Procollagen VII Self-Assembly Depends on Site-Specific Interactions and Is Promoted by Cleavage of the NC2 Domain with Procollagen C-Proteinase[†]

Morgana Colombo,[‡] Raymond J. Brittingham,[‡] John F. Klement,[‡] Ireneusz Majsterek,[‡] David E. Birk,[§] Jouni Uitto,[‡] and Andrzej Fertala*[,‡]

Department of Dermatology and Cutaneous Biology, Jefferson Medical College, and Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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ABSTRACT: Procollagen VII is a homotrimer of 350-kDa proα1(VII) chains. Each chain has a central collagenous domain flanked by a noncollagenous amino-terminal NC1 domain and a carboxy-terminal NC2 domain. After secretion from cells, procollagen VII molecules form antiparallel dimers with a 60 nm overlap. These dimers are stabilized by disulfide bonds formed between cysteines present in the NC2 domain and cysteines present in the triple-helical domain. Electron microscopy has provided direct evidence for the existence of collagen VII dimers, but the dynamic process of dimer formation is not well understood. In the present study, we tested the hypothesis that, during dimer formation, the NC2 domain of one procollagen VII molecule specifically recognizes and binds to the triple-helical region adjacent to Cys-2625 of another procollagen VII molecule. We also investigated the role of processing of the NC2 domain by the procollagen C-proteinase/BMP-1 in dimer assembly. We engineered mini mouse procollagen VII variants consisting of intact NC1 and NC2 domains and a shortened triple helix in which the C-terminal region encompassing Cys-2625 was either preserved or substituted with the region encompassing Cys-1448 derived from the N-terminal part of the triple-helical domain. The results indicate that procollagen VII self-assembly depends on site-specific interactions between the NC2 domain and the triple-helical region adjacent to Cys-2625 and that this process is promoted by the cleavage of the NC2 by procollagen C-proteinase/BMP1.

Procollagen VII is a homotrimer of proα1(VII) chains, each chain containing a large central triple-helical collagenous domain flanked by the amino-terminal NC1 domain of 140 kDa and the carboxy-terminal NC2 domain of 30 kDa (*1*). The overall structure and organization of these domains are highly conserved in mouse and human proteins (*1*−*3*). The triple-helical domain consists of -Gly-X-Y- repeats, in which the X position is frequently occupied by proline and Y by hydroxyproline (*1*, *4*, *5*). Unlike in fibrillar procollagens, the continuity of the -Gly-X-Y- repeats is interrupted by noncollagenous motifs, which are thought to facilitate the flexibility of the molecule (*1*, *4*−*6*). Procollagen VII self-assembles into highly organized anchoring fibrils, which extend from the lamina densa of the epidermal basement membrane into the underlying dermal connective tissue and take part in securing the epidermal−dermal adhesion (*7*, *8*). The importance of anchoring fibrils in stabilizing the cutaneous basement membrane zone is demonstrated by alterations of these fibrils as a result of mutations in a group of heritable blistering diseases, collectively known as the dystrophic forms of epidermolysis bullosa (DEB), which can be inherited in an autosomal dominant (DDEB) or an autosomal recessive (RDEB) pattern (*9*, *10*). Over 300 distinct mutations in COL7A1 have been identified, and these mutations have occurred within the entire length of the proα1(VII) polypeptide (*11*). Despite the growing number of known collagen VII mutations, the pathogenic pathways leading from the gene defect to dermal−epidermal tissue separation and blistering of the skin have remained elusive.

The process of a procollagen VII dimer formation is central for the formation of anchoring fibrils. It has been demonstrated that the dimers isolated from human skin overlap in an antiparallel fashion at their carboxy termini and that they are stabilized by a disulfide bond formed between Cys-2634[1] located in the triple-helical region close to the C-terminus and Cys-2802 or Cys-2804 located in the NC2 domain of the docking procollagen VII molecule (*4*, *6*, *12*). In support of the significance of the region adjoining Cys-2634 is the analysis of COL7A1 mutations in DEB, which shows a cluster of mutations localized in this region. In one example of RDEB, a Gly2576Arg substitution, in conjunction with a nonsense mutation at Glu2858X in the NC2 domain resulted in a moderately severe phenotype, and electron microscopy

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* Corresponding author. Tel: 215-503-0113. Fax: 215-503-5788. E-mail: Andrzej.Fertala@jefferson.edu.

[‡] Department of Dermatology and Cutaneous Biology, Thomas Jefferson University.

[§] Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University.

[1] The numbers indicate amino acid positions in the proα1(VII) chain where residue 1 is the methionine encoded by the translation initiation codon.

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analysis of the proband's skin revealed poorly formed anchoring fibrils (*13*). In a case of DDEB reported in a patient with a Gly2623Cys substitution, profound morphologic changes in anchoring fibrils were found (*14*). In other examples, Arg2610Ser, Arg2622Gln, Gly2653Arg, and Gly2674Arg substitutions resulted in mild DDEB (Database of DEBRA Molecular Diagnostics Laboratory, Jefferson Medical College, Dr. Ellen Pfender, personal communication). These data suggest that mutations in the region critical for dimer formation may change the structure of specific sites required for collagen VII/collagen VII interaction, thereby altering the kinetics of assembly or completely aborting dimer formation.

It has been demonstrated that the NC2 domain of procollagen VII is processed by procollagen C-proteinase/BMP-1[2] (PCP), an enzyme that also catalyzes the cleavage of C-terminal propeptides of procollagens I, II, and III (*15*−*18*). The role of processing the procollagen VII NC2 domain in formation of the anchoring fibrils, however, is not known. To study the role of specific regions of procollagen VII in the process of dimer formation, we created an experimental system consisting of mini variants of recombinant mouse procollagen VII in which the specific C-terminal region encompassing Cys-2625 was either preserved or substituted with the region encompassing Cys-1448 derived from the N-terminal part of the triple-helical domain.

