Single Amino Acid Substitutions in Procollagen VII Affect Early Stages of Assembly of Anchoring Fibrils*

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Procollagen VII is a homotrimer of 350-kDa pro-
α1(VII) chains, each consisting of a central collagenous
domain flanked by the noncollagenous N-terminal NC1
domain and the C-terminal NC2 domain. After secretion
from cells, procollagen VII molecules form anti-parallel
dimers with a C-terminal 60-nm overlap. Characteristic
alignment of procollagen VII monomers forming a dimer
depends on site-specific binding between the NC2 do-
main and the triple-helical region adjacent to Cys-2634
of the interacting procollagen VII molecules. Formation
of the intermolecular disulfide bonds between Cys-2634
and either Cys-2802 or Cys-2804 is promoted by the
cleavage of the NC2 domain by procollagen C-protein-
ase. By employing recombinant procollagen VII variants
harboring G2575R, R2622Q, or G2623C substitutions
previously disclosed in patients with dystrophic epider-
molysis bullosa, we studied how these amino acid sub-
stitutions affect intermolecular interactions. Binding
assays utilizing an optical biosensor demonstrated that
the G2575R substitution increased affinity between mu-
tant molecules. In contrast, homotypic binding between
the R2622Q or G2623C molecules was not detected. In
addition, kinetics of heterotypic binding of all analyzed
mutants to wild type collagen VII were different from
those for binding between wild type molecules. More-
over, solid-state binding assays demonstrated that
R2622Q and G2623C substitutions prevent formation of
stable assemblies of procollagen C-proteinase-processed
mutants. These results indicate that single amino acid
substitutions in procollagen VII alter its self-assembly
and provide a basis for understanding the pathomecha-
nisms leading from mutations in the COL7A1 gene to
fragility of the dermal-epidermal junction seen in pa-
tients with dystrophic forms of epidermolysis bullosa.

Adhesion of the epidermis to the underlying dermis is a
requirement for the integrity of skin. The dermal-epidermal
junction consists of a number of interconnected macromole-
cules that form several attachment complexes critical for epi-
dermal/dermal adhesion (1). Hemidesmosomes, located within
the cutaneous basement membrane zone on the epidermal side,
consist of the intracellular proteins, plectin (2–4) and the 230-
kdA bullous pemphigoid antigen (5–7), as well as transmem-
brane proteins, the 180-kdA bullous pemphigoid antigen also
known as collagen XVII (8, 9), and the α6β4 integrin (10, 11).
Extending from the hemidesmosomes are anchoring filaments
that bridge the hemidesmosomes with the basement mem-
brane lamina densa. The anchoring filaments are composed
primarily of the extracellular domains of the collagen XVII and
lamins 5, 6, and 7 (12). Contiguous with the anchoring fila-
ments beneath the lamina densa are centrosymmetrically
cross-striated fibers, the anchoring fibrils, composed of collagen
VII (13). The anchoring fibrils form an extended network that
interacts with fibrous elements within the papillary dermis
and secure the association of the lower part of lamina densa to
the underlying mesenchyme (14).

Procollagen VII is a homotrimer consisting of an extended
central triple-helical collagenous domain flanked by the N-termi-
nal NC1 domain of 140 kDa and the C-terminal NC2 domain of
30 kDa (15). The triple-helical domain consists of -Gly-X-Y-
repeats, in which the X position is frequently occupied by proline
and the Y position by hydroxyproline (15–17). The contribution
of procollagen VII to the mechanical stability of the dermal-
epidermal junction depends on the ability of single molecules to
self-assemble into highly ordered anchoring fibrils. The self-as-
cmple of collagen VII includes an enzymatic cleavage of procoll-
gen to collagen, and it depends on the binding between specific
regions of the interacting molecules (18–20).

During the initial stages of anchoring fibril assembly, pro-
collagen VII molecules form dimers that overlap in an antipa-
rallel fashion at their C termini, and the dimers are stabilized
by a disulfide bond formed between Cys-2634,1 located in the
triple-helical region close to the C terminus, and either Cys-
2802 or Cys-2804, located in the NC2 domain of the docking
procollagen VII molecule (16, 21, 22). By employing genetically
modified recombinant procollagen VII variants, we have dem-
strated previously that formation of collagen VII-anchoring
VII dimers depends on the binding of an intact NC2 domain
with the region encompassing Cys-2634, and we postulated
that this binding depends on an electrostatic interaction (16).
We have also postulated that stabilization of the dimer by
formation of disulfide bonds is initiated by the enzymatic cleav-
age of the NC2 domain with procollagen C-proteinase (PCP),2
also known as bone morphogenetic protein 1 (PCP/BMP-1) (see
Fig. 1) (18).

