The Recombinant Expression of Full-length Type VII Collagen and Characterization of Molecular Mechanisms Underlying Dystrophic Epidermolysis Bullosa*

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Type VII collagen is a major component of anchoring fibrils, attachment structures that mediate dermal-epidermal adherence in human skin. Dystrophic epidermolysis bullosa (DEB) is an inherited mechano-bullous disorder caused by mutations in the type VII collagen gene and perturbations in anchoring fibrils. In this study, we produced recombinant human type VII collagen in stably transfected human 293 cell clones and purified large quantities of the recombinant protein from culture media. The recombinant type VII collagen was secreted as a correctly folded, disulfide-bonded, helical trimer resistant to protease degradation. Purified type VII collagen bound to fibronectin, laminin-5, type I collagen, and type IV collagen and also supported human dermal fibroblast adhesion. In an attempt to establish genotype-phenotype relationships, we generated two individual substitution mutations that have been associated with recessive DEB, R2008G and G2749R, and purified the recombinant mutant proteins. The G2749R mutation resulted in mutant type VII collagen with increased sensitivity to protease degradation and decreased ability to form trimers. The R2008G mutation caused the intracellular accumulation of type VII collagen. We conclude that structural and functional studies of in vitro generated type VII collagen mutant proteins will aid in correlating genetic mutations with the clinical phenotypes of DEB patients.

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‡ The abbreviations used are: BMZ, basement membrane zone; NC1 and NC2, NH2- and COOH-terminal noncollagenous domains, respectively, of type VII collagen; DEB, dystrophic epidermolysis bullosa; ECM, extracellular matrix; CMV, cytomegalovirus; PMSF, phenylmethylsulfonyl fluoride; MMP, matrix metalloproteinase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; TH, triple-helical; PTC, premature termination codon; WISH, human amniotic epithelial cell.
Type VII collagen represents less than 0.001% of the total collagen extracted from human skin (26). This has impeded purification of sufficient amounts of native type VII collagen for functional studies. Furthermore, a major problem in studying the biological functions of type VII collagen has been the necessity of using denaturing or proteolytic conditions to release type VII collagen from supramolecular complexes with other extracellular macromolecules during purification procedures. In the current study, we used an efficient eukaryotic recombinant approach to engineer and generate type VII collagen molecules in sufficient amounts for biological characterization and further functional analysis. Further, we introduced two individual substitution mutations, R2008G and G2749R, into the type VII collagen expression vector using site-directed mutagenesis. These two mutations have been reported to be associated with severe, mutilating Hallopeau-Siemens type of recessive DEB (24, 25). We demonstrated that these mutations affect the type VII collagen homotrimer formation, secretion, folding, and stability. Our structural and functional studies of the mutated molecules may yield novel insights into the molecular pathomechanisms underlying DEB disease.

EXPERIMENTAL PROCEDURES

Cell Culture—The human embryonic kidney cell line 293 (ATCC, Rockville, MD) was routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum. Human fibroblasts from neonatal foreskin were initiated into culture as described previously (27). The cells were subcultured and passaged in DMEM supplemented with 10% fetal bovine serum and used in the attachment assays between passages 3 and 6.

Expression Vector Construction and Transfection—The eukaryotic expression vector pRC/CMV (Invitrogen, San Diego, CA), which contains a cytomegalovirus (CMV) promoter and enhancer, was used to express human type VII collagen cDNA in 293 cells. The full-length human type VII cDNA was assembled from CMV/NC1, three cDNA clones described previously (18), and four newly generated cDNA fragments by standard subcloning techniques and recombinant PCR. These four fragments, corresponding to the collagenous domain of human type VII collagen cDNA, were generated by reverse transcription-PCR amplification using human amniotic epithelial cells (WISH) cDNA as a template and oligonucleotides SUR1 (CCCTGCGCTAAGGCGTTC/ACACAAGCCTTCCCCAGGGGC), S2/R2 (AAGGGTGACCTGGGAGGAGTG/GCACCCTTTAGCGTCGTG), S3/R3 (GAGAGCCTG/CGAACGAGGGGTGGCAGACCGCTCCTGAC), and S4/R4 (GTAGACGGAGCTGGGAGCAGGTGACATGGGCTTGGTGG/CGC/CTTTGGAGCAGGTGACATGGGCTTGGTGGC) as primers based on published sequences (12). The cycling conditions were 94 °C for 5 min, followed by 40 cycles of 95 °C, 1 min; 57 °C, 1 min; and 72 °C, 2 min, and a 7-min extension at 72°C. The PCR product was subcloned into a TA vector (Invitrogen), DNA sequence analysis performed, and the sequence compared with the published sequence to confirm its identity (12). The construction of full-length type VII collagen cDNA utilized the overlapping internal restriction sites including Accl, BsrII, and SacII from each cDNA fragment. The correct ligation and in-frame insertion of various DNA fragments were confirmed by DNA sequence analysis.