We show that procollagen VII self-assembly depends on the interaction between an intact NC2 domain and a specific region of the triple-helical domain. We also show that formation of procollagen VII dimers is promoted by processing of the NC2 domain by PCP.

EXPERIMENTAL PROCEDURES

DNA Constructs Encoding Procollagen VII Mini Variants. The full-length mouse procollagen VII cDNA (FL-VII) was initially cloned into the pSoCMVIN vector (Roche Institute of Molecular Biology) (*19*). DNA constructs encoding the following mini procollagen VII variants were engineered: (i) a variant with a 1042 amino acid deletion conserving the C-terminal Cys-2625 but without the N-terminal Cys-1448 (described as Cys −N +C), (ii) a variant with a 1215 amino acid deletion conserving the N-terminal Cys-1448 but without the C-terminal Cys-2625 (described as Cys +N −C), and (iii) a variant with a 1234 amino acid deletion conserving neither cysteine (described as Cys −N −C) (Figures 1 and 2).

To engineer the DNA construct encoding Cys +N −C, the *Stu*I restriction enzyme was used to create an in-frame deletion in the FL-VII construct. To create the DNA constructs encoding the Cys −N +C and the Cys −N −C variants, the FL-VII was digested with the *Sfi*I restriction enzyme. Cleavage of the FL-VII occurred at nucleotide positions 4307 and 7430,[3] and single-stranded DNA overhangs were digested with the S1 nuclease (Invitrogen Corp.). In all constructs, a DNA region spanning the junction site was sequenced with the use of an internal sequencing primer (5′ggtgccagtgaacagggt3′).

[2] Abbreviations: BMP-1, bone morphogenetic protein 1; pN-collagen, procollagen in which C-terminal propeptide has been processed by procollagen C-proteinase.

[3] The numbers indicate nucleotide positions where nucleotide 1 is the "A" in the ATG starting codon of the mouse procollagen VII cDNA.

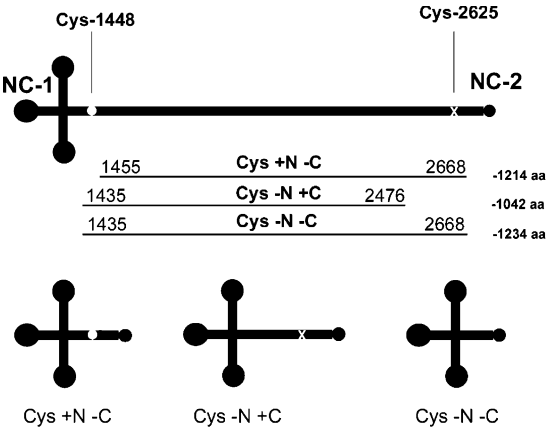

FIGURE 1: Schematic drawing of the mini procollagen VII variants developed to study self-assembly of procollagen VII. Upper panel: Schematic of full-length procollagen VII with the position of the Cys-1448 indicated by a white circle and the position of the Cys-2625 indicated by a white X. Note that both cysteines are located within the triple-helical domain. Middle panel: Regions deleted in the mini procollagen VII variants. The beginning and end of the deletions and the number of deleted amino acids (right) are indicated. Lower panel: Schematic structures of mini procollagen VII variants. Symbols: Cys +N −C, mini procollagen VII containing Cys-1448 but lacking Cys-2625; Cys −N +C, mini procollagen VII containing Cys-2625 but lacking Cys-1448; Cys −N −C, mini procollagen VII lacking both Cys-1448 and Cys-2625.

Transfection of Mammalian Cells and Selection of Positive Clones. Engineered DNA constructs cloned into the pcDNA3.1 vector (Invitrogen Corp.), which contains a G418 resistance gene and the cytomegalovirus promoter, were used to transfect human embryonal kidney cell line 293 (ATCC, CRL-1573) by use of the calcium phosphate precipitation method (ProFection, Promega). The G418-resistant clones were collected and analyzed independently for secretion of procollagen VII variants. In brief, the clones were grown in six-well cell culture plates. Upon reaching confluency, the cells were cultured for 24 h in serum-free medium supplemented with L-ascorbic acid phosphate magnesium salt *n*-hydrate (WAKO Inc., Japan) at a concentration of 40 μg/mL. Proteins secreted into the media were precipitated with 5% poly(ethylene glycol) (MW 8000; Sigma-Aldrich), and pellets were resuspended in 40 μL of 0.1 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 25 mM EDTA, and 0.02% NaN_3. Proteins were then separated in a 7.5% polyacrylamide gel. The presence of procollagen VII variants was determined by Western blot analysis with the use of monoclonal anti-NC1 antibody (LH7.2; Sigma-Aldrich) and secondary anti-mouse IgG conjugated to horseradish peroxidase. Bands corresponding to the procollagen VII variants were visualized by chemiluminescense (ECL kit; Amersham Biosciences, Inc.).

