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*J Immunol* 2010; 185:4938-4947; Prepublished online 22 September 2010;
doi: 10.4049/jimmunol.1001524
http://www.jimmunol.org/content/185/8/4938

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Ectodomain Sheding Generates Neoepitopes on Collagen XVII, the Major Autoantigen for Bullous Pemphigoid

Wataru Nishie,*† Stephanie Lamer,‡ Andreas Schlosser,§ Emilia Licaret,e,*§ Claus-Werner Franzke,* Silke C. Hofmann,* Joanna Jackow,* Cassian Sitaru,* and Leena Bruckner-Tuderman*‡

As a type II transmembrane protein in basal keratinocytes, collagen XVII provides stable adhesion between epidermis and dermis in the skin. Its ectodomain can be shed from the cell surface, and autoantibodies in certain blistering diseases preferentially recognize the shed form. Major epitopes of collagen XVII are clustered within the juxtamembranous noncollagenous 16th A domain, and ectodomain shedding occurs within this region, suggesting that cleavage generates neoepitopes. However, the candidate cleavage sites have been controversial, and the mechanism of neoepitope generation is unclear. In this study, we investigated cleavage sites in the noncollagenous 16th A domain to understand the generation of neoepitopes and their pathological role. Polyclonal Abs recognizing the stretch Leu524-Gly532 preferentially reacted with the shed ectodomain, but not with the full-length form, indicating that a neoepitope was localized at this site. The neoepitope-specific Ab fixed complement and induced granulocyte-dependent blister formation in normal human skin. The physiological cleavage sites were identified using mass spectrometry. N termini were found at Asp514, Leu 524, Glu 525, and Gly 526, among which Asp 514 and Glu 525 were blocked by acetylation and pyroglutaminate. In silico prediction of B cell epitopes indicated that the antigenicity of the Leu 524-Gly 532 region increased substantially after shedding, regardless of the cleavage sites. Correspondingly, neoepitopes were found in the skin and blister fluids of patients with bullous pemphigoid, and bullous pemphigoid sera reacted with the peptide Leu524-Gly532. Taken together, these data demonstrate that physiological shedding of collagen XVII generates neoepitopes, which may serve as a target of blister-inducing autoantibodies. The Journal of Immunology, 2010, 185: 4938–4947.

Near 2% of mammalian genes encode proteases, and processing of proteins is a crucial event (1). Among different types of proteolysis, the release of extracellular domains of transmembrane proteins, mainly catalyzed by proteases of a disintegrin and metalloprotease (ADAM) family, is known as ectodomain shedding (2). Ectodomain shedding is involved in functions of a variety of essential transmembrane proteins such as TNF-α, TGF-α, epidermal growth factor receptor ligands (2, 3), or transmembrane collagens (4, 5). All of type XIII, XVII, XXIII, and XXV collagens as well as ectodysplasin A are transmembrane proteins in type II orientation, with an intracytoplasmic N terminus and an extracellular C terminus, and their ectodomains can be shed. The shedding occurs within juxtamembranous noncollagenous domains, and is usually mediated by furin and/or ADAMs (4, 5). However, the biological significance of these processes is still unclear.

Transmembrane collagen XVII, also known as BP180 or BPAG2, is widely expressed in skin (6–8), cornea (7, 9), teeth (10), buccal and esophageal mucous membranes (7), brain (11), placenta, and umbilical cord (7). Among these tissues, collagen XVII plays the most important role in the skin, where it is a component of hemidesmosomes in basal keratinocytes and provides stable adhesion between the epidermis and the dermis. Dysfunction of collagen XVII due to genetic or acquired diseases results in blistering phenotypes, as in junctional epidermolysis bullosa or bullous pemphigoid (BP), respectively (4, 8, 12).

The ectodomain of collagen XVII is constitutively shed from the cell surface in vitro (4, 13, 14). The shedding can be suppressed by a furin inhibitor, decanoyl-RVKR-chloromethyl ketone, and there is actually a tribasic furin cleavage motif, -RIRR-, within the noncollagenous 16th A (NC16A) domain (13). Although initial experiments suggested furin-mediated cleavage on collagen XVII, furin itself cannot cleave it in vitro (15). Several lines of evidence, including inhibitor and overexpression studies, suggested that ectodomain shedding of collagen XVII is mediated by ADAMs (15–17).

First, ADAM17 was thought to be the main sheddase for collagen XVII, but a recent study suggested that ADAM10 and 9 are mainly involved (18). Thus, the mechanisms of shedding of collagen XVII, including the responsible sheddase(s), must still be elucidated in detail.

The question of the precise sheddase cleavage site in collagen XVII has been addressed by several investigations, which have yielded varying results. One study used protein extracted from normal human skin and suggested N-terminal cleavage at Ala531 (19). Next, different N termini at Leu524 and Ala528 were identified...
in immortalized HaCat keratinocyte and DJM-1 cell lines (20). In addition, structural modeling of ADAM17 with phosphorylated collagen XVII predicted Leu444 to be a cleavage site (21). In contrast, a further study showed that a mAb with an epitope within the amino acid stretch of Ser412 to Arg523 recognized the shed ectodomain of collagen XVII (22), indicating that ectodomain shedding of collagen XVII could occur at different position(s), probably in a context-dependent manner.

