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Immunopathology and Infectious Disease

The Cartilage Matrix Protein Subdomain of Type VII Collagen Is Pathogenic for Epidermolysis Bullosa Acquisita

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Epidermolysis bullosa acquisita (EBA) is an acquired bullous disease of the skin characterized by IgG autoantibodies against type VII (anchoring fibril) collagen. We previously defined four immunodominant antigenic epitopes within the noncollagenous 1 (NC1) domain of type VII collagen. In this study, we produced an additional recombinant fusion protein from the NC1 domain corresponding to the N-terminal 227 amino acids (residues 1 to 227), which contains homology with cartilage matrix protein (CMP). Using enzyme-linked immunosorbent assay and immunoblot analysis, we tested sera from EBA patients (n = 32), bullous systemic lupus erythematosus patients (n = 3), bullous pemphigoid patients (n = 15), and normal humans (n = 12). Twenty-six of 32 EBA sera and two of three bullous systemic lupus erythematosus sera reacted with the CMP domain, whereas none of the control sera did. Affinity-purified anti-CMP EBA antibodies injected into hairless mice produced the clinical, histological, immunological, and ultrastructural features of EBA. F(ab′)$_2$ fragments generated from anti-CMP EBA autoantibodies did not induce disease. Our studies provide the first evidence that EBA autoantibodies to the CMP subdomain of NC1 are pathogenic and induce blister formation. This is the first antigenic epitope on type VII collagen demonstrated to be a pathogenic target for EBA autoantibodies. ***(Am J Pathol 2007, 170:2009–2018; DOI: 10.2353/ajpath.2007.061212)***

Epidermolysis bullosa acquisita (EBA) is a severe, chronic, subepidermal bullous disease of the skin and mucosa characterized by skin fragility, blisters in trauma-prone sites, scarring with milia formation, and nail dystrophy.[1] It is a prototypic autoimmune disease in which EBA patients have *in vivo* tissue-bound and circulating IgG autoantibodies directed against type VII collagen, a major component of anchoring fibrils, structures that anchor the epidermis onto the dermis.[2–8] EBA autoantibodies bind to type VII collagen within anchoring fibrils. EBA patients have a diminution of normal anchoring fibrils and subsequent epidermal-dermal disadherence. The clinical appearance of EBA patients and the histology of their cutaneous lesions are often very reminiscent of hereditary dystrophic epidermolysis bullosa. These two diseases are etiologically unrelated but share the common feature of decreased anchoring fibrils. In the case of inherited dystrophic epidermolysis bullosa, the cause of decreased or absent anchoring fibrils is a genetic defect in the gene that encodes for type VII collagen.[9,10]

Type VII collagen is composed of three identical α chains, each consisting of a 145-kd central collagenous triple-helical segment characterized by repeating Gly-X-Y amino acid sequences, flanked by a large 145-kd amino-terminal noncollagenous domain (NC1), and a small 34-kd carboxyl-terminal noncollagenous domain (NC2).[6–8,11,12] Within the extracellular space, type VII collagen molecules form anti-parallel, tail-to-tail dimers stabilized by disulfide bonding through a small carboxyl-terminal NC2 overlap between two type VII collagen molecules. The anti-parallel dimers then aggregate laterally to form anchoring fibrils with large globular NC1 domains at both ends of the structure. Sequence analysis of the NC1 domain revealed multiple submodules with homology to adhesive proteins.[13] These include a segment with homology to CMP, nine consecutive fibronectin type III-like repeats (FNIII), and a segment with homology to the

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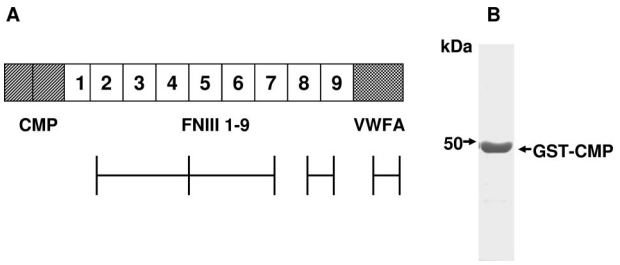

Figure 1. Domain organization, four immunodominant epitopes for EBA autoantibodies within the NC1 domain of human type VII collagen, and expression of recombinant CMP. **A:** The deduced 1253-amino acid sequence of the NC1 domain revealed motifs with homology to known adhesive proteins. CMP, cartilage matrix protein; FNIII 1–9, nine fibronectin type III-like repeats; VWF-A, the A domain of von Willebrand factor. Bottom shows the four previously identified immunodominant epitopes recognized by EBA sera. **B:** Ten percent SDS-PAGE and Coomassie Blue staining of recombinant GST-CMP protein purified from bacteria. The positions of the 50-kd molecular weight marker and the GST-CMP fusion protein are indicated.

A domain of von Willebrand factor (VWF-A) (Figure 1A). We and others have shown that NC1 interacts with various extracellular matrix components including fibronectin, laminin-5, type I collagen, and type IV collagen.[14–17] Therefore, the NC1 domain may facilitate binding of type VII collagen to other basement membrane zone (BMZ) and matrix components. These matrix interactions are thought to stabilize the adhesion of the BMZ to the underlying dermis.

Using a panel of recombinant fusion proteins or fragments of type VII collagen, we and others have shown previously that EBA autoantibodies recognize four major antigenic epitopes confined to the FNIII and VWF-A subdomains of NC1.[18–20] At that time, the amino terminus of NC1 had not been cloned or characterized. Moreover, none of the EBA autoantibodies to the identified antigenic epitopes was shown to be pathogenic.

The pathogenicity of rabbit anti-type VII collagen antibodies in the induction of EBA has been established in animal models by passively transferring immune rabbit antibodies against type VII collagen into hairless mice.[21,22] Recently, we immunized rabbits and raised a high titer antiserum to the NC1 domain of human type VII collagen. We injected the antibody into hairless immunocompetent mice, and the mice developed a bullous eruption that had many of the features of EBA patients.[21] Another recent study by Sitaru and colleagues[22] showed that the injection of rabbit polyclonal antibodies to the NC1 domain of mouse type VII collagen into adult mice also induced subepidermal skin blisters reminiscent of human EBA. More recently, we affinity-purified anti-NC1 autoantibodies from EBA patients' sera and injected them into hairless mice. The animals developed a subepidermal bullous disease with clinical, histological, immunological, and ultrastructural features similar to human EBA.[23] These results provide evidence that human EBA autoantibodies to the NC1 domain of type VII collagen are pathogenic and capable of inducing epidermal-dermal separation of skin.

