The Carboxyl Terminus of Type VII Collagen Mediates Antiparallel Dimer Formation and Constitutes a New Antigenic Epitope for Epidermolysis Bullosa Acquisita Autoantibodies*

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Type VII collagen, the major component of anchoring fibrils, consists of a central collagenous triple-helical domain flanked by two noncollagenous domains, NC1 and NC2. The NC2 domain has been implicated in catalyzing the antiparallel dimer formation of type VII procollagen. In this study, we produced the entire 161 amino acids of the NC2 domain plus 186 amino acids of adjacent collagenous domain (NC2/COL) and purified large quantities of the recombinant NC2/COL protein. Recombinant NC2/COL readily formed disulfide-bonded hexamers, each representing one antiparallel dimer of collagen VII. Removal of the collagenous helical domain from NC2/COL by collagenase digestion abolished the antiparallel dimer formation. Using site-directed mutagenesis, we found that mutation of either cysteine 2802 or cysteine 2804 alone within the NC2 domain blocked antiparallel dimer formation. In contrast, a single cysteine mutation, 2634, within the collagenous helical domain had no effect. A generated methionine to lysine substitution, M2798K, that is associated with recessive dystrophic epidermolysis bullosa, was unable to form antiparallel dimers. Furthermore, autoantibodies from epidermolysis bullosa acquisita patients also reacted with NC2/COL. We conclude that NC2 and its adjacent collagenous segment mediate antiparallel dimer formation of collagen VII. Epidermolysis bullosa acquisita autoantibodies bound to this domain may destabilize anchoring fibrils by interfering with antiparallel dimer assembly leading to epidermal-dermal dis-adherence.

Type VII collagen, a genetically distinct member of the collagen family, is found within the basement membrane zone beneath stratified squamous epithelium (1, 2). Type VII collagen is a major component of anchoring fibrils, attachment structures within the basement membrane between the epidermis and dermis of human skin (3, 4). In inherited forms of dystrophic epidermolysis bullosa (DEB),1 anchoring fibrils are diminutive and/or reduced in number (5–7). In addition to inherited DEB, epidermolysis bullosa acquisita (EBA) is an acquired autoimmune form of epidermolysis bullosa. EBA is characterized by circulating and tissue-bound IgG autoantibodies to type VII collagen (8, 9). Like DEB, ultrastructural studies have demonstrated a dramatic paucity of anchoring fibrils in EBA skin (10). These observations suggest that type VII collagen plays an important role in maintaining epidermal-dermal adherence. Type VII collagen has been cloned, and a genetic linkage has been established between inherited DEB and mutations in the gene that encodes for type VII collagen, COL7A1 (11–14). There have been over 100 distinct COL7A1 gene mutations identified in patients with DEB, and these mutations have occurred within NC1, NC2, and the helical domain (15, 16).

Type VII collagen is composed of three identical α chains, each consisting of a 145-kDa central collagenous triple-helical segment, flanked by a large 145-kDa amino-terminal non-collagenous domain (NC1), and a smaller 34-kDa carboxyl-terminal non-collagenous domain (NC2) (4, 17). In the extracellular space, individual type VII collagen molecules form antiparallel tail-to-tail dimers stabilized by disulfide bonding through a small carboxyl-terminal overlap (NC2), and a portion of the NC2 domain is proteolytically removed (18, 19). The antiparallel dimers then aggregate laterally in a nonstaggered manner to form anchoring fibrils (4).

NC2 consists of 161 amino acids. The NC2 domain has a segment that is homologous with the Kunitz-type protease inhibitor (12). It also contains four potential sites for phosphorylation by casein kinase I and II (12). The overall conservation between mouse and human NC2 domain sequences is 90% at the amino acid level. The relatively high degree of conservation of this segment is highlighted by the presence of 67-amino acid residues spanning the junction of the collagenous and NC2 domains which is 100% conserved between hamster, mouse, and human sequences (20). It has been proposed that this region contains the cleavage site for proteolytic removal of the NC2 domain. This region also contains a pair of cysteines in amino acid positions 2802 and 2804 which are likely to be involved in the disulfide-bond linkages that stabilize antiparallel dimers. In addition, there is a conserved single cysteine residue in amino acid position 2634 within the adjacent collagenous domain which may be involved in interchain disulfide bonding (20).

