

Immunohistochemical and Mutation Analyses Demonstrate that Procollagen VII Is Processed to Collagen VII through Removal of the NC-2 Domain

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Abstract. Collagen VII is the major structural constituent of anchoring fibrils in the skin. It is synthesized as a procollagen that is larger than the collagen deposited in the tissue. In this study, we investigated the conversion of procollagen VII to collagen VII in human skin and in cutaneous cells in vitro and identified the propeptide using domain-specific antibodies. For this purpose, two bacterial fusion proteins containing unique sequences of the carboxy-terminal globular NC-2 domain of procollagen VII were prepared, and polyclonal antibodies raised against them. Immunoblotting showed that the anti-NC-2 antibodies reacted with procollagen VII isolated from cultured keratinocytes, but not with collagen VII extracted from the skin. Immunohistochemical experiments with the NC-2 antibodies revealed a strong reaction in cultured keratinocytes, but the basement membrane zone of normal skin remained negative. The staining could not be rendered positive by chemical or

enzymatic unmasking of potential hidden epitopes in the skin, indicating that most of the NC-2 domain is absent from normal skin. In contrast, a positive staining with NC-2 antibodies was observed in the skin of a patient with dystrophic epidermolysis bullosa, who carried a 14-bp deletion at one of the intron-exon junctions of the collagen VII gene. This aberration led to an in-frame skipping of exon 115 from the mRNA and eliminated 29 amino acids from the NC-2 domain which include the putative cleavage site for the physiological processing enzyme, procollagen C-proteinase. The results indicate that in normal human skin, the removal of the NC-2 domain from procollagen VII precedes its deposition at the dermal-epidermal junction. Furthermore, they suggest that an aberration in the procollagen VII cleavage interferes with the normal fibrillogenesis of the anchoring fibrils.

ANCHORING fibrils extend from the lamina densa of the epidermal basement membrane to the papillary connective tissue, thus ensuring tight cohesion between the basement membrane and the dermis in the skin. The main structural protein of the fibrils is collagen VII, a homotrimeric collagen containing three identical $\alpha 1(\text{VII})$ chains (4, 5, 31). This collagen is synthesized mainly by epidermal keratinocytes (20, 30). The primary synthetic product is larger than the collagen deposited in the tissue, and has been called procollagen VII in analogy to other members of the collagen protein family (5, 18, 20). Each $\alpha 1$ chain of procollagen VII consists of a long central triple-helical domain with a typical collagenous -Gly-X-Y-

repeat sequence, a large globular NH_2 -terminal NC-1 domain, and a small globular COOH-terminal NC-2 domain (24, 25). Based on the cDNA-derived amino acid sequence, the following molecular masses have been calculated for the three procollagen VII domains: NC-1, 133 kD; collagenous domain, 145 kD; and NC-2, ~18 kD (7, 12). Rotary shadowing electron micrographs of anchoring fibrils from tissue extracts suggested that NC-2 cleavage is necessary for correct assembly of collagen VII (24, 25). During fibrillogenesis, collagen VII molecules form antiparallel tail-to-tail dimers with a small carboxy-terminal overlap and with the amino termini pointing outwards (5, 24, 31). The dimers then aggregate laterally in a nonstaggered manner into the anchoring fibrils. Although not all small globular domains were removed from collagen VII dimers isolated from epidermoid carcinoma cell lines, most dimers from amnion extracts contained only the large glob-

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ular end (25). These observations led to the assumption of COOH-terminal cleavage during the assembly and processing of the molecules in the extracellular space (24, 25). However, other investigators suggested that the procollagen to collagen conversion occurs through cleavage of amino-terminal sequences (22). Thus, the knowledge about the location and timing of the proteolytic process that converts procollagen to collagen VII is limited and controversial. In addition, a specific procollagen N- or C-proteinase catalyzing this process has not yet been identified.

In dystrophic epidermolysis bullosa (EBD)¹, a genetic blistering disorder of the skin, minor trauma induces separation of the epidermis from the dermis. This tissue separation occurs below the basement membrane, at the level of the anchoring fibrils. Electron microscopic studies have revealed a variety of structural abnormalities of anchoring fibrils in EBD (2, 11). In contrast to normal skin, where the anchoring fibrils are slender and cross-striated, EBD skin can present with broad and wispy fibrils without crossbanding, with very short fibrils, or no fibrils at all. These observations, together with immunochemical and cell biological studies defined collagen VII as a candidate molecule for abnormalities in EBD (see 3), and in line with these data, mutations have been identified in the gene for collagen VII, COL7A1 (8) in several EBD patients (6, 14, 34).

The aim of the present study was to characterize the physiological processing of procollagen VII and to identify the peptide domain that is removed during this process. We show that procollagen VII synthesized by human keratinocytes in vitro contains all three structural domains. During the processing, a large part of the NC-2 domain is cleaved off and is not present in the collagen VII molecules deposited in normal human skin. This is further attested by the fact that the NC-2 domain is retained in the skin of an epidermolysis bullosa patient who carries a splice-site mutation of the collagen VII gene that causes elimination of the putative NC-2 cleavage site encoded by exon 115.

Materials and Methods

Preparation and Purification of Bacterial Fusion Proteins

To obtain antibodies recognizing the NC-2 domain of procollagen VII, two different portions of the NC-2 domain were expressed as prokaryotic fusion proteins. Fusion protein NC2-10 involved amino acids 2780–2944 and fusion protein NC2-7 amino acids 2837–2944 of procollagen VII (7) (Fig. 1). The corresponding cDNA was amplified by reverse transcriptase (RT)-PCR from RNA from normal human keratinocytes. Briefly, poly A+ RNA was reverse transcribed to cDNA using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Boehringer-Mannheim GmbH, Mannheim, Germany) and processed for cDNA amplification. 40 PCR cycles were run on a thermocycler using a PCR amplification kit (Perkin Elmer Corp., Ueberlingen, Germany) with primers listed in Table I. The amplification conditions for both primer pairs consisted of a denaturing step at 95°C for 45 s, primer annealing at 60°C for 30 s, and extension at 72°C for 45 s. The final extension was prolonged to 10 min. All reactions were performed in a final reaction vol of 50 µl. Resulting amplicates were digested with SfiI and NotI, run on an agarose gel, electroeluted, and

subcloned in the SfiI/NotI restriction sites of pDS9 cassette (35). The resulting constructs were sequenced over the ligation sites to confirm correct in-frame cloning of the procollagen VII cDNA fragments.

