Some, but Not All, Glycine Substitution Mutations in COL7A1 Result in Intracellular Accumulation of Collagen VII, Loss of Anchoring Fibrils, and Skin Blistering*

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COL7A1 gene mutations cause dystrophic epidermolysis bullosa, a skin blistering disorder. The phenotypes result from defects of collagen VII, the major component of the anchoring fibrils at the dermo-epidermal junction; however, the molecular mechanisms underlying the phenotypes remain elusive. We investigated naturally occurring COL7A1 mutations and showed that some, but not all, glycine substitutions in collagen VII interfered with biosynthesis of the protein in a dominant-negative manner. Three point mutations in exon 73 caused glycine substitutions G2006D, G2034R, and G2015E in the triple helical domain of collagen VII and interfered with its folding and secretion. Confocal laser scanning studies and semiquantitative immunoblotting determined that dystrophic epidermolysis bullosa keratinocytes retained up to 2.5-fold more procollagen VII within the rough endoplasmic reticulum than controls. Limited proteolytic digestions of mutant procollagen VII produced aberrant fragments and revealed reduced stability of the triple helix. In contrast, the glycine substitution G1519D in another segment of the triple helix affected neither procollagen VII secretion nor anchoring fibril function and remained phenotypically silent. These data demonstrate that collagen VII presents a remarkable exception among collagens in that not all glycine substitutions within the triple helix exert dominant-negative interference and that the biological consequences of the substitutions probably depend on their position within the triple helix.

Anchoring fibrils attach the epidermal basement membrane of the skin to the underlying dermal connective tissue (1, 2). They represent polymers of collagen VII, a large homotrimeric protein with a central triple helix and flanking amino- and carboxyl-terminal globular domains. Epidermal keratinocytes synthesize and secrete collagen VII as a triple helical precursor (procollagen VII) into the extracellular matrix. After secretion, procollagen VII undergoes proteolytic trimming to collagen VII (3) and assembles to polymers (1). This is a multistep process during which collagen VII monomers first form disulfide-bonded antiparallel dimers and then laterally aggregate into anchoring fibrils, which interact with laminin 5 to secure the dermo-epidermal adhesion (1, 4). Further stabilization of anchoring fibrils and presumably of intermolecular aggregates is achieved through cross-linking by transglutaminase-2 (5). Anchoring fibrils are functionally deficient in hereditary dystrophic epidermolysis bullosa (DEB), a heterogeneous group of bulbous skin disorders (for reviews, see Refs. 6 and 7) with mechanically induced blistering and scarring of the skin. In the most severe forms of the disease, both collagen VII protein and anchoring fibrils are absent from the skin (8), whereas in milder forms, collagen VII is expressed, but the morphology of the anchoring fibrils may be altered (7, 9).

Mutations in COL7A1 encoding collagen VII have been disclosed in both recessive and dominant DEB subtypes (10–17). In recessive subtypes, homozygous or compound heterozygous mutations leading to premature termination codons underlie very severe skin blistering and scarring (18), whereas homozygous or compound heterozygous missense mutations cause milder phenotypic manifestations. In dominant DEB (DDEB), only about a dozen mutations have been identified, most of them causing substitution of a glycine in the triple helical domain of collagen VII (12, 17, 19, 20). However, despite a growing number of known collagen VII mutations, the biological consequences of these mutations and the pathogenic pathways from the gene defect to dermo-epidermal tissue separation in the skin have remained elusive.

Glycine substitution mutations in other collagen genes underlie heritable connective tissue diseases, such as osteogenesis imperfecta, chondrodysplasias, certain subtypes of Ehlers-Danlos syndrome, or Alport’s syndrome (for reviews, see Refs. 21–24). These mutations cause pathologic phenotypes through dominant-negative interference. Therefore, the prediction was that glycine substitution mutations in COL7A1 had similar effects. Surprisingly, however, molecular genetic analyses of a number of DEB families disclosed several compound heterozygous glycine substitution mutations in COL7A1 that did not cause a pathologic phenotype in obligate carriers (20).