The NC2 domain of type VII collagen has been implicated in initiating the triple-helical assembly of type VII collagen and catalyzing the antiparallel dimer formation of procollagen (2).
Recombinant Noncollagenous Domain (NC2) of Type VII Collagen

However, the exact function of the NC2 domain is not known. This is at least partly due to the extremely low quantities of procollagen VII in skin and cell culture. Furthermore, there has been a very limited amount of NC2 available because of its susceptibility to nonspecific proteolytic degradation during biochemical purification. Therefore, there has been great difficulty obtaining sufficient quantities of pure NC2.

The major antigenic epitopes for autoantibodies in patients with EBA and bullous systemic lupus erythematosus (BSLE) reside within the NC1 domain of type VII collagen (21–23). However, recently a novel EBA subgroup with a milder clinical presentation was defined in children with tissue bound and circulating autoantibodies targeting only the triple-helical central domain of type VII collagen (24). These data suggest that EBA may exhibit a wider, more heterogeneous spectrum of autoantibody reactivities than previously assumed.

In this study, we prepared a cDNA construct for the NC2 domain plus its immediately adjacent collagenous domain (COL). This construct was used in an eukaryotic expression system to produce large quantities of purified NC2/COL, a 50-kDa carboxyl terminus of the type VII collagen α-chain. We demonstrate herein that purified NC2/COL readily forms disulfide-bonded hexameric aggregates, each representing one anti-parallel dimer of collagen VII. We also demonstrate that the adjacent COL domain is essential for the formation of antiparallel dimers. Using site-directed mutagenesis, we found that mutating either cysteine 2802 or cysteine 2804 within the NC2 domain inhibits antiparallel dimer formation. In contrast, a single cysteine mutation, cysteine 2634, within the collagenous domain had no effect. Furthermore, a methionine to lysine substitution mutation, M2798K, within the NC2 domain also caused an inability to form antiparallel dimers. Interestingly, this mutation has been reported to be associated with recessive DEB.

EXPERIMENTAL PROCEDURES

Expression Vector Construction and Transfection—The eukaryotic expression vector pRC/CMV (Invitrogen, San Diego, CA) which contains the cytomegalovirus (CMV) promoter and enhancer was used to express a chimeric cDNA (ECOL, 1). The cDNA comprises a 16-amino acid signal peptide sequence derived from human type VII collagen, a 186-amino acid COL domain, and the entire 161-amino acid NC2 domain.

To generate the expression vector, a 1.8-kilobase pair of COL and NC2 cDNA fragment was released from pGEX-PCR10 by BamHI/EcoRI digestion (21) and made blunt-ended by DNA polymerase I (Klenow fragment) following a standard protocol. This fragment was then ligated to the KoxI-digested and blunt-ended pRC/CMV to generate a construct designated CMV/NC2*. Then, a cDNA fragment containing signal peptide, COL domain, and NC2 domain was released from CMV/NC2* by NruI/MaeI digestion and ligated into the NruI/MaeI (blunt-ended) pRC/CMV vector, resulting in the final expression construct as shown in Fig. 1. We designated the protein product of this construct NC2/COL. All the constructs were verified by DNA sequencing. The expression vector was used to transfect a human embryonic kidney cell line 293 (ATCC, Rockville, MD) using Lipofectin (Life Technologies, Inc., Gaithersburg, MD), and stable clones were selected using 500 μg/ml amphotericin B and 50 μg/ml G418/ml as previously described (25).

For Northern blot analysis, total RNA was extracted using guanidine isothiocyanate-CsCl density gradient centrifugation as previously described (26). The total RNA was size fractionated and transferred to a nylon membrane (GeneScreen Plus, DuPont, Boston, MA) and hybridized to a 32P-labeled (random-primer labeling kit, BMB, Indianapolis, IN) 1.4-kilobase pair human type VII collagen cDNA probe containing NC2/COL domain.