The fusion proteins were produced essentially as described by Stüber et al. (33). The constructs were transformed into M15(pREP4) cells, and the transformed cells were grown in Luria broth (LB) medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Expression of the fusion proteins was induced by the addition of isopropyl β-thiogalactopyranoside (Boehringer-Mannheim Corp.) to a final concentration of 2 mM. 3 h after induction, cells were centrifuged at 6,000 g for 10 min, and the pellet was extracted with 6 M guanidine-HCl in 100 mM sodium phosphate, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride for 1 h at 4°C. The solution was cleared of debris by centrifugation at 10,000 g for 10 min, and the supernatant was directly applied to a 5-ml Ni²⁺-NTA agarose (QIAGEN Inc., Düsseldorf, Germany) affinity column. After washing with 8 M urea in 100 mM sodium phosphate and 10 mM Tris, pH 8.0, the fusion proteins were eluted stepwise by lowering the pH to 6.3 and 5.9. Fractions were analyzed by SDS-PAGE and the fusion proteins were dialyzed against 4 M guanidine-HCl.

Antibodies

For immunization of New Zealand White rabbits, the fusion proteins were ethanol precipitated, suspended in PBS, and mixed with Freund's adjuvant according to standard immunization protocols (13). The antibodies were affinity purified on nitrocellulose strips containing fusion proteins that were separated by SDS-PAGE and electrotransferred as described previously (4, 20). Polyclonal affinity-purified antibodies to the triple-heli-

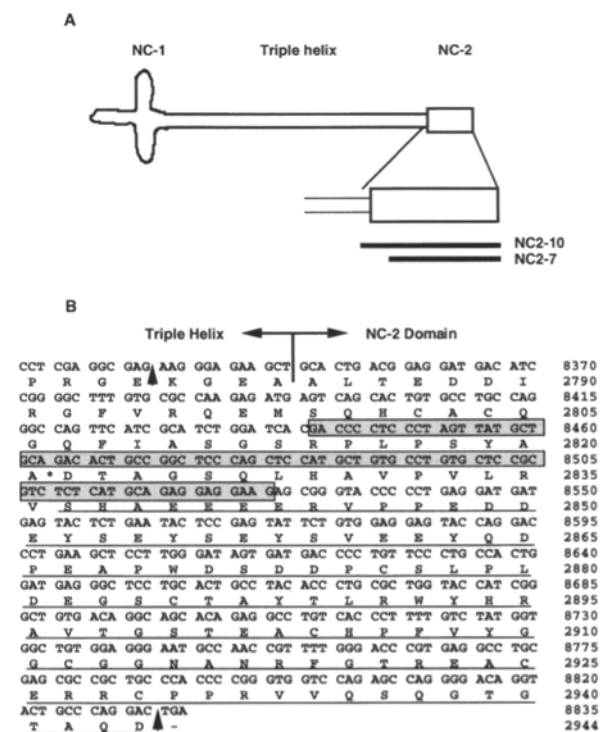


Figure 1. Location of the fusion proteins within the NC-2 domain of procollagen VII. (A) A schematic representation of the domain structure of procollagen VII. The areas of the NC-2 domain corresponding to fusion proteins NC2-10 and NC2-7 are indicated by black bars. (B) The amino acid and nucleotide sequence of the NC-2 domain (7). Vertical line with the double arrow marks the border between the triple-helical domain and the NC-2 domain. Arrowheads define fusion protein NC2-10. Amino acids covered by fusion protein NC2-7 are underlined. Asterisk (*) shows the putative procollagen C-proteinase cleavage site. Nucleotides of exon 115 are shaded.

1. Abbreviations used in this paper: EBD, dystrophic epidermolysis bullosa; IF, immunofluorescence staining; KGM, keratinocyte growth medium; RT, reverse transcriptase.

Table 1. Primers Used to Amplify Procollagen VII Coding Sequences

Primers for production of recombinant fusion proteins*

NC2-10 forward:
5'TTGGCCTTATTGGCCATTGAAGGTCGTAAGGGAGAAGCTGCACTGAC 3'
NC2-7 forward:
5'TTGGCCTTATTGGCCATTGAAGGTCGTTCTCATGCAGAGGAGGAAGA 3'
NC2-reverse:
5'ATGCGGCCGCTCAGTCCTGGGCAGTACCT 3'

Primers for mutation analysis[‡]

Col11F: 5'AGGACGAATTCTTGTGCGCCAAGAGATGAGTC 3'
Col10R: 5'CATGCAAGCTTGTCCCCTGGCTCTGGACCAC 3'
ColE114: 5'CCAGTTCATCGCATCTGGATCAC 3'

The PCR conditions for the primers are described in Materials and Methods.

*Primer NC2-reverse was used in combination with both NC2-10 forward and NC2-7 forward.

[‡]The internal restriction sites are underlined: an EcoRI site in Col11F and a HindIII site in Col10R.

cal domain of human collagen VII (4), and the mAb LH-7.2 (23) to the NC-1 domain of collagen VII were used as controls. The FITC- or biotin-labeled second antibodies were obtained from Dakopatts, Glostrup, Denmark, peroxidase-labeled goat anti-rabbit antibodies from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, and streptavidin peroxidase and FITC-labeled streptavidin from Boehringer-Mannheim Corp.