The importance of a correct structure of the anchoring fibrils

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1 The numbers indicate amino acid positions in the pro-α1(VII) chain where residue 1 is the methionine encoded by the translation
initiation codon.

2 The abbreviations used are: PCP, procollagen C-proteinase; DEB,
dystrophic form of epidermolysis bullosa; DDEB, dominant DEB;
RDEB, recessive DEB; PBS, phosphate-buffered saline; BSA, bovine
serum albumin; WT, wild type; AP, alkaline phosphatase.
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For the integrity of the cutaneous basement membrane zone is demonstrated by the occurrence of pathological changes within the dermal-epidermal junction resulting from mutations in the COL7A1 gene encoding human procollagen VII. Over 300 distinct mutations in COL7A1 have been identified 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 (23, 24).

Even though the mutations have occurred within the entire length of the procollagen VII α1 chain (25), some are grouped in the region adjoining Cys-2634. For example, in a case of RDEB described by Shimizu et al. (26), a G2575R substitution, in conjunction with a nonsense mutation E2857X in the NC2 domain, resulted in a moderately severe phenotype characterized by poorly organized anchoring fibrils. In contrast, the G2623C substitution identified in a patient with DDEB led to significant changes in morphology of anchoring fibrils (27).

To study how the mutations occurring in the region of procollagen VII encompassing Cys-2634 influence intermolecular interactions, we employed recombinant mini-procollagen VII (mProVII) variants in which mutations found in DEB patients were introduced. The G2575R, R2622Q, and G2623C mProVII mutants were engineered and employed in binding studies by using an optical biosensor. Results indicate that mutations in the region encompassing Cys-2634 change the structure and physicochemical properties of sites critical for the collagen VII-collagen VII interaction, thereby altering the kinetics of assembly or completely precluding dimer formation.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs Encoding mProVII Mutants—**Procollagen VII mutants were engineered by introducing single base substitutions into cDNA encoding mini mouse procollagen VII (mProVII), as described in detail by Colombo et al. (18) (Fig. 2). Deleting the central region of procollagen VII enabled site-directed mutagenesis, which was not possible to achieve with the use of the full-length cDNA, which is characterized by a high content of cytosine and guanine, a feature that makes gene engineering difficult. The mutations in codons corresponding to those encoding amino acid residues 2575, 2622, and 2623 of the human procollagen VII were generated using the QuickChange™ site-directed mutagenesis kit (Stratagene). The G2575R mutant was created by changing the -GGC- codon for glycine to the -TGC- codon for arginine. The R2622Q mutant was created by changing the -AGG- codon for arginine to the -CAG- codon for glutamine. The G2623C mutant was created by changing the -AGC- codon for glycine to the -TGC- codon for cysteine. Fidelity of mutation sites in DNA constructs was verified by nucleotide sequencing.

**Transfection of Mammalian Cells and Selection of mProVII-positive Clones—**DNA constructs cloned into the pcDNA3.1 vector (Invitrogen), which contains a G418 resistance gene and the cytomegalovirus promoter, were used to transfect human embryonic kidney cell line 293 (ATCC, CRL-1573) by use of the calcium phosphate precipitation method (ProFect™, Promega). The G418-resistant clones were collected and analyzed independently for secretion of the mProVII mutants, as described by Colombo et al. (18). In brief, the clones were grown in 6-well cell culture plates, and upon reaching confluence, the cells were cultured for 24 h in serum-free medium supplemented with l-aspartic 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% polyethylene glycol (M, 8000; Sigma), and pellets were resuspended in 40 μl of 0.1 M Tris-HCl, pH 7.4, containing 0.4 mM NaCl, 25 mM EDTA, and 0.02% NaN₃. Proteins were then analyzed by electrophoresis in 7.5% polyacrylamide gels. The presence of mProVII variants in the analyzed samples was determined by Western blot analysis with the polyclonal anti-NC1 antibody (NC1AF7) and second-ary anti-rabbit IgG conjugated to horseradish peroxidase (18). Bands corresponding to the procollagen VII α1 chains were visualized by chemiluminescence (ECL kit; Amersham Biosciences).