Site-directed Mutagenesis—Site-directed mutagenesis was performed on the NC2/COL cDNA inserts in pRC/CMV vector using a QuikChange® site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA) according to the manufacturer’s instructions. Briefly, a pair of complementary primers with 39 bases was designed, and mutation to change cysteine to serine or methionine to lysine was placed in the middle of the primers. A double cysteine mutant was created by changing cysteine at a given site to serine and using this mutant DNA as a template in the next round of mutagenesis. Parental cDNA inserted in pRC/CMV was amplified using Pyrococcus furiosus DNA polymerase with these primers for 12 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the parental DNA with DpnI, the amplified DNA with nucleotide substitution incorporated was transformed into Escherichia coli (XL1-Blue). The mutations were confirmed by automated DNA sequencing.

Protein Purification and Analysis—For Western blot analysis, clonal cell lines resistant to G418 were grown to confluence, the medium was changed to serum-free medium, and the cultures were maintained for an additional 24 h. The media were collected, concentrated 10-fold by Centricon-30 (Amicon, Beverly, MA), and subjected to 10% SDS-PAGE. Proteins were then electrotransferred onto a nitrocellulose membrane.

The presence of recombinant NC2/COL was detected with a rabbit polyclonal antibody prepared against the NC2/COL domain of type VII collagen followed by a horseradish peroxidase-conjugated goat anti-recombinant IgG and enhanced chemiluminescence detection reagent (Amersham Corp.) (27).

For large-scale purification of recombinant NC2/COL, serum-free media were equilibrated to 5 mM EDTA, 50 μM phenylmethylsulfonyl fluoride, and 50 μM N-ethylmaleimide, and precipitated with 400 mg/ml ammonium sulfate at 4 °C overnight with stirring. Precipitated proteins were collected by centrifuging at 13,000 rpm for 1 h, resuspended and dialyzed in buffer A containing 500 mM NaCl, 25 mM Tris-HCl, pH 7.8, and 1 mM EDTA and passed over a Q-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in the same buffer. Elution was then carried out with a linear gradient from 0.5 to 1.0 mM NaCl of appropriate volume size. The proteins that eluted between 0.8 and 1.1 mM NaCl were pooled and further purified on a Superdex 200 (HR 10/30, Pharmacia) equilibrated in 25 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 200 mM NaCl.

Protease-inhibitor Assay—The inhibitory activity of recombinant NC2/COL for various proteases was examined. We tested whether NC2/COL could inhibit selected proteases from degrading recombinant NC1 domain of type VII collagen. The recombinant NC1 (200 μg/ml) as substrate was dissolved in 50 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 2 mM CaCl2 as described previously (28). The proteases (2 μg/ml) were incubated with inhibitors in 10- or 50-fold excess (by molar) for 1 h at 37 °C prior to the addition of recombinant NC1. Digestions were allowed to incubate for 4 h at 37 °C and then analyzed by SDS-PAGE.

Digestion with Collagenase—Purified recombinant NC2/COL (5 μg) was digested with 50 μg/ml collagenase type VIII from Clostridium histolyticum, pH 7.4, 150 mM NaCl, 10 mM CaCl2, and 2 mM N-ethylmaleimide and then subjected to 10 units of bacterial collagenase (clostridiopeptidase A, type III, Sigma) digestion at 37 °C for 1 h.

Enzymelinked Immunosorbent Assay (ELISA) Using Recombinant NC2/COL—For testing IgG reactivity with NC2/COL, EBA and BSLE sera were used. The diagnosis of EBA was based on clinical findings, characteristic histology, demonstration of linear IgG deposits at the dermal-epidermal junction by direct immunofluorescence, and demonstration of IgG deposits at the dermal floor of the patients skin when dermal-epidermal junction was fractured though the lamina lucida by transillumination (29). Patient sera and control sera (human IgG, Control, E) 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 5% nonfat dry milk (1:200). The strips were then washed as was used as antigen to coat 96-well microtiter plates (Immulon-4, Dynach Laboratory Inc., Alexandria, VA) (31).

