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Context-Dependent Regulation of Collagen XVII Ectodomain Shedding in Skin

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Pemphigoid is a common autoimmune blistering disorder in which autoantibodies target transmembrane collagen XVII (COL17), a component of hemidesmosomes in basal keratinocytes. The ectodomain of COL17 can be cleaved from the cell surface within the juxtamembranous extracellular NC16A domain, and, interestingly, certain autoantibodies of pemphigoid patients preferentially react with the shed ectodomain. These findings suggest that COL17 ectodomain shedding generates neoepitopes on the shed form; however, the regulatory mechanism of the shedding in *in vivo* skin and the pathogenicity of the neoepitope-targeting antibodies still are uncertain. To address these issues, we produced rabbit antibodies specifically reacting with N-terminal cleavage sites of the shed COL17 ectodomain. The antibodies showed that certain amounts of the human COL17 ectodomain are cleaved physiologically at Gln⁵²⁵ in *in vivo* skin. In contrast, migrating human keratinocytes cleave COL17 at Leu⁵²⁴ but not at Gln⁵²⁵. The passive transfer of antibodies reacting with an N-terminal cleavage site of the mouse COL17 ectodomain into neonatal wild-type mice failed to induce blister formation, even though the antibodies bound to the dermal-epidermal junctions, indicating that cleavage site-specific antibodies have reduced or absent pathogenicity for blister formation. This study shows the ectodomain shedding of COL17 to be a physiological event in *in vivo* human skin that probably generates nonpathologic epitopes on the cleavage sites. (*Am J Pathol* 2015, 185: 1361–1371; <http://dx.doi.org/10.1016/j.ajpath.2015.01.012>)

Bullous pemphigoid (BP), a major pemphigoid subtype, is a common autoimmune blistering skin disease in which autoantibodies (autoAbs) target two hemidesmosomal components: transmembrane collagen XVII (COL17)/BP180 and the plakin family protein BP230.^{1–5} Previous studies have shown that COL17 is the major autoantigen not only for BP but also for other pemphigoid disorders, including linear IgA bullous dermatosis, mucous membrane pemphigoid, pemphigoid gestationis, and lichen planus pemphigoides.³ The major epitopes for BP autoAbs tightly cluster within the juxtamembranous noncollagenous NC16A domain of COL17, and the majority of IgG autoAbs react with this region.^{6,7} Passive transfer experiments of rabbit IgG⁸ and autoAbs from BP patients⁹ into wild-type (WT) or transgenic neonatal mice have shown pathogenic roles of Abs to the NC16A domain in blister formation.

COL17 is a type II–oriented transmembrane glycoprotein whose N-terminus and C-terminus are in the cytoplasm and the extracellular matrix (ECM), respectively.^{10,11} As is true for

other transmembrane collagen family members, the extracellular domain of COL17 can be cleaved from the cell surface *in vitro* by a disintegrin and metalloproteases 9, 10, and 17,^{12,13} and, interestingly, the ectodomain shedding occurs within the NC16A domain, which contains major epitopes for BP autoAbs.^{14,15} In addition, autoAbs from linear IgA bullous dermatosis¹⁶ and certain BP patients¹⁷ are known to react preferentially with the shed ectodomain of COL17, but do not react with the full-length form of COL17. This unique characteristic also was observed in several monoclonal Abs (mAbs) directing the ectodomain of COL17,^{11,18–20} suggesting that neoepitopes appeared on the shed ectodomain of COL17. Among them, mAb 123, which reacts specifically with the shed COL17 ectodomain, has been shown to induce subepidermal blister formation on unfixed human frozen skin *in vitro*.¹⁸

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However, the precise pathomechanism for the generation of neoepitopes after COL17 cleavage has not been elucidated.

Recently, we identified physiological N-terminal cleavage sites of human COL17 within the NC16A domain *in vitro*: Asp⁵¹⁴, Leu⁵²⁴, Gln⁵²⁵, and Gly⁵²⁶.¹⁵ In addition, rabbit IgG Abs targeting the cleavage site of Leu⁵²⁴ (Ab HK139) react specifically with the shed COL17 ectodomain, indicating that the cleavage of the molecule actually generates neoepitopes. Furthermore, incubation of cryosections of normal human skin (NHS), neutrophils, and Ab HK139 induced dermal–epidermal separation *in vitro*, suggesting pathogenic roles for the neoepitope-specific Abs in blister formation.¹⁵ However, the Ab HK139 showed mottled staining at the dermal–epidermal junction (DEJ) of NHS; thus, Leu⁵²⁴ may be a minor cleavage site in a physiological setting and a different cleavage site (or sites) may be involved *in vivo*. In addition, it still is uncertain whether neoepitope-specific Abs to COL17 are pathogenic for blister formation *in vivo*.

Here, we tried to determine whether COL17 is cleaved physiologically and whether the ectodomain shedding of COL17 produces neoepitopes in *in vivo* skin, and we investigated the pathogenic roles of Abs targeting the neoepitopes at the cleavage sites in blister formation. We found that novel Abs targeting a physiological cleavage site showed that the ectodomain of human COL17 is able to be shed and that the cleaved ectodomain is present at the DEJ even in a physiologically steady setting. In contrast, the cleavage site or sites of COL17 ectodomain are targeted differentially when keratinocytes migrate in cultured keratinocytes as well as in *in vivo* wound skin. Finally, passive-transfer experiments using Abs targeting a candidate N-terminal physiological cleavage site of the mouse COL17 ectodomain failed to induce blister formation in mice, suggesting that the immune reaction to the neoepitopes on the physiological cleavage sites is not pathogenic.