**Purification of mProVII Variants—**Cells expressing mProVII mutants were grown in serum-free medium supplemented with l-aspartic acid phosphate magnesium salt n-hydrate. 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-aminobenzamide. On average, 3 liters of culture media were collected for 7 consecutive days. Subsequently, proteins were concentrated by precipitation with 300 mg/ml of ammonium sulfate. mProVII mutants were purified by ion exchange chromatography as described (18, 28, 29). In brief, the precipitate was resuspended and dialyzed against chromatography loading buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 1 mM p-aminobenzamide). Following dialysis, insoluble material was removed by centrifugation at 10,000 rpm for 30 min and discarded, and the supernatant was passed over a Q-Sepharose column (Amersham Biosciences). 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.
To separate low molecular weight proteins collected from the Q-Sepharose column in some preparations, the concentrated samples were further purified by size exclusion high pressure liquid chromatography. The samples were run at 1 ml/min through a size exclusion column (G3000SW, 2.15 $\times$ 60 cm, Tosohas, Japan) connected to a high pressure liquid chromatography system equipped with a UV detector and fraction collector (Pro Star$^\text{TM}$, Varian, Inc.). Peak fractions containing purified mutant mProVII were collected and analyzed by PAGE.

**Transmission Electron Microscopy of mProVII Molecules**—For rotary shadowing, the recombinant mProVII mutants were dissolved in 0.5 M acetic acid at a concentration of 50 $\mu$g/ml. Rotary shadowing was done as described previously (18, 30). Procollagen molecules were examined with the Hitachi 7000 transmission electron microscope (Hitachi, Inc.). Photographs were taken at magnification $\times 30,000$ or $\times 40,000$.

**Biosensor Assays of Kinetics of Homotypic and Heterotypic Binding between mProVII Variants**—Intermolecular interaction assays were carried out using an optical biosensor (Iasys; Affinity Sensors, UK).

To bind covalently purified mProVII variants, described as acceptors, onto the surface of a sensor, carboxyylate groups present on the surface were activated by injection of a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.4 M N-ethyl-3-(dimethylaminopropyl)carbodiimide (Pierce). Recombinant mProVII at concentration of 100 $\mu$g/ml dissolved in PBS was added to biotinylated mProVII molecules; (iv) between G2623C mutant molecules; and (v) between WTm-ProVII and each of the G25275R, R2622Q, or G2623G mutant molecules.

Cuvettes with immobilized collagens were primed with PBST at 25 °C for 15 min. A 200-$\mu$l sample containing free mProVII dissolved in PBST was added to the cuvette, and then the association phase was recorded. Subsequently, the sample was removed; analyte-free PBST was added to the cuvette, and the dissociation phase was recorded. After each assay, the surface of the cuvette was regenerated by washing with 10 mM HCl, followed by equilibration with PBST. During each regeneration cycle, attention was paid to completely remove surface-bound analyte, and the washing continued until a response equal to a baseline value was reached.

**Dynamic Analysis of Mutations on Collagen VII Self-assembly**—To study these interactions, we employed a modified experimental system utilizing immobilized nonbiotinylated mProVII acceptors and free biotinylated mProVII interactants (18). In brief, with the 8-well plastic strips (Nalgene Nunc) were coated with nonbiotinylated mProVII acceptors and then blocked with PBS containing 1% solution of bovine serum albumin (BSA) dissolved in PBS. Excess BSA was removed, followed by binding of the cuvette with the Tween 20 (PBST), followed by three consecutive washes with 10 mM HCl. After re-equilibration with PBST, the cuvettes were ready for the binding assays. In addition, a control cuvette with immobilized BSA was prepared by employing the same procedure.

**Effects of Mutations on Collagen VII Self-assembly**—To analyze effects of mutations on formation of homotypic and heterotypic assemblies, mProVII variants were employed in solid-state binding assays utilizing biotinylated mProVII molecules as described (18). Purified mProVII variants were biotinylated using sulfo-N-hydroxysuccinimidobiotin (EZ-Link$^\text{TM}$ Sulfo-NHS-biotin, Pierce) (18). To analyze biotinylated proteins, samples containing 0.5 $\mu$g of procollagen VII variants were electrophoresed on a 7.5% polyacrylamide gel and then electroblotted onto a nitrocellulose membrane, blocked with 5% biotin-free non-fat dry milk (Bio-Rad) followed by a wash with 0.05 M ethanolamine-HCl, pH 8.5. Nonspecific binding sites were blocked by the injection of 200 $\mu$g/ml dissolved in PBS containing 1% BSA. The following homotypic and heterotypic interactions were analyzed separately: (i) between wild type mProVII (WTmProVII) molecules; (ii) between mProVII and rhCVD V1; (iii) between G2575R mutant molecules; (iv) between G2623C mutant molecules; and (v) between WTm-ProVII and each of the G25275R, R2622Q, or G2623G mutant molecules.