In this study, an additional recombinant fusion protein corresponding to the N-terminal 227 amino acids (residues 1 to 227) of NC1 and homologous to CMP was generated and analyzed by immunoblot and enzyme-linked immunosorbent assay (ELISA) for reactivity with autoantibodies from 32 EBA patients. We found that 26 of 32 EBA sera and two of three bullous systemic lupus erythematosus (BSLE) sera reacted with the CMP domain in both assays. We then affinity-purified anti-CMP autoantibodies from the serum of one EBA patient and injected them intradermally into adult immunocompetent hairless mice. The injected autoantibodies consistently induced a subepidermal blistering disease resembling the clinical, histological, and immunological features of human EBA. These results demonstrate that EBA autoantibodies to the CMP subdomain of type VII collagen are pathogenic and likely play an important role in the induction of epidermal dermal disadherence featured in EBA.

Materials and Methods

Patients and Sera

Serum samples were collected from 32 patients with EBA. These EBA patients had 1) an active, chronic, mechanobullous disorder; 2) subepidermal blisters as assessed by routine light microscopy of lesional skin; 3) IgG deposits detected at the dermal-epidermal junction (DEJ) by routine direct immunofluorescence (DIF); 4) IgG deposits localized to the dermal floor of the patient's skin when the DEJ was fractured through the lamina lucida by treatment with 1 mol/L NaCl[24,25]; 5) immunoreactivity to the NC1 domain of type VII collagen by ELISA and immunoblot analysis[26]; and 6) IgG deposits detected within the sublamina densa region of the DEJ using direct immunoelectron microscopy.[27]

Because sera from patients with BSLE have been shown to have autoantibodies to type VII collagen,[28] three sera from BSLE patients were also tested. Control serum samples were collected from 12 normal individuals (normal human sera, NHS) and 15 patients who had clinical, histological, and immunofluorescence findings consistent with the diagnosis of BP. Patient and normal control sera were stored frozen at −20°C before analysis.

Plasma was collected from one patient with EBA during the early phase of their disease (before treatment). This patient was plasmaphoresed for therapeutic purposes, which generated large volumes of plasma rich in anti-CMP antibodies. This EBA patient met the EBA criteria outlined above. In addition, she had both IgG and C3 deposits detected at the DEJ by routine DIF and indirect immunofluorescence (IIF) titers ranging from 1:1280 to 1:5120 as analyzed on salt-split normal human skin substrate. The study was conducted according to Declaration of Helsinki Principles.

Construction and Expression of Recombinant Fusion Proteins Corresponding to NC1 Subdomains

The fragment corresponding to the 700-bp CMP subdomain of human type VII collagen cDNA was generated by

reverse transcriptase-polymerase chain reaction amplification using human amniotic epithelial cell (WISH) cDNA as a template as described previously.[14] The insert was then subcloned into a TA vector and pGEX expression vector (Pharmacia, Inc., Piscataway, NJ), as modified by Dr. George Giudice, Medical College of Wisconsin, Milwaukee, WI.[29] The correct ligation and in-frame insertion of the DNA fragment was confirmed by DNA sequence analysis. Bacterial fusion proteins corresponding to discrete segments within the NC1 domain of type VII collagen were developed and purified by a glutathione-Sepharose 4B column (Pharmacia, Uppsala, Sweden) as described.[18] These fusion proteins included CMP (residues 1 to 227), FP1 (residues 201 to 602), FP3 (residues 595 to 826), FP7 (residues 814 to 1028), and FP8 (residues 1022 to 1253).

ELISA Using CMP

Ninety-six-well microtiter plates (Immulon-4; Dynatch Laboratory Inc., Alexandria, VA) were coated with purified glutathione *S*-transferase (GST)-CMP at a concentration of 1.5 μg/ml (0.15 μg/well) in 20 mmol/L carbonate buffer, pH 9.3, overnight at 4°C. ELISA was performed as previously described.[26] The patients' sera dilutions ranged from 1:100 to 1:1250.

Immunoblotting of Recombinant Fusion Protein CMP

Purified GST-CMP protein (100 ng/well) was run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then electrotransferred to a nitrocellulose membrane. Cut strips of nitrocellulose were blocked for 60 minutes at room temperature with 10% nonfat dry milk or overnight at 4°C with 5% bovine serum albumin in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.1% Tween 20 (TTBS). After washing with TTBS buffer, the strips were incubated for 1 hour at room temperature with individual patient sera or control sera diluted in TTBS with 1% bovine serum albumin (1:100). The strips were then washed as before with TTBS three times. The immunoreactivity was detected with a horseradish peroxidase-conjugated goat anti-human IgG (Organon Teknika-Cappel, Durham, NC) diluted in TTBS with 1% bovine serum albumin (1:5000) for 30 minutes at room temperature and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Mice

SKH1 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and hosted at the University of Southern California Facility. These are hairless mice with an intact immune system. Four- to 10-week-old animals were injected with affinity-purified anti-CMP antibodies or flow-through IgG (depleted of reactivity to CMP) from the EBA patients' sera or control IgG fractions from the sera of normal human patients at the same IgG concentrations. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Preparation and Characterization of IgG Fractions