Neoepitopes are newly appeared antigenic sites on cleaved protein fragments, which are not present in their uncleaved precursors (23). Consequently, neoepitope-specific Abs have been used as a powerful tool to directly monitor cleavage events (1, 23). Neoepitopes seem to be involved in human autoimmune diseases such as systemic lupus erythematosus (24) and especially in autoimmune blistering diseases of the pemphigoid group, including BP, pemphigus, and linear IgA dermatosis (LAD). In BP and LAD, it is well known that IgG or IgA class autoantibodies preferentially react with the shed ectodomain of collagen XVII (25, 26). Furthermore, several Abs specifically targeting the shed ectodomain of collagen XVII have been reported in the literature (16, 19, 22, 26). These findings indicate that shedding of collagen XVII generates neoepitopes within the shed ectodomain, which may play a role in the pathogenesis of autoimmune blistering diseases. However, it remains unclear whether ectodomain shedding of collagen XVII can actually generate neoepitopes that are involved in blister formation. To elucidate these questions, we generated Abs to candidate neoepitopes on the shed ectodomain of collagen XVII (25, 19, 26). Furthermore, several Abs specifically targeting the shed ectodomain of collagen XVII have been reported in the literature.

Materials and Methods

Cell culture

Primary normal human keratinocytes (NHKs) were isolated from skin samples obtained from healthy volunteers after fully informed consent. NHKs were cultured in serum-free keratinocyte growth medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen, Darmstadt, Germany). Cells up to the fourth passage were used for this study. Freshly prepared ascorbic acid was added into the culture medium (final concentration 50 µg/ml) every 48 h to allow hydroxylation of collagen and proper triple helix formation (13, 15). Flp-in 293 host cells, were precipitated with ethanol, as described (15).

Production of Abs to candidate neoepitopes on the shed ectodomain of collagen XVII

Based on previously described candidate cleavage sites on collagen XVII (19–21), four different short peptides, 7–9 aa in length, and with nonblocked N termini, were prepared (Fig. 1A). The peptides were coupled with key-hole limpet hemocyanin, and two New Zealand white rabbits per peptide were immunized (25). Abs were affinity purified with the antigenic peptides coupled to AP-Amino TOYOPEARL (Tosho Bioscience, Stuttgart, Germany). Briefly, serum diluted with PBS was incubated with peptide-bound beads, and after extensive washing, bound Abs were eluted with 0.1 M glycine (pH 2.5), followed by immediate neutralization with 1 M Tris-HCl (pH 7.4). Purified Abs were dialyzed against PBS, and the concentration was measured by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Schwerte, Germany). Affinity purification was also performed against N-terminally acetylated Leu24-Gly32 peptide.

Generation of recombinant ectodomain of human collagen XVII

To generate a recombinant ectodomain of collagen XVII, corresponding to aa 490–1407, which can be secreted from stably expressing cells into the culture medium, PCR was first performed using primers His-ect-F, 5′-CAACGCTTGGAGGTAGAACGATACCTC-3′ (underlined; HindIII), and His-ect-R, 5′-ATGCCGCCGCGTTCACCACATGTCCTAC-3′ (underlined; NotI), using human collagen XVII cDNA as template (a gift of L. Borradori). After digestion with restriction enzymes, the PCR fragment was inserted into the HindIII to NotI sites of pSecTag2HygroB vector (Invitrogen), designated as Ect-pSec. In addition, human COL17A1 cDNA was introduced into the NotI site of pcDNA5/FRT (Invitrogen), designated as COL17dcDNA-pcDNA5. Finally, a neo-clal-digested fragment from Ect-pSec was introduced into sites digested by the same enzymes of COL17cDNA-pcDNA5 to produce a vector with the Ig k-chain leader sequence and subsequent sequences encoding the collagen XVII ectodomain, designated as pSec-Ect-pcDNA5. A hexa-histidine fragment, in which an EcoRV site has been incorporated for further study, was introduced into the HindIII site of pSec-Ect-pcDNA5 using oligonucleotides 5′-AGCTTCATCATCATCATCATGATATA-3′ and 5′-AGCTACATCATGATGATGATGATGATG-3′ (underlined). NheI-digested COL17cDNA-pcDNA5, and the pcDNA5/FRT empty vector as control were cotransfected with pOG44 (Invitrogen) into Flp-in 293 host cells, and stably expressing cells were selected under 200 µg/ml hygromycin B (Invitrogen). The medium proteins, including His-ect, 490–532 His-ect, or that from control cells, were precipitated with ethanol, as described (15).

Immunoblotting, immunostaining, and ELISA

For immunoblotting, subconfluent NHKs or transformed 293 cells were lysed for 30 min on ice in a buffer containing 1% Nonidet P-40, 0.1 M NaCl, 25 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 1 mM Pefabloc (13). The samples were separated on SDS-PAGE on 7% polyacrylamide gels, followed by transfer onto nitrocellulose membrane. Abs including mouse mAb NC16a-1 and NC16a-3 directed to different portions on NC16a domain of collagen XVII (22) and rabbit polyclonal Abs described above were used. After incubation with a HRP-conjugated secondary Ab, signals were visualized by ECL-plus (GE Healthcare, München, Germany). For immunohistochemistry, skin specimens were mounted and snap frozen in OCT compound. The following samples were processed: normal human skin, 1 M NaCl-split skin, skin of a collagen XVII-deficient patient with junctional epidermolysis bullosa and mutations in the COL17A1 gene (11) (NM_000494) (12), and skin of blistered patients (11) (NM_000494) (12) and of healthy volunteers (n = 23) and of healthy volunteers (n = 23) using BP180 ELISA kit (MBL, Nagoya, Japan). For the peptide ELISA, 96-well plates were coated with 20 µg/ml peptide, and the absorbance was measured at 450 nm by microtiter plate readers (Bio-Rad, Hercules, CA).