The expression vector was used to transfect the human embryonic kidney cell line 293 (ATCC, Rockville, MD) using Lipofectin (Invitrogen) as described previously (18). Stable clones were selected using 500 µg of G418/ml (18).

Protein Purification and Analysis—For immunoblot analysis, clotlon cells resistant to G418 were grown to confluence, the medium was changed to serum-free medium, and the cultures were maintained for an additional 24 h. The media were collected; equilibrated to 5 mM EDTA, 50 µM N-ethylmaleimide, and 50 µM phenylmethylsulfonyl fluoride (PMSF); concentrated 10–15-fold (Centricon-100, Amicon, Beverly, MA); and subjected to 6% SDS-PAGE. Proteins were then electrotransferred onto nitrocellulose membrane. The presence of recombinant type VII collagen was detected with the polyclonal antibody to the NCI and NC2 domains of type VII collagen, respectively (28), followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence detection reagent (Amersham Biosciences, Inc.).

For large scale purification of recombinant collagen, serum-free media were equilibrated to 5 mM EDTA, 50 µM PMSF, and 50 µM N-ethylmaleimide and precipitated with 300 mg/ml ammonium sulfate at 4 °C overnight with stirring (4). Precipitated proteins were collected by centrifuging at 1.2 × 10^6 g/min for 1 h, resuspended, and dialyzed in Buffer A (65 mM NaCl, 25 mM Tris-HCl, pH 7.8). Following dialysis, insoluble material was collected by centrifugation at 1.6 × 10^6 g/min for 20 h, and the pellet resuspended with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM PMSF. The solution was clarified as above, and the supernatant, S1 was passed over a Q-Sepharose column (Amersham Biosciences, Inc.) equilibrated in the same buffer. Elution was then carried out with a linear gradient from 0.2 to 1.0 M NaCl of appropriate volume size. The type VII collagen was eluted at 1 M NaCl.

Site-directed Mutagenesis—Site-directed mutagenesis was performed on type VII collagen cDNA in the pRC/CMV vector using a commercial kit (QuiKChange™ site-directed mutagenesis kit, Stratagen Inc., La Jolla, CA) according to the manufacturer’s instructions. Briefly, a pair of complementary primers with 39 bases was designed, and a mutation to change glycine to arginine (G2749R) or arginine to glycine (R2008G) was placed in the middle of the primers. Parental cDNA inserted in pRC/CMV was amplified using *Pyrococcus furiosus* DNA polymerase with these primers for 16 cycles in a DNA thermal cycler (PerkinElmer Life Sciences). After digestion of the parental DNA with DpnI, the amplified DNA with nucleotide substitution incorporated was transformed into *Escherichia coli* (XL1-Blue). The mutations were confirmed by automated DNA sequencing.

Protein Binding Assay—The binding of soluble type VII collagen and NC1 to immobilized ligands was studied using a colorimetric enzyme-linked antibody assay as previously described (18). Briefly, multwell plates (96 wells, Dynatech Laboratory Inc., Alexandria, VA) were coated overnight with 100 µg/ml of antibody K140-Sepharose as described (31). Protein Purification and Analysis was described above, the wells were blocked with PBS containing 0.5% Tween 20. Coated wells were subsequently incubated with 2 µg of purified recombinant type VII collagen or NC1 overnight at 4 °C. The binding of type VII collagen to ECM proteins was detected using an affinity-purified anti-NC1 antibody at a dilution of 1:500, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:400) (Organon Teknika-Cappel, Durham, NC). The development of a colorimetric reaction using 5-bromo-4-chloro-3-indolyl phosphate and molybdate as a substrate (Bio-Rad) was measured by spectrophotometry at 405 nm (Labsystems Multiskan MultiSorb).

