Noncollagenous (NC1) Domain of Collagen VII Resembles Multidomain Adhesion Proteins Involved in Tissue-Specific Organization of Extracellular Matrix

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Type VII collagen (C7) is a stratified squamous epithelial basement membrane protein composed of three identical alpha chains, each consisting of a 145-kDa amino-terminal noncollagenous (NC1) domain and a 145-kDa carboxyl-terminal collagenous domain. Morphologic and biochemical studies have shown that tissue-specific aggregates of C7 dimers called anchoring fibrils may contribute to epithelial basement membrane organization and adherence by interacting with extracellular matrix (ECM) proteins such as type IV collagen. In this study, we cloned a cDNA encoding most of the NC1 domain of C7. The deduced amino acid sequence revealed motifs characteristic of multidomain ECM proteins that contribute to the tissue-specific organization of ECM

including a region of 7½ sequential fibronectin type III (Fn III) homology repeats, a potential collagen-binding region homologous to the A domain of von Willebrand factor (vWf) and an RGD sequence. A purified C7 fusion protein containing these motifs specifically bound to type IV collagen in a functional assay. These results suggest that regions within the NC1 domain of C7 mediate interactions with lamina densa and dermal ECM proteins including type IV collagen. Structural mutations and autoepitopes in these regions may represent mechanisms for the development of defective basement membrane organization and adherence in genetic and autoimmune forms of epidermolysis bullosa. *J Invest Dermatol* 99:691–696, 1992

C7 molecules are composed of three identical alpha chains, each consisting of a 145-kDa amino-terminal noncollagenous (NC1) domain and a 145-kDa carboxyl-terminal collagenous domain [1,2]. C7 is distributed in the epithelial basement membrane of stratified squamous epithelia in morphologically distinctive, tissue-specific structures called anchoring fibrils [3]. Anchoring fibrils are composed of unstaggered lateral aggregates of C7 dimers that are formed by the anti-parallel association of the triple helical, carboxyl-terminal collagenous domains of two C7 molecules. The antiparallel arrangement of C7 dimers gives anchoring fibrils a sym-

metrical structure with the collagenous domains at the center and the NC1 domains at both ends [4]. The NC1 domains at the ends of anchoring fibrils associate with the lamina densa subregion of the epithelial basement membrane and with dermal structures resembling fragments of lamina densa called anchoring plaques. That arrangement forms an extensive sub-lamina densa network of anchoring fibrils interwoven with dermal interstitial collagen fibers [5,6]. The relationships between anchoring fibrils, the lamina densa, and anchoring plaques, and between anchoring fibrils and other components of the basement membrane adhesion complex, suggest anchoring fibrils contribute to the organization of the basement membrane region and to lamina densa-dermal adherence [6,7]. Furthermore, the data suggest the NC1 domains of C7 dimers interact with extra-cellular matrix (ECM) proteins [6]. That view is supported by studies indicating the NC1 domain may mediate the binding of C7 to type IV collagen, a major component of the lamina densa and anchoring plaques [8].

A role for C7 in lamina densa-dermal adherence is also supported by studies showing that abnormal expression of anchoring fibrils and C7, and autoantibodies to C7 are features of genetic and acquired forms of epidermolysis bullosa that are characterized by defective lamina densa-dermal adherence [9]. A recessively inherited form is associated with a severe adherence defect, absent anchoring fibrils, and reduced expression of immunoreactive C7. A less severe dominantly inherited form is characterized by reduced numbers of abnormal-appearing anchoring fibrils. An adherence defect similar to that observed in dominantly inherited disease occurs in the autoimmune disease epidermolysis bullosa acquisita (EBA), which is associated with autoantibodies that recognize a proteolytic fragment of C7 that contains the NC1 domain [10].