Dermal-epidermal separation assay

Dermal-epidermal separation assays were performed using Abs, as previously described (28). The ability of Abs to activate human leukocytes and induce dermal-epidermal separation was assessed, as described previously (28).

Isolation of physiologically shed ectodomain of collagen XVII

NHK culture medium was concentrated by Amicon Ultrafilter (150 kDa; Millipore, Bedford, MA), and the proteins were precipitated with 50% saturated ammonium sulfate after dialysis against 20 mM EM EDTA, the proteins were dissolved in Laemmli sample buffer and separated on SDS-PAGE with 7% acrylamide gel under nonreducing conditions and without boiling. As the native trimerized form of collagen XVII ectodomain
ectodomain (arrows), whereas Ab 83460 equally bound to both shed and full-length forms (arrowheads) on immunoblotting. Reactivity of Ab 83459 to identified both His-ect and Δ490–532 His-ect proteins. Ab 83457 only reacted to recombinant His-ect and lost the reactivity to Δ490–532 His-ect proteins lacking epitope for Ab 83459, suggesting specificity of this Ab to the epitope. D, Distinctive staining patterns of the neoepitope-specific Ab 83457 and the anti-Collagen XVII mAb NC16A-1. E, Abs 83457 and 83458 preferentially reacted with the shed ectodomain of collagen XVII (arrows), whereas Ab 83460 only bound to both shed and full-length forms (arrowheads) on immunoblotting. Reactivity of Ab 83459 to shed ectodomain of collagen XVII was very faint (data not shown). C, mAb NC16A-1 reacted only to recombinant His-ect protein, whereas mAb NC16A-3 recognized both His-ect and Δ490–532 His-ect proteins. Ab 83457 only reacted to recombinant His-ect and lost the reactivity to Δ490–532 His-ect proteins lacking epitope for Ab 83459, suggesting specificity of this Ab to the epitope. D, Distinctive staining patterns of the neoepitope-specific Ab 83457 and the mAb NC16A-1. Ab 83457 stained the dermal-epidermal junction in a linear fashion, whereas mAb NC16A-1 showed intensive basolateral cytoplasmic staining, with only slight linear signals along the dermal-epidermal junction. E, Ab 83457 stained the epidermal side of salt-split skin. Star: spontaneous blister. Scale bar, 40 μm Original magnification ×400. TM, transmembrane.

FIGURE 1. A schematic representation of collagen XVII and characterization of Abs targeting neoepitopes. A, Collagen XVII is a type II integral transmembrane protein, with an intracellular N terminus and an extracellular C terminus. The ectodomain can be shed from cell surface through cleavage within the NC16A domain. Below the scheme, the amino acid sequence of the NC16A domain, spanning aa 490–566, is delineated. A furin recognition motif, -RIRRS-, is shown in the box. The black bars indicate the peptides used to generate different Abs to target candidate cleavage sites indicated with red arrowheads. The gray bars show the epitopes of mAbs produced previously (22). B, Abs 83457 and 83458 preferentially reacted with the shed ectodomain of collagen XVII (arrows), whereas Ab 83460 equally bound to both shed and full-length forms (arrowheads) on immunoblotting. Reactivity of Ab 83459 to shed ectodomain of collagen XVII was very faint (data not shown). C, mAb NC16A-1 reacted only to recombinant His-ect protein, whereas mAb NC16A-3 recognized both His-ect and Δ490–532 His-ect proteins. Ab 83457 only reacted to recombinant His-ect and lost the reactivity to Δ490–532 His-ect proteins lacking epitope for Ab 83459, suggesting specificity of this Ab to the epitope. D, Distinctive staining patterns of the neoepitope-specific Ab 83457 and the mAb NC16A-1. Ab 83457 stained the dermal-epidermal junction in a linear fashion, whereas mAb NC16A-1 showed intensive basolateral cytoplasmic staining, with only slight linear signals along the dermal-epidermal junction. E, Ab 83457 stained the epidermal side of salt-split skin. Star: salt-induced artificial blister. F, Ab 83457 did not stain collagen XVII-deficient skin of a patient with junctional epidermolysis bullosa. The arrows indicate the position of the dermal-epidermal junction. Star: spontaneous blister. Scale bar, 40 μm Original magnification ×400. TM, transmembrane.
standard settings for the Agilent Q-TOF and the Agilent ion trap. Mascot Server 2.2 was used for database searching using a small custom database containing the protein sequence of collagen XVII. All relevant MS/MS spectra were validated manually.

**Prediction of B cell epitopes on shed ectodomain of collagen XVII**

Based on the disclosed cleavage sites, continuous B cell epitopes on cleaved fragments and the entire NC16A domain (spanning aa 490–566) were predicted, and the antigenic propensity scores were calculated by COBEpro software (29).