The ligands used for binding included purified type I and type IV collagens (Collaborative Biomedical Product, Bedford, MA). Laminin 1 was routinely prepared from the EHS tumor as described previously (29). Fibronectin was prepared from human plasma as described previously (30) and was used in the binding assay from Laminin 1 with the generous gift of Dr. Peter Marinkovich (Stanford University, Stanford, CA). Human laminin 5 was purified from collagenase-solubilized human amniotic membranes by antibody affinity chromatography using monoclonal antibody K140-Sepharose as described (31).

Cell Adhesion Assay—Evaluation of cell attachment to ECM proteins was performed as described previously (32). Briefly, 96-well microtiter plates were coated with 100 µg/ml of each ECM protein in 100 µM carbonate buffer, pH 9.3. Each well was then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline, 0.05% Tween 20. Coated wells were subsequently incubated with 2 µg of purified recombinant type VII collagen or NC1 overnight at 4 °C. The binding of type VII collagen to ECM proteins was detected using an affinity-purified anti-NC1 antibody at a dilution of 1:500, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:400) (Organon Teknika-Cappel, Durham, NC). The development of a colorimetric reaction using 5-bromo-4-chloro-3-indolyl phosphate as a substrate (Bio-Rad) was measured by spectrophotometry at 405 nm (Labsystems Multiskan MultiSorb).

Protease Digestion—Purified recombinant type VII collagen or mutant type VII collagen was incubated with trypsin (Sigma) at an enzyme-to-substrate ratio of 1:10 by weight in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl at 14 °C for 2 h or with pepstatin in 0.1% acetic acid at 4 °C for 2 h or overnight and then analyzed by SDS-PAGE, followed by immunoblot analysis with a polyclonal antibody against the collagenous domain of type VII collagen. For collagenase digestion, purified recombinant type VII collagen (5 µg) was dialyzed against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl2, and 2 mM N-ethylmaleimide and then incubated with 30 units of type VII collagenase (elastodiodeptidase A, type III, Sigma) digestion at 37 °C for 1 h.

Preparation and Purification of MMP-2—MMP-2 was purified by a previously published procedure (33). Briefly, human dermal fibroblasts were embedded in type I collagen lattice (1 × 10^6 cell/ml) and cultivated in serum-free DMEM supplemented with tumor necrosis factor-α at 2 ng/ml. After culture for 72 h, the conditioned medium was collected.
and cleared by centrifugation. MMP-2 was purified by affinity chromatography through gelatin-Sepharose-4B (Amersham Biosciences, Inc.). The MMP-2 generated by this procedure contains both the active 62-kDa monomer and the 72-kDa latent forms. The identity of the proteinase was confirmed by Western blot, and purity was examined by staining with Coomassie Blue R-250.

Immunofluorescence Staining—293 cells were plated in TissueTek chamber slides (Nunc, Inc., Naperville, IL) on polylysine at 37 °C for 18 h. Cells were immersed in periodate-lysine-paraformaldehyde fixative for 10 min at room temperature, washed several times with PBS to remove fixative, and then permeabilized and blocked by incubating in PBS with 3% BSA, 1% saponin, and 10% normal goat serum for 15 min at room temperature. The cells were incubated with an affinity-purified polyclonal antibody to the NC1 domain of type VII collagen at a dilution of 1:200 in a humidified chamber for 2 h, then washed three times with PBS, 1% saponin, counterstained with a fluorescein isothiocyanate-conjugated goat antibody to rabbit IgG (1:200 dilution) for 1 h (Organon Teknika-Cappel) and washed. The cells were then examined and photographed with a Zeiss epiluminating immunofluorescence microscopy.

RESULTS

Recombinant Production of Type VII Collagen—The entire cDNA sequence coding for the type VII collagen was cloned into the eukaryotic expression vector, pRC/CMV, as shown in Fig. 1 and used to transfect human 293 cells. The collagen expression was initially examined by transient transfection of human 293 embryonic kidney cells. Western blot analysis with polyclonal antibodies against the NC1 and the NC2 domains of type VII collagen detected the expression of a 290-kDa recombinant doublet secreted into the serum-free medium (Fig. 2, lanes 2 and 5). These proteins were absent in the control vector-transfected 293 cells (Fig. 2, lanes 1 and 4). The recombinant type VII collagen co-migrated with authentic type VII collagen prepared from WISH cells (compare Fig. 2, lanes 2 and 3). Triple-helical assembly of collagens often requires hydroxylation and the presence of ascorbic acid. As shown in Fig. 2, in the absence of ascorbic acid, the type VII collagen was secreted as a 290-kDa monomer (lane 6) under nonreducing conditions, whereas, in the presence of ascorbic acid, it was secreted as a 900-kDa protein, corresponding to the association of three type VII collagen monomers (lane 7).