Immunofluorescence Staining (IF)

IF staining of 5- μ m cryosections of adult human skin or semiconfluent human keratinocytes on coverslips was carried out with standard techniques. For demasking of potentially hidden epitopes at the basement membrane zone, the cryosections were air dried, rinsed with TBS, and treated with 0.1 M acetic acid, 8 M urea, or 0.1% trypsin for 30 min at room temperature, or with 2% hyaluronidase for 1 h at room temperature. After these treatments the sections were washed extensively with TBS before IF staining.

Keratinocyte Cultures

Keratinocytes were cultured in serum-free keratinocyte growth medium (KGM) (Gibco Life Technologies, Eggenstein, Germany) supplemented with bovine pituitary extract and EGF as described previously (20). For IF, cells were seeded on glass coverslips and grown to subconfluence. Two days before the staining or extraction of the cells, bovine pituitary extract and EGF were omitted from the medium, and 50 μ g/ml ascorbate, and in some experiments 5 ng/ml TGF- β , were added daily.

For some experiments, keratinocytes were grown on an extracellular matrix produced by fibroblasts. For this purpose, fibroblasts were cultivated in DMEM/10% FCS to confluency and then incubated for 2–3 d in serum-free DMEM daily supplemented with 50 μ g/ml ascorbate. The fibroblasts were then removed by treating the cultures with 0.1% Triton X-100 in PBS twice for 10 min. The remaining extracellular matrix was washed extensively with PBS and preincubated in KGM, and keratinocytes were seeded onto the preexisting matrix.

Extraction of Procollagen and Collagen VII

For extraction of procollagen VII, the confluent keratinocyte layer was directly solubilized in a buffer containing 8 M urea, 2% SDS, 0.1 M 1,4-dithioerythritol, 0.1 M Tris-HCl (pH 6.8), and a mixture of proteinase inhibitors at a final concentration of 1 mM Pefabloc (Merck, Darmstadt, Germany), 10 mM EDTA, 20 mM *N*-ethylmaleimide, and 100 mM ϵ -aminocaproic acid as described (20). Extraction of collagen VII from skin was carried out after splitting the skin through the lamina lucida of the basement membrane with 1 M NaCl in TBS for 48 h (9) in the presence of proteinase inhibitors. The epidermis was mechanically separated from the dermis, and the newly exposed dermal layer was extracted with the above extraction buffer for 2 min at 95°C (4).

Electrophoresis and Immunoblotting

The samples were separated by SDS-PAGE under reducing conditions on polyacrylamide gradient gels. After electrotransfer onto nitrocellulose in the presence of 0.1% SDS, the nitrocellulose sheet was incubated with the

first antibodies overnight and with either peroxidase-labeled goat anti-rabbit IgG for 2 h, or biotin-labeled swine anti-rabbit IgG for 4 h, and streptavidin peroxidase for 2 h. 4-chloro-1-naphtol was used as chromogen.

Epidermolysis Bullosa Patient

The female patient was born with blisters over the trunk and the extremities. The family history was free of skin diseases or genetic disorders. Blistering activity diminished over the years resulting in a localized EBD by the age of 12 yr. The blisters healed with scarring and development of milia, Pasini papules, and nail dystrophy. The patient thus presented with the characteristic clinical phenotype of localized dystrophic EB. Biopsies were obtained from clinically unaffected perilesional skin. The diagnosis was confirmed with antigen mapping (2) and electron microscopy using standard techniques.

Detection and Characterization of a Mutation in the COL7A1 Gene

Total RNA was isolated from EBD and control fibroblasts with TRIzol reagent according to the manufacturer's specifications (GIBCO BRL, Gaithersburg, MD). The RNA was dissolved in RNase-free water in a concentration of 1 μ g/ μ l. Deoxyoligo-(dT)₁₈-primed cDNA synthesis was carried out with SuperScriptTMII reverse transcriptase essentially as described by the manufacturer (GIBCO BRL) but with the following exceptions; RNA was denatured with 100 pmol deoxyoligo(dT) at 90°C for 10 min before primer extension which was performed at 50°C for 1 h.

Collagen VII-specific primers were deduced from Greenspan, 1993 (12) and Christano et al., 1994 (7); These sequence data are available from EMBL/GenBank under accession number L23982). The primer sequences are listed in Table I. Primers Col11F and Col10R flanking the NC-2-encoding DNA sequence were designed with internal restriction sites. An internal primer ColE114 was designed for restriction fragment length polymorphism (RFLP) analysis and for DNA sequencing of genomic clones. RT-PCR was performed with primers Col11F and Col10R with the following cycling conditions: denaturation at 95°C for 4 min and then 34 cycles with 95°C for 1 min, 59°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. PCR on genomic DNA was performed with primers Col11F and Col10R essentially as described above, but with 32 cycles and with extension time of 2 min and 30 s. The PCR products were purified with Qiaex particles (QIAGEN Ltd., Dorking, UK) before EcoRI/HindIII digestion and ligation into EcoRI/HindIII-treated M13-mp19. Recombinant clones were propagated on JM101. cDNA clones were sequenced with the -40 M13 universal primer, whereas genomic clones were sequenced with primer ColE114. DNA sequencing was carried out with standard techniques using kit reagents (United States Biochemical Corp., Cleveland, OH).

For RFLP analyses, PCR was performed on genomic DNA with primers ColE114 and Col10R with initial denaturation for 4 min at 95°C followed by 30 cycles with 94°C for 1 min, 62°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were digested with PstI and SphI overnight and separated on a 3.5% agarose gel according to the manufacturer's specifications (MetaPhor; FMC BioProducts, Rockland, ME).

Results

Expression of Procollagen VII NC-2 Fragments in Bacteria

Bacterial fusion proteins corresponding to the NC-2 domain of procollagen VII were designed on the basis of the cDNA sequence and deduced amino acid sequence (7, 12) as shown in Fig. 1. The larger fragment, NC2-10, covered 165 amino acids and corresponded to the entire globular sequence plus the four last amino acids of the triple-helical domain (amino acid residues 2780–2944 of procollagen VII, or nucleotides 8338–8832 of the cDNA, numbering according to ref. 7). The shorter fragment, NC2-7, was designed to correspond to the most COOH-terminal sequence of this globular domain, covering the last 108 carboxy-terminal amino acids of the procollagen, residues 2837–2944 (nucleotides 8509–8832 of the cDNA). Even if the physiological C-proteinase cleavage site was not located immediately adjacent to the border of the triple helical domain, antibodies to this latter sequence should still exclusively recognize the propeptide (see Fig. 1).