In an attempt to establish genotype-phenotype relationships and to elucidate the different biological consequences in COL7A1 mutations, we identified glycine substitution mutations in COL7A1 exons 44 and 73 and investigated the biological and clinical phenotypes of the mutations. Whereas a glycine substitution mutation in exon 44 remained biologically...
and clinically silent, three glycine substitution mutations in exon 73 interfered with the biosynthesis and function of collagen VII in a dominant-negative manner.

EXPERIMENTAL PROCEDURES

Probands—The diagnosis of DDEB (6, 7) in probands 1–3 was based on 1) the pedigree consistent with dominant inheritance; 2) history of mechanically induced skin blistering and scarring since infancy; 3) clinical observations of skin blisters, scarring, milia, and nail dystrophy at trauma-exposed body sites, such as hands, feet, and knees; 4) electron microscopic findings of dermo-epidermal tissue separation below the lamina densa of the skin basement membrane and/or paucity and altered morphology of the anchoring fibrils; 5) antigen mapping (25) of blistered skin regions that revealed structural antigens of the dermo-epidermal junction at the blister roof; and 6) positive immunofluorescence staining of collagen VII using different domain-specific antibodies. In family 1, the affected individuals included the 48-year-old male proband (1-1) and his 8-year-old son (1-2). A biochemical and morphological study on collagen VII expression in vitro of proband 1-1 was published earlier (26), at a time when the genetic basis of DDEB was not yet known. In family 2, the proband was a 64-year-old male (2-1). His father and brother were deceased, but were reported to have had similar blistering tendency. No other family member had a skin disease or a genetic disorder. The affected members of family 3 included the 7-year-old proband (3-1), his 5-year-old brother (3-2), the 36-year-old mother (3-3), the 33-year-old aunt (3-4), and the 65-year-old grandfather (3-5). The father and grandfather of 3-5 as well as 13 other relatives in five generations reportedly suffered from similar mechanically induced skin blistering. Proband 4 was the clinically unaffected father of a compound heterozygous epidermolysis bullosa child with two different glycine substitution mutations in COL7A1.2

Mutation Detection—Genomic DNA was isolated from peripheral blood or cultured cells using the Easy-DNATM kit (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. Balanced primer pairs were used for PCR amplification of exons directly from genomic DNA (GenBankTM accession number L23982), and the products were examined for heteroduplex formation by conformation-sensitive gel electrophoresis (27). Heteroduplex bands were detected in the amplimers corresponding to exon 73. Primers used for amplification of exon 73 were as follows: sense primer, 5'-GGGTGTAGCTGTACAGCAC-3' (nucleotides 23399–23419); and antisense primer, 5'-CCCTCTTCCCTCACTCTCCT-3' (nucleotides 23684–23704) (20). For PCR, 100 ng of genomic DNA were used as template, and amplification conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s in a Perkin-Elmer 9800 thermal cycler. The size

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of the PCR product was 286 base pairs. The PCR products were sequenced by an automated sequencer (Genome Express, Grenoble, France). The detection of the mutation in exon 44 has been described elsewhere.2

Keratinocyte Cultures—Keratinocytes obtained by trypsinization of skin biopsies were cultured in serum-free, low calcium keratinocyte growth medium (28) supplemented with bovine pituitary extract and epidermal growth factor (keratinocyte growth medium, Life Technologies, Inc.). Prior to analyses, cells at an early passage received 50 μg/ml L-ascorbate for 48 h (28, 29).

Antibodies and Immunofluorescence Staining—The polyclonal antibodies to the triple helical and carboxyl-terminal (NC-2) domains of human collagen VII were produced as described (3, 30). The monoclonal antibody LH-7.2 to the amino-terminal (NC-1) domain of collagen VII was a kind gift from Dr. I. Leigh (London Hospital, London) (31). The monoclonal antibody to protein-disulfide isomerase and the fluorescein isothiocyanate-labeled anti-rabbit antibodies were purchased from Dako (Glostrup, Denmark). The Texas Red-labeled polyclonal anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). For immunofluorescence (IF) staining, subconfluent cells on coverslips were permeabilized and fixed with absolute methanol at −20 °C and incubated at room temperature with the first antibody overnight and the second antibody for 1 h (26). Preparations were mounted in Mowiol (Hoechst, Hoechst, Germany) and photographed using an inverted confocal scanning microscope (IBBE, Leitz, or an LSM 410, Carl Zeiss, Oberkochen, Germany) combined with two HeNe lasers (543/633 nm) and one argon laser (488 nm) for multicolor fluorescence.