Materials and Methods

Cell Culture

Primary normal human epidermal keratinocytes (NHEKs) isolated from neonatal foreskin (Lonza, Basel, Switzerland) were cultured in keratinocyte growth medium (Lonza). To isolate primary mouse keratinocytes (NMKs), neonatal WT mice on a C57BL/6J background (Clea, Tokyo, Japan) were sacrificed, and whole-skin sections were incubated with 0.25% trypsin EDTA (Life Technologies, Carlsbad, CA) for 1 hour at 37°C. After the epidermal layer was scraped and incubated with 10% fetal calf serum in phosphate-buffered saline (PBS; Life Technologies), primary NMKs were seeded on plastic with Cnt-57 medium supplemented with bovine pituitary extract (Cellntec, Bern, Switzerland). Cells up to the fourth passage were used.

Generation of Abs

Rabbit polyclonal Abs targeting the N-terminal cleavage site Gln⁵²⁵ of human COL17 (Ab Hu-pyro-Glu) were produced by

immunizing two New Zealand White rabbits with peptides corresponding to eight amino acids.¹⁵ Because N-terminal Gln⁵²⁵ is cyclized physiologically to pyroglutamate (pyro-Glu) *in vitro*,¹⁵ the peptides pyro-Glu-Gly-Met-Ala-Pro-Ala-Ala-Gly-Cys were synthesized chemically using L-pyroglutamic acid (Hokudo, Sapporo, Japan). The peptides were coupled with keyhole limpet hemocyanin through carboxyl terminal Cys with m-maleimidobenzoyl-N-hydroxysuccinimide ester.¹⁵ Whole IgG from the immunized rabbit serum first was affinity-purified with protein A Sepharose (ProteNova, Takamatsu, Japan) according to the manufacturer's protocol. A portion of the whole IgG Abs were immunoabsorbed using chemically synthesized R7 peptide (Asp⁵²² to Gln⁵⁴⁵ of human COL17; Hokudo) to eliminate Abs that react with the full-length COL17. Briefly, whole IgG Abs were dialyzed to PBS, followed by incubation with R7 peptide-coupled, NHS-activated High Performance (GE Healthcare, Little Chalfont, UK), as previously published.^{15,21} Flow-through samples containing unbound Abs were collected, and the concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). The immunoabsorbed Ab Hu-pyro-Glu was used for experiments in this study unless otherwise specified. Rabbit Abs against the NC16A domain (Glu⁴⁹⁰ to Arg⁵⁶⁶ of human COL17, Ab Hu-NC16A)²¹ targeting the cleavage site Leu⁵²⁴ (Ab HK139)¹⁵ and the C-terminus (Val¹¹⁹² to Pro¹⁴⁹⁷ of human COL17, Ab 09040)²² were produced as previously described.

Rabbit polyclonal Abs targeting a candidate N-terminal cleavage site of mouse COL17, Gln⁵³² (Ab Mo-pyro-Glu), were produced by immunizing rabbits with the synthetic peptides pyro-Glu-Ala-Glu-Ala-Pro-Ser-Leu-Gly-Cys (Hokudo). The whole Abs first were prepared by using protein A Sepharose as described earlier, and they were affinity-purified further with antigenic peptides by using the SulfoLink Immobilization Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Precise information about epitopes for these Abs are illustrated in Figure 1. To produce rabbit Abs against the mouse NC14A domain of COL17 (Ab Mo-NC14A), glutathione *S*-transferase (GST)-conjugated mouse NC14A protein (GST-NC14A) was prepared as previously described.⁹ Briefly, PCR products encoding 75–amino acid NC14A polypeptides (from Glu⁴⁹⁹ to Arg⁵⁷³ of mouse COL17) were amplified using the primers mNC14A-F (forward, 5'-CCGAATTTCGAGGAGGTAAGAAAGCTG-AA-3'; the EcoRI restriction site is underlined) and mNC14A-R (reverse, 5'-GGGCGGCCGCTCTGAGATT-CCCGTTCTCCT-3'; the NotI restriction site is underlined), and cDNA from mouse keratinocytes. The 243-bp PCR product was digested by EcoRI and NotI, followed by integration into the pGEX-6P-1 vector (GE Healthcare). After DNA sequences were confirmed, the expression vector was transformed into *Escherichia coli* (BL21; GE Healthcare). The cells were lysed by B-PER protein extraction reagent (Thermo Fisher Scientific), and GST-NC14A protein was purified by GSTrap affinity column (GE Healthcare) according to the manufacturer's

Figure 1 The physiological N-terminal cleavage site Gln⁵²⁵ of human collagen XVII (COL17) is highly conserved among different species. **A:** **Black arrow** and dashed lines illustrate amino acid sequences of juxtamembraneous NC16A domain of COL17. Physiologically, the ectodomain of human COL17 is cleaved at different sites within the NC16A domain, including at Asp⁵¹⁴, Leu⁵²⁴, Gln⁵²⁵, and Gly⁵²⁶ (**red arrowheads**). Epitopes of antibodies (Abs) HK139 and Hu-pyro-Glu are shown. The N-terminal Gln⁵²⁵ of the immunized peptides for the Ab Hu-pyro-Glu has been changed chemically into pyro-Glu (**asterisk**). **B:** The N-terminal cleavage site Gln⁵²⁵ for human COL17 (**black arrow** indicating **red letters**) is highly conserved among different species, suggesting that Gln⁵²⁵ is a vital amino acid. The corresponding epitope of immunized peptides to produce the Ab Mo-pyro-Glu is shown (black bar). The N-terminal Gln of the immunized peptides for the Ab also was changed chemically into pyro-Gln (**asterisk**). TM, transmembrane.

instructions. The correct expression of GST-NC14A as a GST-fusion protein was confirmed by digesting it with PreScission Protease (GE Healthcare). Similarly, recombinant mouse NC14A protein with C-terminal hexahistidine and herpes simplex virus tag sequences (NC14A-His•herpes simplex virus) was produced using the same primers and the pET-44a (+) vector (Novagen, Madison, WI). For the control, an empty vector was used. To obtain the Ab Mo-NC14A, four New Zealand White rabbits were immunized with the purified GST-NC14A protein, and the whole IgG was affinity-purified as described earlier. The specificity of the Ab Mo-NC14A to the NC14A domain of mouse COL17 was assessed by immunoblotting using NC14A-His•herpes simplex virus protein.