**In vitro digestion of recombinant collagen XVII by blister fluid from BP patients**

Collagen XVII-expressing Flp-in 293 cells transfected with COL17cDNA-pcDNA5 were treated with 0.1 mM furin inhibitor I (Calbiochem, San Diego, CA) overnight, and cell lysates were coupled with a cation exchange column (Vivapure S; Sartorius Stedim, Edgewood, NY). Full-length collagen XVII was eluted with a gradient of 0.4–1.0 M NaCl, followed by dialysis against PBS. A total of 10 μl collagen XVII was incubated with 1 μl blister fluid obtained from three BP patients for 1 h at 37˚C. To discern the responsible proteases, protease inhibitors including 10 mM EDTA, 1–5 mM Pefabloc, 3–50 μM Marimastat (British Biotechnology, Oxford, U.K.), 10 μg/ml chymostatin (Calbiochem), 70 μg/ml α2-antiplasmin (Calbiochem), and 100 μg/ml soybean trypsin inhibitor (Calbiochem) were added to the enzyme reaction.

**Purification of Ab 83457**

Purified recombinant full-length collagen XVII was coupled with NSH-activated Sepharose (GE Healthcare) and used to immunoadsorb Ab 83457 to eliminate minimal reactivity with the full-length form of collagen XVII.

**Immunoprecipitation of ectodomain of collagen XVII from blister fluid of BP patients**

The ectodomain of collagen XVII was immunoprecipitated from blister fluid of BP patients with Ab 83457 and normal rabbit IgG as a control (22). Briefly, 100 μl blister fluid was dialyzed against PBS and precleared with 100 μl protein G Dynabeads (Invitrogen). After extensive washing, the sample was incubated with 10 μg Ab 83457 or normal rabbit IgG overnight at 4˚C, and then reacted with 100 μl protein G Dynabeads for 1 h at room temperature. The samples were eluted with SDS sample buffer and separated by SDS-PAGE, and the ectodomain of collagen XVII was detected by immunoblotting using mAb NC16A-3.

**Results**

**Abs target neoepitopes in the shed ectodomain of collagen XVII**

In this study, we used 7- to 9-aa-long peptides to generate Abs, which recognize shedding-associated epitopes in collagen XVII (Fig. 1A). Three of four different Abs to target candidate cleavage sites worked well (Fig. 1B). Abs 83457 and 83458 preferentially reacted with the shed ectodomain, whereas Ab 83460 bound equally well to both the shed and the full-length forms in cell lysates of NHKs (Fig. 1B). The reactivity of Ab 83459 was very faint, and this Ab was not used. These findings suggest that Abs 83457 and 83458 target neoepitopes in the shed ectodomain of collagen XVII. The specific reactivity of Ab 83457 with the shed ectodomain of collagen XVII did not change when N-terminally acetylated Leu524-Gly532 peptide was used for affinity purification of the Ab (data not shown). Ab 83457 reacted with the recombinant...
full-length His-ect, but not with the Δ490–532 His-ect deletion mutant (Fig. 1C), verifying the specificity of this Ab and reactivity with a collagen XVII epitope. Correct expression of His-ect and Δ490–532 His-ect was also confirmed with the reactivity of mAb NC16A-1 and NC16A-3 (Fig. 1C).

Of the newly generated Abs, only Ab 83457 recognized native collagen XVII in normal human skin in immunofluorescence staining; it produced linear signals at the dermal-epidermal junction. In comparison, the mAb NC16A-1, which recognizes an epitope in close vicinity, showed intensive basolateral cytoplasmic staining in addition to a slight linear signal along the dermal-epidermal junction (Fig. 1D). Although collagen XVII is a transmembrane protein, substantial expression can be observed in the cytoplasm of basal keratinocytes (14, 16). Thus, the specific linear staining pattern of Ab 83457 indicates that collagen XVII present in the cytoplasm does not contain neoepitopes, and that the shed ectodomain is actually present along the dermal-epidermal junction, as a consequence of proteolytic cleavage on the basal cell surface. The specificity of Ab 83457 was corroborated by the fact that it stained the epidermal side on salt-split skin (Fig. 1E) and remained negative on collagen XVII-deficient skin of a patient with junctional epidermolysis bullosa and the mutations 1392G>A/p.W464X in the COL17A1 gene (Fig. 1F).

Collagen XVII neoepitope-specific Ab fixed complement and induced dermal-epidermal separation in cryosections of human skin

A major diagnostic and pathogenic feature of pemphigoid IgG autoantibodies is their capacity to activate the complement system (27, 28). To assess the capacity of rabbit IgG Abs recognizing different forms of collagen XVII to bind complement, an in vitro complement-fixation assay was performed using pAb-NC16A and Ab 83457. Both Abs bound to the dermal-epidermal junction of normal human skin and showed complement-fixing activity (Fig. 2a, 2b, 2d, 2e). In contrast, the IgG fraction from a preimmune rabbit did not stain the dermal-epidermal junction nor show complement-binding capacity (Fig. 2c, 2f).

Several lines of evidence indicate that recruitment and activation of granulocytes by autoantibodies after binding to the dermal-epidermal junction are a prerequisite for blister formation in pemphigoid diseases (27, 28). Therefore, to characterize the granulocyte-activating ability of the collagen XVII neoepitope-specific Ab, we used an ex vivo Ab-induced granulocyte-dependent assay having as readout dermal-epidermal splits in cryosections of normal human skin (27, 28). Incubation of cryosections with the pAb-NC16A (Fig. 2g) and Ab 83457 (Fig. 2h), but not with the control IgG (Fig. 2i), resulted in subepidermal cleavage when coincubated with leukocytes from healthy donors.