Immunoblotting of Recombinant NC2/COL—Purified recombinant NC2/COL protein (100 ng/well) was subjected to 10% SDS-PAGE, then electrotransferred to a nitrocellulose membrane. Cut strips of the nitrocellulose were blocked for 1 h at room temperature with 5% nonfat dry milk in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (TTBS). After washing with TTBS buffer, the strips were incubated for 1 h at room temperature with individual patient sera or control sera diluted in TTBS. The strips were then washed as was done for the NC2/COL assay, then incubated with 1.0 μg/ml goat anti-human IgG (Organon Teknika-Cappel, Durham, NC) diluted in TTBS in the presence of 5% nonfat dry milk (1:200). The strips were then washed as was used as antigen to coat 96-well microtiter plates (Immulon-4, Dynach Laboratory Inc., Alexandria, VA) (31).

Immunohistochemical Detection of Recombinant NC2/COL in Normal and DEB Skin—Purified recombinant NC2/COL (100 ng) was lyophilized and then dissolved in 100 μl of 0.1 M sodium phosphate buffer pH 7.4. A single cutaneous biopsy was placed in 25 μl of this solution and then incubated at 37 °C for 24 h in a humidified incubator. The biopsies were then washed and air-dried for 8 h. The biopsies were then incubated in buffer A containing 2% normal donkey serum (NDS), followed by 2% NDS, and then directly applied to normal human skin sections which were fixed and paraffin-embedded. For the positive control, normal skin sections were incubated with rabbit anti-NC2/COL (1:200) antibody. Normal mouse skin sections were incubated with normal rabbit serum (1:200) as a negative control. The sections were then incubated with 1.0 μg/ml goat anti-rabbit IgG (Organon Teknika-Cappel, Durham, NC) diluted in TTBS followed by 1.0 μg/ml rhodamine-conjugated donkey anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted in TTBS. The skin sections were then washed and mounted in glycerol mounting media and viewed with a Zeiss Axioskop microscope.

Immunoblotting of Normal Skin Sections—Normal skin sections were prepared and incubated as outlined in the previous section. The immunoreactivity was detected with a horseradish peroxidase-conjugated goat anti-human IgG, and the protein was visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).
Northern hybridization of the NC2/COL stably transfected 293 cell clones demonstrated abundant amounts of a 1.5-kilobase pair exogenous mRNA which corresponds in size to that of the cDNA insert (Fig. 1C, lane 2). No hybridization, even after longer exposure, was observed with parental 293 cell RNA, indicating that there is very little or no production of endogenous type VII collagen in 293 cells (lane 1).

**Purification of NC2/COL Domain of Type VII Collagen**—The recombinant NC2/COL was purified from serum-free culture medium from a high expressing 293 cell clone by a two-step procedure. An ammonium sulfate precipitate of the conditioned medium proteins (Fig. 2, lane 1) was passed over a Q-Sepharose column. Most of the NC2/COL proteins eluted from the column in high salt fractions (0.8–1 M NaCl) (lane 2). Further purification was achieved using molecular sieve chromatography on a Superdex-200 (lane 3). The final purified yields were 2–5 mg/liter of culture medium.

**Formation of Hexamers or Antiparallel Dimers by NC2/COL**—Previous studies suggested that dimerization is catalyzed by binding of the NC2 domain to a specific region of the triple helix (2). We assessed the ability of recombinant NC2/COL to assemble into disulfide-bonded oligomers. Fig. 3 shows that prior to reduction, the recombinant NC2/COL migrated as a disulfide-bonded 300-kDa hexamer, equivalent to one antiparallel dimer (that is, a molecular dimer) (lane 3). Reduction effectively dissociated the aggregate into a 50-kDa monomer of NC2/COL (lane 1). These results indicate that the recombinant NC2/COL is capable of forming a hexameric complex such as those that comprise antiparallel dimers consisting of six α-chains of type VII collagen.