Both plasmid constructs with the cDNA for NC-2 fragments could be efficiently expressed in bacteria. The fusion proteins were soluble in 6 M guanidin-HCl and could be purified to homogeneity from cell extracts with a one-step Ni²⁺-affinity chromatography. Usually 1 liter of bacterial cultures yielded ~4–20 mg of the respective fragment. Interestingly, the mobility on SDS-PAGE of both fragments was slower than expected based on their molecular size (Fig. 2). The larger protein has a calculated molecular mass of ~20 kD and the smaller one ~14 kD. However, they migrated with apparent molecular masses of 33 and 25 kD, respectively.

Differential Reactivity with Procollagen and Collagen VII in Immunoblots

Polyclonal antibodies were raised against NC2-10 and NC2-7 fragments and affinity purified on fusion proteins adsorbed to nitrocellulose strips. As expected, the antibodies cross-reacted with either polypeptide in ELISA and immunoblot assays (Table II). However, they showed different reactivity to procollagen and collagen VII isolated from cultured keratinocytes or skin (Fig. 3). In immunoblots, antibodies to the fusion protein NC2-10 showed positive staining with procollagen VII from keratinocytes, collagen VII from the skin, as well as pepsin-treated collagen VII, which contains the triple-helical domain with only short noncollagenous sequences. In contrast, antibodies to the shorter NC2-7 fragment recognized procollagen

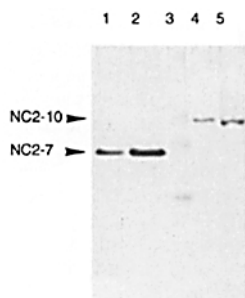


Figure 2. Migration of fusion proteins on SDS-PAGE. The fusion proteins were run on an SDS-PAGE with a 10–15% gradient gel. Lanes 1 and 2: NC2-7. Lanes 4 and 5: NC2-10. Lane 3: globular molecular mass markers from top to bottom: 49.5, 32.5, 27.5, and 18.5 kD. Positions of these markers are also indicated on the right.

Table II. Immunoreactivity of Domain-specific Antibodies to Procollagen VII

Antigen/Tissue	Antibodies*				
	NC2-7	NC2-10	Triple-helical	LH-7.2	EBA
IF staining					
Keratinocytes	+	+	+	+	+
Skin	–	+	+	+	+
Immunoblot					
Procollagen VII (Keratinocyte extract)	+	+	+	+	+
Collagen VII (Skin extract)	–	+	+	+	+
Triple-helical domain [‡]	–	+	+	–	–
NC-1 domain [§]	–	–	–	+	+
NC2-7	+	+	–	–	–
NC2-10	+	+	+	–	–

* Polyclonal antibodies to the triple-helical domain were raised against pepsinized collagen VII (4). Monoclonal antibody LH-7.2 (23) and the EBA autoantiserum (10) recognize the amino-terminal NC-1 domain.

[‡] The triple-helical domain was obtained with pepsin digestion of procollagen VII.

[§] The NC-1 domain was obtained with digestion of procollagen VII with purified bacterial collagenase.

+, positive reaction; –, negative reaction.

VII, but neither collagen VII nor pepsin-treated collagen VII (Fig. 3). Control antibodies to the triple-helical domain reacted with procollagen VII, collagen VII, and pepsin-treated collagen VII. The mAb LH-7.2 to the NC-1 domain and autoimmune sera from patients with epidermolysis bullosa acquisita, an acquired blistering skin disease with autoantibodies reactive with the NC-1 domain (10), recognized both the procollagen and the collagen forms, indicating that the NC-1 domain is part of the mature collagen VII molecule in the tissue (Table II).

Immunostaining of Normal Control Skin

In IF staining of normal control skin, antibodies to the longer fusion protein NC2-10 produced a positive linear

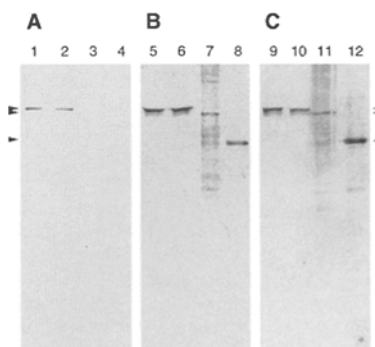


Figure 3. Specificity of fusion protein antibodies. For immunoblotting, the antigens were separated on an SDS-PAGE with a 3–15% gradient gel, and the blot was stained with antibodies to fusion protein NC2-7 (A), to the triple-helical domain of collagen VII (B), and to fusion protein NC2-10 (C). Lanes 1, 2, 5, 6, 9, 10: keratinocyte extract. Lanes 3, 7, 11: skin extract. Lanes 4, 8, 12: pepsinized collagen VII (triple-helical fragment). Arrowheads from top to bottom point to procollagen VII, collagen VII, and to the triple-helical fragment, respectively.

fluorescence at the dermal-epidermal junction, similar to the reaction seen with antibodies to the triple-helical domain of collagen VII (Table II). In contrast, the reaction with antibodies to the shorter fusion protein NC2-7 remained negative, indicating that all or the majority of epitopes contained in the fragment NC2-7 were absent from normal human skin (Fig. 4). This pattern was verified in skin from 50 different normal individuals of different ages. Only in a few cases was a very faint staining observed. Unmasking of potential hidden epitopes with 8 M urea, 0.1 M acetic acid, 0.1% trypsin, or 2% hyaluronidase did not render positive staining in normal skin. As expected, cultured human keratinocytes stained positively with both antibodies indicating presence of newly synthesized procollagen VII in the cells (Fig. 4).