**MS revealed different shedding sites within the NC16A domain of collagen XVII**

The fact that collagens spontaneously fold into a triple helix allowed us to obtain a highly purified shed ectodomain of collagen XVII for MS analysis. On SDS-PAGE, the triple-helical shed ectodomain migrated at ∼360 kDa (13, 14), and the excision of this band eliminated contaminating proteins smaller than 300–330 kDa. The band was subjected to a second SDS-PAGE under denaturing conditions, which presented the distinct unfolded 120-kDa monomer (Fig. 3A, 3B). The efficiency of this procedure was very high, and 40 ml culture medium was sufficient to generate the 120-kDa Coomassie blue-stained band seen in Fig. 3B.

Multiple LC-MS/MS analyses with different proteases detected several candidates for N-terminal peptides of the shed ectodomain (Table I). Among them, two peptides with a free N-terminal amino group starting with Leu524 and Gly526 were highly likely to be physiological cleavage sites. For differentiation between true N-terminal peptides (generated by a protease in vivo) and peptides generated by a protease during in-gel digestion, the samples were acetylated before second SDS-PAGE. This enabled us to block all free N termini, including unblocked cleavage sites. As a result,

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**FIGURE 3.** Proteomics strategy for determination of sheddase cleavage sites in collagen XVII using LC-MS/MS. The physiologically shed ectodomain of collagen XVII in NHK culture medium was concentrated and subjected to SDS-PAGE under nondenaturing conditions. The trimeric ectodomain migrated at ∼360 kDa. Therefore, the gel bands in this migration position were excised, and the proteins were electroeluted for further analysis. A, Immunoblotting of electroeluted samples. Under nondenaturing conditions (without boiling), the trimeric forms of the shed ectodomain of collagen XVII only penetrate into the upper gel. In contrast, under denaturing conditions (after boiling), a distinct 120-kDa band corresponding to the monomeric form of shed ectodomain is seen. B, The Coomassie blue-stained 120-kDa band, corresponding to the monomeric form of the shed ectodomain under denaturing conditions, was cut and subjected to MS analysis. C, In the schematic representation of collagen XVII, the cleavage sites identified by MS are indicated with arrows within the NC16A sequence. Notably, the cleavage sites were not associated with the furin consensus motif (box), and the cleavage sites, Leu524, Gln525, and Gly526, were tightly clustered. The N termini of the fragments starting at Asp514 and Gln525 were blocked by acetylation (star) and pyroglutamate (stars), respectively. TM, transmembrane.
acetylated peptides starting at Leu$^{524}$ and Gly$^{526}$ were successfully detected (Table I), indicating these N termini are cleavage sites without modifications (Fig. 3C).

Interestingly, the N terminus of a peptide starting at Asp$^{514}$ was naturally blocked by acetylation. This novel, to our knowledge, cleavage site clarified why mAb NC16A-1, with epitopes located N-terminally of Leu$^{524}$ (Fig. 1A), had been shown to react with the shed ectodomain of collagen XVII (22). Several attempts to define the N termini of the shed ectodomain by Edman degradation have failed in the past. The present experiments explained this by demonstrating that the N terminus of the fragment starting at Asp$^{514}$ was blocked by acetylation and the N terminus of the peptide starting at Gln$^{526}$ was also blocked by pyroglutamate. The fragment starting at Gln$^{525}$ was detected by trypsin and thermolysin digestion. Trypsin digestion showed low chymotrypsin-like activity (data not shown); therefore, it was not possible to exclude the possibility for Gln$^{525}$ to be an artificial terminus. However, thermolysin digestion also could detect this fragment, and elastase has been reported to cut after Leu with $\sim$20% probability (30), suggesting that Gln$^{525}$ is a true cleavage site.

### Table I. Candidate N termini of shed ectodomain of collagen XVII detected by MS

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Modification of Samples before SDS-PAGE</th>
<th>Acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>pyro-QGMAPAAGADLDK</td>
<td>acetyl-LQGMAPAAGA</td>
</tr>
<tr>
<td>Elastase</td>
<td>acetyl-DSMDRIEKDRGLQGM</td>
<td>acetyl-LQGMAPAAGADLDKI</td>
</tr>
<tr>
<td></td>
<td>GMAPAAGADLDKI</td>
<td>acetyl-GMAPAAGADLDKI</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>pyro-QGMAPAAGADLDKIG</td>
<td>acetyl-LQGMAPAAGADLDKIG</td>
</tr>
</tbody>
</table>

Acetyl, N terminus has been blocked by acetylation; pyro-Q, N-terminal glutamine has been blocked by pyroglutamate.

### FIGURE 4. Prediction of B cell epitopes on NC16A domain of collagen XVII before and after ectodomain shedding. Net votes for epitopes around Leu$^{524}$ to Gly$^{532}$ of the Ab 83457 were totally negative on long sequences covering entire NC16A domain. In contrast, distinctive epitopes appeared in this region when shedding of the ectodomain was included in the prediction. The appearance of the novel (to our knowledge) epitopes did not depend on one particular cleavage site.
In silico prediction of neoepitopes in each of the shed ectodomains

Detection of four different, distinct N termini on shed ectodomains of collagen XVII eliminated the previous discrepancies regarding N termini and immunoreactivity of several Abs. In addition, the preferential reactivity of Ab 83457 with the shed ectodomain of collagen XVII proved that distinct neoepitopes were generated by the shedding. The next question was how neoepitopes developed after shedding of collagen XVII. To address this, we used the COBEpro software (29) to predict continuous B cell epitopes within the NC16A domain of collagen XVII. This showed that predicted antigenic propensity scores were very low in the middle and C-terminal portions of the intact NC16A domain, spanning amino acids Leu$^{224}$-Arg$^{266}$. However, when the newly detected cleavage sites Asp$^{514}$ and Leu$^{524}$ were included as N termini, the antigenic propensity scores rose substantially in two regions, Asp$^{514}$-Gly$^{526}$ and Met$^{527}$-Ile$^{538}$, suggesting that these stretches refold upon shedding and become highly antigenic. These neoepitopes are recognized by the mAb NC16A-1 and Ab 83457, respectively (Fig. 4).

