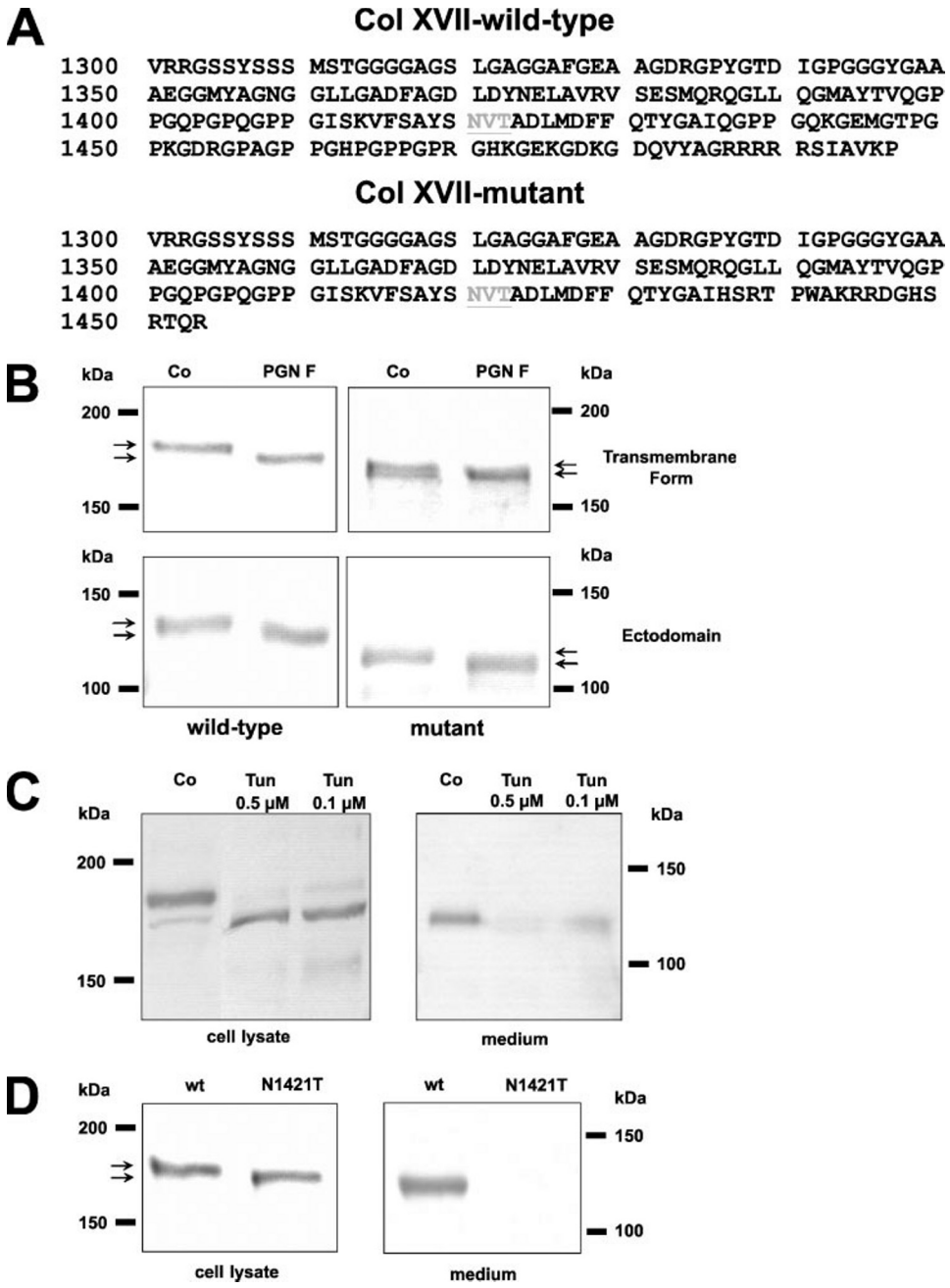

FIGURE 4. **Deletion of the distal C terminus of collagen XVII (*Col XVII*) impairs its *N*-glycosylation.** *A*, comparison of the amino acid sequences of the C termini of wild-type and mutant collagen XVII, starting with valine at position 1301. Wild-type collagen XVII contains one NVT sequon for *N*-glycosylation at positions 1421–23, 77 amino acids away from the distal C terminus. C-terminal deletion in the mutant collagen XVII does not alter the NVT sequon but shortens its distance to the C terminus from 77 to 34 amino acids. *B*, deglycosylation of collagen XVII with PNGase F (*PGN F*). Lysates of COS-7 cells transfected with either wild-type or mutant collagen XVII were treated with PNGase F before immunoblot analysis. The wild-type 180-kDa collagen XVII band was completely sensitive to PNGase F digestion and yielded a band of about 175 kDa (*upper left panel*, *arrows*). The double band at 170 kDa representing mutant collagen XVII yielded in a single band after incubation with PNGase, indicating that C-terminally truncated collagen XVII was partially glycosylated (*upper right panel*). In contrast, the shed ectodomain of both transfectants was sensitive to PNGase F digestion, as indicated by a motility shift of about 5 kDa (*lower panels*). *Co*, control cells. *C*, inhibition of *N*-glycosylation of normal collagen XVII by tunicamycin. Transiently transfected COS-7 cells were treated with 0.1 or 0.5 μM tunicamycin (*Tun*) for 48 h, washed twice with PBS, and cultured in serum-free medium for 5 h to determine their shedding activity. Cell lysates and concentrated media were analyzed by immunoblotting with the antibody Endo-2 for the transmembrane form and antibody NC16A for the ectodomain. Tunicamycin treatment inhibited glycosylation of the full-length collagen XVII molecules (*left panel*) and prevented ectodomain shedding in a concentration-dependent manner (*right panel*). *D*, immunoblot analysis of a *N*-glycosylation-deficient collagen XVII mutant. The *N*-linked glycosylation consensus site in collagen XVII was disrupted by site-directed mutagenesis of the critical asparagine residue ^{1421}NVT^{1423} into threonine ^{1421}TVT^{1423} (N1421T). COS-7 cells were transiently transfected with cDNA of wild-type and N1421T collagen XVII and cultured for 48 h. The shedding activity was determined after two PBS washing steps and a 6-h culture in serum-free medium. Cell lysates and concentrated media were analyzed by immunoblotting with the antibody Endo-2 for the transmembrane form and antibody NC16A for the ectodomain. The cells transfected with N1421T have exclusively expressed the unglycosylated collagen XVII form with a higher mobility when compared with the wild-type form (*left panel*) and failed to release any ectodomain into the culture medium (*right panel*).