Immunoblotting and Immunohistochemistry

For immunoblotting, subconfluent NHEKs or NMKs were lysed for 30 minutes on ice in a buffer of 1% Nonidet P-40, 0.1 mol/L NaCl, 25 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, and 1 mmol/L Pefabloc SC (Sigma, St. Louis, MO).¹² Medium proteins were precipitated by ethanol.¹² To address spontaneous cyclization of N-terminal Gln⁵²⁵ into pyro-Glu in the human COL17 ectodomain, a sterilized culture medium of NHEKs was incubated at 37°C for 2, 4, or 9 days before ethanol precipitation. The ECM proteins were prepared as described elsewhere.^{23,24} Briefly, the cell layer was incubated at room temperature with 20 mmol/L NH₄OH solution after being washed with PBS. After all of the cells had detached, the ECM was washed thoroughly with PBS and dissolved in SDS-polyacrylamide gel electrophoresis sample buffer. The samples were separated on SDS-polyacrylamide gel electrophoresis 7% polyacrylamide gels, followed by transfer onto a nitrocellulose membrane. The following Abs were used:

anti-herpes simplex virus-tag Ab (A00624; Genscript, Piscataway, NJ), the rabbit polyclonal Abs described earlier, and mouse mAb NC16A-3 directing the NC16A domain of human COL17²⁰ (provided by Prof. Leena Bruckner-Tuderman, Freiburg Institute for Advanced Studies, Freiburg im Breisgau, Baden-Württemberg, Germany). After incubation with a horseradish peroxidase-conjugated secondary Ab, signals were visualized by ECL-plus (GE Healthcare). For indirect immunofluorescence (IIF) microscopy, skin specimens of NHS, skin from a COL17-lacking epidermolysis bullosa patient,²⁵ normal mouse skin (NMS), mouse Col17-lacking mice skin,⁹ and NHEKs cultured on coverslips for 18 hours were used. For IIF of wounded skin, three skin ulcer samples taken after surgery from a 26-year-old man, a 68-year-old man, and a 69-year-old man were used (one from each individual), from whom pilomatricoma on the left upper arm, the left inguinal lymph nodes, and malignant melanoma on the left sole had been removed surgically 25, 37, and 35 days before, respectively. For some experiments, human or mouse skin sections were incubated with 1 mol/L NaCl to produce artificial blisters for 14 and 24 hours at 4°C, respectively. The skin specimens were mounted and snap-frozen in optimal cutting temperature compound (Tissue-Tek O.C.T.; Sakura, Alphen aan den Rijn, the Netherlands), and 5-μm cryosections were incubated with antiserum, purified rabbit polyclonal IgG Abs, or serum from rabbit Ab-injected mice for 1 hour at room temperature and detected with 1:1000 diluted Alexa488-conjugated anti-rabbit IgG (Life Technologies) and propidium iodide (Wako, Osaka, Japan). For direct immunofluorescence microscopy, Alexa488-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-mouse C3 (MP Bio-medicals, Solon, OH) were used. IIF on cultured NHEKs was performed using rhodamine phalloidin (Life Technologies) as previously described,²⁴ and images were observed by

Figure 2 Ectodomain shedding of human collagen XVII (COL17) in cultured primary normal human epidermal keratinocytes (NHEKs) and in *in vivo* human skin. **A:** Western blot using cell lysate (L) and culture medium protein (M) from NHEKs shows that the antibody (Ab) Hu-pyro-Glu weakly reacts only with the 120-kDa shed ectodomain (arrow) and not with the 180-kDa full-length form of COL17. In contrast, Abs 09040 and Hu-NC16A, which target the C-terminus and the NC16A domain, respectively, react with both the full-length and the shed forms. The Ab Hu-NC16A reacts more strongly with the 120-kDa shed ectodomain than with the full-length form. **B:** Immunoprecipitation using Abs Hu-NC16A and Hu-pyro-Glu before and after immunoadsorption using synthesized R7 peptide spanning Asp⁵²² to Gln⁵⁴⁵. Immunoprecipitation samples from cell lysate and culture medium of NHEKs were detected by Ab 09040 and monoclonal Ab NC16A-3, respectively. The Ab Hu-pyro-Glu before immune adsorption weakly reacts with full-length COL17, whose reactivity was abolished after immunoadsorption of the Abs. The reactivity of the Ab Hu-pyro-Glu to the shed ectodomain also was abolished after the immunoadsorption. **C:** Immunoreactivity of the Ab Hu-pyro-Glu on normal human skin does not differ before versus after immunoadsorption (Ab concentration, 0.005 μ g each). **D:** Treatment of NHEK culture medium with recombinant human glutaminy-peptide cyclotransferase. Western blot shows an increase in the 120-kDa COL17 ectodomain detected by Ab Hu-pyro-Glu in medium protein treated with glutaminy-peptide cyclotransferase, the reactivity by monoclonal Ab NC16A-3 is unchanged. The Ab Hu-pyro-Glu can immunoprecipitate small amounts of COL17 ectodomain from nontreated culture medium (3 mL) of NHEKs; in contrast, the Ab immunoprecipitates increase 120-kDa COL17 ectodomain from the glutaminy-peptide cyclotransferase-treated medium protein; detected by monoclonal Ab NC16A-3. **E:** Spontaneous cyclization of N-terminal Gln⁵²⁵ of the shed COL17 ectodomain into pyro-Glu. Long-term incubation of NHEK culture medium at 37°C produces increased amounts of COL17 ectodomain, which is detectable by immunoadsorbed Ab Hu-pyro-Glu of COL17 in a time-dependent manner. In contrast, reactivity of the 120-kDa Ab NC16A-3 is decreased, indicating that certain amounts of COL17 ectodomain are degraded during incubation (a stripped membrane was used). **F:** The Ab Hu-pyro-Glu fails to react with skin sections obtained from a COL17-lacking epidermolysis bullosa patient. The Ab reacts with the epidermal side of artificial blisters (stars) of NHS induced by pre-incubation with 1 mol/L NaCl for 14 hours. Scale bars: 50 μ m (**C** and **F**). IP, immunoprecipitation.