Protein Extraction and Immunoblot Analyses—The culture medium and the cell layer were extracted separately. The medium was precipitated with ethanol in the presence of proteinase inhibitors (28), and the cell layer was extracted with a neutral buffer containing 0.1 M NaCl, 0.020 M Tris-HCl, pH 7.4, 1% Nonidet P-40, and a mixture of proteinase inhibitors (32). The samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using domain-specific collagen VII antibodies (3). Semiquantitative immunoassays were used to determine relative amounts of collagen VII in the cells and the medium (28).

Protein Digestion—The stability of procollagen VII from control and DDEB keratinocytes was analyzed by limited pepsin or sequential pepsin/trypsin digestions. For a limited pepsin digestion of procollagen and DDEB keratinocytes was analyzed by limited pepsin or sequential antibodies (3). Semiquantitative immunoassays were used to determine inhibitors (32). The samples were subjected to SDS-polyacrylamide gel electrophoresis using standard methods as described (7, 17).

Electron Microscopy—Electron microscopy of biopsy samples of intact normal appearing skin from probands 1-1 and 3-1 was performed using standard methods as described (7, 17).

RESULTS

Identification of Mutations—Direct sequencing of the 286-base pair PCR product of exon 73 disclosed point mutations in all probands (Fig. 1). The affected members of family 1 were heterozygous for a 6017G→A transition (G2006D), family 2 for a 6100G→A transition (G2034R), and family 3 for a 6044G→A transition (G2015E). The sequence variations did not represent neutral polymorphisms since they were not found in healthy first-degree relatives or in 100 normal chromosomes by dideoxynucleotide sequencing. Mutation G2006D was recently also found in a Japanese patient with epidermolysis bullosa patient (35). Proband 4 was heterozygous for a 4556G→A transition in exon 44 (G1519D), as described elsewhere.2

Effect of the Mutations on Procollagen VII Production in Keratinocytes in Vitro—Immunofluorescence staining with antibodies to collagen VII demonstrated a weak intracellular granular staining in normal control keratinocytes (Fig. 2) that colocalized with protein-disulfide isomerase, a resident protein in the endoplasmic reticulum (data not shown). In contrast, two of the three DDEB keratinocyte strains (1-1 and 2-1) revealed a clearly increased intracellular collagen VII content as evidenced by a strong IF signal (Fig. 2 and Table I). A certain proportion of 1-1 (26) and 2-1 keratinocytes exhibited confluent intracellular granular patches. These were consistent with accumulation of collagen VII in dilated cisternae of the rough endoplasmic reticulum as evidenced by colocalization with protein-disulfide isomerase (data not shown). Use of domain-specific antibodies against the NC-1, NC-2, and triple helical domains demonstrated that the retained material consisted of procollagen VII molecules. These observations were in concert with previous investigations on proband 1-1 keratinocytes, which accumulated collagen VII in the rough endoplasmic reticulum as shown by IF and immunoelectron microscopy (26). In 3-1 cells, only a slight increase was observed.

The results of immunohistochemistry on keratinocyte extracts were consistent with the IF staining. A single 320-kDa band representing the pro-α1(VII) polypeptide chain was found in control and DDEB cell media and extracts, but quantitative differences emerged (Fig. 3). The procollagen VII bands derived from 2-1 and 3-1 cell extracts were stronger than controls, whereas the bands derived from the media of the cultures appeared similar. A semiquantitative immunoblot assay using water-soluble glycine substitutions result in a change in the amino acid sequence of the protein. The glycine residue is a critical component of the triple helix structure of collagen. Substitution of glycine can disrupt the helical structure, leading to the misfolding and accumulation of the mutant collagen protein within the cells. The accumulation of these abnormal proteins within the cells is thought to contribute to the skin blistering and other symptoms associated with these conditions. The experiments involving digestion with pepsin and trypsin suggest that the specific processing of collagen VII may also play a role in the disease phenotype. The use of domain-specific antibodies in immunohistochemistry and immunoblotting allows for a more detailed analysis of the distribution and expression of collagen VII variants. These techniques are essential for understanding the underlying molecular basis of these skin disorders.
control extract without enzyme (Co) pepsin/trypsin digestion. Keratinocyte extracts from controls (lane 1) and the mutant (Mut) procollagen from proband 2-1 (lane 4) remained stable during control incubation without pepsin and trypsin. Both pepsin digestion alone (lane 2) and the combined pepsin/trypsin digestion (lane 3) removed the globular domains of normal procollagen VII and resulted in the appearance of two bands on the blot, corresponding to the intact triple helical domain and its carboxyl-terminal half, the P1 fragment. In contrast, in the case of proband 2-1, the triple helical fragment of procollagen VII was resistant to pepsin treatment alone (lane 5), but was completely cleaved during the combined pepsin/trypsin digestion (lane 6). In addition, the double band of the P1 fragment created by pepsinization appeared less intensive after pepsin/trypsin digestion (lane 6). The trypsin sensitivity of the mutant collagen VII demonstrates reduced stability of the triple helix as compared with controls, indicating incomplete protein folding.