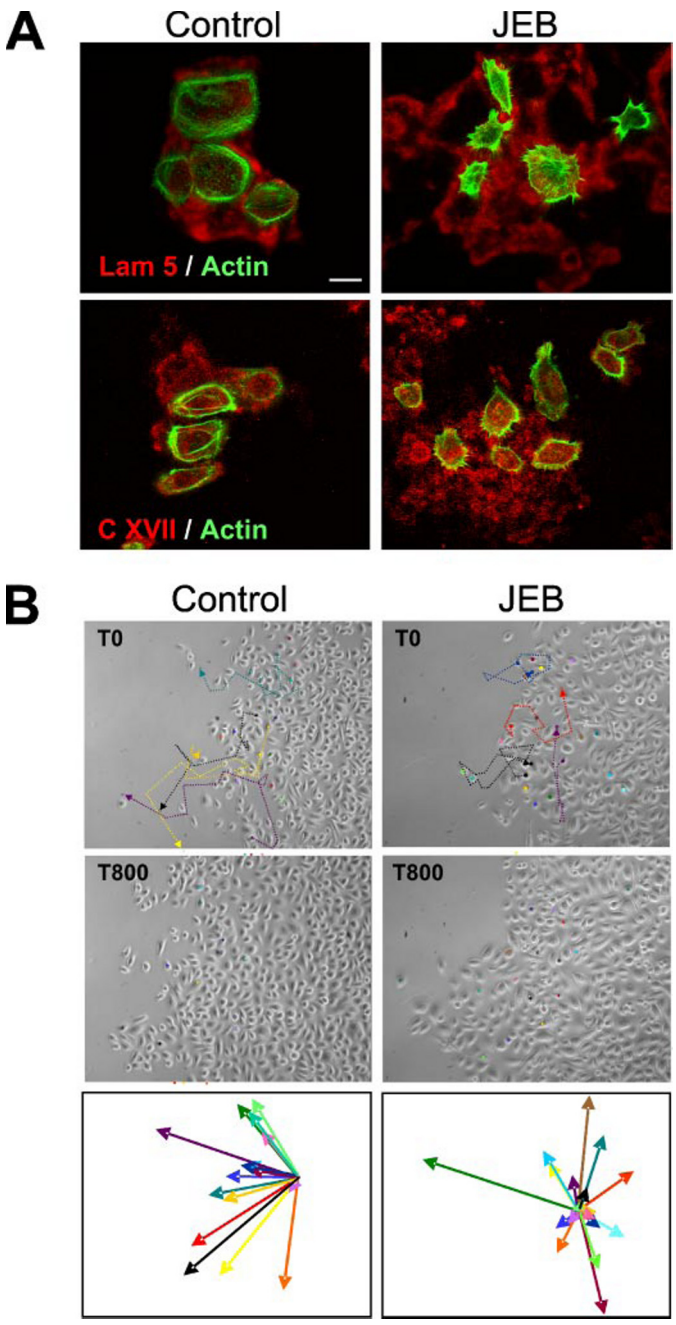

FIGURE 5. **Scattered matrix deposition and altered migration of JEB keratinocytes.** *A*, control (*left panels*) and JEB keratinocytes (*right panels*) were grown on glass coverslips for 24 h and processed for double immunofluorescence staining with fibrillar actin (*green*) to visualize the cell bodies and with laminin 5 (*Lam 5*) (*upper panels*) or collagen XVII (*C XVII*) (*lower panels*). Laser scanning confocal microscopy images recorded at the interface between cells and the culture support show that in cultures of control cells, laminin 5 and collagen XVII are deposited in a concentric manner underneath and in well limited areas in the vicinity of the cells (*left panels*). In contrast, laminin 5- and collagen XVII-rich prints are scattered on the culture support, away from JEB keratinocytes (*right panels*). *Bars*, 20 μm. *B*, 12 h after seeding as single colonies in the center of tissue culture wells; movement of keratinocytes was monitored for 800 min by time-lapse videomicroscopy. Phase contrast photographs of the cells are shown at the onset (*T0*) and end (*T800*) of the recordings. Tracks representing migration of single cells are drawn with *colored lines* on phase contrast images captured at T0 for control (*upper left panel*) and JEB keratinocytes (*upper right panel*). Wind rose plots (*lowest panels*) of the net linear translocations of single cells (linear distance covered between T0 and T800) show oriented and random movement for control and JEB keratinocytes, respectively.

protein in neuronal cells (21). In that case, *N*-glycosylation was abolished when the sequons were less than 60 residues from the C terminus. Initial *N*-glycosylation is essential for correct trafficking, folding, and sorting of a number of membrane proteins (14), and its lack leads to retention in the ER and to proteasomal degradation in association with disease processes. This has been demonstrated for membrane proteins, such as fukutin-related protein, PHEX, Slc11a1, or SURx in muscular dystrophy (28), X-linked hypophosphatemia (29), innate macrophage resistance to pathogen infections (30), or persistent hyperinsulinemic hypoglycemia of infancy (31).

In the case of collagen XVII, the importance of *N*-glycosylation for cell surface integration was confirmed by both mutagenesis of the *N*-glycosylation site and tunicamycin treatment. Both methods led to dramatically reduced glycosylation of collagen XVII and abolished the release of the ectodomain from the cell surface, indicating that non-glycosylated collagen XVII does not meet the ER criteria for further transit