fluorescence microscopy (IX-51; Olympus, Tokyo, Japan) or confocal microscope (FX81, Olympus).

Immunoprecipitation

Immunoprecipitation was performed using rabbit polyclonal Abs as described earlier, normal rabbit polyclonal IgG (37415; Abcam, Cambridge, UK), cell lysates, or medium of cultured NHEKs and NMJs. Briefly, 50 μ L of protein-G Sepharose (GE Healthcare) was coupled with 50 μ g of Abs Hu-pyro-Glu, Hu-NC16A, or normal rabbit IgG, followed by incubation with cell lysates of NHEKs or 1 mL of culture medium for 1 hour at room temperature. After extensive washing of the beads with PBS containing 0.1% NP-40, bound proteins were eluted by boiling for 5 minutes with 1 $\times$ SDS sample buffer. To assess whether the N-terminus of the shed COL17 ectodomain was cyclized from

Gln⁵²⁵ into pyro-Glu, 3 mL of NHEK culture medium was concentrated and buffer was exchanged to 25 mmol/L HEPES, pH 7.0, using an Amicon Ultra Centrifuge Filter Unit (30 kDa; Millipore, Darmstadt, Germany). The concentrated culture medium was treated with 1 μ g of recombinant human glutaminy-peptide cyclotransferase (6368-ZN; R&D Systems, Minneapolis, MN) for 24 hours at 37°C followed by immunoprecipitation using Ab Hu-pyro-Glu. TruBlot (Rockland Immunochemicals, Limerick, PA) was used as a secondary Ab.

Passive Transfer of the Cleavage-Specific Abs into Mice

Neonatal and 8-week-old WT C57BL/6J mice were used. The neonatal mice received a single intraperitoneal injection of 250 μ g/g/body weight of the Ab Mo-pyro-Glu ($n = 6$), which had been affinity-purified with peptide beads, or

Figure 3 Ectodomain shedding of collagen XVII (COL17) in migrating keratinocytes. **A:** The cleaved ectodomain of COL17 in the extracellular matrix (arrows in left column, green in right column) of the primary normal human epidermal keratinocytes is detectable by both the antibodies (Abs) targeting the NC16A domain (Ab Hu-NC16A) and the cleavage site of Leu⁵²⁴ (Ab HK139), whereas not detectable by the Ab Hu-pyro-Glu and normal rabbit IgG as control. Red: Rhodamine phalloidin. **B:** Western blot using extracellular matrix samples (E) and culture medium protein (M) of normal human epidermal keratinocytes shows detection of the 120-kDa cleaved ectodomain of COL17 in the extracellular matrix by Ab Hu-NC16A and the Ab HK139 but no detection by Ab Hu-pyro-Glu. Scale bars: 25 μ m (**A**).

2000 μ g/g/body weight of whole IgG containing the Ab Mo-NC14A ($n = 4$). Forty-eight hours after the injection, back skin was evaluated separately by two investigators for detachment by gentle rubbing.^{9,21} For adult mice, 500 μ L of antiserum containing the Ab Mo-pyro-Glu was injected subcutaneously each day for 5 consecutive days, and skin disease was assessed 3 days after the last injection ($n = 4$). For the control, preimmune serum or normal whole-rabbit IgG (Abcam) was used.

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University. This study was approved by the Ethics Committee of Hokkaido University, and fully informed consent was obtained from all patients and healthy volunteers for the use of their materials.

Results

Gln⁵²⁵ of COL17 Is a Highly Conserved, Physiological, N-Terminal Cleavage Site in Normal Human Skin

We previously reported that different cleavage sites cluster within the NC16A domain of COL17 (Figure 1A).¹⁵ Although amino acid sequences covering the NC16A domain show significant differences among different species,^{10,26} the cleavage site Gln⁵²⁵ is highly conserved (Figure 1B), indicating that it is a functionally important amino acid. Interestingly, our previous studies showed that the N-terminal Gln⁵²⁵ is unique because its N-terminus is cyclized naturally by changing from Gln into pyro-Glu after cleavage *in vitro*.¹⁵ To assess whether human COL17