Purification and Characterization of Structural Properties of Recombinant Type VII Collagen—The recombinant type VII collagen was purified from serum-free cell culture medium of stably transfected 293 cells, which contained 2–5 μg/ml type VII collagen. The ammonium sulfate precipitate of the conditioned medium proteins (Fig. 3, lane 1) was sequentially solubilized with salt (Fig. 3, lane 2) and then passed over a Q-Sepharose column. Most of type VII collagen proteins eluted from the column in the high salt fraction (1 M NaCl) (lane 3). When the purified type VII collagen was subjected to 4–12% SDS-PAGE under nonreducing condition (Fig. 3B), it migrated with an apparent molecular mass of 900 kDa, the expected size of a trimer. Under reducing conditions, however, all the trimers converted into monomers.

The demonstration that type VII collagen assembled to form S-S bonded trimers in vitro suggests, but does not prove, that these composites are stable and have a proper triple-helical conformation in their collagenous domain. To examine this question further, purified type VII collagen was treated with trypsin and pepsin and then subjected to immunoblot analysis using a polyclonal antibody against the triple-helical domain. Because type VII collagen is very sensitive to proteolysis, a low
a polyclonal antibody against the triple-helical domain. This result again indicates that the recombinant type VII collagen has a relaxation in the helical part of the molecules similar to that seen in authentic human type VII collagen.

Interaction of Type VII Collagen with ECM Proteins—We reported previously that the NC1 domain of type VII collagen interacts with fibronectin, laminin-5, type I collagen, and type IV collagen (18). Therefore, we examined the ability of purified type VII collagen to bind immobilized ECM components. To compare the binding affinity between NC1 and type VII collagen, purified recombinant NC1 was also included in the binding assays. As shown in Fig. 5, like NC1, type VII collagen binds to fibronectin, laminin-5, type I collagen, and type IV collagen. Little or no binding occurred with laminin-1. The ECM binding affinities appeared the same when NC1 and type VII collagen were compared with the exception of the affinity for fibronectin. In this case, type VII collagen bound more avidly than NC1. This is consistent with our previous study that mapped a second fibronectin binding site within the collagenous domain (36).

Cell Adhesion Activity of Type VII Collagen—We demonstrated previously that the NC1 domain of type VII collagen promotes the adhesion of human dermal fibroblasts (32). Therefore, we evaluated the ability of recombinant type VII collagen to support fibroblast adhesion. Purified recombinant type VII collagen, NC1, and other ECM proteins were immobilized to plastic wells and assayed for their ability to mediate the attachment of human fibroblasts (Fig. 6). Both recombinant type VII collagen and NC1, similar to collagen types I and IV, and fibronectin, promoted vigorous attachment of human dermal fibroblasts. The adhesion of fibroblasts to control wells coated with BSA was negligible. Further, type VII collagen appears to be a better cell attachment agent than NC1. We previously constructed a recombinant type VII collagen “mini-gene” comprising the sequences encoding for the entire noncollagenous globular domains (NC1 and NC2, respectively) and half of the central helical collagenous domain (37). Purified “minicollagen” was also included in the adhesion assay. As shown in Fig. 6, the minicollagen supported fibroblast attachment to levels similar to that generated by NC1.

Glycine Substitution G2749R Is Sensitive to Proteolytic Digestion—Previous studies have identified the homozygous glycine to arginine missense mutation at the amino acid residue
2749 in the patients with recessive DEB (25). To investigate the molecular defect underlying this mutation, site-directed mutagenesis was used to generate the same mutation in the type VII collagen expression construct. As shown in the immunoblot analysis in Fig. 7, transfection of the G2749R construct into 293 cells resulted in the secretion of a 290-kDa type VII collagen at a similar level as the wild type (compare lanes 1 and 2). Under nonreducing conditions, the G2749R mutation protein product runs as a 290-kDa monomer and a 900-kDa trimer (lane 4). In contrast, wild type VII collagen all runs as a 900-kDa trimer (lane 3). These data indicated that a glycine substitution at 2749 affects the ability of type VII collagen to form trimers or decreases the stability of the trimers that have been formed.