to Golgi. However, a minor fraction of mutated molecules in JEB cells must have been normally glycosylated since about 15% of mutant collagen XVII was found on the cell surface. This is also supported by the resistance of the mutant collagen XVII ectodomain against endoglycosidase H treatment, which indicates normal intracellular maturation of these molecules. Taken together, the results demonstrate that the *N*-glycosylation site in the C terminus of collagen XVII is important for the maturation and plasma membrane targeting of the molecule.

Mutations in the *COL17A1* gene result in weakening of the basal keratinocyte-basement membrane anchorage and tissue separation in JEB skin (32, 33). Since laminin 5 and collagen XVII are functional ligands, the amount of collagen XVII required for adequate epidermal-dermal adhesion in the skin poses an intriguing question. Here we demonstrate that about 15% of the normal amount is not sufficient. On the other hand, heterozygous carriers of *COL17A1* null mutations, who have about 50% of the normal collagen XVII levels in the skin, are clinically unaffected (3, 4), indicating that 50% of the normal level is sufficient for adequate stabilization of the dermal-epidermal junction. The molecular details and regulation of collagen XVII-laminin 5 ligand interactions are not yet fully understood. The interactions are likely to vary upon changing biological context, such as steady state adhesion *versus* proliferation and migration. One possibility is that collagen XVII is needed for correct organization of laminin 5 in the extracellular matrix (20). The scattered, poorly organized laminin 5 matrix observed here and, previously, with collagen XVII-deficient keratinocytes (20), favors this hypothesis. In addition, collagen XVII may coordinate keratinocyte behavior within a group of cells in certain situations, *e.g.* during wound healing. Using *in vitro* wounding assays, we previously reported that collagen XVII-deficient cells are hypermobile and close a scratch wound faster than controls (20). Conversely, wound closure is slower in the presence of exogenously added ectodomain of collagen XVII (2). Here we provide experimental evidence that diminished plasma membrane integration of collagen

XVII is associated with uncoordinated keratinocyte movements, an observation congruent with disturbed wound healing in JEB (34). Taken together, the findings underline the role of collagen XVII as a glycosylated cell surface protein and basement membrane ligand, which plays an important role in epidermal cell adhesion and motility.

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