Purification of Procollagen VII Variants. For large-scale purification, 293 cells expressing procollagen VII variants were grown in serum-free medium supplemented with L-ascorbic acid phosphate magnesium salt *n*-hydrate (*20*, *21*). After 24 h, cell culture media were collected, filtered through a 1.6 μm glass fiber filter (Millipore) to remove cell debris, and supplemented with 5 mM EDTA and 1 mM *p*-aminobenzamidine. Culture media were collected for 7 days consecutively, and subsequently, proteins were concentrated by precipitation with 300 mg/mL ammonium sulfate. To

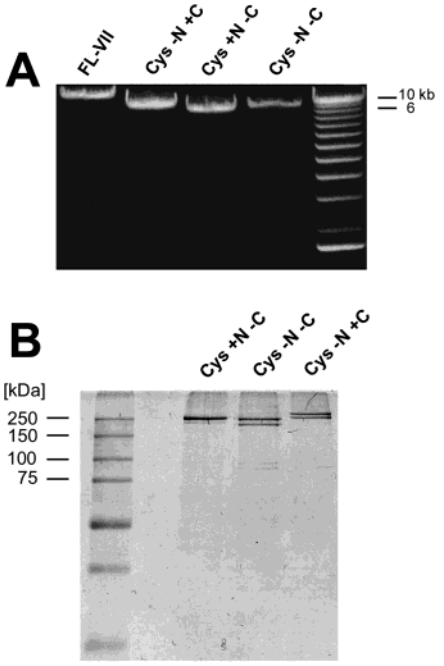

FIGURE 2: Analysis of mini procollagen VII variants. Panel A: Linearized DNA constructs consisting of truncated mouse procollagen VII cDNA cloned into mammalian expression vector pcDNA3.1. The sizes of the DNA constructs are as predicted. Nucleotide sequencing further confirmed that the sequence and orientation of the inserts in pcDNA 3.1 are correct. Symbols: FL-VII, full-length mouse procollagen VII cDNA; Cys −N +C, truncated mouse procollagen VII cDNA containing the sequence encoding Cys-2625 and the adjacent region but devoid of the sequence encoding Cys-1448 with adjacent region, which was deleted; Cys +N −C, truncated mouse procollagen VII cDNA containing the sequence encoding Cys-1448 and adjacent region and in which the sequence encoding Cys-2625 with adjacent region was deleted; Cys −N −C, truncated mouse procollagen VII cDNA in which the sequence encoding Cys-1448 and Cys-2625 with adjacent regions were deleted. The right lane shows DNA size markers. Panel B: Electrophoretic analysis of purified mini procollagen VII variants. The purified proteins were analyzed by polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue. Some of the procollagen variants migrated as double bands, a result of variation in posttranslational modifications (*20*). The left lane shows markers of molecular mass. Symbols are the same as in panel A.

purify procollagen VII variants, pellets were resuspended and dialyzed against a chromatography loading buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 1 mM *p*-aminobenzamidine). Following dialysis, insoluble material was removed by centrifugation at 10000 rpm for 30 min and discarded, and the supernatant was passed over a Q-Sepharose column (Amersham Biosciences, Inc.). Proteins bound to the column were eluted with a linear gradient ranging from 0.2 to 1.0 M NaCl. Fractions were analyzed by electrophoresis in a 7.5% polyacrylamide gel. Peak fractions were combined and concentrated 25-fold by ultrafiltration (YM-100; Millipore).

Biotinylation of Procollagen VII Variants. A portion of purified mini procollagens was biotinylated with use of sulfo-*N*-hydroxysuccinimidobiotin (EZ-Link Sulfo-NHS-biotin; Pierce) that at neutral pH specifically reacts with primary amines. To analyze biotinylated samples, proteins were separated in a 7.5% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane, blocked with 5% biotin-free nonfat dry milk (Bio-Rad) dissolved in 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.1% Tween-20 (TBST). Next, the membrane was incubated for 2 h at room temperature with avidin conjugated with horseradish peroxidase (Bio-Rad), and the proteins were visualized by chemiluminescence.

Digestion of Procollagen VII Variants with Bacterial Collagenase and PCP. For collagenase digestion (collagenase III; Sigma-Aldrich), 1 μg of purified protein was incubated with 1 unit of bacterial collagenase in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$ at 30 °C for 1 h. For cleavage with PCP, 1 μg of the procollagen VII variants in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$ was incubated at 37 °C with 0.3 unit of PCP isolated from chick embryo tendons. One unit of enzyme was defined as the amount of enzyme that processes 1 μg of procollagen VII in 1 h of incubation at 37 °C. Products of enzymatic digestion with bacterial collagenase and PCP were analyzed by polyacrylamide gel electrophoresis and by Western blotting with the use of anti-NC1 polyclonal antibodies (NC1AF7). The NC1AF7 antibodies were generated by Zymed Laboratories, Inc. (San Francisco, CA), using the synthetic peptide RGYRLEWRRESGLETPQ derived from the N-terminus of the NC1 domain of mouse procollagen VII as an antigen. The NC1AF7 antibody was also tested for its ability to interact with nondenatured procollagen VII variants. For this assay, 50 ng of mini procollagen VII variants was applied directly onto a nitrocellulose membrane, and proteins were visualized by chemiluminescense, as described earlier. In separate experiments, the specificity of the PCP was determined by analysis of the NC1 domain. For this assay, the PCP-processed procollagen VII variants were further digested with collagenase, and the integrity of the NC1 domain was analyzed by Western blot with the use of the NC1AF7 antibody.

Electron Microscopy of Procollagen VII Variants. For rotary shadowing, the recombinant procollagen VII variants were dissolved in 0.5 M acetic acid at a concentration of 50 μg/mL. Rotary shadowing was done as previously described (*22*). Photographs were taken at 30000× or 40000× magnification.

Procollagen VII/Procollagen VII Binding Assay. The following interactions were studied: (i) Cys +N −C/Cys +N −C, (ii) Cys −N +C/Cys −N +C, and (iii) Cys −N −C/Cys −N −C. To study these interactions, we designed an experimental system with the use of plastic-immobilized nonbiotinylated mini procollagen VII variants and nonimmobilized biotinylated mini procollagens. Wells of the eight-well plastic strips (Nalge Nunc) were coated with purified NC1AF7 antibodies at 37 °C for 2 h, followed by washing with PBS containing 0.05% Tween-20 (PBST). The nonspecific sites were blocked with 5% nonfat, biotin-free milk in PBST. Next, the wells were washed with PBST, followed by the addition of nonbiotinylated mini procollagen VII variants at a concentration of 10 μg/mL. The nonbiotinylated procollagens were allowed to saturate the antibody-coated wells for 18 h at 4 °C. Subsequently, the wells were washed with PBST, followed by a wash with 0.05 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.15 M NaCl.