To examine whether the adjacent COL domain is required for the hexamer formation, we subjected purified recombinant NC2/COL to collagenase digestion. As also shown in Fig. 3, digestion of NC2/COL by bacterial collagenase converted the 50-kDa NC2/COL into a 32-kDa fragment under reducing conditions (lane 2). This apparent molecule mass corresponds to that expected for the NC2 domain of type VII collagen. Under nonreducing conditions, the 32-kDa NC2 runs as a 64-kDa dimer (D) and a 100-kDa trimer (T), but not as a hexameric complex of 192 kDa (lane 4). The NC2 domain is capable of forming trimers, but not hexamers. Therefore, the 186-amino acid collagenous sequences adjacent to NC2 are essential for the formation of hexamers and the initiation of antiparallel dimers.

To determine more directly if NC2/COL formed hexameric structures akin to antiparallel dimers, we subjected purified recombinant protein to rotary shadowing. Rotary-shadowed images of the recombinant NC2/COL showed an extended overlapping rod COL domain with two distinct small globular fold-
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Involvement of the collagenous domain in the formation of antiparallel dimers. Purified recombinant NC2/COL was either untreated (lanes 1 and 3) or treated with bacterial collagenase (lanes 2 and 4) and analyzed by 8–16% SDS-PAGE followed by immunoblot analysis with a polyclonal antibody to NC2 domain. Proteins were either reduced with 2-mercaptoethanol (lanes 1 and 2) or nonreduced (lanes 3 and 4). The positions of molecular weight markers, monomer (M), dimer (D), trimer (T), and hexamer (H) of NC2/COL and NC2 are indicated. The migration of full-length type VII collagen served as a 300-kDa marker.

Identification of Cysteine Residues Involved in Antiparallel Dimer Formation—To further identify the cysteine residues involved in the formation of the disulfide linkages to stabilize the antiparallel type VII collagen dimers, we generated four site-directed Cys->Ser mutant constructs and transfected them into 293 cells. As shown in the immunoblot analysis in Fig. 3A, transfection with C2802S, C2804S, and C2802S/C2804S constructs resulted in the secretion of the 50-kDa NC2/COL at a similar level as wild type (compare lanes 2–5 to 1). Under nonreducing conditions, the protein product of two single cysteine mutations within the NC2 domain: C2802S/C2804S runs as a 50-kDa monomer, a 100-kDa dimer, and a 150-kDa trimer (lanes 8 and 9). A double cysteine mutation within the NC2 domain (C2802S/C2804S) runs predominately as a 50-kDa monomer, but some 100-kDa dimers are also observed (lane 10). In contrast, a single cysteine mutation within the collagenous domain (C2803S) runs as a 300-kDa hexamer similar to the wild type (lanes 6 and 7). Thus, both cysteine residues at amino acid 2802 and 2804 within the NC2 domain are vital for hexamer (antiparallel dimer) formation.

Formation of Antiparallel Dimer Is Impaired by a Methionine to Lysine Mutation—Previous studies have identified a homozygous methionine to lysine missense mutation at amino acid residue 2798 in a patient with recessive DEB (32). To investigate the molecular defect underlying this mutation, appropriate control sera (12 NHS, 10 BP) were analyzed to set an appropriate threshold for this assay. All the control sera were shown previously to react with the NC1 domain of type VII collagen with and without the presence of purified recombinant NC2/COL added at 10- or 50-fold excess. Recombinant NC1 domain of type VII collagen was selected as the protein substrate simply because of its large size (145 kDa) and the presence of many protease cleavage sites. As shown in Fig. 6, degradation of NC1 by either trypsin or chymotrypsin was completely protected by a 50-fold excess of aprotinin. However, no protective effect could be observed with recombinant NC2/COL added at 10-fold excess (lanes 3 and 7) or 50-fold excess (lanes 4 and 8). The same lack of inhibitory activity of NC2/COL was also found for thrombin and plasmin (data not shown).