actually is cleaved at this site and whether the N-terminus was changed from Gln⁵²⁵ to pyro-Glu⁵²⁵ *in vivo*, we produced rabbit Abs (Ab Hu-pyro-Glu) targeting the modified cleavage site (Figure 1A). Western blot showed that the Ab Hu-pyro-Glu reacts weakly with the 120-kDa shed ectodomain and does not react with the 180-kDa full-length form of COL17 (Figure 2A). However, immunoprecipitation studies using high doses of the Ab and plenty of NHEK cell lysate showed that the Ab was able to immunoprecipitate small amounts of full-length COL17 (Figure 2B). Therefore, we immunoadsorbed the Ab using synthesized R7 peptide (Asp⁵²² to Gln⁵⁴⁵),²¹ which does not contain Gln⁵²⁵ as an N-terminal cleavage site, and the treatment eliminated the reaction to full-length COL17 (Figure 2B). The ability of Ab Hu-pyro-Glu to immunoprecipitate the 120-kDa COL17 ectodomain also was abolished after immunoadsorption (Figure 2B), indicating that the shed COL17 ectodomain with pyro-Glu N-terminus was not present or was present only in small amounts in the NHEK culture medium. Immunoreactivity of the Ab Hu-pyro-Glu to NHS was not reduced after immunoadsorption (Figure 2C); thus, we speculated that N-terminal cyclization from Gln⁵²⁵ into pyro-Glu of the shed COL17 ectodomain may occur preferentially *in vivo* and that the cleaved molecule in the NHEK culture medium had noncyclized N-terminal Gln⁵²⁵. To address these hypotheses, we treated a culture medium of NHEKs with recombinant human glutaminyl-peptide cyclotransferase, an enzyme that cyclizes N-terminal Gln into pyro-Glu.²⁷ After the treatment, Ab Hu-pyro-Glu showed increased reaction to the shed COL17 ectodomain (Figure 2D), indicating that the shed COL17 ectodomain

Figure 4 Ectodomain shedding of collagen XVII (COL17) in ulcers after surgery. **A:** Ulcers after surgery from case 1 (a 26-year-old man, 25 days after the surgery). In contrast to the antibodies (Abs) Hu-NC16A and HK139, signals at the dermal-epidermal junction (DEJ) by the Ab Hu-pyro-Glu are diminished at the leading edge and at the re-epithelialized hypertrophic epidermis (regions 3 and 2, respectively; **arrowheads**). Note the gradual reduction of Ab Hu-pyro-Glu staining at the DEJ in the transitional region from normal to hypertrophic skin (region 1, **arrows**). The **lowest panel** is a high magnification of the Ab-Hu-pyro-Glu in region 1. **B:** Case 2 (a 68-year-old man, 37 days after the surgery). Signals at the DEJ by the Ab Hu-pyro-Glu are diminished at the leading-edge epidermis (region 3). The **lowest panel** is a high magnification of the Ab-Hu-pyro-Glu in region 2, in which there is a gradual reduction of the Ab Hu-pyro-Glu staining at the DEJ in the transitional hypertrophic skin (**arrowheads**). In case 2, the Ab Hu-NC16A shows expression patterns similar to those of the Ab Hu-pyro-Glu. **C:** Case 3 (a 69-year-old man, 35 days after the surgery). Note the gradual reduction of the Ab Hu-pyro-Glu staining at the DEJ in the transitional region from normal to hypertrophic skin (region 1, **arrowheads**). The **lowest panel** is a high magnification of the Ab-Hu-pyro-Glu in region 1. Dermal staining by Abs HK139 in regions 2 and 3 in case 2, and by Ab Hu-pyro-Glu in region 3 in both cases is attributed to cross-reactivity to unknown dermal products that probably are associated with inflammation. Note that the normal skin region is lacking in case 3 (**C**). Green represents Abs; red represents propidium iodide.

with noncyclized N-terminal Gln⁵²⁵ is present in the NHEK culture medium. In addition, the Ab Hu-pyro-Glu precipitated increased amounts of COL17 ectodomain from the NHEK culture medium treated with glutaminy-peptide cyclotransferase (Figure 2D), proving specific binding of the Ab to the cyclized pyro-Glu N-terminus of the shed molecule. Furthermore, spontaneous cyclization of N-terminal Gln⁵²⁵ in culture medium after incubation at 37°C was confirmed (Figure 2E). These findings prove that a majority of the COL17 ectodomain in the NHEK culture medium has a noncyclized Gln⁵²⁵ N-terminus and that the Ab Hu-pyro-Glu reacts specifically with the pyro-Glu N-terminal shed COL17 ectodomain. The immunoadsorbed Ab Hu-pyro-Glu failed to react with skin sections obtained from a patient with epidermolysis bullosa but lacking COL17 (Figure 2F). This confirmed that the reaction was specific to human COL17. Interestingly, the Ab Hu-pyro-Glu showed positive staining on the epidermal side of artificial blisters of NHS induced by pre-incubation with 1 mol/L NaCl for 14 hours (Figure 2F), suggesting the presence of an unknown binding molecule or molecules that anchor the shed COL17 ectodomain in the ECM. The reactivity of the Abs to the epidermal side was diminished when the incubation time of NHS with 1 mol/L NaCl was increased to 24 hours to induce artificial blisters (not shown), indicating that epitopes of the Ab Hu-pyro-Glu probably are cleaved off by internal proteases.