Interactions between mini procollagen VII variants were studied in five experimental groups. In all groups, biotinylated samples in 0.05 M HEPES, pH 7.4, containing 0.15 M

NaCl and 10 mM $CaCl_2$ were used at a concentration of 20 μg/mL. In experiments involving the use of PCP, 0.3 unit of enzyme/μg of mini procollagen VII was used. In the first group, biotinylated mini procollagen variants were added to wells containing corresponding nonbiotinylated interactants. The samples were preincubated for 4 h at room temperature. Subsequently, PCP was added into the wells, and the samples were incubated at 30 °C for 18 h. In the second group, biotinylated mini procollagen variants were added to wells containing immobilized nonbiotinylated interactants, and at the same time without preincubation, PCP was added to the samples. The samples were then incubated for 18 h at 30 °C. The third group was similar to the second, but EDTA at a concentration of 15 mM was added at the beginning of incubation to inhibit the activity of the PCP. In the fourth group, immobilized nonbiotinylated procollagen variants were incubated with PCP for 4 h at 30 °C before the addition of the biotinylated interactants. Next, the biotinylated variants and a new aliquot of PCP were added, and incubation was continued for 18 h at 30 °C. In the fifth group, the immobilized nonbiotinylated mini procollagens were preincubated for 4 h with *N*-ethylmaleimide (NEM), a compound that binds to free sulfhydryl groups of cysteines but, unlike dithiothreitol or β-mercaptoethanol, does not significantly inhibit the activity of PCP (*16*). After preincubation with NEM the biotinylated interactants and PCP were added to the well, and the samples were incubated for 18 h at 30 °C.

After incubation, the excess of nonbound biotinylated procollagens was washed out with high-stringency PBST, in which the NaCl concentration was increased to 0.5 M. Next, the samples were incubated for 2 h at room temperature with streptavidin conjugated with alkaline phosphatase (ImmunoPure AP-streptavidin; Pierce) diluted 1:20000 in PBST. Subsequently, wells were washed extensively with PBST, followed by three washes with 0.05 M carbonate−bicarbonate buffer (pH 9.6). To detect the nonbiotinylated collagen VII/biotinylated collagen VII/AP-streptavidin complex, 3 mM *p*-nitrophenyl phosphate in carbonate−bicarbonate buffer was used as a substrate for alkaline phosphatase. Relative amounts of bound biotinylated procollagens were determined colorimetrically at 405 nm with the use of a plate reader (Benchmark, Bio-Rad). Because the amount of biotin bound to procollagen VII variants is a function of their molecular mass, data were corrected to account for these differences. The following controls were used: (i) samples prepared in a similar way but without biotinylated procollagen VII variants, (ii) samples with biotinylated variants but without AP-streptavidin, (iii) wells coated only with the NC1AF7 antibody or 5% milk, and (iv) wells coated with rabbit IgG (Sigma-Aldrich). To analyze whether the amount of different procollagen variants bound to the wells coated with the NC1AF7 antibody was the same in all analyzed samples, we employed biotinylated proteins. The amount of these proteins bound to the wells was determined colorimetrically with use of AP-streptavidin. In all experiments, data were collected from three independent experiments and evaluated by employing a computer program (GraphPad Prizm program; GraphPad Software Inc.).

Computer Analysis of the Regions Adjacent to Cys-1448 and Cys-2625. To determine the physicochemical properties of the regions surrounding Cys-1448 and Cys-2625, computer analysis of these regions was performed by employing a modeling program (Sybyl 6.6; Tripos, Inc., St. Louis, MO) installed on an Octane computer station (Silicon Graphics, Inc.). The Gly-1431/Gly-1466 and the Gly-2608/Pro-2643 regions spanning the Cys-1448 or Cys-2625, respectively, were analyzed. The models of collagen VII fragments were generated as described earlier for modeling of the collagen II triple helix (*23*, *24*). The electron density surface, which represents an isosurface of electron densities, was calculated (*25*). The electrostatic potential (EP; expressed as kilocalories per mole per electron) and lipophilicity potential (LP) of the surfaces were then evaluated.

Table 1: Amino Acid Sequence of Sites Created as a Result of the In-Frame Deletions in Mouse Procollagen VII cDNA

procollagen VII variant	amino acid sequence at junction site
Cys +N −C	EDGGPGL/IGP
Cys −N −C	SPGPQG/LIGP
Cys −N +C	PGPQG/PGQEG

RESULTS

Development and Expression of DNA Constructs for Mini Procollagen VII Variants. In-frame deletions were created in the FL-VII construct to express mini procollagen VII variants containing Cys-1448 but lacking Cys-2625 (Cys +N −C), containing Cys-2625 but lacking Cys-1448 (Cys −N +C), or lacking both Cys-1448 and Cys-2625 (Cys −N −C) (Figures 1 and 2). Protein sequences of the junction regions in procollagen VII variants are presented in Table 1.

Differences in the electrophoretic mobility of recombinant procollagen VII variants (Figure 2) corresponded to the differences between their predicted molecular masses of 195, 193, and 211 kDa for Cys +N −C, Cys −N −C, and Cys −N +C, respectively. Some proteins migrated as two bands (Figure 2), a phenomenon previously reported in other recombinant procollagen VII variants expressed in 293 cells and attributed to differences in posttranslational modifications (*20*). As expected, biotin-specific signals were absent in nonbiotinylated mini procollagen VII variants, and the method used to identify biotinylated samples enabled us to detect less than 5 ng of protein (Figure 3). As determined by densitometry of bands representing equimolar amounts of procollagen VII variants, the degree of biotinylation per α chain was the same in all analyzed samples.