Cuvettes with immobilized collagens were primed with PBST at 25 °C for 15 min. A 200-$\mu$l sample containing free mProVII dissolved in PBST was added to the cuvette, and then the association phase was recorded. Subsequently, the sample was removed; analyte-free PBST was added to the cuvette, and the dissociation phase was recorded. After each assay, the surface of the cuvette was regenerated by washing with 10 mM HCl, followed by equilibration with PBST. During each regeneration cycle, attention was paid to completely remove surface-bound analyte, and the washing continued until a response equal to a baseline value was reached.

**Intermolecular Interaction Assays**—For rotary shadowing, the recombinant mProVII mutants were dissolved in 0.5 M acetic acid at a concentration of 50 $\mu$g/ml. Rotary shadowing was done as described previously (18, 30). Procollagen molecules were examined with the Hitachi 7000 transmission electron microscope (Hitachi, Inc.). Photographs were taken at magnification $\times 30,000$ or $\times 40,000$.
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RESULTS

Analysis of mProVII Variants—The system utilizing human embryonic kidney 293 cells employed here produced about 0.5 mg of purified recombinant mProVII variants per 3-liter batch. Analysis of purified procollagens by PAGE revealed the presence of single bands corresponding to the mini pro-α1(VII) chains, indicating that mutant mini procollagens were secreted from cells as intact proteins (Fig. 3A). Furthermore, the mProVII mutants appeared to be correctly folded, as electron microscopy of individual molecules demonstrated the presence of characteristic subunits of the NC1 domain and extended triple-helical region flanked with a C-terminal globular NC2 domain (Fig. 3B) (21). The contour length of the triple helical domain of the mProVII variants was 134.3 ± 11.5 nm (mean ± S.E., n = 15), a value similar to that for WTmProVII (18). Electron microscopy of rotary shadowed molecules demonstrated that the mProVII mutants existed as nonaggregated monomers. Analysis of electrophoresed biotinylated samples by densitometry of biotin-positive bands representing equimolar amounts of procollagen VII variants showed that amount of biotin per α chain was similar in all analyzed samples (not shown).

mProVII-mProVII Biosensor Binding Assays—An optical biosensor employed by us in binding kinetics assays utilizes resonant mirror technology and takes advantage of the optical evanescent wave. Even though different instruments for optical biosensing exist, their common characteristic is that they all utilize surface-confined electromagnetic fields for the precise and real time measurement of the refractive index of a sample in the immediate vicinity of the sensor surface (34). In binding assays ligands are immobilized on a surface of a sensor and reversibly interacting free binding analytes are added in solution. Binding of the free analyte to the surface-immobilized interactant results in mass accumulation and leads to changes in the refractive index. This change is recorded by an instrument’s optical sensors as the association phase. When solution of free analyte is removed from the binding surface and is substituted with a solvent, the surface-bound analyte dissociates. Consequently, the refractive index on the sensor surface decreases, which is recorded as the dissociation phase. Because changes in signal are proportional to changes in the mass of surface-bound analyte, the time course of the signal reflects binding kinetics.

The main advantages of binding studies carried out with the use of biosensors over conventional solid-state binding assays include continuous real time measurements, a variety of techniques for immobilizing interactants, and high sensitivity. Because of the high sensitivity, however, biosensors are not well suited for experiments involving complex, multicomponent systems consisting of more than two purified macromolecules. For this reason experiments involving mProVII variants and PCP were carried out using solid-state binding assays (see below).

An optical biosensor was used to determine the kinetics of homotypic binding between WTmProVII, between G2575R, R2622Q, and G2623C amino acid substitutions. Concentrations of free WTmProVII and G2575 mutant were 5.14 × 10⁻⁸, 1.01 × 10⁻⁸, 2.02 × 10⁻⁸, and 3.88 × 10⁻⁸ M. Concentrations of free R2622Q mutant were 1.39 × 10⁻⁸, 1.71 × 10⁻⁸, 6.85 × 10⁻⁹, and 1.11 × 10⁻⁹ M. Concentrations of free G2623C mutant were 1.88 × 10⁻⁸, 3.76 × 10⁻⁹, 5.63 × 10⁻⁹, and 9.39 × 10⁻¹⁰ M. The samples were added into a biosensor cuvette containing a sensor chip coated with an analogous mProVII variant, and the association phase was recorded. Next, biosensor chips were washed with solvent, followed by recording of the dissociation phase. Middle panels include graphic representation of the residuals of all curves fitted to the data points collected by a biosensor for WTmProVII-WTmProVII and G2575R-G2575R interactions.