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Abbreviations

- BSA: bovine serum albumin
- C7: collagen type VII
- EBA: epidermolysis bullosa acquisita
- ECM: extra-cellular matrix
- ELISA: enzyme-linked immunosorbent assay
- Fn III: fibronectin III
- GCG: Genetics Consulting Group
- IPTG: isopropyl-B-D-thiogalactopyranoside
- NC1: non-collagenous domain 1
- SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis
- vWf: von Willebrand factor

In this study, we cloned a cDNA encoding most of the NC1 domain of C7. The putative NC1 domain contains at least three different sequence motifs found in ECM adhesion proteins that are believed to influence the supramolecular organization of ECM and basement membranes. A purified fusion protein encoded by the cDNA specifically bound to type IV collagen *in vitro*. These results establish C7 as a member of a growing number of multidomain ECM adhesion proteins that contribute to tissue-specific organization of ECM and basement membranes and provide additional evidence that the NC1 domain may interact with type IV collagen and possibly other lamina densa and anchoring plaque matrix proteins.

MATERIALS AND METHODS

cDNA Library Construction and Screening Random-primed cDNA was synthesized from WISH cell mRNA using the cDNA Synthesis System Plus kit (Amersham), and cloned into Lambda Zap II (Stratagene) according to the manufacturer's instructions. The library contained 1.0×10^6 independent clones with an average insert size of 2.2 kb. Recombinant phage were plated on *Escherichia coli* strain XL-1 blue cells, and fusion protein synthesis was induced with isopropyl-B-D-thiogalactopyranoside (IPTG)-saturated nitrocellulose filters [11]. Fusion proteins were screened with an EBA patient serum (E007) containing previously characterized IgG autoantibodies to a proteolytic fragment of C7 containing the NC1 domain [10]. The serum was adsorbed twice with *E. coli* lysates and diluted 1:250 in 0.15 M NaCl buffered with 0.01 M Tris-HCl, pH 7.6, before screening. Positive clones were identified by staining with an alkaline phosphatase-conjugated secondary antibody (rabbit anti-human IgG, Stratagene).

Immunologic Analysis of Positive Clones Positive clones were screened for NC1 epitopes by Western immunoblotting native and collagenase-digested C7 with antibodies that had been epitope-selected on confluent phage plaques. Epitope selection, extraction, and digestion of tissue C7 and immunoblotting were performed as previously described [10,12]. Positive clones were also screened by immunoblotting fusion proteins obtained by subcloning the cDNA into the expression vector pGEMEX1 (Promega-Biotech Inc.) with EBA patients' sera; a previously characterized monoclonal to a proteolytic fragment of C7 containing the NC1 domain, L3d; and affinity-purified EBA autoantibodies prepared as previously described [13]. All sera were adsorbed and diluted as noted above for E007. A rabbit antibody to the 26-kDa pGEMEX1 bacterial gene 10 protein (Novogen Inc.) was used to localize recombinant proteins in immunoblots.

Preparation and Sequencing of CVII-1 cDNA Positive clones were plaque purified and recombinant plasmids rescued in pBluescript (Stratagene). Plasmid DNA was mapped using established methods [14]. Restricted cDNA fragments were purified by electrophoretic elution from agarose gels and subcloned into M13 mp18/19 phagemids (Pharmacia) or pGEMEX1 plasmids using instructions provided by the manufacturers. Fragments were sequenced bi-directionally by the dideoxy chain-termination method using a series of internal primers [15].

Sequence Analysis The CVII-1 DNA sequence was assembled and DNA and amino acid sequences were analyzed using version 6.0 of the Genetics Consulting Group (GCG) Sequence Analysis Software Package. Searches of NBRF protein, SwissProt, and GenBank databases were performed using the FASTA program [16].

Fusion Protein Binding to Type IV Collagen A glutathione transferase fusion protein containing nts 1-2255 of the C7 cDNA was expressed in pGEX2 (Pharmacia) and purified on glutathione sepharose according to instructions of the supplier. ELISA wells were coated with 0.03 μ g of mouse type IV collagen (Collaborative Biomedical Products) or equivalent amounts of BSA, type I collagen, or type III collagen in 0.1 M carbonated buffer, pH 8.0, overnight and subsequently blocked with bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-0.05% Tween-20. This type IV collagen preparation was 99% pure by sodium dodecylsulfate-