BP blister fluid generates 120-kDa ectodomain of collagen XVII in vitro

To assess the possibility that BP blister fluids can hydrolyze collagen XVII, in vitro digestions were performed. Incubation of recombinant collagen XVII with blister fluid of three BP patients (BP13, BP29, and BP87) yielded a stable 120-kDa ectodomain detectable with the mAb NC16A-3 and Ab 83457 (Fig. 5A). Control blister fluids obtained from suction blisters in healthy volunteers also generated minor amounts of the 120-kDa ectodomain (Fig. 5A), consistent with our previous report showing that blister fluids contain proteolytic enzymes (22).

For further characterization of the enzymes hydrolyzing collagen XVII, different protein inhibitors were employed. Among them, 5 mM Pefabloc inhibited the digestion moderately (Fig. 5B). Because Pefabloc is a broad serine protease inhibitor, chymostatin, α2-antiplasmin, and soybean trypsin inhibitor were also tested. As a result, α2-antiplasmin and soybean trypsin inhibitor moderately inhibited the hydrolysis (Fig. 5B), implicating involvement of plasmin and trypsin-like enzymes in the digestion of collagen XVII. Although mAb NC16A-3 reacted intensively with the hydrolyzed 120-kDa ectodomain of collagen XVII, the neoepitope-specific Ab 83457 did not considerably change its reactivity between physiological (without blister fluid) and pathological (with blister fluid) setting (Fig. 5A, 5B). This suggests that the cleavage by different proteases in the blister fluid may occur at different sites, although further details remain to be elucidated.

Collagen XVII neoepitopes in BP

The above data suggested the involvement of collagen XVII neoepitopes in the pathogenesis of BP. The next question was whether autoantibodies in BP patients actually react with the neoepitopes and whether ectodomains containing neoepitopes exist in tissues. ELISAs demonstrated that a number of BP sera reacted with the peptide Leu$^{524}$-Gly$^{532}$, indicating that the neoepitope is recognized by circulating Abs in BP patients (Fig. 6A). Furthermore, the neoepitope-specific Ab 83457 immunoprecipitated the ectodomain from blister fluids of two BP patients (Fig. 6B). These observations delivered evidence for in vivo emergence of neoepitopes within the ectodomain of collagen XVII and presence of autoantibodies to these neoepitopes in at least some patients with BP.

Adsorption of the Ab 83457 with recombinant full-length collagen XVII abrogated its minor reactivity with the full-length form (Fig. 7A). In immunofluorescence staining, the purified Ab specifically reacted with perilesional, still nonblistering skin areas in all three BP patients (Fig. 7B), indicating involvement in early lesions. In addition, intracytoplasmic staining was observed in two patients, and a mottled staining was seen along the dermal-epidermal junction in one patient, possibly corresponding to the released ectodomain of collagen XVII (Fig. 7B). Control IgG failed to yield any signals (data not shown).

Discussion

In this study, we disclosed distinctive neoepitopes and what we believe are novel cleavage sites on the NC16A domain of collagen XVII. In silico prediction of B cell epitopes indicated that neoepitopes were evoked by ectodomain shedding, and in vitro tests demonstrated that the neoepitope-specific Ab could induce blister formation in human skin. Furthermore, the presence of neoepitopes was demonstrated in BP patients. On one hand, circulating autoantibodies in BP sera recognized the neoepitope-containing peptide Leu$^{524}$-Gly$^{532}$ in ELISAs. On the other hand, the neoepitope-containing ectodomain of collagen XVII was present in blister fluids of BP patients. Blister fluids contained proteases that hydrolyzed full-length collagen XVII into the 120-kDa ectodomain in vitro. Finally, a distinctive expression pattern of neoepitopes was disclosed in lesional skin of BP patients. These results suggest
a strong association between ectodomain shedding and evolution of neoepitopes on the NC16A domain of collagen XVII.

The neoepitope Leu\(^{524}\)-Gly\(^{532}\) on the NC16A domain lies adjacent to one of the cleavage sites and includes the N terminus at Leu\(^{524}\) (20). Ectodomain shedding of collagen XVII is catalyzed by ADAM proteases, especially by ADAM17 (15, 17, 21), and 9, and 10 (15, 18). ADAMs do not require a specific cleavage consensus sequence, but cleave their transmembrane substrates at a defined length from the cell surface (17, 31). Thus, it is highly likely that the main collagen XVII cleavage sites lie within the region Leu\(^{524}\)-Gly\(^{532}\). The fact that immunoreactivity of Ab 83457 did not significantly change upon affinity purification with an N-terminally acetylated peptide may point to Gln\(^{525}\) as the major N-terminal cleavage site, which has been blocked by pyroglutaminate. This would explain why Ab 83457 could also react with the blocked neoepitopes.