![Fig. 3. Analysis of mProVII variants. A. electrophoretic analysis of purified mProVII variants. The purified proteins were analyzed by PAGE and visualized by staining with Coomassie Blue. B. transmission electron microscopy of rotary-shadowed mProVII variants. The white arrow indicates the Cys-2634 region in which mutations were introduced. WTmProVII, wild type mini procollagen VII; G2575R, R2622Q, and G2623C, mini procollagen VII mutants with single amino acid substitutions at indicated positions; mini-pro-α1(VII), mini procollagen VII α chains. Molecular mass markers are indicated in the left lane. Bar, 100 nm.](image)

![Fig. 4. Association and dissociation curves illustrating kinetics of the homotypic binding between WTmProVII molecules and between the molecules harboring G2575R, R2622Q, and G2623C amino acid substitutions. Concentrations of free WTmProVII and G2575 mutant were 5.14 × 10⁻⁸, 1.01 × 10⁻⁸, 2.02 × 10⁻⁸, and 3.88 × 10⁻⁸ M. Concentrations of free R2622Q mutant were 1.39 × 10⁻⁸, 1.71 × 10⁻⁸, 6.85 × 10⁻⁹, and 1.11 × 10⁻⁹ M. Concentrations of free G2623C mutant were 1.88 × 10⁻⁸, 3.76 × 10⁻⁹, 5.63 × 10⁻⁹, and 9.39 × 10⁻¹⁰ M. The samples were added into a biosensor cuvette containing a sensor chip coated with an analogous mProVII variant, and the association phase was recorded. Next, biosensor chips were washed with solvent, followed by recording of the dissociation phase. Middle panels include graphic representation of the residuals of all curves fitted to the data points collected by a biosensor for WTmProVII-WTmProVII and G2575R-G2575R interactions.](image)
other collagen types (35–37), were found to be optimal with respect to minimizing such undesirable factors as heterogeneity of the analyzed ligand, steric hindrance of binding to neighboring sites, mass transport limitations, or formation of multivalent aggregates of the analytes.

The results show that interaction between WTmProVII molecules was characterized by lower affinity than that calculated for G2575R-G2575R binding (Fig. 4 and Table I). The difference between these affinities is attributed to faster association of the G2575R mutants in comparison to the WTmProVII molecules. In contrast to homotypic interactions between the WTmProVII or between G2575R mutant molecules, no homotypic binding between R2622Q or G2623C mProVII mutants was detected (Fig. 4).

Because of slow association rates, heterotypic interactions between intact WTmProVII and intact mProVII mutants were characterized by a low affinity (Fig. 5 and Table I). Nevertheless, although there was no detectable homotypic interaction between R2622Q or G2623C mutants (Fig. 4), these proteins were able to interact with the WTMProVII (Fig. 5 and Table I), but because of slow association rate, these heterotypic interactions were also characterized by low affinities (Table I).

Because of a decrease in the response of the biosensor, indicating dissociation of complexes (Fig. 4 and Fig. 5), binding between all analyzed mProVII molecules is considered a reversible process. Neither WTmProVII nor mProVII mutants bound to the BSA-coated control sensor (not shown), indicating accurateness of measured interaction between analyzed mProVII variants.

Kinetic parameters were obtained by evaluating experimental data from a biosensor with a global fitting method. The best fit for all analyzed samples, as illustrated by low and randomly distributed residuals, was obtained with a simple bimolecular interaction model (Fig. 4 and Fig. 5).

**Binding between PCP-processed mProVII Variants**—It has been demonstrated that procollagen VII molecules form dimers stabilized by disulfide bonds and that this process is promoted by cleavage of the NC2 domain with PCP (see Fig. 1) (18–21, 38). To analyze whether interacting mProVII variants were able to form stable complexes, we employed solid-state binding assays utilizing human recombinant PCP, plastic-immobilized nonbiotinylated acceptors, and biotinylated free analytes (18).