Figure 1. Western blots. A: Western immunoblots of epitope selected L3d (lanes 1 and 2), epitope-selected E007 (lanes 3 and 4), unselected E007 serum (lanes 5 and 6), and unselected L3d culture supernate (lanes 7 and 8) against tissue C7 (lanes 1, 3, 5, and 7) and collagenase-digested tissue C7 (lanes 2, 4, 6, and 8). E007 serum was diluted 1:250. Epitope-selected E007, L3d, and L3d culture supernate were used undiluted. Forty micrograms total protein was loaded in each well. Markers are present for the Mr 290-kDa band of tissue C7 and the Mr 145-kDa band of collagenase-digested tissue C7. B: Western immunoblots of pGEMEX1 CVII-1 fusion proteins (lanes 1, 3, and 5) and pGEMEX1 nonrecombinant negative controls (lanes 2, 4, and 6) with rabbit antibodies to the bacterial gene 10 protein (lanes 1 and 2), L3d (lanes 3 and 4), and E007 (lanes 5 and 6). The rabbit antibody to gene 10 recognizes both the recombinant protein (140 kDa) and the gene 10 protein (40 kDa) in fusion preparations but only gene 10 protein in nonrecombinant controls (lane 2). L3d and E007 recognize the fusion protein in lanes 3 and 5, respectively, but do not react with the gene 10 protein (lanes 4 and 6). C: Western immunoblots of pGEMEX1 CVII-1 fusion protein with L3d (lane 1), E007 (lane 2), affinity-purified E007 (lane 3), seven additional EBA sera containing autoantibodies to C7 (lanes 4-10), and two EBA sera without circulating autoantibodies to C7 (lanes 11 and 12). L3d, E007, affinity-purified E007, and seven autoantibody-positive EBA sera recognize a 140-kDa fusion protein.

polyacrylamide gel electrophoresis (SDS-PAGE), consisted of two bands of 185 and 170 kDa, and was negative for laminin contamination by Western immunoblotting. Coated wells were subsequently incubated with 50 μ l of 1-24 μ g/ml pGx2.4 or glutathione transferase in PBS-Tween at room temperature for 1 h. Binding of pGx2.4 to substrate proteins was detected with L3d or E007 using alkaline phosphatase-conjugated secondary antibody as described above.

RESULTS

Cloning of cDNA Encoding the NC1 Domain of C7 Immunoscreeing of 2.5×10^6 plaques from a human WISH cell lambda gt11 library with EBA serum E007 identified one positive plaque, denoted CVII-1. Preliminary rescreening of purified CVII-1 plaques showed strong reactivity with several other EBA sera and a monoclonal antibody (L3d) that recognizes a proteolytic fragment of C7 containing the NC1 domain (results not shown). To confirm the identity of this cDNA, E007 and L3d antibodies were epitope selected on CVII-1 plaques and immunoblotted against tissue C7 (Mr 290 kDa) and a proteolytic fragment of C7 containing the NC1 domain (Mr 145 kDa) derived by collagenase digestion. The results (Fig 1A, lanes 1-8) showed epitope-selected

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collagen (results not shown). Selective binding of the fusion protein to type IV collagen suggests that a type IV collagen-binding site may reside in the corresponding region of the NC1 domain of C7. However, the absence of saturable binding indicates that binding kinetics are complex and not those of classic receptor-ligand interactions and raises the possibility that the interactions observed in this study are nonspecific. Further studies to define this important issue and the specific interactions between NC1 fusion proteins and epithelial matrix proteins are clearly needed.

DISCUSSION

In the current studies, we have shown that C7 is a multidomain molecule with structural features to a group of collagenous and noncollagenous proteins that contain Fn III repeats, A domains, RGD sequences, or combinations of the three motifs. Most of the proteins in this group have a restricted tissue distribution and are believed to be involved in cell-matrix and matrix-matrix interactions that contribute to the tissue-specific supramolecular organization of the ECM and perhaps to basement membrane organization and adhesion. Examples include cartilage matrix protein that contains two A domains and may contribute to cartilage structure by associating with type II collagen and proteoglycans [28]; type VI collagen that contains all three motifs and may facilitate adherence of nonepithelial basement membranes to stroma and contribute to a network of interstitial collagen fibers by bridging types I and III collagens [26,29]; and undulin, a noncollagenous protein containing Fn III repeats and A domains that appears to cross-link collagen fibrils to form collagen bundles [30].