NC2/COL Is Targeted by Autoantibodies in Bullous Diseases—Previously, we and others have identified the 4 immunodominant epitopes within the NC1 domain of type VII collagen for EBA and BSLE autoantibodies (21–23). To investigate whether NC2/COL contained any antigenic epitopes for EBA and BSLE autoantibodies, recombinant purified NC2/COL was used as an antigen in an ELISA and immunoblot analysis. We then tested sera from 24 EBA patients and 5 BSLE patients. All of these sera were shown previously to react with the NC1 domain of type VII collagen (31). The results of the NC2/COL ELISA are shown in Fig. 7 as a scatter plot. Twenty-two negative control sera (12 NHS, 10 BP) were analyzed to set an appropriate threshold for this assay. All the control sera showed very low reactivity in the ELISA with values less than 0.2 optical density. We set 0.25 optical density as the threshold for the ELISA (horizontal line in Fig. 7). In contrast to the controls, 4 of the 24 EBA sera and 4 of the 5 BSLE sera exhibited reactivity with the recombinant NC2/COL in the ELISA with optical density values ranging from 0.5 to 1.6.

To confirm the ELISA, the EBA and BSLE sera that were positive in the ELISA were further analyzed by immunoblotting with NC2/COL. As shown in Fig. 8, sera from 3 EBA patients (lanes 3–5) and 3 BSLE patients (lanes 8–10) strongly recognized the 50-kDa recombinant NC2/COL while serum from 1 EBA patient (lane 6) and 1 BSLE patient (lane 7)
demonstrated very weak reactivity. These two sera also had low optical density values in the ELISA. Control normal human sera (lanes 1 and 2) did not react with NC2/COL. Taken together, these data show strong concordance between the results of the ELISA and the immunoblot analysis.

**DISCUSSION**

In the present study, we developed a versatile eukaryotic expression system that allowed us to join the 161-amino acid of NC2 domain plus 186 amino acids of the adjacent collagenous domain of type VII collagen to a potent signal peptide sequence derived from the NC1 domain of type VII collagen. This approach directs the recombinant protein products to the secretory pathway, facilitating their purification. The efficient production and secretion of recombinant NC2/COL was achieved in a human cell line 293, which showed no endogenous production of type VII collagen. The production of NC2/COL has allowed us to obtain sufficient quantities of the NC2/COL segment for structural and functional studies.

The cDNA sequence analysis of the NC2 domain reveals the presence of a region homologous with the large family of Kunitz-type serine protease inhibitors. There is no evidence to date, however, that the NC2 actually can serve as a protease inhibitor. We tested the recombinant NC2/COL and NC2 for inhibitory activity against trypsin and trypsin-like proteases. "Fig. 6. Inhibition of NC1 cleavage by trypsin and chymotrypsin with protease inhibitors and NC2/COL. The cleavage products from either trypsin (lanes 1–4) or chymotrypsin (lanes 5–8) were separated by 6% SDS-PAGE followed by Coomassie Blue staining. Inhibitors were added as follows: lanes 1 and 5, none; lanes 2 and 6, 10-fold excess of aprotinin; lanes 3 and 7, 10-fold excess of NC2/COL (by molar); lanes 4 and 8, 50-fold excess of NC2/COL. The position of intact NC1 is indicated.

"Fig. 7. Scatter plot representation of ELISA results using recombinant NC2/COL. Patient and control sera (as indicated along the horizontal axis) (1:200 dilution) were incubated with immobilized purified NC2/COL and the bound antibodies were detected with an alkaline phosphatase-conjugated antibody against human IgG whole molecule. Each sample was run in triplicate and the points plotted on this graph are the average of the Abs obtained from study sera. Similar results were obtained in three other independent studies.

"Fig. 8. Immunoblot of the recombinant NC2/COL proteins by EBA and control sera. The purified recombinant NC2/COL (100 ng/well) protein was separated on 10% SDS-PAGE and transferred to nitrocellulose membranes before incubation with sera at a dilution of 1:200 and horseradish peroxidase-conjugated anti-human IgG (1:5000) followed by ECL detection. The location of 50 kDa of recombinant NC2/COL is indicated."