Conversion of Procollagen to Collagen VII Is Efficient In Vivo but Inefficient In Vitro

Extracts of cultured human keratinocytes always contained procollagen VII but no collagen VII, and conversely, skin extracts contained collagen VII but no procollagen VII (Fig. 5 A). The extraction procedure included pretreatment of the monolayer cell cultures with ascorbic acid in serum-free low-calcium medium for 48–72 h. During this time, no significant conversion of procollagen to collagen VII took place. Addition to the cultures of substances that are known to enhance conversion of other procollagens or other precursor proteins to their respective tissue forms, such as 0.1% dextran sulphate, 1.5 mM calcium, or 50 μ M ZnCl₂ (1, 28, 29), did not stimulate conversion of procollagen VII. Neither did culturing of kerati-

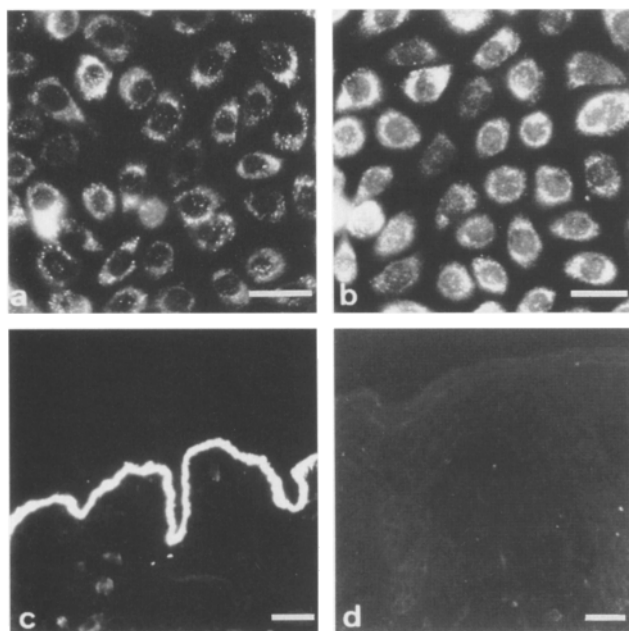


Figure 4. IF staining with fusion protein antibodies. Human keratinocytes (a and b) and normal control skin (c and d) were stained with antibodies to the triple-helical domain (a and c) and to fusion protein NC2-7 (b and d). Both antibodies stained keratinocytes. The control antibodies recognized collagen VII at the basement membrane zone of the skin, but the reaction with antibodies to NC2-7 remained negative, indicating absence of the NC-2 domain from normal skin. Bars, a and b, 10 μ m; c and d, 50 μ m.

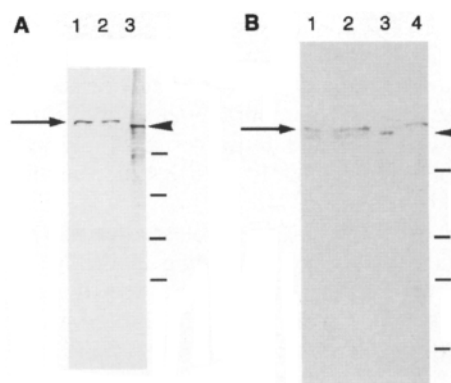


Figure 5. Procollagen to collagen VII conversion in vitro. Cell and skin extracts were separated on SDS-PAGE with a 3–15% (A) or 3–10% (B) gradient gel, and immunoblotted with antibodies to the triple-helical domain of collagen VII. (A) Procollagen VII isolated from keratinocytes grown on plastic (lanes 1 and 2) and collagen VII isolated from the skin (lane 3). In the skin, only collagen VII is present, implying efficient conversion of procollagen in vivo. (B) In extracts of keratinocytes grown for 48 h on an extracellular matrix produced by fibroblasts (lanes 1 and 2), some conversion of procollagen VII (lane 4) to collagen VII (lane 3) can be seen. Arrow marks procollagen, and the arrowhead marks collagen VII. On the right, molecular mass markers are, from top to bottom, 205, 117, 80, and 50 kD.

nocytes on plates coated with heparan sulphate or heparan sulphate proteoglycan, treatment of keratinocyte cultures with fibroblast extracts, or coculturing fibroblasts and keratinocytes result in processing of procollagen VII.

Under two different cell culture conditions, a low level of procollagen to collagen VII conversion was observed. When keratinocytes were grown on an extracellular matrix that had been deposited by fibroblasts, some processing of procollagen VII took place within 48 h (Fig. 5 B). Addition of 5 ng/ml of TGF- β to the keratinocyte cultures (21) for 48 h also induced conversion to some extent (not shown). Quantitation of the procollagen/collagen VII ratio in the immunoblots was not attempted, but the intensity of the collagen VII bands was always clearly less than that of procollagen VII bands, suggesting that maximally 20–30% of the procollagen in these cultures was processed to collagen VII.

For skin organ culture experiments, the tissue was metabolically labeled with [³⁵S]methionine/cysteine in a methionine- and cysteine-free KGM medium overnight at 37°C. The tissue was extracted with the chaotropic extraction buffer described in Materials and Methods, and the extracts subjected to SDS-PAGE, autoradiography, and immunoblotting. In all experiments only collagen VII, but no procollagen VII was found (not shown), indicating that the processing occurs remarkably faster in the skin than in monolayer cell cultures. The factors required for an efficient processing in vitro remain unknown at the present time.

Retention of the NC-2 Domain in the Skin of an EB Patient with a Mutation in the COL7A1 Gene

In contrast to normal skin, a bright fluorescence was observed with antibodies to the NC2-7 fusion protein in the skin of a patient with localized EBD. This indicated that

the COOH-terminal NC-2 domain was retained in the skin of this patient (Fig. 6). Ultrastructural analysis of clinically unaffected skin showed paucity of anchoring fibrils. On long stretches of the basement membrane zone, no clearly defined fibrils could be discerned. However, at other locations anchoring fibrils with a slightly diffuse structure and crossbanding could be seen (Fig. 7).