To assess further the stability of the G2749R mutant protein, the purified protein was subjected to limited pepsin and trypsin digestion. As shown in Fig. 8, both pepsin and trypsin treatment of G2749R resulted in the complete digestion of the TH fragment and produced an extremely weak P1 fragment compared with wild type VII collagen (compare lanes 3 and 4 and lanes 5 and 6). Further, trypsin digestion revealed an additional band below P1 fragment. These results demonstrated that the triple-helical domain of the G2749R mutant type VII collagen is less stable against proteolysis than wild type VII collagen. This suggests that a glycine substitution causes abnormal and incomplete folding of the type VII collagen molecule.

**R2008G Substitution Causes Intracellular Retention of Type VII Collagen**—Site-directed mutagenesis was also used to generate an arginine to glycine substitution at the amino acid residue 2008 in the middle of the triple-helical domain. This gene defect has been reported in a patient with recessive DEB who had a homozygous missense mutation (24). Immunofluorescence staining with antibody to type VII collagen showed weak intracellular staining in 293 cells stably transfected with the wild type VII collagen construct (Fig. 9A, WT). In contrast, 293 cells stably transfected with the R2008G mutant revealed markedly increased intracellular type VII collagen staining (R2008G). There was no immunostaining in the parent 293 cells (data not shown). These results were further confirmed by immunoblotting analysis. As shown in Fig. 9B, compared with wild type, there is a reduced amount of secretion of the R2008G mutant protein into the medium (compare lanes 1 and 2). Accordingly, increased amounts of the R2008G mutant protein accumulated inside the cells (compare lanes 3 and 4).

**FIG. 6.** Adhesion of human fibroblasts to type VII collagen and various ECM proteins. Human fibroblasts were plated onto wells coated with collagen type I (C1), collagen type IV (C4), fibronectin (FN), purified recombinant NC1 (NC1), purified type VII minicollagen (C7M), purified type VII collagen (C7) and BSA at a concentration of 20 μg/ml for overnight at 4°C. Attached cells were fixed and stained as described under “Experimental Procedures,” and the optical density at 540 nm was measured as an index of cell adhesion. Each value represents the mean ± S.E. (bar) for triplicate assays.

**FIG. 7.** Formation of trimer by G2749R mutant. 293 cells were transfected with either wild type C7 (WT) or with glycine to arginine substitution (G2749R) mutant, and conditioned media were concentrated and subjected to 8% SDS-PAGE followed by immunoblot analysis with a polyclonal antibody to the NC2 domain. Proteins were either reduced with 2-mercaptoethanol (lanes 1 and 2) or nonreduced (lanes 3 and 4). The positions of molecular size markers, monomer (M) and trimer (T) of type VII collagen are indicated.

**FIG. 8.** G2749R substitution causes reduced stability of mutant type VII collagen against proteolytic digestion. Purified wild type VII collagen (WT) or the glycine to arginine substitution (G2749R) mutant protein was either untreated (lanes 1 and 2) or treated with pepsin (lanes 3 and 4) and trypsin (lanes 5 and 6) and analyzed by 8% SDS-PAGE, followed by immunoblot analysis with a polyclonal antibody to the triple-helical domain. The positions of molecular size markers, 290-kDa type VII collagen (C7), the 200-kDa intact TH domain (TH), and the 120-kDa carboxyl-terminal half of the TH fragment (P1), are indicated.

**DISCUSSION**

The efficient production and secretion of human type VII collagen was achieved by using an eukaryotic expression vector in a human cell line that has no endogenous type VII collagen. The recombinant collagen was secreted in large quantities into the culture medium (2–5 mg/liter), which allowed purification of the protein in milligram quantities for structural and functional studies.

Our data indicate that recombinant type VII collagen is a better adhesive ligand than NC1 for human fibroblast attachment. We demonstrated that a truncated type VII minicollagen, containing the intact noncollagenous NC1 and NC2 and half of the central helical collagenous domain, supported fibroblast attachment at levels similar to those of NC1 alone. These results indicate that the deleted 678 amino acids (residues 1920–2603) within the central collagenous domain contain the additional fibroblast binding site (s).