"Fig. 5. Formation of antiparallel dimers by cysteine mutants. A, 293 cells were transfected with either wild type NC2/COL (WT) or with cysteine mutants, and conditioned media were concentrated and subjected to 8% SDS-PAGE followed by immunoblot analysis with a polyclonal antibody to NC2 domain. Proteins were either reduced with 2-mercaptoethanol (lanes 1–5) or nonreduced (lanes 6–10). The positions of molecular weight markers, monomer (M), dimer (D), trimer (T), and hexamer (H) of NC2/COL are indicated. B, immunoblot analysis of either wild type NC2/COL (WT) or methionine to lysine substitution (M279K). Proteins were either reduced with 2-mercaptoethanol (lanes 1 and 2) or nonreduced (lanes 3 and 4)."
proteases. We were unable to demonstrate any protease inhibitory activity with either NC2/COL or NC2 obtained biochemically from collagenase digestion of NC2/COL (data not shown). All protease inhibitors with the Kunitz motif contain an important region that is responsible for the antiprotease function. This region has a critical lysine or arginine at the P1 site in all protease inhibitors with antitrypsin activity (33, 34). In what would be the homologous NC2 region (residue 2882–2289), there is a threonine (residue 2886) rather than either lysine or arginine. This single amino acid difference may account for the lack of inhibition of trypsin by NC2. Some proteins have a homologous region without a basic residue at the P1 site and yet still have antiprotease activity against chymotrypsin. However, we were unable to demonstrate any anti-chymotrypsin activity in our recombinant NC2. Although we were unable to demonstrate any antiprotease activity in NC2, our studies do not exclude the possibility that the Kunitz motif in NC2 could have an inhibitory activity for a protease that is yet to be identified. Or, perhaps, in vivo NC2 is processed to an active form.

It is of interest that the Kunitz motif in the NC2 domain of type VII collagen is most similar to the motif in the carboxyl-terminal noncollagenous domain of the α1-chain of type VI collagen (20). Although there is lysine at the P1 site in type VI collagen, a recombinant Kunitz domain of the type VI α1-chain did not demonstrate protease inhibitor activity (28). The Kunitz motif in protease inhibitors have also been shown to play a role in binding to active sites. For example, dendrotoxins are speculated to act by binding to some component of the presynaptic membranes. In this regard, it is interesting to note that type VI collagen, like type VII collagen, forms anti-parallel dimers as the first step in the formation of the macromolecular structure (35). Thus, the non-active Kunitz-type serine protease inhibitory motif in both collagens may contribute to the initial intermolecular binding needed for the formation of antiparallel dimers.

The cDNA sequence analysis of the NC2 domain reveals an 88% conservation at the amino acid level between the human and mouse. Furthermore, there is 100% conservation in the 67-amino acid segment spanning the junction of the triple helical and the NC2 domain which contains a pair of cysteine residues. It has been postulated that NC2 within one α-chain may bind to an appropriate site on the triple-helical domain of a second molecule and align cysteine residues, which subsequently becomes stabilized by intermolecular disulfide bonds (2). This model is consistent with the observation that native pepsin-treated type VII collagen dimers do not remain associated following disulfide bond reduction even under physiological conditions (18). In this study, we provide direct evidence that recombinant NC2/COL is capable of forming disulfide-bonded hexamers in vitro. Furthermore, rotary-shadowed images of the recombinant NC2/COL demonstrated directly that the molecules align into hexameric aggregations that represent antiparallel dimers. By subjecting the NC2/COL segment to bacterial collagenase digestion, we showed that the NC2 domain alone was unable to form oligomers larger than a trimer. These results suggest that the 186-amino acid collagenous sequences adjacent to the NC2 domain are required for antiparallel dimer formation.