COL17 Ectodomain Shedding in Migrating Keratinocytes Is Regulated Differentially *in Vitro* Versus *in Vivo*

We previously showed that migrating NHEKs cleave COL17 at Leu⁵²⁴ and that the cleaved ectodomain of COL17 is detectable by the Ab HK139 targeting this cleavage site as migration tracks in the ECM *in vitro*.¹⁵ However, the Ab Hu-pyro-Glu showed no signals in the ECM of cultured NHEKs, whereas both the Ab to the NC16A domain (Ab Hu-NC16A) and the Ab HK139 were able to detect large amounts of COL17 as migration tracks (Figure 3A). Consistent with the IIF findings, Western blot using ECM samples of NHEKs showed that the Ab Hu-pyro-Glu was unable to detect the 120-kDa ectodomain of COL17, whereas both the Ab Hu-NC16A and the Ab HK139 were able to detect that ectodomain (Figure 3B). These results suggest that migrating NHEKs cleave COL17 at sites different from those of the normal steady state *in vitro*. To address this further in an *in vivo* setting, we tested the reactivity of the Abs on ulcerated wound skin after surgery as described in *Material and Methods*. It was found that physiological N-terminal cleavage at Gln⁵²⁵, detectable by the Ab Hu-pyro-Glu, was diminished at the DEJ of the leading-edge epidermis, although slightly reduced but distinct expression of COL17 was detectable by the Ab Hu-NC16A (Figure 4). These results suggest that the ectodomain shedding of COL17 also is regulated

differentially in *in vivo* human wound skin. In addition, different expression patterns were observed in a region intermediate between the re-epithelializing edge and normal skin among different samples. The expression of the Ab Hu-pyro-Glu was much weaker in cases 1 and 3 than in case 2 (Figure 4, A–C). In case 2, the Ab Hu-NC16A showed expression patterns similar to those of the Ab Hu-pyro-Glu (Figure 4B). These differences may reflect the difference in time elapsed after surgery (case 1, 25 days; case 2, 37 days; and case 3, 35 days).

Passive Transfer of Abs to Neoepitopes on Candidate Physiological Cleavage Sites of Mouse COL17 Ectodomain Fails to Reproduce Blistering Disease in Mice

The final question was whether Abs to neoepitopes on the cleavage sites of the shed ectodomain of COL17 are pathogenic for blister formation. Because the cleavage site-specific Ab Hu-pyro-Glu did not react with the COL17-humanized mice skin (not shown), we generated another Ab targeting a candidate physiological cleavage site of mouse COL17. Based on the facts that Gln⁵²⁵ of human COL17 is highly conserved among different species (Figure 1B), we hypothesized that the amino acid Gln⁵³² of mouse COL17, corresponding to the Gln⁵²⁵ of human COL17, may be a major cleavage site of mouse skin *in vivo*. In addition, we speculated that the N-terminal Gln⁵³² may be modified by changing into pyro-Glu, as seen for the human N-terminal Gln⁵²⁵ of COL17. To address these hypotheses, we produced another rabbit Ab, Mo-pyro-Glu, which targets the candidate N-terminal cleavage site of mouse COL17, whose Gln⁵³² has been changed into pyro-Glu (Figure 1B). The Ab Mo-pyro-Glu reacted strongly with the DEJ of the WT mouse skin, although it failed to react with the COL17-lacking mouse (*Col17*^{−/−}) skin (Figure 5A), supporting the idea of specificity of the Ab to mouse COL17. In addition, similar to the Ab Hu-pyro-Glu, the Ab Mo-pyro-Glu reacted with the epidermal side of artificial blisters of NMS pre-incubated with 1 mol/L NaCl for 14 hours (Figure 5A), although its reactivity was lost on skin that had been incubated with 1 mol/L NaCl for 24 hours as observed by Ab Hu-pyro-Glu (not shown). The Ab Mo-pyro-Glu immunoprecipitates the shed mouse COL17 ectodomain (Figure 5B), and Western blotting showed that the Ab Mo-pyro-Glu reacted with the shed ectodomain of mouse COL17 but not with the full-length form (Figure 5, C–E). These results suggest that the Ab Mo-pyro-Glu preferentially targets neoepitopes on the shed mouse COL17 ectodomain, although Western blot using the highly concentrated Ab Mo-pyro-Glu and cell lysate from NMJs showed that the Ab was able to react weakly with the 180-kDa full-length form (not shown).

To address the pathogenic roles of Abs targeting the neoepitopes on the shed COL17 ectodomain in blister formation, we performed passive transfer of the Ab Mo-pyro-Glu and the Ab Mo-NC14A into neonatal and 8-week-old

Figure 5 Pathogenic roles of Abs to neopeptides on physiological cleavage sites of mouse collagen XVII (COL17). **A:** IIF using 2.25 ng/ μ L diluted affinity-purified antibody (Ab) Mo-pyro-Glu shows a strong reaction with the dermal-epidermal junction (DEJ) of the wild-type (WT) mouse skin, but it fails to react with COL17-lacking mouse (*Col17*^{-/-}) skin. The Ab reacts with the epidermal side of 1 mol/L NaCl-induced artificial blisters of normal mouse skin (NMS). Blisters are shown by stars. **B:** Immunoprecipitation of the shed COL17 ectodomain from normal mouse keratinocyte (NMK) culture medium by Mo-pyro-Glu. Ab Mo-pyro-Glu immunoprecipitates the 120-kDa mouse COL17 ectodomain, which was detected by Ab Mo-NC14A. **Input:** concentrated medium protein. **C:** Western blot using cell lysate (L) and culture medium (M) of NMKs. In contrast to the Ab Mo-NC14A reacting with both the full-length form and the shed ectodomain of mouse COL17, the Ab Mo-pyro-Glu only reacts with the shed 120-kDa ectodomain (arrow). The dim band around 150 kDa is a nonspecific signal. **D:** The recombinant GST-NC14A protein is seen to migrate around 37 kDa by Coomassie staining. Small fragments between 25 and 37 kDa are expected to be degraded or truncated proteins, which can be cleaved off by PreScission protease (star) (GE Healthcare). **E:** Western blot using recombinant NC14A-His-HSV protein (lane 1) and control protein from empty pET-44a vector (lane 2). Similar to anti-HSV-tag Abs, the Ab Mo-NC14A reacts with NC14A-His-HSV, which migrates around 80 kDa. **F:** A neonatal C57BL/6 WT mouse 48 hours after receiving an intraperitoneal injection of 250 μ g/g body weight affinity-purified Ab Mo-pyro-Glu. No epidermal detachment was observed (arrows), but direct immunofluorescence (DIF) studies show *in vivo* deposition of rabbit IgG and mouse C3 (white arrowheads) at the DEJ of skin from the injected mice. In contrast, a neonatal C57BL/6 WT mouse that received an intraperitoneal injection of 2000 μ g/g body weight purified whole IgG containing Ab Mo-NC14A shows skin fragility (black arrowheads) associated with *in vivo* deposition of rabbit IgG (white arrowheads), but mouse C3 was not activated. No distinct abnormal findings were observed in the control mice that received normal rabbit IgG (arrows). **G:** An adult C57BL/6 mouse that received subcutaneous injection totaling 2500 μ L of antiserum containing the Ab Mo-pyro-Glu. No phenotypic abnormalities were observed. DIF studies showed *in vivo* deposition of rabbit IgG and mouse C3 (arrowheads) at the DEJ of the back skin of the injected mice. **H:** IIF studies using sera from adult WT mice that were injected with the Ab Mo-pyro-Glu. The treated mice, but not the control mice, had circulating Abs that strongly reacted with the DEJ of NMS at 400 $\times$ dilution. Scale bars: 50 μ m (A); 100 μ m (F–H). HSV, herpes simplex virus; PP, PreScission protease.