RT-PCR analysis of the patient's fibroblast mRNA with primers Col11F and Col10R corresponding to the NC-2 domain produced two PCR fragments, one with the expected length and one shorter product. The apparent difference in the length of the products was 80–90 bp (Fig. 8 A). Sequencing of the cDNA revealed a deletion of 87 bp which corresponded exactly to exon 115, indicating in-frame skipping of this exon (Fig. 8 B). Exon 115 codes for amino acids 2814–2843 of procollagen VII (see Fig. 1). Since the phase of the intron–exon boundaries does not coincide with the reading frame of the cDNA, the exon skipping creates a new codon at the splice junction which codes for glutamine. Thereafter, the reading frame continues normally. The resulting polypeptide is 29 amino acids shorter than the wild-type procollagen VII $\alpha 1$ chain. Using

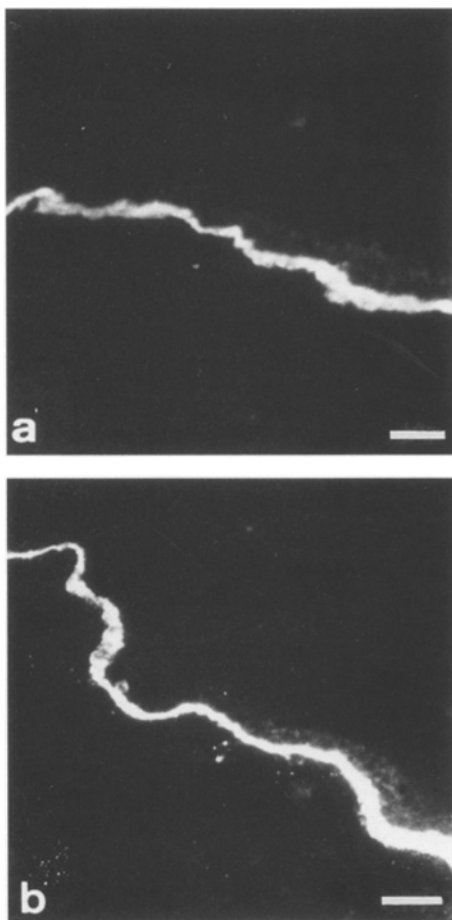


Figure 6. Immunofluorescence staining of the EBD patient's skin. Antibodies to the NC2-7 fusion protein (a) and control antibodies to collagen VII (b) showed a linear fluorescence at the dermo–epidermal basement membrane, indicating that in the patient's skin the NC-2 domain is retained. Normal control skin showed negative reaction with the NC2-7 antibodies, see Fig. 4 d. Bar, 50 μ m.

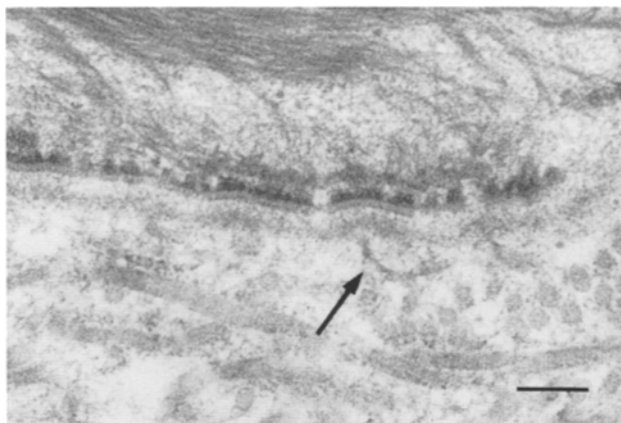


Figure 7. Electron microscopic analysis of the EBD patient's skin. Ultrastructural analysis of clinically uninvolved skin revealed paucity of well-defined, tightly packed anchoring fibrils. Clearly identifiable fibrils appeared somewhat diffuse and lacked a strong crossbanding pattern (arrow). The other components of the dermal–epidermal basement membrane zone appeared normal. Bar, 0.2 μ m.

genomic PCR and restriction enzyme analysis we localized the genetic defect that leads to this exon skipping in the exon–intron junction of exon 115. We showed the aberration is caused by a heterozygous 14-bp deletion in the patient's collagen VII gene (Fig. 9, A and B).

Discussion

Ample evidence exists for the fact that the “cell culture form” of collagen VII is larger than the “tissue form” (5, 20, 24, 25). Rotary shadowing images of partially purified collagen VII preparations isolated from carcinoma cell lines or amnion demonstrated dimeric molecules with or without a small globular domain (24, 25). It seemed, therefore, likely that the carboxy-terminal globular NC-2 domain was removed during the procollagen to collagen conversion, but other evidence based on collagenase digestion of procollagen VII from keratinocyte cultures and collagen VII from human skin pointed to processing at the amino terminus (22). Since domain-specific antibodies to collagen VII were needed for the characterization of procollagen and collagen VII in skin, we prepared antibodies against recombinant collagen VII fragments and used them in immunohistochemical experiments.

Two different recombinant bacterial fusion proteins were produced that corresponded to the amino acid sequences of the NC-2 domain and included in addition a 21–amino acid leader peptide. The mobility of the purified fusion proteins on SDS-PAGE was significantly slower than expected from their deduced amino acid sequences. The larger fusion protein with a calculated molecular mass of ~ 20 kD migrated just below the 32.5-kD globular molecular mass marker, and the shorter protein with a calculated molecular mass of ~ 14 kD just below the 27.5-kD marker. This observation is in line with the electrophoretic mobility estimates of the NC-2 domain isolated from procollagen VII using bacterial collagenase digestion. The apparent molecular mass was 32 kD (25), in spite of the fact that the domain only consists of 161 amino acids as de-