It has been suggested that a pair of cysteine residues at positions 2802 and 2804 within the NC2 domain may be involved in the interchain and intermolecular disulfide bonds which stabilize type VII collagen antiparallel dimers. We directly tested the importance of these cysteine residues by site-directed mutagenesis studies. We showed that a single cysteine mutant protein in either C2802S or C2804S within the NC2 domain failed to form hexamers. Furthermore, mutation of both cysteines 2802 and 2804, but not mutation of either alone, abolished the trimer formation. Interestingly, a single cysteine mutation C2634S within the collagenous domain had no effect on hexamer formation, even though this cysteine has been proposed to be involved in interchain disulfide bonding (20).

In a study by Christiano and colleagues (32), a family with members who had recessive DEB was described in which a gene defect in type VII collagen was identified as a single amino acid substitution from methionine to lysine at residue 2789 within the NC2 domain. Specifically, this substitution was 4 residues upstream from cysteines 2802 and 2804 within the NC2 domain (32). Because of this report and the resultant complete lack of anchoring fibrils in the skin of affected patients, we attempted to duplicate this gene defect in vitro using site-directed mutagenesis. We introduced the same mutation in the NC2/COL construct which rendered the protein product unable to form hexameric, disulfide-bonded aggregates (anti-parallel dimers). It is possible that the replacement of a non-polar amino acid methionine with a positively charged amino acid lysine might interfere with the ability of the two adjacent cysteine residues, 2802 and 2804, to form a disulfide bond and stabilize antiparallel dimers. Such a mechanism could result in deficient formation of type VII collagen antiparallel dimers and assembly of anchoring fibrils. This would lead to a paucity of anchoring fibrils which is characteristic of recessive DEB.

Several biochemical studies employing rotary shadowing and immunoelectron microscopy have suggested that the NC2 domain is cleaved off of type VII collagen α-chains in the process of anchoring fibril formation (4, 17). In addition, using NC2-specific antibody, it was shown that this domain was absent from normal human skin (19). Because of its protease sensitivity and the low yield of purified type VII collagen using biochemical purification methods, detailed studies to identify the peptide bond involved in the physiologic NC2 removal have not been possible. It is interesting that in this study, we repeatedly observed two protein bands of 30 and 20 kDa in our purified fractions of recombinant NC2/COL. Whether this represents the natural cleavage process which occurs in the physiologic removal of NC2 during anchoring fibril assembly is not known.

The IgG autoantibodies in the sera of patients with EBA recognize distinct antigenic epitopes in the NC1 domain (21–23). Nevertheless, at least three EBA patients have been identified with autoantibodies against the helical domain (24). These patients were Japanese children who had the inflammatory, bullous-pemphigoid-like variety of EBA. We examined the immunoreactivity of EBA sera with recombinant NC2/COL using ELISA and Western blot analysis. We found four bullous SLE and four EBA sera that recognized both the NC1 domain and NC2/COL. The reactivity was abolished with collagenase treatment of NC2/COL which suggested that the antigenic epitope is within the 186-amino acid helical collagenous segment immediately juxtaposed to NC2 (data not shown). All of the EBA patients in this study had the classical mechanobullous form of EBA. In our previous study, the majority of EBA patients with classic clinical features had autoantibodies reactive with the NC1 domain of type VII collagen except one patient with bullous SLE who showed immunoreactivity to both NC1 and the collagenous domain (19). In this study, we demonstrate coexistence of autoantibodies to both NC1 and the collagenous domains in a subset of EBA patients.

It has been hypothesized that EBA autoantibodies may contribute to defective epidermal-dermal adhesion by binding to the NC1 domain of type VII collagen. Autoantibodies bound to type VII collagen would interfere with the normal interactions
between type VII collagen and its ECM ligand(s) in the basement membrane zone or the papillary dermis (25, 36). In this study, we identified EBA and BSLE sera that bound to NC2/COL. In this regard, it is interesting to note that we have previously mapped the 187-amino acid collagenous sequences in the NC2/COL construct as a fibronectin-binding site (37). It is possible that perturbation of the type VII collagen-fibronectin interaction may contribute to the pathogenesis of EBA. Alternatively, binding of EBA autoantibodies to the COL domain may destabilize anchoring fibrils by interfering with the antiparallel assembly of procollagen dimers leading to epidermal-dermal disadherence.

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