In a study by Christiano and colleagues (24), a family with
members who had recessive DEB was described in which a gene defect in type VII collagen was identified as a single amino acid substitution from arginine to glycine at residue 2008 within the triple-helical domain. Specifically, this substitution was in exon 73, in close vicinity with the noncollagenous hinge region in the center of the long triple helix. Because of this report and the resultant reduced anchoring fibrils detected in the skin of affected patients, we attempted to duplicate this gene defect in vitro using site-directed mutagenesis. We introduced the same mutation in our type VII collagen expression construct. Our data show that this mutation leads to reduced secretion and intracellular accumulation of the mutant protein. It is interesting to note that three other type VII collagen mutations, G2006D, G2034R, and G2015E, within exon 73 close to the hinge region have also been shown to interfere with protein folding in a dominant negative manner and cause intracellular accumulation of the mutant molecules (38). It is possible that exon 73 may contain amino acid residues critical for the efficient secretion of type VII collagen. Protein suicide can be regarded as a protective mechanism because incorporation of abnormally folded molecules into fibrillar polymers destabilizes the suprastructures and renders them ultrastructurally abnormal and functionally inadequate. Our current data, taken together with others’ previously published work with other mutant proteins (G2006D, G2034R, and G2015E), are consistent with this hypothesis. Such a mechanism could lead to a paucity of anchoring fibrils at the DEJ and explain the ultrastructural observation of reduced and abnormal anchoring fibrils in DEB patients’ skin (24). Because a small amount of R2008G mutant protein (10–15%) was still able to be secreted into the extracellular matrix, it is possible that the R2008G mutation may impair some other important functions of type VII collagen. In this regard, we could not detect effects of the R2008G mutation on type VII collagen stability, trimer formation, or fibronectin binding (data not shown). However, recent studies have identified four unrelated DEB patients with attenuated or negative staining for GDA-J/F3, a new component of human skin BMZ (39, 40). All of these four DEB patients had distinct amino acid substitutions within the RGD motif of residue Arg-2008. This suggests that this residue is important for the affinity between type VII collagen and the GDA-J/F3 protein (39, 40). This interaction may play a role in dermal epidermal adherence.

The homozygous glycine substitution mutation (G2749R) in an affected individual was associated with severe recessive DEB phenotype. In general, the tight packing of collagen triple helices makes them relatively resistant to the effects of proteases. Glycine substitutions within the collagen tripeptide repeat can alter this conformation, and, as a consequence, its function and/or stability. A local disruption in the triple-helical structure can often be monitored by measuring the susceptibility of the collagen to proteolytic cleavage in vitro. In the present study, we demonstrated that the G2749R mutant protein revealed an increased sensitivity to protease and a decreased ability to form trimers. It is interesting to note that the G2749R mutation occurs right before a small interrupted collagenous segment. The substitution of a glycine residue in this short, nonhelical collagenous segment may lead to destabilization and local unraveling of the triple helix. This destabilization is manifested as an increased sensitivity to protease digestion in vitro. Because of this local structural alteration, it is likely that peptide bonds in proximity to the G2749R mutation are more susceptible to proteolysis by extracellular proteases present at the epidermal BMZ. Our finding that the G2749R mutation leads to increased sensitivity to proteolysis and a corresponding decreased steady-state level of type VII collagen in vivo may contribute to the abnormal and the markedly reduced numbers of anchoring fibrils characteristic of recessive DEB. Alternatively, the mutation-associated structural alteration could well have a direct negative impact on type VII collagen function. In this regard, it is interesting to note that the G2749R mutation occurs within the domain that contains a fibronectin binding site. However, purified mutant G2749R protein binds to fibronectin with the same potency as wild type VII collagen (data not shown).

The G2749R mutant protein had reduced ability to form trimers. The reasons why a single glycine substitution can exert such an effect are unclear. However, this mutation occurs within the domain which initiates the triple-helical assembly of type VII collagen and directs antiparallel dimer formation (19). Therefore, it is possible that these amino acids are located within motifs required for triple-helical assembly of type VII collagen.

The expression system used here allows for the production of native, post-translationally modified type VII collagen in quantities sufficient for comprehensive functional and structural studies. Furthermore, the recombinant expression system should be useful in elucidating the effects of human mutations on the structure and function of type VII collagen by site-directed mutagenesis. Studies of this nature may facilitate the interpretation of the naturally occurring molecular defects within type VII collagen associated with DEB. These studies may also provide an understanding of the correlation between genetic mutations in type VII collagen and the resultant DEB clinical phenotypes.

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