It has been a mystery why autoantibodies in autoimmune blistering diseases of the pemphigoid group, especially in LAD, preferentially react with the shed ectodomain of collagen XVII and less with its full-length form (32, 33). Previous investigations have yielded variable results and interpretations. One study showed that autoantibodies in a subgroup of patients with LAD specifically reacted with NC16A domain of collagen XVII (34), which is consistent with the current study. In contrast, in another group of LAD patients, IgA autoantibodies were shown to react selectively with 15th collagenous domain (32), suggesting that development of neoepitopes on collagen XVII could occur in more extended regions. This is supported, for example, by the fact that the mAb NC16A-3, which recognizes the sequence Gln\(^{545}\)-Met\(^{557}\), preferentially reacted with the shed ectodomain of collagen XVII on immunoblotting (22). However, the full scenario of neoepitope evolution and its implications for the pathogenesis of collagen XVII-related disorders remain to be elucidated.

**FIGURE 6.** The neoepitope-containing ectodomain of collagen XVII in BP A, ELISAs showed that whereas most BP sera reacted with the recombinant NC16A domain of collagen XVII, a number of sera also recognized the peptide Leu\(^{524}\)-Gly\(^{532}\), indicating that BP autoantibodies target neoepitopes on the shed ectodomain. B, Ab 83457 immunoprecipitated the 120-kDa neoepitope-containing ectodomain of collagen XVII from blister fluid from two BP patients (arrowheads). This was not the case with control IgG. The blot was detected by mAb NC16A-3.

**FIGURE 7.** Collagen XVII neoepitopes in lesional skin in BP A, Immunoadsorption of Ab 83457 with recombinant full-length collagen XVII abrogated residual reactivity with the full-length form (arrowheads), as shown by immunoblotting using lysates of NHKs with different concentration of Ab 83457 (lanes 1–4, 10–1.25 \(\mu\)g/ml) before and after immunoadsorption. B, Indirect immunofluorescence staining of the skin of three BP patients with 5 \(\mu\)g/ml affinity-purified Ab 83457 (designated as Ab HK139). Different from expression pattern detected by a polyclonal Ab to NC16A, Ab HK139 specifically reacted with the nonblistering areas in the periphery of the lesions in all three BP patients. In addition, the Ab showed intracytoplasmic staining in two patients (BP 09-115 and 09-275), and a mottled staining along the dermal-epidermal junction in one patient (BP 09-115, arrowheads), which may reflect the released ectodomain of collagen XVII. Stars: blister. Scale bar, 160 \(\mu\)m. Original magnification \(\times 100\).
development still remains elusive at least in part. Possible changes in folding of the collagenous triple helix structures within the ectodomain must be considered (15). Although the neighbor of the NC16A domain, the 15th collagenous domain, is quite stable and can form a triple-helical structure by itself when expressed as a recombinant fragment (35, 36), it is still possible that conformational or other changes take place after shedding of the entire ectodomain. This assumption is in line with observations that IgA from LAD serum can also react with full-length collagen XVII, if the incubation temperature is increased (37), because it is well known that collagen triple helices unfold in higher temperatures.

Autoantibodies from patients with BP or epidermolysis bullosa acquisita show complement- and leukocyte-activating capacity, which appear to be main determinants of their pathogenicity (28, 38). In this study, we assessed these two major pathogenic features of the neoepitope-specific Ab 83457. Complement activation was measured by an immunofluorescence microscopy test assessing the classical pathway. The granulocyte-activating capacity of the Ab was evaluated by an ex vivo model of Ab-induced granulocyte-dependent dermal-epidermal separation in cryosections of human skin incubated with the Ab in the presence of leukocytes. These experiments demonstrated that, in analogy to human autoantibodies in BP, the neoepitope-specific Ab 83457 can fix human complement and induce granulocyte activation ex vivo.

Another commonly used method to verify the pathogenicity of autoantibodies is the passive transfer of IgG from patients into mice to test blister induction (39, 40). In the case of BP, this has not succeeded yet, because the amino acid sequences of the epitope region within the NC16A domain differ significantly between humans and animals (39). To overcome the species’ differences, a collagen XVII-humanized mouse has been developed to reproduce the BP phenotype (40, 41), but ectodomain shedding of human collagen XVII in the mouse skin has not yet been precisely analyzed. Therefore, currently, the in vitro dermal-epidermal separation assay is most suitable to verify the ability of the Abs to induce blister formation.

In this study, a novel (to our knowledge) and simple approach allowed determination of the N termini of shed ectodomain of collagen XVII. Previously, low amounts of the shed ectodomain in the skin or in primary cell cultures, tedious protein isolation protocols, and blocked N termini have hampered corresponding efforts (20, 22). Although MS protocols for determining the N termini of abundant proteins are well established (42, 43), this has not been the case for proteins present at low concentrations. To solve this problem, we took advantage of the collagenous nature of the shed ectodomain of collagen XVII that forms a relatively stable trimer (14) and used successive SDS-PAGE runs, which increased the target protein while decreasing background proteins. The high molecular mass trimerized ectodomain was isolated first and then subjected to a second SDS-PAGE under denaturing conditions, which enabled isolation of sufficient amounts of highly purified shed ectodomain for MS analysis. Furthermore, acetylation of the ectodomain labeled free N termini, which could then be defined as cleavage sites by MS. Acetylation of free N termini is a very useful method (42, 43) to distinguish in vitro processed fragments from those digested during in-gel fragmentation for MS. The simple and powerful method described in this work can be easily used to find shedding-generated, to our knowledge, novel N termini in other type II transmembrane collagens and other proteins forming tight complexes.