The patient's plasma was first diluted with antibody binding buffer (20 mmol/L sodium phosphate, pH 7.0) at a 1:5 dilution and then centrifuged at 4000 rpm to remove insoluble particulate material. The supernatants were then subjected to chromatography using a protein G-Sepharose Fast Flow column following the manufacturer's recommendation (Amersham Biosciences, Uppsala, Sweden). Control IgG fractions were prepared in an identical manner from a commercial lot of human gamma globulins obtained from several hundred normal donors (Sigma, St. Louis, MO). IgG fractions from the EBA patient were further affinity-purified using recombinant GST-CMP fusion protein covalently coupled to a CNBr-activated Sepharose 4B column following the manufacturer's instructions (Amersham Biosciences). Affinity-purified anti-CMP EBA antibodies were dialyzed against phosphate-buffered saline, concentrated by Centricon Plus-20 ultrafiltration (Amicon, Lexington, MA) to 20 to 50 mg/ml, filter-sterilized, and stored at −20°C. By IIF, antibody titers ranged from 1:5000 to 1:10,000 on normal human skin, mouse skin, and salt-split skin substrate. The affinity-purified autoantibodies were also assessed by Western blot analyses and ELISA as described.[26]

Preparation of F(ab′)$_2$ Fragments

F(ab′)$_2$ fragments of affinity-purified anti-CMP IgG were prepared by digestion with pepsin as described.[23] Undigested IgG and Fc fragments were removed by affinity chromatography using a protein G-Sepharose Fast Flow column (Amersham Biosciences). Purified F(ab′)$_2$ fragments migrated as a 100-kd band under nonreduced SDS-PAGE. The completeness of digestion was assessed by IIF on mouse skin substrate. The purified F(ab′)$_2$ preparations showed reactivity only with a fluorescein isothiocyanate (FITC)-labeled goat anti-human-F(ab′)$_2$ but not with a FITC-labeled goat anti-human-Fc secondary antibodies.

Administration of EBA Antibodies, Animal Evaluation, and Characterization

SKHl mice were injected intradermally with EBA anti-CMP IgG (n = 8), EBA IgG depleted of reactivity to the CMP domain of NC1 (flow-through fractions from the CMP affinity column) (n = 7), or normal human control IgG (n = 8) once every day for 8 days and observed every day. IgG doses ranged from 10 to 400 μg/g body weight/per day. The animals were photographed daily. Skin erythema, blisters, and erosions were recorded. Mice that developed blisters had skin biopsies from the blisters and nonblistered normal-appearing skin within 0.5 cm of

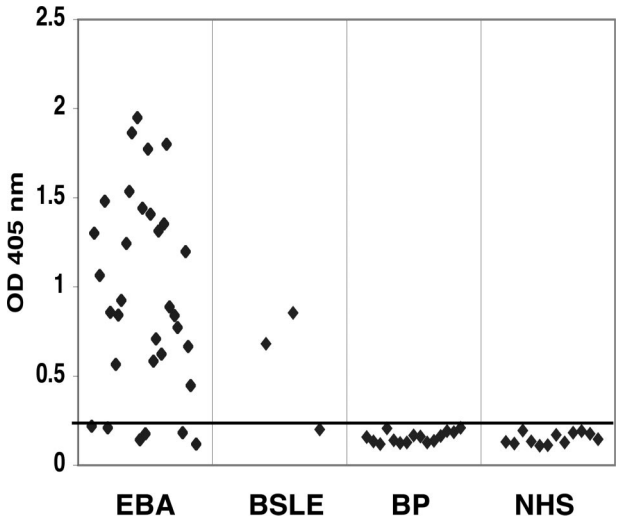

Figure 2. Scatter plot representation of ELISA results using recombinant CMP. Patient and control sera (as indicated along the horizontal axis) (1:200 dilution) were incubated with immobilized CMP subdomain, and the bound antibodies detected with an alkaline phosphatase-conjugated antibody against human IgG. Each sample was run in triplicate. The points plotted on this graph are the average of the OD_{405} obtained from study sera. Similar results were obtained in three other independent experiments.

a blister. Skin samples were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Both lesional and perilesional tissues were subjected to DIF staining as previously described.[2,24] Monospecific FITC-conjugated sera were obtained commercially: goat anti-human IgG (Sigma), monospecific goat anti-mouse C3 (Cappel Laboratories, Durham, NC), goat anti-mouse neutrophils (Cedarlane, Ontario, ON, Canada), and goat anti-human $F(ab')_2$ and Fc (Cappel Laboratories). Photographs of immunolabeled tissues were obtained with a Zeiss Axioplan fluorescence microscope equipped with a Zeiss Axiocam MRM digital camera system (Carl Zeiss, Thornwood, NY).

Results

CMP of NC1 Domain Harbors Epitopes Recognized by EBA Autoantibodies

We previously produced a series of GST fusion proteins encompassing the complete FNIII and VWFA regions of NC1 domain (Figure 1).[18] In this study, we produced an additional NC1 subdomain recombinant fusion protein corresponding to the N-terminal 227 amino acids (residues 1 to 227) of NC1 and homologous to CMP. These recombinant type VII collagen fusion proteins were used to identify regions within NC1 recognized by EBA sera. As shown previously, EBA autoantibodies recognize four major NC1 antigenic epitopes confined within FINIII and VWF-A subdomains of NC1 (Figure 1A).[18] By ELISA, 26 of 32 EBA sera (81%) exhibited reactivity with CMP with optical density (OD) values ranging from 0.4 to 2 (Figure 2). Two of three BSLE sera also reacted with CMP, consistent with previous studies showing that BSLE sera

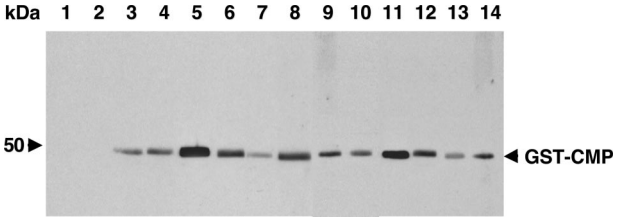

Figure 3. Immunoblot of the recombinant GST-CMP proteins by EBA and control sera. Purified recombinant GST-CMP (100 ng/well) protein was separated on 10% SDS-PAGE and transferred to nitrocellulose membranes before incubation with sera at a dilution of 1:100 and horseradish peroxidase-conjugated anti-human IgG (1:5000) followed by enhanced chemiluminescence detection. **Lane 1**, NHS; **lane 2**, BP; **lanes 3** to **12**, EBA; and **lanes 13** to **14**, BSLE. The positions of the 50-kd molecular weight marker and the GST-CMP fusion protein are indicated.