FIG. 4. G1519D substitution does not lead to intracellular accumulation of procollagen VII in keratinocytes. Cultured keratinocytes of a normal control (A) and proband 4 (B) were stained with antibodies to the NC-2 domain of procollagen VII with IF. The cells of proband 4 exhibited a similar intracellular IF signal as normal keratinocytes, indicating that procollagen VII was not retained intracellularly. The same staining pattern was observed with antibodies to the NC-1 and triple helical domains of procollagen VII. Immunoblotting of keratinocyte cultures showed similar amounts of procollagen VII (Procoll. VII) in cell extracts and media from both the control and proband’s keratinocytes (C). Lane 1, control cell extract; lane 2, cell extract of proband 4; lane 3, control culture medium; lane 4, culture medium of proband 4.

Mutations in Exon 73 Cause Reduced Stability of Mutant Procollagen VII against Proteolytic Digestion—To assess the stability of the mutant procollagen VII retained in the cells, the protein was extracted from control and DEB keratinocytes and probed by limited pepsin digestion or a sequential pepsin/trypsin treatment (33). Since collagen VII is very sensitive to proteolysis (1), a low enzyme concentration and a reaction temperature were chosen for the pepsin digestion. Under these conditions, the globular NC-1 and NC-2 domains were removed from procollagen VII. The triple helical domain mostly resisted chromophores and a spectrophotometric determination of the immune signal (28) revealed a higher cell layer/medium ratio of procollagen VII in DDEB cells than in controls, indicating intracellular accumulation of procollagen VII. When the calculated cell layer/medium ratio was set at 1.0 in controls, it was 2.5 in 2-1 cells and 1.7 in 3-1 cells (Table I).

In contrast to the cells of probands 1-1, 2-1, and 3-1, cultured keratinocytes of proband 4 did not retain procollagen VII, as demonstrated by IF staining and immunoblot analysis (Fig. 4). Semiquantitative assessment of the immunoblot signals revealed a normal cell layer/medium ratio of procollagen VII in the keratinocyte cultures of proband 4, indicating that no intracellular accumulation occurred. The calculated cell layer/medium ratio was 1.0 in both controls and in the cells of proband 4 (Table I).

Mutations in Exon 73 Cause Reduced Stability of Mutant Procollagen VII against Proteolytic Digestion—To assess the stability of the mutant procollagen VII retained in the cells, the protein was extracted from control and DEB keratinocytes and probed by limited pepsin digestion or a sequential pepsin/trypsin treatment (33). Since collagen VII is very sensitive to proteolysis (1), a low enzyme concentration and a reaction temperature were chosen for the pepsin digestion. Under these conditions, the globular NC-1 and NC-2 domains were removed from procollagen VII. The triple helical domain mostly resisted chromophores and a spectrophotometric determination of the immune signal (28) revealed a higher cell layer/medium ratio of procollagen VII in DDEB cells than in controls, indicating intracellular accumulation of procollagen VII. When the calculated cell layer/medium ratio was set at 1.0 in controls, it was 2.5 in 2-1 cells and 1.7 in 3-1 cells (Table I).