Analysis of the Procollagen VII Collagenous Domain by Digestion with Bacterial Collagenase. The sensitivity of mini procollagens to digestion with bacterial collagenase indicated the presence of a collagenous domain in these proteins, as evidenced by a shift in the electrophoretic mobility of processed samples and by the presence of a 140 kDa band corresponding to the collagenase-resistant NC1 domain (Figure 4). In addition, Western blot analysis of the collagenase-digested Cys +N −C variant with the use of the NC1-specific NC1AF7 antibody confirmed the presence of the intact NC1 domain (Figure 4). Similar results were obtained with Cys −N +C and Cys −N −C variants (not shown).

Cleavage of Procollagen VII Variants with PCP. Electrophoresis of the products of the enzymatic cleavage of procollagen VII variants with PCP demonstrated that their molecular mass was consistent with the prediction that the cleavage occurs only at the single cleavage site present in the NC2 domain (Figure 4). Using the NC1-specific NC1AF7

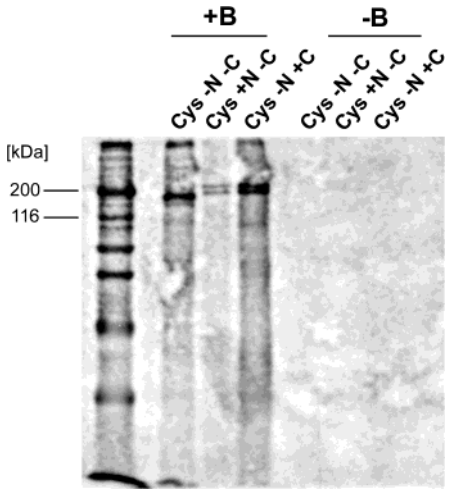

FIGURE 3: Analysis of biotinylated mini procollagen VII variants. Purified proteins were biotinylated at amine groups using sulfo-*N*-hydroxysuccinimidobiotin. The biotinylated proteins were electrophoresed in a 7.5% polyacrylamide gel, transferred onto a nitrocellulose filter, and then detected by avidin conjugated with horseradish peroxidase. Note that only biotinylated proteins are detectable by this method while nonbiotinylated proteins are not. Symbols: +B, biotinylated proteins; −B, nonbiotinylated proteins. The other symbols are the same as in Figure 1.

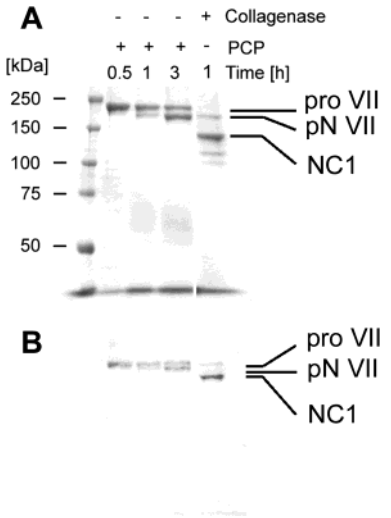

FIGURE 4: Analysis of the products of the cleavage of mini procollagen VII with PCP and with bacterial collagenase. The recombinant protein Cys +N −C was digested with bacterial collagenase for 1 h at 30 °C in 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 10 mM $CaCl_2$. For cleavage with PCP, Cys +N −C was incubated at 37 °C with 0.3 unit/μg procollagen in the same buffer. At the time intervals indicated, sample aliquots were withdrawn and analyzed by polyacrylamide gel electrophoresis. Panel A: Polyacrylamide gel in which protein bands were visualized by staining with Coomassie Blue. Panel B: Products of the cleavage of the Cys +N −C with PCP and bacterial collagenase were analyzed by Western blot with the use of the anti-NC1 polyclonal antibody and a secondary antibody conjugated with horseradish peroxidase. Protein bands were visualized by chemiluminescense. The order of samples in the upper and lower panels is the same. Symbols: pro VII, intact Cys +N −C mini procollagen VII; pN VII, product of the cleavage of Cys +N −C with PCP; NC1, collagenase-resistant N-terminal noncollagenous domain of procollagen VII.

antibodies, Western blot analysis of the product of the cleavage of Cys +N −C with PCP confirmed the presence of the intact NC1 domain in the pN-collagen VII, which further demonstrates specific activity of PCP at a site present in the NC2 domain (Figure 4). The specific activity of PCP was also determined by digestion of the PCP-processed procollagen VII variants with bacterial collagenase. In this experiment collagenase digestion generated the collagenase-resistant NC1 domain, which had the same electrophoretic mobility (not shown) as the NC1 domain seen in Figure 4 and derived by collagenase digestion of the procollagen VII variant not subjected to the PCP cleavage. Similar results were obtained with Cys −N +C and Cys −N −C (not shown).

Electron Microscopy of Mini Procollagen VII Variants. Mini procollagen VII variants were correctly folded, as evidenced by the presence of three subunits of the NC1 domain and extended triple-helical region flanked with a C-terminal globular NC2 domain (Figure 5). Contour lengths of the triple-helical domains of the Cys +N −C, Cys −N −C, and Cys −N +C were 80.0 nm (±1.6 SEM, $n = 16$), 72.5 nm (±4.6 SEM, $n = 9$), and 132.5 nm (±13.5 SEM, $n = 4$), respectively. In comparison, the length of intact procollagen VII is 424 nm (*26*). As expected, the differences in length between analyzed mini procollagens reflect differences in size of deletions generated during the engineering of DNA constructs (see Figure 1). Of note is the observation that all variants existed as nonaggregated monomers.