contain autoantibodies to NC1.[28] In contrast, all control sera (15 BP and 12 NHS) showed background reactivity with CMP with values less than 0.21 OD (0.25 OD is the cutoff for a positive ELISA reading).[26]

The 26 EBA and 2 BSLE sera that reacted with CMP in the ELISA were further analyzed by immunoblot analysis. A representative blot is shown in Figure 3. The 50-kd GST-CMP fusion protein was recognized by 22 EBA sera and two BSLE sera but not by BP or normal human sera at the same 1:100 dilution. Four EBA sera were negative by immunoblot analysis even when the serum dilution was decreased to 1:20. These four EBA sera are those sera with the lowest reactivity to CMP by ELISA. Taken together, these data show that like FINIII and VWF-A subdomains, CMP also contains epitopes recognized by the majority of EBA autoantibodies.

Epitopes within CMP Are Recognized by Pathogenic EBA Autoantibodies

We recently showed that affinity-purified autoantibodies against type VII collagen NC1 from EBA patients' sera induced subepidermal blisters when injected into adult hairless immunocompetent mice.[23] To determine whether pathogenic EBA autoantibodies are reactive with CMP epitopes, we affinity-purified CMP-specific EBA antibodies and analyzed these antibodies by immunoblotting and immunofluorescence. Using a panel of GST fusion proteins encompassing the complete NC1 domain of type VII collagen (Figure 4A),[18] we found that the pathogenic EBA IgG used in this study recognized exclusively only CMP (Figure 4B, lane 2). The antibody reactivity with this antigenic epitope was specific because the EBA IgG did not recognize FP1, FP3, FP7, or FP8 (Figure 4B, lanes 3 to 6). Further, control normal human serum did not react with any of these fusion proteins (data not shown). As expected, the CMP-specific EBA antibodies only reacted with the 50-kd CMP protein and NC1, not with other fusion proteins (Figure 4C.). These data indicate that epitopes recognized by pathogenic EBA IgG reside within the first 227 AA of NC1.

To determine whether EBA-IgG also reacts with murine type VII collagen, we performed an immunoblot using type VII collagen extracted from murine skin. As