C57BL/6J mice. Affinity-purified Ab Mo-pyro-Glu (10.72 μ g/ μ L) with immunized peptides strongly reacted with the DEJ of the NMS, as observed by IIF (Figure 5A) at more than 1:32,000 dilution (not shown). Although the Ab Mo-pyro-Glu in high concentration was able to react weakly with the

180-kDa full-length mouse COL17, we used the Abs for passive-transfer experiments without immunoadsorption because the treatment did not solely change the reactivity of the Ab Hu-pyro-Glu to the NHS, as mentioned earlier. As a result, high-dose passive transfer of the affinity-purified

Ab Mo-pyro-Glu failed to induce skin fragility in any of the recipient mice by 48 hours after injection ($n = 6$) (Figure 5F), although direct immunofluorescence studies showed strong deposition of rabbit IgG and mouse C3 at the DEJ in all recipient mice (Figure 5F). In contrast, three fourths of neonatal mice that had received the Ab Mo-NC14A, which was affinity-purified by protein A (84.29 $\mu\text{g}/\mu\text{L}$), and reacts with NMS at more than 1:12,800 dilution, showed epidermal detachment by gentle mechanical friction (Figure 5F). Interestingly, direct immunofluorescence studies from the diseased mice showed *in vivo* deposits of rabbit IgG at the DEJ, although complement activation was very faint or largely absent (Figure 5F).

For adult mice, we performed the passive transfer of antiserum containing the Ab Mo-pyro-Glu, which reacts strongly with the DEJ of NMS as observed by IIF at more than 1:3200 dilution (not shown). Repeated passive transfer of antiserum containing the Ab Mo-pyro-Glu, totaling 2500 μL per mouse, did not induce any skin disease in recipient WT adult mice ($n = 4$), although *in vivo* deposition of rabbit IgG and mouse C3 was observed at the DEJ of the skin in all of the recipient mice (Figure 5G). In addition, the treated mice had circulating Abs that reacted with the DEJ of the NMS at more than 400 $\times$ dilution (Figure 5H). Control mice that received the same dose of preimmune rabbit serum showed no phenotypical abnormalities, and no rabbit IgG or mouse C3 deposition was observed in the skin (not shown).

Discussion

COL17 is a key molecule for maintaining stable adhesion at the DEJ. Innate and acquired dysfunctions of the molecule, respectively, lead to the blistering disorders of non-Herlitz junctional epidermolysis bullosa¹⁰ and pemphigoid diseases.³ These blistering diseases suggest that COL17 plays a vital role in the stability of the skin. In contrast, here we showed that certain amounts of the COL17 ectodomain are cleaved physiologically even in steady-state normal human skin. COL17 is known to be expressed not only at the DEJ but also basolaterally and in the cytoplasm.^{11,15} However, the cleaved COL17 ectodomain detected by Abs targeting cleavage sites of the molecule (Ab Hu-pyro-Glu) was observed exclusively at the DEJ. This finding suggests that ectodomain shedding occurs only beneath the basal keratinocytes, probably within the hemidesmosomes. Similar findings have been reported for mAb 1337, another ectodomain-specific mAb reacting with bovine COL17.¹¹

Interestingly, we found that a majority of N-terminal Gln⁵²⁵ of the shed COL17 ectodomain in NHEK culture medium was not cyclized into pyro-Glu. This finding was unexpected because, in a previous study, we identified pyro-Glu⁵²⁵ as a major N-terminal cleavage site by mass spectrometry analyses.¹⁵ The reason pyro-Glu was identified as a major N-terminal cleavage site probably is because N-terminal Gln⁵²⁵

spontaneously cyclized into pyro-Glu during repetitive sample preparation for mass spectrometry. Spontaneous N-terminal conversion from Glu to pyro-Glu is observed commonly in various proteins, including human IgG Abs.²⁸ In contrast to *in vitro* culture systems, the present study showed that *in vivo*, the shed COL17 ectodomain in normal human skin has a cyclized pyro-Glu N-terminus, although it is uncertain whether the N-terminal cyclization of Gln⁵²⁵ occurs spontaneously or enzymatically *in vivo*.