The activity of each batch of PCP was tested by using recombinant human procollagen II and mProVII variants as substrates (18, 39). As expected, all analyzed procollagen variants (G2575R, R2622Q, and G2623C not shown) were correctly processed as electrophoresis of the products of the enzymatic cleavage with PCP demonstrated that their molecular masses were consistent with the prediction that the cleavage occurs only at the single cleavage site present in the NC2 domain (Fig. 6). Furthermore, in agreement with results from biosensor binding assays, the solid-state binding assays (Fig. 7) demonstrated formation of the following complexes of the PCP-processed mProVII variants: (i) WTmProVII-WTmProVII complex, and (ii) WTmProVII-G2575R complex.

In agreement with the results of biosensor binding assays of homotypic interactions nonprocessed R2622Q or G26223C mutant molecules, formation of stable homotypic assemblies was not detected in the samples consisting of PCP-processed R2622Q or G26223C mutants (Fig. 7). Solid-state binding assays demonstrated formation of only a limited number of stable complexes as a result of heterotypic interactions between WTmProVII and R2622Q or G2623C mutants (Fig. 7). As expected, there was no formation of stable complexes in the absence of PCP in any of analyzed groups (not shown) (18). In addition, there were no biotin-positive signals in control samples (not shown).

**Computer Analysis of the Regions of mProVII Variants Adjacent to Mutation Sites**—By using a computer-modeling program, we analyzed fragments of mProVII encompassing Gly-2575, Arg-2622, and Gly-2623 sites, and we studied how amino acid substitutions at these sites change the physicochemical characteristics of adjoining regions. Even though computer-assisted molecular modeling only allows for approximation of physicochemical parameters of analyzed fragments, the results presented in Table II suggest that the G2575R and R2622Q substitutions change the electrostatic potentials of the regions encompassing the mutation sites. In contrast, the electrostatic potential did not change in the G2623C mProVII mutant (Table II). There were no significant differences between the lipophilic potentials of the regions encompassing mutation sites and the lipophilic potentials of the corresponding regions in the WTmProVII (Table II). It should be noted that we have demonstrated that, similarly to the Cys-2634 residue (18), formation of the intramolecular disulfide bonds between Cys-2623 residues is energetically permitted.

**DISCUSSION**

By employing recombinant G2575R, R2622Q, and G26223C mutants, we studied how changing the amino acid sequence of the region neighboring the Cys-2634 affects formation of procollagen VII assemblies. The G2575R mutation was documented previously in a patient with RDEB with moderate severity who had also inherited the E2857X nonsense mutation from an unaffected mother (26). Microscopic analysis of the skin of the patient revealed a reduced number of anchoring fibrils. The R2622Q1 and G2623C (27) substitutions were found in patients with DDEB, respectively. Analysis of the skin of these patients revealed significant pathological changes in the number and morphology of anchoring fibrils, but the molecular basis underlying these changes is not known. By employing an optical biosensor, we determined the kinetics of binding between wild type procollagen VII and studied how single amino acid substitutions change the interaction between mutant molecules.

**Homotypic Interaction between WTmProVII Molecules**—We determined that the $K_s$ for the binding between WTmProVII molecules is $4.0 \times 10^{-3}$ M. In comparison to a $K_s$ of $6.5 \times 10^{-8}$ M reported for the binding between other collagenous proteins, procollagen II and collagen IX, the procollagen VII-procollagen VII interaction is characterized by high affinity (35). Results also indicate that binding between intact procollagen VII molecules is a reversible process characterized by a rapid dissociation phase, suggesting that the major energy of binding is contributed to by a fast on rate. Furthermore, reversibility of the binding indicates that the complexes formed from intact procollagen VII molecules were not covalently linked. This result supports previously published observations suggesting that the formation of intermolecular disulfide bonds in a collagen VII-collagen VII dimer requires cleavage of the NC2 domain.
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**FIG. 5.** Association and dissociation curves illustrating kinetics of the heterotypic binding between WTm-ProVII molecules and the molecules harboring G2575R, R2622Q, or G2623C amino acid substitutions. In each panel, the curves represent heterotypic interaction between immobilized WTm-ProVII and free mutants at concentrations \(2.2 \times 10^{-7}, 4.4 \times 10^{-7}, 8.8 \times 10^{-7}\), and \(1.6 \times 10^{-7}\) M. Lower panels are graphic representations of the residuals of all curves fitted to the data points collected by a biosensor.

**FIG. 6.** Analysis of the cleavage of procollagen II and mProVII with recombinant PCP. The recombinant proteins were incubated at 30 °C with 0.3 units of PCP/µg of procollagen. At time intervals indicated, sample aliquots were withdrawn and analyzed by PAGE. Pro-II, recombinant procollagen II; mProVII, WTmProVII variant; pN-mVII and pN-II, products of the cleavage of procollagen VII and procollagen II with PCP in which N-terminal propeptides are intact. Molecular mass markers are indicated in the left lane.