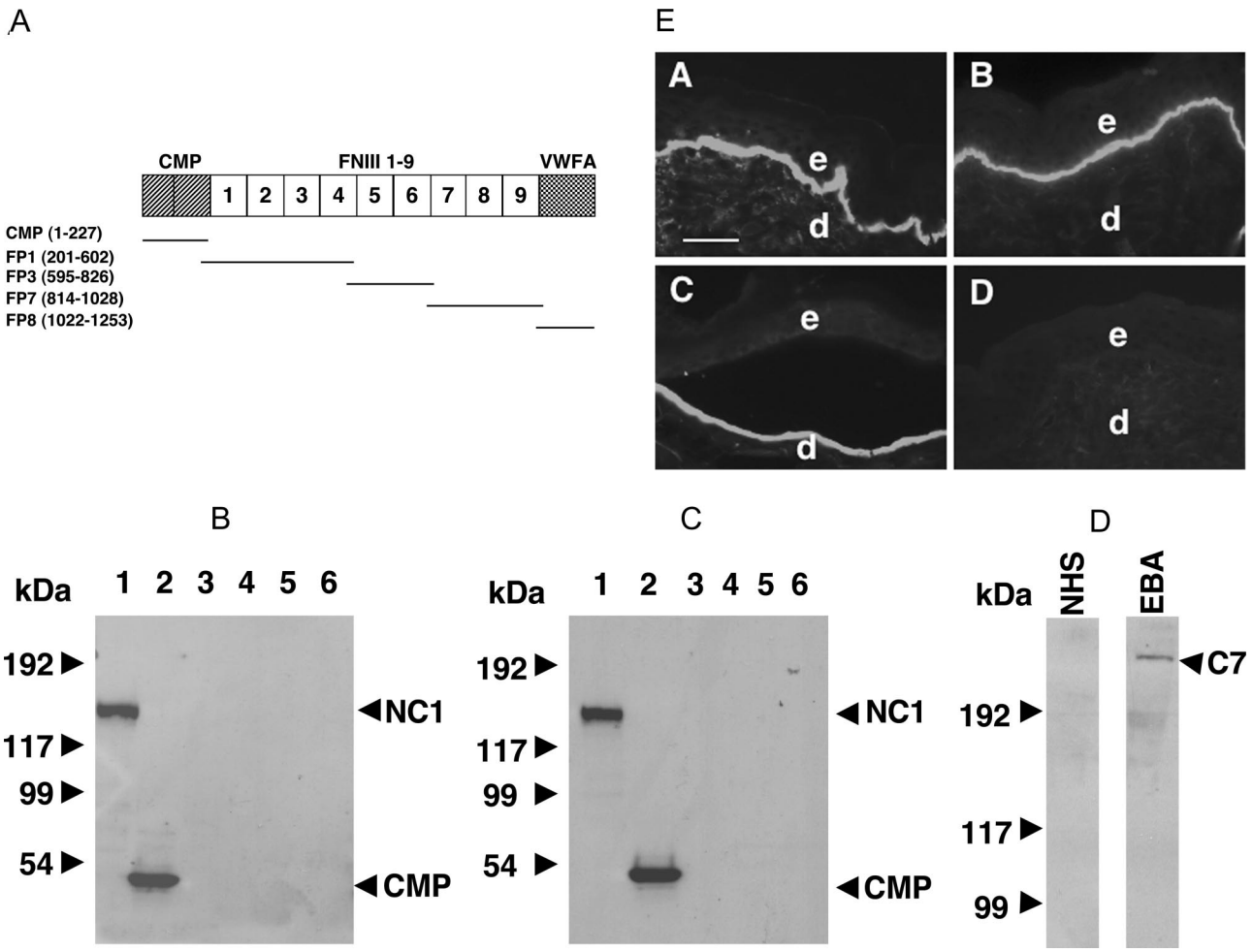

Figure 4. Epitope mapping of anti-type VII collagen EBA antibodies. **A:** Schematic diagram of the recombinant fusion proteins encompassing the complete NC1 domain of type VII collagen. Four deletion mutant recombinant proteins along with CMP are shown in the schematic with the amino acid residue numbers indicated. **B** and **C:** Purified recombinant NC1, as well as five other recombinant fusion proteins (400 ng/well) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The transferred proteins were incubated with EBA antibodies before (**B**) and after (**C**) affinity purification at a dilution of 1:100 and horseradish peroxidase-conjugated anti-human IgG (1:5000) followed by enhanced chemiluminescence detection. **Lanes 1**, **2**, **3**, **4**, **5**, and **6** are NC1, CMP, FP1, FP3, FP7, and FP8, respectively. The locations of the 145-kd recombinant NC1, CMP, and molecular weight markers are indicated. **D:** Protein extracts from mouse skin were separated on 6% SDS-PAGE and immunoblotted with affinity-purified anti-CMP antibodies (EBA) or normal human sera (NHS) at a dilution of 1:100. Please note that a polypeptide of ~290-kd murine type VII collagen α chain was recognized by EBA autoantibodies only. The locations of the 290-kd mouse type VII collagen and molecular weight markers are indicated. **E:** Immunolabeling of mouse and human skin with the affinity-purified anti-CMP EBA antibodies. Immunofluorescence staining was performed on human skin (**A**), mouse skin (**B**, **D**), and salt-split human skin (**C**). The tissue was labeled with EBA antibodies diluted 1:1000 and a FITC-conjugated goat anti-human IgG. Note that the affinity-purified anti-CMP EBA antibodies strongly labeled the BMZ of both mouse and human skin (**D**) and the dermal floor of salt split human skin (**A–C**). In contrast, flow-through IgG depleted of reactivity to CMP did not stain the BMZ of the mouse skin. d, dermis; e, epidermis. Scale bar, 200 μm.

shown in Figure 4D, EBA-IgG, but not control normal human sera, specifically recognized the murine 290-kd type VII collagen α chain. Because pathogenic EBA autoantibodies bind to type VII collagen in skin, we determined whether the purified anti-CMP EBA antibodies reacted with skin by IIF. As shown in Figure 4E, the anti-CMP antibodies strongly stained the BMZ of both human and mouse skin at dilution titers of more than 1:10,000. The control IgG fraction purified from normal human sera did not label either mouse or human skin (data not shown). In addition, like pathogenic EBA autoantibodies, the anti-CMP antibody labeled the dermal side of salt-split human skin (Figure 4EC). Finally, EBA IgG depleted of reactivity to the CMP subdomain (flow-through fractions from CMP affinity column) did not stain mouse skin (Figure 4ED).

Mice Injected with Anti-CMP Antibodies Develop EBA

To test whether anti-CMP autoantibodies are pathogenic, we intradermally injected SKH1 mice with affinity-purified anti-CMP antibodies daily at a dose of 50 μg/g body weight per day. The characteristics of the anti-CMP EBA antibodies are outlined in Table 1. As shown in Figure 5, animals injected with anti-CMP EBA antibodies (n = 8) showed blisters as early as 8 days after the initial injection (Figure 5, A and C). With time, these blisters ruptured and additional erosions, crusted lesions, and blisters were observed on the body nearby the injection area, as well as the ears in some mice (Figure 5, B, D, and E). Four of eight (50%) injected mice lost their nails (Figure 5, F). As

Table 1. Characterization of the Affinity-Purified Anti-CMP EBA Antibodies Used and the Incidence of Disease in Injected Mice

Source of IgG	IIF Titer*	Dose of IgG injected (μg/g of body weight per day)	Number of mice with skin lesions†
EBA patient	10,000	10 to 50	8/8
Control IgG‡	0	20 to 400	0/8
Flow-through IgG§	0	20 to 400	0/7

*Titers of concentrated affinity-purified anti-CMP EBA antibodies tested against normal mouse skin.

†The numerator denotes the number of mice in which skin lesions developed; the denominator denotes the total number of mice that received injections.

‡Normal human serum.

§Flow-through IgG from the flow-through fractions of the CMP affinity column in which the reactivity to CMP subdomain was depleted.

summarized in Table 1, cutaneous lesions occurred in all eight experimental mice given anti-CMP EBA antibodies. In contrast, none of the eight animals injected with identical or higher doses of control IgG developed any cutaneous lesions. Furthermore, none of the seven animals injected with EBA IgG depleted of reactivity to the CMP subdomain of NC1 (flow-through fractions from CMP affinity column) developed skin lesions. Two experimental mice exhibited weight loss after the 8-day injection period. We sacrificed these mice and performed histology of various organs. We observed erosions in the oral mucosa and the upper one-third of the esophagus (data not shown). Many, but not all, patients with EBA have involvement of the oral mucosa and upper one-third of the esophagus.

Histological examination of lesional skin of the diseased mice showed an EBA-like DEJ separation (Figure 6Aa). There was also a moderate dermal inflammatory infiltrate consisting mostly of mononuclear cells and a few neutrophils. In contrast, mice injected with similar concentrations of control normal human IgG had a normal epidermis and dermis without any pathological alteration (Figure 6Ab). Further, injection of flow-through IgG into mice did not show any disease pathology (data not shown).

DIF of perilesional and lesional skin demonstrated that all of the mice injected with anti-CMP EBA antibodies had human IgG deposits along the BMZ (Figure 6B). Human anti-CMP IgG deposits were also detected in the ear, oral mucosal, and esophagus of the injected mice. In contrast, all seven mice that received EBA IgG depleted of reactivity to the CMP subdomain did not show any deposits of human IgG, along the BMZ in skin.