Digestion of recombinant full-length collagen XVII with blister fluids from BP patients produced the 120-kDa ectodomain, which could be moderately inhibited by α2-antiplasmin and soybean trypsin inhibitor. Plasmin has been suggested to play a role in early blistering skin lesions of BP (44) and in the generation of the LAD autoantigen, which is also a digestion product of ectodomain of collagen XVII (22). Thus, based on the above arguments, the 120-kDa neoepitope-containing ectodomain seems to be involved in the pathogenesis of BP. However, in vivo expression of the neoepitopes in lesional BP skin has remained unknown. In this study, we used affinity-purified Ab83457, which has no reactivity with full-length collagen XVII, to demonstrate a distinctive expression pattern of the neoepitopes in the periphery of blisters, in nonblistering areas, indicating association with early BP lesions. The reactivity was not seen in overt blistering, probably due to loss of epitopes through nonspecific tissue proteolysis in inflamed skin. Another possibility is that the neoepitope-containing ectodomain diffuses into the blister fluid. This is supported by the observation that Ab 83457 immunoprecipitated the ectodomain from BP blister fluids. In some instances, Ab 83457 revealed intracytoplasmic staining in nonblistering skin. Internalization of collagen XVII has been suggested in BP (45), which may be a possible mechanism for this particular finding.

Taken together, we deliver clear evidence for shedding-generated neoepitopes on the NC16A domain of collagen XVII, which are highly likely to be involved in the pathogenesis of autoimmune blistering skin diseases, such as BP or LAD.

Acknowledgments
We thank Margit Schubert for technical assistance and Dr. Cristina Has and Dr. Ying-Hong He for help with the experiments and for critical suggestions.

Disclosures
The authors have no financial conflicts of interest.

References
25. Schumann, H., J. Baetge, K. Tasanen, F. Wojnarowska, H. Schäcke, D. Zillikens,
22. Hofmann, S. C., U. Voith, V. Schöau, L. Sorokin, L. Bruckner-Tuderman, and
28. Sitaru, C., E. Schmidt, S. Petermann, L. S. Munteanu, E. B. Bröcker, and
27. Sitaru, C., J. Powell, G. Messer, E. B. Bröcker, F. Wojnarowska, and
membrane metalloproteolytic cleavage of L-selectin (CD62l) by the epidermal
specificity protease clastase for large-scale phosphoproteome analysis. Anal.
Chem. 80: 9526–9533.
33. Nishie, W., D. Sawamura, K. Natsuga, S. Shinkuma, M. Goto, A. Shibaki,
32. Nie, Z., Y. Nagata, S. Joubeh, Y. Hirako, K. Owaribe, Y. Kitajima, and
K. Owaribe. 2003. Extracellular cleavage of bullous pemphigoid antigen 180kDa collagen and its in-
34. Schumann, H., J. M. Mascaro, Jr., L. A. Diaz, L. Bruckner-Tuderman,
E. B. Bröcker, and G. J. Giudice. 1999. Autoantibodies in a subgroup of patients
with linear IgA disease react with the NC16a domain of BP180. J. Invest.
NC16A domain of collagen XVII plays a role in triple helix assembly and sta-
36. Tasanen, K., J. A. Eible, M. Aumailley, H. Schumann, J. Baetge, H. Tu,
P. Bruckner, and L. Bruckner-Tuderman. 2000. Collagen XVII is destabilized by
a glycosylation substitution mutation in the cell adhesion domain Col15. J. Biol. Chem.
275: 3093–3099.
37. Pas, H. H., G. J. Kloorsterhuis, M. C. de Jong, and M. F. Jonkman. 2001. False-
egative results in immunoblot assay of serum IgA antibodies reactive with the
180-kDa bullous pemphigoid antigen: the importance of primary incubation
38. Shimanovich, I., S. Mihai, G. J. Oostingh, T. T. Ienchuk, E. B. Bröcker,
and gelatinase B are required for dermal-epidermal separation induced by
autoantibodies from patients with epidermolysis bullosa acquisita and bullous
pemphigoid. J. Pathol. 204: 519–527.
and G. J. Giudice. 1993. A passive transfer model of the organ-specific autoimmune
disease, bullous pemphigoid, using antibodies generated against the hemides-
40. Nishie, W., D. Sawamura, M. Goto, K. Ito, A. Shibuki, R. J. McMillan, K. Sakai,
41. Nishie, W., D. Sawamura, K. Natsuga, S. Shinkuma, M. Goto, A. Shibuki,
neonatal autoimmune blistering skin disease model induced by maternally
42. McDonald, L., D. H. L. Robertson, J. L. Hurst, and R. J. Beynon. 2005. Posi-
tional proteomics: selective recovery and analysis of N-terminal proteolytic
43. McDonald, L., and R. J. Beynon. 2006. Positional proteomics: preparation of
amo-terminal peptides as a strategy for proteome simplification and charac-
44. Kramer, M. D., and J. Reimertz. 1993. The autoimmune blistering skin disease
bullous pemphigoid: the presence of plasminogen 2-antiplasmin complexes in skin
45. Kitajima, Y., M. Nojiri, T. Yamada, Y. Hirako, and K. Owaribe. 1998. In-
ternalization of the 180 kDa bullous pemphigoid antigen as immune complexes
in basal keratinocytes: an important early event in blister formation in bullous