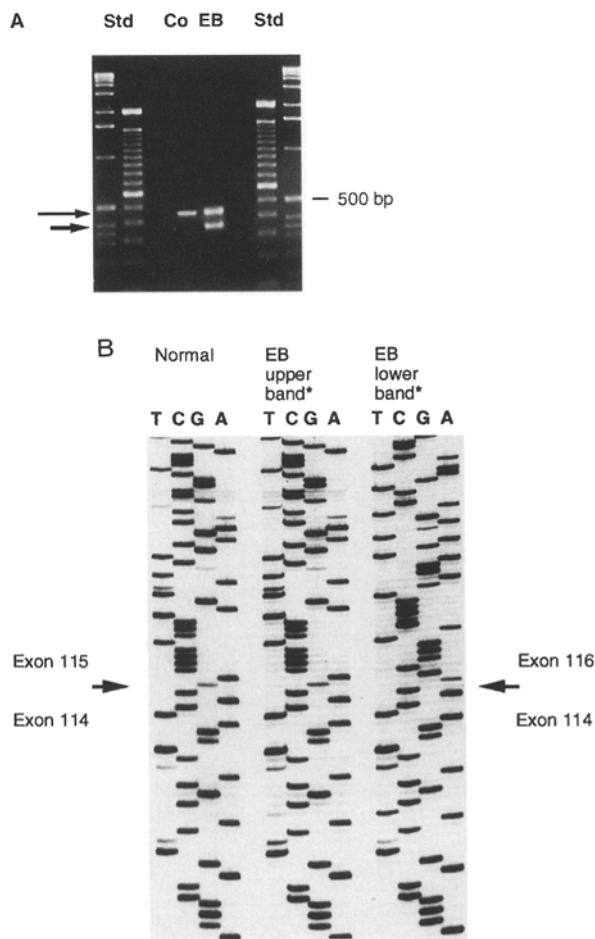


Figure 8. Analysis of the EBD patient's collagen VII cDNA. (A) Agarose gel electrophoresis of products of RT-PCR of mRNA with primers Col11F and Col10R. *Std*, molecular weight markers; *Co*, control mRNA; *EB*, EBD patient's mRNA. The patient's sample showed two PCR products, one of the expected size of 439 kb (*slender arrow*), and one that was ~80–90 bp shorter (*fat arrow*). On the outmost lanes as size markers a 1-kb DNA ladder (Gibco Life Technologies), and on the next lanes a 100-bp ladder, from 1,500 to 100 bp. (B) Nucleotide sequence of the cDNA. (Left) a normal control sequence. (Middle) sequence of the upper band of lane EB in A (*), the patient's normal band. (Right) sequence of the lower band of lane EB in A (*), the patient's abnormal band. Note that in the cDNA sequence of the lower band, exon 116 follows exon 114, indicating skipping of exon 115.

duced from the cDNA sequence (7, 12). Posttranslational modifications could explain this apparent discrepancy. The NC-2 domain contains four potential phosphorylation sites, but no potential sites for glycosylation (7, 12). It is not known whether the NC-2 domain is phosphorylated. However, the most likely explanation seems to be the highly acidic nature of this domain which has a theoretical isoelectric point value of 4.3 (12), since overestimation of apparent size by SDS-PAGE has previously been reported for proteins rich in acidic residues (19).

Antibodies to the longer fusion protein NC2-10 that corresponds to the entire NC-2 domain plus the four last amino acid residues of the triple helix reacted with procollagen VII, collagen VII, and even with pepsinized collagen

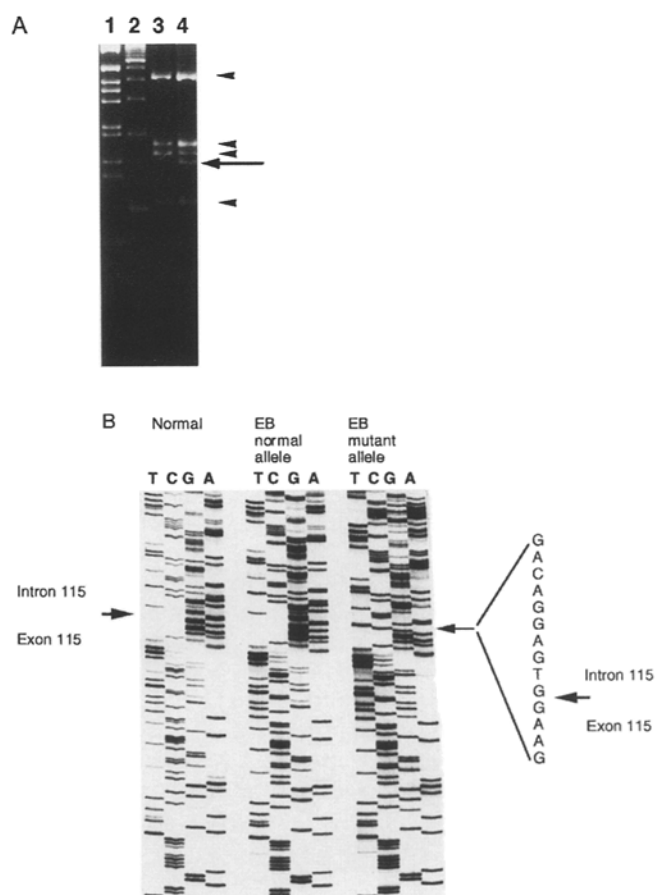


Figure 9. Deletion mutation in the collagen VII gene. (A) Genomic PCR products were generated with primers ColE114 and Col10R, digested with PstI and SphI, and separated on a high resolution agarose gel. Lane 1: size markers, 1-kb DNA ladder; lane 2: size markers, a 100-bp ladder, from 1,500 to 100 bp; lane 3: genomic DNA from a healthy unrelated control person; lane 4: genomic DNA of the EBD patient. In the control, the restriction enzyme digestion generated four fragments: 422, 183, 166, and 105 bp (arrowheads on the right, from top to bottom). In the patient's sample, an additional band of ~150 bp (*arrow*) reflected a deletion in the gene. (B) Characterization of the mutant allele. DNA sequence of genomic DNA from a normal control individual (*left panel*), of the EB patient's normal allele (*middle panel*) and the EB patient's mutant allele (*right panel*). The 14 bp deleted from the mutant allele are shown between the brackets (*slender arrow*). The exon 115–intron 115 junctions are indicated with fat arrows.