In addition to deposits of human IgG, in six of eight experimental mice, continuous deposits of murine C3 complement were also observed at the BMZ (Figure 6C). Neutrophils were detected within the dermal inflammatory infiltrate (Figure 6C). In contrast, there were no murine C3 deposits at the BMZ or neutrophils in the dermis of the mice receiving equivalent amounts of

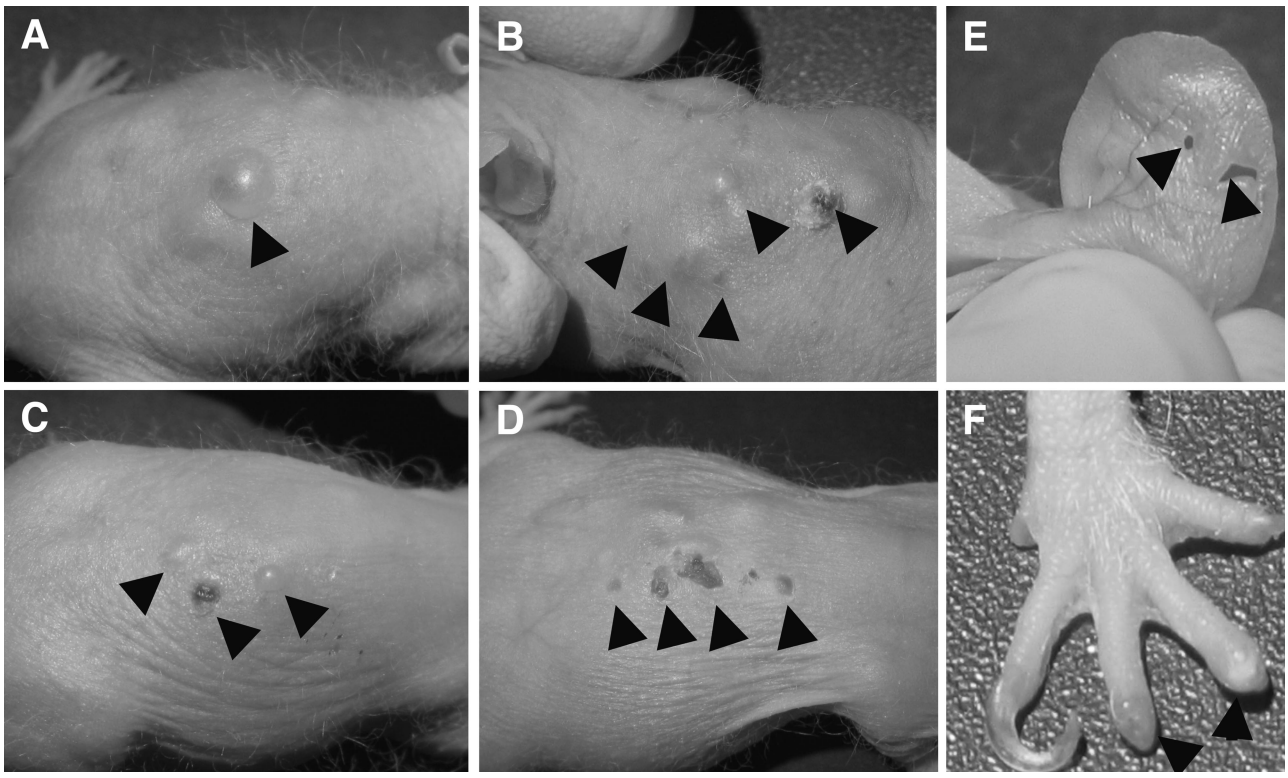

Figure 5. Clinical appearance of SKH1 mice injected with anti-CMP EBA antibodies. **A–F:** SKH1 mice were injected intradermally with EBA antibodies at 50 μg/g body weight once every day for 8 consecutive days. Eight to 12 days after the injections were started, animals developed numerous blisters, erosions, and crusts forming from ruptured blisters (**A** and **B**, days 8 and 12 after eight injections; **C** and **D**, days 8 and 13 after eight injections). **E:** Lesions were observed on the animals' ears at 14 days after the initial injections (eight injections). **F:** Nail loss was also observed in some mice.