The precise role of Fn III repeats and A domains in the interactions between these proteins has not been completely elucidated, but there is evidence that A domains in vWf and other ECM proteins can bind different collagen types [26,27,29,30]. There is also emerging evidence that Fn III repeats may mediate biologically significant protein-protein interactions as indicated by recent studies showing that Fn III repeats in the ECM protein tenascin influence cell behavior [31].

Based on the current studies, we conclude that C7 is a member of the superfamilies of ECM adhesion proteins containing Fn III and A domain homology regions and RGD sequences. These structural similarities and functional studies showing binding interactions between type IV collagen and a CVII-1 fusion protein support a role for C7 in organization of the epithelial basement membrane of stratified squamous epithelia and lamina densa-dermal adherence, and support a role for interactions between C7 and type IV collagen in those functions. If the Fn III and A domain homology regions are involved in protein-protein interactions required for C7 to mediate lamina densa-dermal adherence, then they may also be potential targets for structural mutations and autoepitopes in inherited and acquired blistering diseases associated with defective lamina densa-dermal adherence. Strong support for a genetically determined abnormality in C7 in at least one large pedigree with dominant dystrophic epidermolysis bullosa has recently been obtained from studies linking the disease to the C7 gene [32]. Defective lamina densa-dermal adherence is also a feature of EBA, a disease previously shown to be characterized by autoantibodies to a proteolytic fragment of C7 containing the NC1 domain [10,33]. In this study, we have shown that C7 autoantibodies from seven EBA patients were able to recognize fusion proteins corresponding to the NC1 domain of C7, leading us to speculate that those autoantibodies may contribute to defective lamina densa-dermal adhesion by the relatively novel pathomechanism of targeting functional epitopes in an important matrix adhesion protein. Thus, autoantibodies to the NC1 domain of C7 may interfere with the normal interactions between C7 and its ligand(s) in the ECM. In preliminary studies, we have found that C7 autoantibodies from several patients recognize at least two distinct epitopes on the fusion protein encoded by CVII-1. Both epitopes are in the region of the fusion protein that contain Fn III repeats but one lies very close to the type IV collagen-binding A domain. Studies to define and characterize the relationships between functional and autoepitopes are in progress.

In summary, we have cloned a cDNA encoding 887 amino acid residues of noncollagenous sequence immediately N-terminal of a previously cloned collagenous domain of C7. This effectively doubles the amount of reported noncollagenous sequence and helps establish that the position of the NC1 domain of C7 is N-terminal rather than C-terminal to the collagenous domain. Initial biochemical studies had indicated that the 145-kDa NC1 domain was C-terminal and the 30-kDa NC2 domain was N-terminal of the collagenous domain. A previous cloning study questioned that relationship by showing a partial noncollagenous sequence with an estimated size of approximately 45 kDa N-terminal of the collagenous sequence [2]. The present study demonstrates that the size of the noncollagenous region N-terminal of the collagenous domain is at least 90 kDa, and convincingly demonstrates that this region is not the NC2 domain. The results of this study also report for the first time that the NC1 domain of C7 has a multidomain structure with repetitive, sequential Fn III repeats and a potential collagen-binding A domain homology region. These findings and studies indicating that a fusion protein containing most of the CVII-1 cDNA may bind collagen IV provide structural evidence that complements previous biochemical and morphologic evidence that the NC1 domain of C7 cross-links lamina densa and anchoring plaque matrix proteins, thus contributing to lamina densa-dermal adherence. Finally, these studies unequivocally demonstrate that the sequence of NC1 encoded by CVII-1 cDNA contains a major epitope region(s) recognized by C7 autoantibodies and supports previous immunochemical studies showing that EBA antibodies recognize epitopes in a proteolytic fragment of C7 containing the NC1 domain.

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