**FIG. 7.** Effect of cleavage of the NC2 domain with PCP on formation of stable mProVII complexes. Nonbiotinylated mProVII variants were immobilized in plastic wells, and biotinylated mProVII variants were added to the wells followed by cleavage with PCP. Binding of biotinylated samples was detected colorimetrically with AP-streptavidin. WTm-ProVII, wild type mini procollagen VII; G2575R, R2622Q, and G2623C, mini procollagen VII mutants with single amino acid substitutions at indicated positions.

Homotypic and Heterotypic Interactions of the G2575R Mutant—Introduction of the G2575R substitution did not affect the ability of mutant molecules to interact, but the kinetics of interaction changed. We determined that the higher affinity for binding between G2575R molecules was predominantly a result of a faster on rate. Because the G2575R substitution causes a significant increase of the electrostatic potential of the region adjoining the mutation site (see Table II), we hypothesize that the fast rate of binding between the G2575R mutant molecules was because of the formation of an additional binding site at the mutation region. 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, is very acidic with pI of about 4.3 (40). Thus, the strong negative charge of the NC2 domain, which in native procollagen VII is critical for binding to the Cys-2634 site, in the G2575R mutant could also lead to binding to the region encompassing the Arg-2575 mutation site, thereby causing formation of abnormal aggregates. Because a similar increase in affinity between wild type collagen IX and mutant collagen II harboring R519C5 was demonstrated previously (35), creation of an additional binding site could be a common aberration occurring as a consequence of single amino acid substitutions in collagenous proteins.

The heterotypic interaction between the G2575R mutant and WTmProVII was characterized by a lower affinity than those calculated for the homotypic interactions between WTmProVII or between G2575R mutant molecules, and this difference was due to lower on rate. Based on binding studies, it is not clear what the precise mechanism of the WTmProVII-G2575R interaction is; the results suggest, however, that some of the WTm-ProVII-G2575R assemblies are held together by a “one-point” attachment created by the binding of WTmProVII to the Arg-2575 site. If this prediction is correct, these results indicate that the most stable procollagen VII assemblies are formed by a homotypic “two-point” binding between NC2 domains and regions within triple-helical domains of interacting molecules. It is predicted that these assemblies are further stabilized by

| Mutation region | Range of EP of analyzed region | Range of LP of analyzed region |
|-----------------|-------------------------------|-------------------------------|
| Gly-2575        | -123.8 to 210.7              | -0.113 to -0.058              |
| Arg-2575        | -89.0 to 264.7               | -0.112 to -0.056              |
| Arg-2622/Gly-2623 | -211.7 to 323.3               | -0.104 to -0.053              |
| Gln-2622        | -234.5 to 281.5              | -0.106 to -0.054              |
| Cys-2623        | -210.0 to 323.9              | -0.103 to -0.05                |

5 Location of amino acids in the collagen II, where residue "1" is the first glycine in the collagen triple helix.
VII. line residues in the triple-helical domain of human procollagen VII. White horizontal bars indicate regions of the lowest frequency of occurrence of hydroxyproline residues. The bar in the middle portion of the triple helix (residues 1940–1978) corresponds to the 89-amino acid noncollagenous region interrupting continuity of the triple helix. Arrows indicate the approximate positions of mutations and the position of cysteine 2634 that takes part in formation of the intermolecular disulfide bonds between WTmProVII molecules.

interaction of overlapping C-terminal fragments of the triple-helical domains.

Homotypic and Heterotypic Interactions of the R2622Q and R2623C Mutants—In contrast to the G2575R mutant, homotypic binding between the R2622Q or the G2623C mutant molecules was not detected. The inability of the R2622Q and the G2623C mutants to associate indicates that these mutations cause changes in procollagen VII molecules that prevent the formation of dimers, therefore inhibiting assembly of anchoring fibrils.

Unlike the G2575R substitution, the R2622Q and G2623C substitutions are located at a close proximity to Cys-2634, within the region that most likely serves as a critical binding site for the NC2 domain during dimer formation (Fig. 1) (18, 38). Based on molecular modeling, we determined that the R2622Q substitution causes a significant decrease in the electrostatic potential of the mutation region (Table II). Consequently, we postulate that the inability of the R2622Q mutant to assemble was most likely a result of changes in the electrostatic interaction between the region encompassing R2622Q/Cys-2634 and the NC2 domain. Because the G2623C substitution does not change the electrostatic potential or hydrophilic potential of the mutation site (Table II), it is likely that the mechanism preventing interaction between the G2623C mutant molecules differs from that suggested to alter the binding between G2575R or between R2622Q mutants.