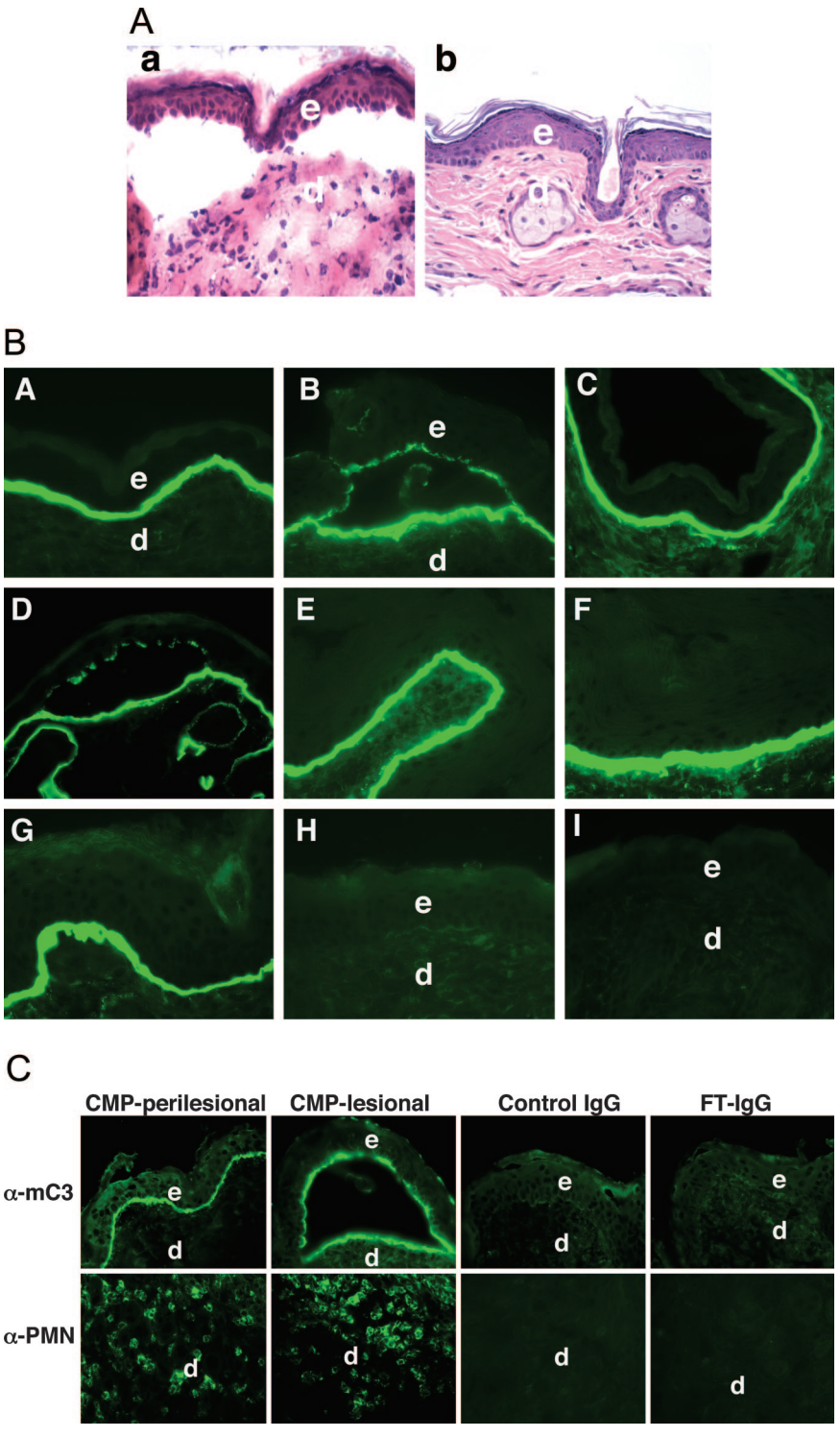

Figure 6. Histological and immunological examination of lesional skin of SKH1 mice injected with anti-CMP EBA antibodies. **A:** Histological appearance of skin lesions induced by EBA autoantibodies. H&E staining of lesional murine skin (**a**) showed separation of the epidermis (e) from the dermis (d). No epidermal-dermal separation was detected in mice receiving equivalent amounts of normal human control IgG (**b**). **B:** Immunofluorescence analysis of SKH1 mice injected with anti-CMP EBA antibodies. Cryosections of perilesional and lesional skin as well as other organs were labeled with FITC-conjugated goat anti-human IgG. Note the mice injected with anti-CMP EBA antibodies had *in situ* deposits of human IgG at the BMZ of perilesional and lesional skin (**A** and **B**), perilesional and lesional ear lesions (**C** and **D**), esophagus (**E** and **F**), and oral mucosal (**G**). In contrast, mice receiving equivalent amounts of flow-through IgG depleted of reactivity to CMP (**H**) or purified control normal human IgG (**I**) had no deposits of human IgG. **C:** Immunofluorescence analysis was performed with FITC-conjugated goat anti-mouse C3 antibody (α-mC3) and FITC-conjugated goat anti-mouse neutrophil antibody (α-PMN). Note linear deposits of murine C3 at the BMZ of perilesional and lesional skin in mice injected with anti-CMP EBA antibodies. Please also note neutrophils in the dermis. In contrast, in mice receiving equivalent amounts of purified control normal human IgG (control IgG) or flow-through IgG depleted of reactivity to CMP (FT-IgG), no murine C3 deposits or neutrophils were detected.

control normal human IgG (control IgG) or flow-through IgG depleted of reactivity to the CMP subdomain (FT-IgG).

F(ab′)$_2$ Fragments of Anti-CMP Antibodies Do Not Induce Blisters

To determine whether complement activation was required for the induction of blisters and nail loss in the mice injected with anti-CMP antibodies, we prepared F(ab′)$_2$ fragments of the affinity-purified anti-CMP antibodies by removing the complement-binding, ie, Fc domains, of the antibodies. We injected F(ab′)$_2$ fragments into SKH1 mice using equimolar doses as intact anti-CMP IgG. In contrast to the experiments with complete anti-CMP IgG, the skin of mice injected with F(ab′)$_2$ did not develop clinical blisters or any lesions (Figure 7A). When the DIF analysis was performed in

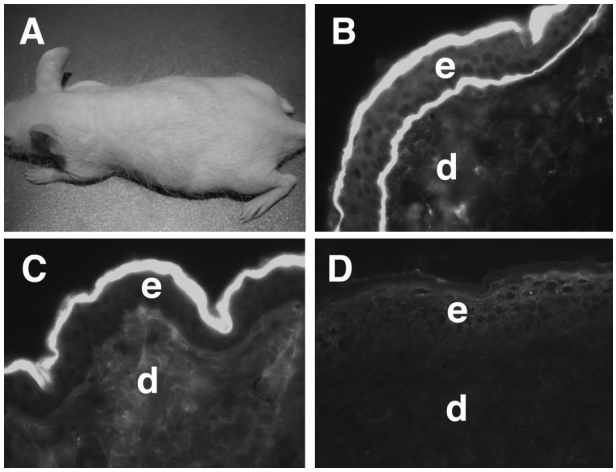

Figure 7. Clinical and immunological examination of SKH1 mice injected with the $F(ab')_2$ fragments generated from anti-CMP EBA antibodies. **A:** Clinical appearance of skin injected with $F(ab')_2$ fragments at 40 μg/g body weight once every day for 8 consecutive days showed no signs of skin blisters. **B–D:** Immunofluorescence analysis of SKH1 mice skin injected with $F(ab')_2$ fragments of anti-CMP antibodies. Cryosections of injected skin were labeled with FITC-conjugated goat anti-human Fab (**B**), goat anti-human Fc (**C**), and goat anti-mouse C3 (**D**). Note the strong binding of $F(ab')_2$ fragments to the BMZ but the absence of Fc and murine C3 complement within the BMZ. e, epidermis; d, dermis.

the skin of the injected mice, the skin exhibited $F(ab')_2$ at the DEJ but no human IgG Fc or murine complement C3 (Figure 7, B–D). As expected, $F(ab')_2$ fragments prepared in an identical manner from control human IgG and injected into the mice did not induce lesions (data not shown).