Formation of Mini Procollagen VII Complexes. We studied the assembly of immobilized nonbiotinylated mini procollagen VII variants with nonimmobilized, biotinylated mini procollagen VII variants in the presence of PCP. Because the procollagen VII variants were immobilized through the interaction of their NC1 domains with the NC1-specific antibodies bound to a plastic surface, the spatial orientation and amount of the immobilized procollagens were uniform in all analyzed samples.

To study the procollagen VII/procollagen VII interaction in detail, five sets of experiments were planned. In the first set, the biotinylated samples were preincubated with the immobilized nonbiotinylated variants, and the procollagens were cleaved by PCP. The Cys −N +C variant formed the most stable assemblies (Figure 6). Similar results were obtained with the second set of experiments, in which slow cleavage of mini procollagens with PCP was started without a preincubation phase. As determined by Student's *t*-test, in the first and the second group the differences between the Cys −N −C or the Cys +N −C variant and the Cys −N +C variant were statistically significant. In the third set of experiments, the enzymatic activity of PCP was inhibited by use of EDTA. In this case, the amount of biotinylated procollagen VII bound to a surface was low in all analyzed samples. To study the role of the intact NC2 domain in the docking of procollagen VII molecules, in the fourth set of experiments prior to adding the biotinylated counterparts the immobilized nonbiotinylated procollagen VII variants were processed by PCP. As indicated in Figure 6, in this experiment all procollagen VII variants failed to form stable assemblies. To determine whether sulfhydryl groups were involved in the stabilization of collagen VII complexes, NEM was used to block these groups present in mini procollagen VII variants. Blocking of the sulfhydryl groups caused a significant reduction in stable complexes formed from nonbiotinylated and biotinylated Cys −N +C procollagen variants (Figure 6). As in other experiments, the amount of detected biotinylated Cys +N −C and Cys −N −C was

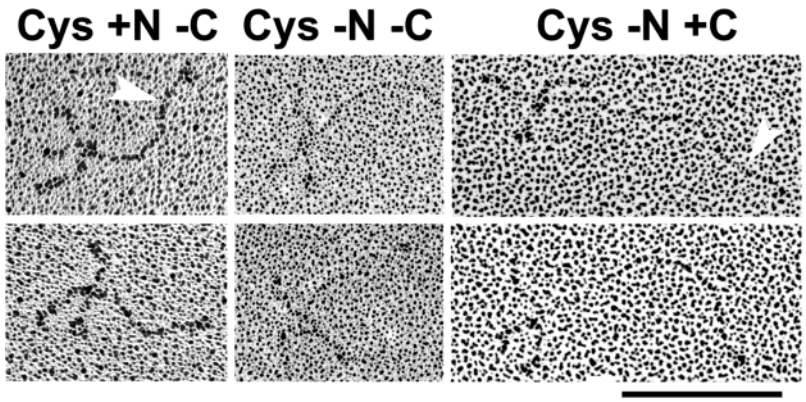

FIGURE 5: Transmission electron microscopy of rotary-shadowed mini procollagen VII variants. Contour lengths of the triple-helical domains of the Cys +N −C, Cys −N −C, and Cys −N +C were 80.0 nm (±1.6 SEM, $n = 16$), 72.5 nm (±4.6 SEM, $n = 9$), and 132.5 nm (±13.5 SEM, $n = 4$), respectively. The differences in length among the analyzed mini procollagens reflect differences in the size of deletions generated during engineering of DNA constructs (see Figure 1). All variants were present as nonaggregated monomers. White arrows indicate an approximate position of cysteines present in the triple-helical domains of the engineered procollagen VII variants. Multidomained NC1 domain and globular NC2 domains are apparent. Symbols are the same as in Figure 1. Bar = 100 nm.

significantly lower and was comparable with background that most likely resulted from the nonspecific adhesion of AP-streptavidin.

Computer Analysis of the Regions of Collagen VII Adjacent to Cys-1448 and Cys-2625. Using a computer modeling program we analyzed physicochemical characteristics of the regions of procollagen VII encompassing Cys-1448 and Cys-2625. The results shown in Figure 7 suggest that the positions of cysteines in the collagen triple helix allow for the formation of an intramolecular disulfide bond and that the third cysteine may take part in the formation of a dimer. The analysis of surface properties (Figure 8) of these regions demonstrated significant differences in the EP. The region encompassing Cys-1448 was characterized by EP ranging from −418 to +41 kcal mol^{-1} e^{-1}. In contrast, the region encompassing Cys-2625 had significantly higher EP, ranging from −108 to +283 kcal mol^{-1} e^{-1}. There was no significant difference in the LP of the analyzed regions, and the values for the fragment encompassing Cys-1448 ranged from −0.05 to −0.09 and for the region surrounding Cys-2626 from −0.07 to −0.1.