VII. Therefore, these antibodies were not useful for distinction between procollagen and collagen VII. Instead, the antibodies to the shorter fusion protein NC2-7 that covered the COOH-terminal two-thirds of the NC-2 domain, exclusively recognized procollagen VII. These antibodies did not react with normal control skin from 50 individuals, indicating that all of the sequences included in NC2-7 were removed during the physiological processing before deposition of collagen VII onto the dermo-epidermal basement membrane in the skin.

The reaction patterns of these antibodies also deliver information about the physiological cleavage site. This is likely to be localized within a 57–amino acid segment between residues 2780 and 2837 of procollagen VII, since

these amino acids distinguish the NC2-10 fragment from NC2-7 (see Fig. 1). Antibodies to the longer fusion protein react with collagen VII, but antibodies to the shorter one do not. Within this region resides an Ala-Asp peptide bond at position 2821–2822, the only such bond within the NC-2 domain. The procollagen C-proteinase that cleaves the carboxy-terminal propeptides from procollagens I, II, and III also cleaves an Ala-Asp peptide bond (15). The amino acid sequences flanking the 2821–2822 peptide bond in procollagen VII are also similar to those found around the cleavage site in procollagens I, II, and III (16, 17), making this Ala-Asp bond a candidate cleavage site for procollagen VII C-proteinase.

Identification of the naturally cleaved peptide bond using protein chemical analysis such as NH₂-terminal sequencing of the cleavage product has not been possible because of the extremely low quantities of procollagen VII and collagen VII in cell cultures, and susceptibility of this protein to unspecific proteolytic degradation during extensive purification procedures. However, we present here genetic evidence that supports the assumption of the cleavage site within the first 53 amino acids of the NC-2 domain, probably at position 2821–2822. A deletion in the COL7A1 gene that causes in-frame skipping of exon 115 (see Fig. 1) results in lack of processing of procollagen VII. Exon 115 encodes amino acids 2814–2843 within the NC-2 domain. Within this sequence resides the putative cleavage site for the physiological procollagen C-proteinase, an Ala-Asp bond at position 2821–2822.

In vitro, the conversion of procollagen to collagen VII is very slow and also dependent on experimental conditions. Some conversion takes place in keratinocytes cultured on a matrix that has been produced by fibroblasts, but no conversion occurs in cultures on plastic with or without addition of dextran sulphate, a substance that has been successfully used to enhance processing of procollagen I or profibrillin I in fibroblast cultures (1, 29). Analogous very slow conversion has been observed for laminin 5 (kalinin), another structural component of the dermo-epidermal basement membrane zone that is synthesized as a high molecular weight precursor and processed before formation of suprastructures in vivo (26). Also laminin 5 processing takes place in skin organ cultures, but not in monolayers. A possible explanation for the efficient conversion of laminin 5 or procollagen VII in organ cultures is a high local concentration of the processing proteinases in the tissue.

The function of the NC-2 domain is not known. Interestingly, the amino acid sequence of the NC-2 domain contains a Kunitz-type proteinase inhibitor motif (12). Therefore, it would be tempting to speculate about a feedback inhibition exerted by the cleaved propeptide on the specific C-proteinase or other inhibitory activity towards tissue proteinases. A similar Kunitz-type inhibitor motif is found in the COOH-terminus of the $\alpha 3$ chain of collagen VI, which is also proteolytically processed before tissue deposition (27). However, a recombinant Kunitz domain of collagen VI $\alpha 3$ chain did not show proteinase inhibitor activity for several proteolytic enzymes (27).

Since it was postulated that the NC-2 domain mediates dimerization of procollagen VII before polymerization into anchoring fibrils, the postulate also predicted that mutations will affect processing of the collagen VII (25). Here

we present a heterozygous deletion mutation that affects this process in a patient with localized EBD. Since the proband is the only affected individual in the family, it remains to be seen if this mutation is recessive or dominant. Abnormal COOH-terminal processing of procollagen VII might be analogous to other hereditary collagen diseases, Ehlers-Danlos syndromes type VII A and B, in which NH₂-terminal processing of procollagen I is inhibited due to genetically altered amino acid sequence of the pro $\alpha 1$ (I) or pro $\alpha 2$ (I) chains (32). It was speculated that for sterical reasons the molecules containing pro α (I) chains would interfere with fibrillogenesis and cross-linking of collagen I. The anchoring fibrils in the skin of the present EBD proband were reduced in number and exhibited diffuse crossbanding, but not other gross morphological alterations. This ultrastructural finding is not specific, but could indicate defective lateral aggregation of collagen VII dimers. The exact molecular mechanisms leading to this ultrastructure remain an open question at present. The deletion of 29 amino acids and the retention of the NC-2 domain of procollagen VII could be involved in the pathogenesis of the present case through sterical hindrance of collagen VII fibrillogenesis, through interference and destabilization of dimer formation, or perhaps have no effect at the formation and stability of anchoring fibrils. Future characterization of the same or similar mutations in other families with more family members and affected individuals will help clarify the exact role of the NC-2 domain for the dimerization of procollagen VII and during fibrillogenesis and maturation of the anchoring fibrils.

The authors gratefully acknowledge the expert technical assistance of Ms. Margit Schubert and the generous support of Professor T. Luger, Chairman of the Department of Dermatology, University of Münster.

This work was supported by grants 31-30933.91 and 32-27165.89 from the Swiss National Science Foundation, grants Br 1475/1-1 and Br 1475/2-1 from the Deutsche Forschungsgemeinschaft, and a grant from the Roche Research Foundation. D. R. Zimmermann is supported by the Krebsliga des Kantons Zürich. D. U. Kalinke was a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

Received for publication 10 May 1995 and in revised form 4 July 1995.

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