It is well characterized that migrating activated keratinocytes express distinct adhesion molecules, such as integrin $\alpha 3\beta 1$ and $\alpha 2\beta 1$, at the basement membrane zone *in vitro*.^{29,30} In addition, wound keratinocytes are known to express these distinctive integrins.³¹ These findings suggest that different molecules are involved in keratinocyte migration. Previously, we showed that migrating keratinocytes cleave and leave the ectodomain of COL17 in the ECM as seen by the migration track *in vitro*, which was shown by Ab HK139, which targets the N-terminal cleavage site at Leu⁵²⁴.¹⁵ This observation was associated somewhat with pathologic settings because we observed actively migrating keratinocytes. In contrast, here we showed that the Ab Hu-pyro-Glu did not react with the migration tracks of cultured NHEKs. Therefore, it is likely that the N-terminal cleavage of COL17 at Gln⁵²⁵ is associated with a physiological setting, rather than a pathologic one, which suggests that regulation of COL17 cleavage depends on the setting.

Alternatively, different cleavage sites on COL17 merely may be a result of different proteases involved under different settings, including normal steady phase or wound healing. However, what is the physiological function of COL17 ectodomain shedding in normal skin? It has been postulated that COL17 ectodomain shedding plays physiological roles associated with the migration, proliferation, and differentiation of basal keratinocytes.¹³ However, there has been no direct evidence for this. One possible physiological role may relate to the differentiation of basal keratinocytes because they must detach from the ECM to differentiate into the spinous and cornified layers. Alternatively, normal epidermis may be a more dynamic organ that communicates actively with ECM proteins through cell-surface receptors including COL17, whereby the ectodomain shedding of the molecule may be essential even in a normal setting.

The present study also suggests the presence of binding proteins of cleaved COL17 ectodomain in the ECM because IIF studies of cleavage site-specific Abs on 1 mol/L NaCl-split skin showed that the cleaved COL17 ectodomain remains on the epidermal side of artificial blisters. The C-terminus of COL17 binds with laminin 332 *in vitro*.^{24,32} Because laminin 332 always is present on the dermal side of 1 mol/L NaCl-split skin,^{3,33} the present data indicate that another binding molecule(s) on the epidermal side is present in the ECM, which binds with the shed COL17 ectodomain more strongly than laminin 332. A candidate molecule is integrin $\alpha 6$, which is known to be able to bind with the

ectodomain of COL17.^{34,35} Alternatively, additional cleavage may occur in the C-terminus of COL17 when artificial blisters form by 1 mol/L NaCl. In any case, the present data suggest that the shed ectodomain of COL17 binds with another molecule(s) at the DEJ.

The ectodomain shedding of COL17 can evoke neoepitopes on the shed forms, which can be targeted by autoAbs of pemphigoid patients.^{16,17} Previously, we showed that linear B-cell epitopes have significantly increased antigenicity around cleavage sites within the NC16A domain, regardless of the physiological cleavage sites predicted *in silico*.¹⁵ However, the detailed pathomechanism of neoepitope generation has not been elucidated, and the pathologic role of autoAbs to neoepitopes still is uncertain *in vivo*. This study showed that modified N-terminal cleavage sites whose Gln has been changed into pyro-Glu produce distinct neoepitopes at the N-terminal cleavage sites. We previously generated transgenic COL17-humanized mice for which the mouse COL17 was replaced by human COL17. The mice enabled us to reproduce blistering disease by passively transferring Abs to human COL17.^{9,21} Although the COL17-humanized mouse skin expresses full-length human COL17, the Ab Hu-pyro-Glu did not stain at the DEJ. Similarly, the cleavage site-specific Ab HK139 targeting N-terminal Leu⁵²⁴ did not react with the COL17-humanized mouse skin (not shown, unpublished data). It still is uncertain why these Abs lost their reactivity to human COL17 in the transgenic mice. One possible mechanism is that the cleavage of human COL17 in the COL17-humanized transgenic mouse skin may be different from that in NHS.

In this study, we produced rabbit Abs targeting a candidate cleavage site of the mouse COL17 ectodomain (Ab Mo-pyro-Glu), which preferentially react with the shed mouse COL17. The passive transfer of the Ab Mo-pyro-Glu failed to induce blistering diseases in neonatal and adult WT mice, even though injected rabbit IgG bound with mouse COL17 and activated mouse C3 *in vivo*. Previous studies have shown that *in vivo* deposition of Abs to the NC16A domain (the NC14A domain for mouse COL17) of COL17 associated with complement activation is a hallmark of neonatal BP models,^{8,36} although we recently found that complement activation is not necessary for blistering diseases in mice.^{21,22,37} In any case, we found no evidence that Abs targeting a candidate physiological N-terminal cleavage site are pathogenic *in vivo*. However, epitopes can be changed significantly in regions far from the cleavage sites. For example, the mAb NC16A-3, whose epitope is located at least 19 amino acids C-terminus from the physiological cleavage site within the NC16A domain of COL17, reacts more strongly with the shed ectodomain than with the full-length form.²⁰ In addition, the 15th collagenous domain is known to be a candidate epitope for autoAbs from linear IgA bullous dermatosis patients,³⁸ which suggests that the conformation of the COL17 ectodomain may change drastically after cleavage within the NC16A domain. Therefore, our study does not rule out the possibility that Abs reacting

with other neoepitopes far from the physiological cleavage sites may be pathogenic for blister formation.

In summary, we showed that the ectodomain of COL17 is cleaved physiologically *in vivo* in human skin, which produces distinct neoepitopes at the cleavage sites with pyroglutamate N-termini. Although rabbit Abs to the shed ectodomain bind with mouse COL17 and activate murine complements *in vivo*, blistering disease is not introduced, suggesting that an immune reaction to the neoepitopes on the physiological N-terminal cleavage sites would not be pathogenic.

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