DISCUSSION

To study the role of specific domains of procollagen VII in the process of self-assembly, we engineered mini mouse procollagen VII variants containing intact NC1 and NC2 domains and the triple-helical domain in which the C-terminal region encompassing Cys-2625 was either preserved or substituted with the region adjoining Cys-1448 derived from the N-terminal part of the triple-helical domain. These mini procollagens were employed in self-assembly assays with the use of biotinylated procollagen VII counterparts. Our results suggest that self-assembly of procollagen VII consists of two major steps. As demonstrated in experiments in which immobilized procollagen variants were preincubated with PCP that cleaves the NC2 domain, the number of stable aggregates formed with biotinylated molecules was low. Therefore, we propose that the first step of procollagen VII self-assembly depends on the presence of the intact NC2 domain. We determined that the amount of collagen VII aggregates was very low in all experiments that used the Cys +N −C variant, in which the Cys-2625 and the adjacent C-terminal region of the triple helix were substituted with the nonspecific region containing Cys-1448. Thus, we also propose that the docking of two procollagen VII monomers not only requires intact NC2 but also depends on the interaction of this domain with the region encompassing the Cys-2625. It is not apparent why the Cys +N −C failed to form stable aggregates, but we demonstrated that the physicochemical properties of the region adjacent to Cys-1448 differ from those of the region adjacent to Cys-2625. The EP of the region encompassing the Cys-2625 is highly positive, while a strongly negative EP characterizes the region adjacent to the Cys-1448. This difference is most likely responsible for the inability of the Cys +N −C molecules to specifically interact with a NC2 domain and form stable dimers. In support of this hypothesis is the fact that the NC2 domain, which has structural similarities to domains found in Kunitz-type serine protease inhibitors and in the C-terminal globular domain of the collagen VI α3 chain, is very acidic with a p*I* of about 4.3 (*27*−*29*). The strongly negative charge of the NC2 domain may be critical for its site-specific electrostatic interaction with the region adjoining Cys-2625 and characterized by high electrostatic potential.

Since procollagen VII is a homotrimer, it contains three cysteines at amino acid positions 1448 and 2625. Only two collagen VII monomers, however, form a disulfide-stabilized complex, which suggests that the other two cysteines could be engaged in an intramolecular bond formation. Molecular modeling of the regions adjacent to Cys-1448 and Cys-2625 demonstrated that formation of intramolecular disulfide bonds is possible in both regions (see Figure 7). Hence, lack of the ability of the Cys +N −C to form stable assemblies cannot be attributed to the steric hindrance of the free sulfhydryl group of the Cys-1448.

The significance of the site-specific interactions in self-assembly of homotypic fibrillar collagens was suggested by Prockop and Fertala (*30*), who determined that telopeptides of one collagen I molecule bind to a narrow region of another collagen monomer during the initial stages of fibril assembly and that this interaction is critical for correct alignment of collagen monomers. Site-specific interactions also regulate the orientation of collagen monomers in a fibril. For example,

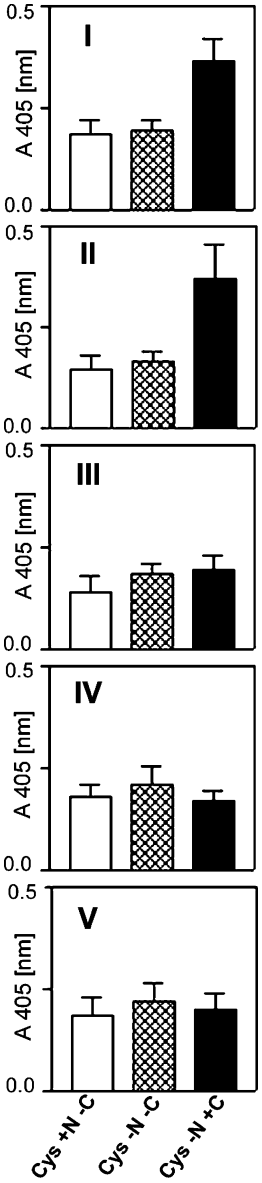

FIGURE 6: Formation of stable procollagen VII complexes. Nonbiotinylated mini procollagen VII variants were immobilized by binding of the NC1 domain to the plastic-bound anti-NC1 polyclonal antibodies, and consequently, biotinylated procollagen VII variants were added to the wells. Procollagens were allowed to interact, and the biotinylated procollagens were detected in colorimetric assays with the use of AP-streptavidin. The following experimental groups were designed: (I) Biotinylated mini procollagen variants were added to wells containing corresponding nonbiotinylated interactants. The samples were preincubated for 4 h at room temperature. Next, PCP was added into wells, and the samples were incubated at 18 h at 30 °C. (II) Biotinylated mini procollagen variants were added to wells containing immobilized nonbiotinylated interactants, and at the same time, without preincubation, PCP was added to the samples. The samples were incubated for 18 h at 30 °C. (III) The group is similar to the second one (II) except that EDTA at a concentration of 15 mM was added at the beginning of incubation to inhibit the activity of PCP. (IV) Immobilized nonbiotinylated procollagen variants were incubated with PCP for 4 h at 30 °C before the addition of the biotinylated interactants. Next, the biotinylated variants and PCP were added, and incubation was continued for 18 h at 30 °C. (V) Immobilized nonbiotinylated mini procollagens were preincubated for 4 h with *N*-ethylmaleimide (NEM), a compound that binds to free sulfhydryl groups of cysteines but does not significantly inhibit the activity of PCP (*16*). After preincubation with NEM, the biotinylated interactants and PCP were added to the well, and the samples were incubated for 18 h at 30 °C. The values are means derived from three independent experiments (±SEM). As determined by Student's *t*-test, in binding experiments I and II, the differences between Cys −N +C and Cys +N −C ($p = 0.04$ and $p = 0.02$, respectively) and between Cys −N +C and Cys −N −C ($p = 0.03$ and $p = 0.03$, respectively) are statistically significant.