Analysis of the distribution of hydroxyproline residues that are critical for the integrity of a collagen triple helix (41, 42) revealed that, assuming all proline residues in the Y position of the Gly-X-Y sequence are hydroxylated, the frequency of occurrence of hydroxyproline residues in the region encompassing Cys-2634 is lowest in the entire procollagen VII triple-helical domain (Fig. 8). The lack of hydroxyproline residues in the central region of the procollagen VII reflects the fact that this particular region represents an interruption in continuity of the -Gly-X-Y- triplets, thus it is not considered “collagen-like” (15). Because the low frequency of occurrence of hydroxyproline residues in any collagen region causes such a region to undergo micro-unfolding at physiological temperature (41, 43), the region encompassing Cys-2634 is characterized by a uniquely open structure. We postulate that the open structure of the Cys-2634 site, together with its physicochemical properties, is critical for binding of the NC2 during formation of the procollagen VII-procollagen VII dimer. In support of such a notion is the fact that in collagen I the region surrounding matrix metalloproteinases 1 cleavage site is also characterized by a low frequency of occurrence of hydroxyproline residues, and as a result, it serves as the binding site for a number of collagenous and noncollagenous macromolecules present in the extracellular matrix (44). The formation of intramolecular disulfide bonds between collagen α chains would lead to alterations of micro-unfolding of the mutation site, thereby preventing this region from being recognized as the binding site for the NC2 domain.

Even though the R2622Q and G2623C mutants were not able to form homotypic assemblies, they were able to interact with WTmProVII (Fig. 5). The ability of these mutant molecules to interact with WTmProVII is consistent with the postulated dominant-negative interference as a mechanism of these autosomal dominant mutations in DDEB.

Intermolecular Interactions between PCP-processed mProVII Variants—The first critical step in the formation of anchoring fibrils is binding between procollagen VII molecules, but stabilization of dimers by disulfide bonds has to follow in order to generate a mechanically competent structure. In the experimental system employed here, formation of disulfide-stabilized mProVII assemblies was initiated by cleavage of interacting mProVII variants with PCP as described (18).

In homotypic binding assays, the formation of stable G2575R-G2575R assemblies indicates that some of the G2575R mutant monomers were able to interact through NC2 domain-Cys-2634 binding in a correct way. In contrast, enzymatic cleavage of R2622Q and G2623C mutants did not result in formation of stable homotypic complexes. Heterotypic solid-state binding assays, in addition to biosensor analyses of binding between intact proteins, showed that WTmProVII forms stable complexes with the G2575R mutant, indicating the ability of this mutant to form functional assemblies.

The R2622Q and G2623C mutants formed only a limited number of heterotypic complexes with WTmProVII. As demonstrated by biosensor assays, binding between WTmProVII and R2622Q or G2623C mutants is permissible, but limited number of stable complexes further suggests that these mutations cause profound structural changes, significantly altering formation of functional heterotypic assemblies.

By employing an experimental model consisting of homozygous procollagen VII mutants, we demonstrated that procollagen VII consists of functional domains that play critical roles in the assembly of anchoring fibrils. Even though kinetic parameters of binding between heterozygous mutant molecules found in actual patients may differ from those reported here, we postulate that single amino acid substitutions occurring in the critical binding domains could alter early stages of assembly of anchoring fibrils. The studies on the early stages of self-assembly of mutant procollagens, together with clinical data on affected patients, suggest that the effects of single amino acid substitutions in procollagen VII on the structure and function of the anchoring fibrils are the result of a complex interplay between effects occurring at molecular and perhaps supramolecular levels of their organization.

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FIG. 8. Schematic of the frequency distribution of hydroxyproline residues in the triple-helical domain of human procollagen VII. White horizontal bars indicate regions of the lowest frequency of occurrence of hydroxyproline residues. The bar in the middle portion of the triple helix (residues 1940–1978) corresponds to the 89-amino acid noncollagenous region interrupting continuity of the triple helix. Arrows indicate the approximate positions of mutations and the position of cysteine 2634 that takes part in formation of the intermolecular disulfide bonds between WTmProVII molecules.
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Single Amino Acid Substitutions in Procollagen VII Affect Early Stages of Assembly of Anchoring Fibrils
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