Discussion

In this study, we demonstrate that the majority of autoantibodies from EBA patients recognize the CMP subdomain of type VII collagen. Twenty-six of 32 EBA sera and two of three BSLE sera were reactive with CMP. More importantly, anti-CMP antibodies from EBA serum, when passively transferred into hairless, immunocompetent mice, induced a subepidermal bullous disease similar to EBA. Like many EBA patients, the diseased mice often had loss of nails and mucosal involvement. In addition, like EBA patients, these mice had tissue-bound IgG deposits at the BMZ of their skin, mouth, and esophagus. Taken together, the mice injected with anti-CMP EBA antibodies exhibited clinical, histological, and immunological features akin to patients with EBA.

Mice injected with anti-CMP EBA antibodies also exhibited very similar features to the mice in our previous passive transfer studies using human anti-EBA autoantibodies purified from an NC1 affinity column. The only difference is that the amount of anti-CMP EBA antibodies used to induce the disease (10 to 50 μg/g body weight per day) was approximately half the amount needed in our previous studies (20 to 100 μg/g body weight per day). It is likely that pathogenic anti-type VII collagen antibodies are highly enriched by affinity purification on a CMP affinity column. The fact that affinity-purified human EBA sera against CMP could produce the full-blown manifestations of EBA in animals suggests that one of the main pathogenic epitopes of type VII collagen resides within the CMP subdomain.

Our conclusion that CMP harbors a major pathogenic epitope is further confirmed by the finding that EBA IgG antibodies depleted of activity to CMP (ie, the flow-through fractions from the CMP affinity column) failed to induce any lesions in the mice. Our previous epitope mapping studies identified four distinct, independent epitopes within the NC1 domain recognized by EBA sera.[18] Three of the four epitopes are clustered to the FINIII. The fourth EBA antigenic epitope is located within the VWF-A. However, in those studies the CMP subdomain had not yet been cloned. Therefore, it was not available for epitope mapping. Further, none of the EBA autoantibodies to any of these four epitopes were demonstrated to be pathogenic. In the present study, we identified CMP as a prominent immunodominant site and demonstrated that the CMP epitope is pathogenic, the first pathogenic epitope identified to date. It should be mentioned that rabbit anti-NC1 antibodies used in our previous passive transfer studies recognized multiple epitopes including the four previously identified epitopes plus the CMP subdomain (data not shown). Because we injected mice with total anti-NC1 antibodies, we did not know in that study which epitope(s) was responsible for the disease induction.[21] Similarly, in the study reported by the Sitaru and colleagues,[22] they used a mixture of rabbit anti-mouse type VII collagen antibodies immunized from three peptides (residues 97 to 200, 479 to 587, and 757 to 967) within the NC1 domain to induce disease in the animals. Two of those peptides were generated using FINIII, the previously identified antigenic epitopes for EBA patient sera and another one consisting of half of the CMP subdomain (amino acid residues 97 to 200). However, which of these antigenic epitopes was actually involved in the disease pathogenesis was not identified because a mixture of rabbit anti-mouse type VII collagen antibodies was used for the passive transfer experiments.

It is well known that an antigenic epitope is not always the same as a pathogenic epitope. For example, in pemphigus paraneoplastica, there are numerous autoantibodies to plakin proteins (eg, desmoplakin, periplakin, and envoplakin) that are markers of the disease but do not cause skin bullae. That is, they are antigenic marker autoantibodies but not pathogenic. The significance of our present studies is underscored by that fact that our patient sera exclusively recognized only the CMP subdomain of type VII collagen and yet could induce full-blown EBA when passively transferred into animals. Because six of the 32 EBA (19%) sera tested in the present study did not react with CMP, it is likely that EBA autoantibodies that target NC1 subdomains other than CMP may also be pathogenic and involved in the disease development. This possibility can be tested directly by passive transfer experiments with affinity-purified EBA autoantibodies against other NC1 subdomains. However, EBA is a rare disease, and our limited availability of large quantities of patient sera needed for this kind of study prevents us from addressing this issue at the present time.

Anchoring fibrils are reduced in the skin of EBA patients, but the underlying mechanism leading to this reduction is unknown.[27,30] One possible mechanism is that binding of autoantibodies to type VII collagen may target functional epitopes on the NC1 domain and interfere with its adhesive function. This could perturb critical direct interactions between type VII collagen and other extracellular components within the DEJ or high papillary dermis such as type IV collagen, laminin-5, or fibronectin.[14–17] In this regard, we and others have shown that NC1 binds to laminin-5, type IV collagen, and fibronectin.[14–17] These interactions may be necessary for keeping the DEJ intact.

Another possible mechanism for autoantibody-induced blisters in EBA is that binding of EBA autoantibodies to type VII collagen invokes an inflammatory cascade that results in proteolytic degradation of matrix components within the DEJ that are essential for dermal-epidermal adherence, including type VII collagen itself. In previous studies with the passive transfer EBA animal models, we and others[22,23] have shown that complement fixation likely plays a role in the pathogenesis of the murine EBA model. In the present study, we also showed that affinity-purified anti-CMP EBA IgG, but not flow-through IgG (depleted of CMP reactivity), fixed murine C3 complement and recruited neutrophils when injected into mice. We further demonstrated that injection of mice with pepsin-derived $F(ab')_2$ fragments prepared from human anti-CMP EBA antibodies did not produce clinical or histological EBA lesions. These data suggest that the Fc domains of anti-CMP EBA antibodies are needed for subepidermal blister formation. Because antibody-specific activation of the complement system is mediated via the Fc portion of IgG molecules, our results indicate that anti-CMP EBA antibodies induce subepidermal blisters and other EBA features in mice via the activation of the complement system.

In summary, this study identifies CMP as a previously unrecognized new antigenic epitope recognized by most EBA patient sera. Further, we demonstrate that EBA autoantibodies directed against the CMP subdomain are pathogenic when passively transferred into hairless immunocompetent mice. The characterization of pathogenically active autoantibodies directed against the CMP subdomain should provide valuable molecular tools to dissect the molecular and immunological mechanisms of subepidermal blister formation in EBA. The further fine mapping of the pathogenic epitope to a smaller region with the 227 AA CMP subdomain may also facilitate the development of effective therapy for EBA such as peptide therapy.

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