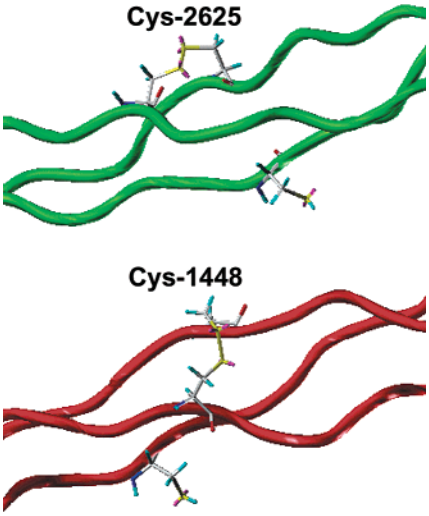

FIGURE 7: Molecular models of the triple-helical regions encompassing Cys-2625 and Cys-1448 residues. Energy-minimized models show the probable structure of the intramolecular disulfide bonds present in the Cys −N +C (upper panel) and the Cys +N −C (lower panel) procollagen VII variants.

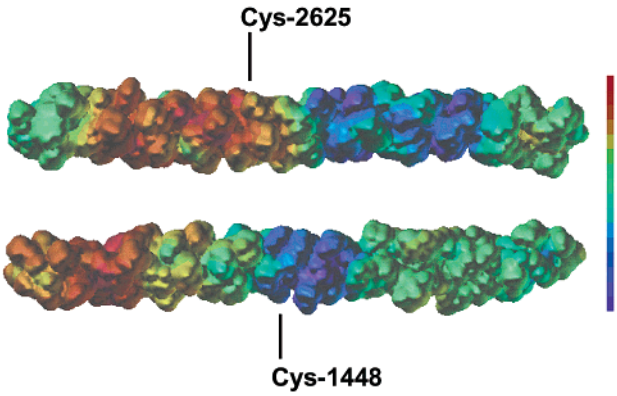

FIGURE 8: Electrostatic potential (EP) of the surfaces of the mini procollagen VII variants containing Cys-2625 and Cys-1448. Electron density surfaces of about 7000 Å^2 were generated using a computer modeling program, and the EP of the analyzed fragments was calculated. The EP of the depicted fragments ranged from −108 to +283 kcal mol^{-1} e^{-1} and from −418 to +41 kcal mol^{-1} e^{-1} for the regions encompassing Cys-2625 and Cys-1448, respectively.The color code panel at the right indicates differences in the EP: red, highly positive EP; blue, highly negative EP. Positions of cysteines in the computer model are indicated.

in collagen II fibrils formed de novo, monomers are pointed with their C-termini toward the tips of fibrils (*31*). The fact that the collagen VII monomers point toward the ends of a dimer with their N-termini is yet another example of site-specific interactions controlling the spatial organization of collagenous proteins.

The importance of the site-specific interactions in the assembly of collagens is best illustrated by observations that some of the mutations in collagen genes alter the structure and function of connective tissues. For example, it has been demonstrated that mutations in a collagen I site critical for specific binding with telopeptides of other collagen molecules lead to formation of abnormal fibrils (*32*). Similarly, some of mutations in COL7A1 could alter the formation of the collagen VII/collagen VII dimer, leading to profound changes in the structure of the dermal−epidermal junction.

We also demonstrated that stable aggregates were formed only when enzymatic activity of PCP was not blocked with

EDTA and when sulfhydryl groups of cysteines were not blocked with NEM. We propose, therefore, that the second step in the formation of anchoring fibrils is stabilization of the dimers by formation of disulfide bonds and that this step is promoted by enzymatic processing of the NC2 domain by PCP. The role of PCP in self-assembly of fibrillar procollagens has been well established (*18*, *33*, *34*). It has been demonstrated that cleavage of the C-propeptides of procollagen I promotes aggregation of collagen during fibril formation by decreasing the solubility of monomers containing C-propeptides from about 1000 to about 1 μg/mL (*35*) and by eliminating steric constraints imposed by bulky globular domains (*35*, *36*). As demonstrated by Hojima et al. (*37*), the activity of PCP is strongly enhanced when C-propeptides are close to each other in procollagen aggregates. This observation may suggest that the processing of the NC2 domains in vivo could serve as a checkpoint for correct alignment of procollagen VII molecules whose NC2 domains in a dimer and in the assemblies of dimers are close to each other. Cleavage of procollagen VII by PCP removes the negatively charged Kunitz-type serine protease inhibitor-like region of the NC2 domain (*15*). Since recognition of procollagen VII molecules most likely depends on electrostatic interaction, enzymatic removal of this domain in the experimental group in which NC2 domain was processed by PCP prior to adding biotinylated counterparts could explain the lack of formation of stable assemblies (see Figure 6). Because PCP is critical for the formation of stable collagenVII/collagen VII assemblies and, as demonstrated earlier, for the processing of laminin 5 (*38*, *39*), this enzyme and other enzymes with similar specific activities (*15*) may be key regulators in the assembly of the anchoring complex.

Our results support previous implications that Cys-2634 in human procollagen VII, which is homologous to the Cys-2625 in mouse protein, is critical for stabilization of collagen VII dimers (*4*). The results, however, contradict the findings by Chen et al. (*40*), who demonstrated that mutating the Cys-2634 in a construct consisting of the NC2 domain and 186 amino acids of the adjacent triple-helical domain does not prevent formation of the disulfide-stabilized dimers in the cell culture. Due to the absence of the Cys-2634 residue in such a mutant, however, any dimer formed could only be held together by disulfide bonds formed between cysteines present in the NC2 domains. Such a dimer would not form a typical 60 nm overlap observed in native collagen VII; therefore, it could preclude normal assembly of anchoring fibrils.

In conclusion, our results demonstrate that the assembly of anchoring fibrils is a complex process dependent on an interaction of the NC2 domain with a specific region of the triple-helical domain and that this process is promoted by enzymatic cleavage of the NC2 domain catalyzed by PCP. Moreover, our results provide a basis for understanding the consequences of the mutations in the procollagen VII gene that may alter collagen VII self-assembly and contribute to the structural weakness of the dermal−epidermal junction observed in dystrophic forms of epidermolysis bullosa.

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