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digestion, except for a partial cleavage into the P1 and P2 fragments (Fig. 3B) at the site of the hinge region in the center of the helical domain, as described by Burgeson (1). In the case of mutant 3-1, pepsin treatment led to a fragment pattern similar to that of controls, but mutant 2-1 yielded a double band of the C-terminal P1 fragment, reflecting decreased stability of the triple helix in mutant procollagen VII (Fig. 3B). In addition, the cell extracts were subjected to a combined, limited pepsin/trypsin digestion (Fig. 3C). To define conditions under which the triple helix of normal collagen VII loses its resistance to this combined proteolytic digestion, the trypsin incubations were carried out at different temperatures. In controls, the triple helical fragment lost its stability around 35 °C. Subsequently, the stability of normal and mutant collagen VII was compared at 33 °C for the trypsin digestion. No significant difference between pepsin digestion alone and the pepsin/trypsin combination was observed in controls at 33 °C, i.e. both the triple helical region and the P1 fragment were visualized on immunoblots (Fig. 3C). In contrast, collagen VII from 2-1 cells was less stable. As shown in Fig. 3C (lane 6), the triple helical fragment was completely digested, and the double band of the P1 fragment appeared weaker. These observations demonstrate that the triple helix of the mutant collagen VII molecules from 2-1 cells is less stable against proteolysis, suggesting that the glycine substitution causes abnormal, incomplete folding of the collagen. Cell extracts of probands 1-1 and 1-2 were not available for these analyses.

Abnormalities of Anchoring Fibrils in the Skin—The ultrastructure of anchoring fibrils in DDEB skin was altered. Electronic microscopic examination of the dermo-epidermal basement membrane zone in 1-1 and 3-1 skin (Fig. 5) showed a reduced number of anchoring fibrils as compared with normal control skin (Fig. 5a). In 1-1, patches of some normal appearing and some slender, narrow anchoring fibrils along the dermo-epidermal junction were observed (Fig. 5b), but long stretches of the basement membrane zone were devoid of fibrils. In 3-1, only rudimentary fibril-like structures could be discerned (Fig. 5c).

DISCUSSION

Since early studies on folding of fibrillar collagens with long uninterrupted triple helices, i.e. collagens I–III, had shown that the presence of a glycine in every third amino acid position in the polypeptide is a prerequisite for formation and secretion of a stable triple helix (for review, see Ref. 23), it has been generally assumed that glycine substitutions prevent adequate folding of all collagens by dominant-negative interference. We now demonstrate that collagen VII presents an exception to this rule in that not all glycine substitutions lead to abnormal folding of the molecule. We show that naturally occurring glycine substitutions in the polypeptide are a prerequisite for formation and secretion of only a fraction of the molecules are consistent with the above hypothesis. They also explain the ultrastructural observations of a reduced number and abnormal appearance of anchoring fibrils in the skin (Fig. 5). In concurrence, a quantitative ultrastructural study showed that, in DDEB skin, the number of anchoring fibrils was reduced to 0–30% of normal (37).

In view of the above considerations, the finding that glycine substitution G1519D did not interfere with folding and secretion of procollagen VII was unexpected. The evaluation of procollagen VII synthesis and secretion in keratinocytes of proband 4 was comparable to that of controls, an observation consistent with the lack of a clinical phenotype in the proband. The mutation obviously causes pathologic consequences only in combination with another gene defect, as exemplified by the daughter of proband 4. The child was compound heterozygous for G1519D and a second maternal glycine substitution and presented with skin blistering at birth.2 Clinical and genetic
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The substitution was localized close to the amino terminus of the collagen XI triple helix. It was important. The silent glycine substitution was that the position of the glycine substitution within the collagen XI triple helix was important. The silent glycine substitution within a long uninterrupted triple helical segment is indicated by the arrow. The location of the G1519D substitution within a long uninterrupted collagenous segment or close to the amino terminus of the collagen molecule may have allowed incorporation of mutated molecules into collagen fibrils and permitted some residual function. Like collagen VII, collagen XI is not a classical fibrillar collagen consisting of uninterrupted -Gly-X-Y- repeat sequences, but has an interrupted triple helix and globular domains. Therefore, the rules found for the effects of glycine substitutions in fibrillar collagens may not apply without exception to other collagen types, and it is likely that further examples of mutations causing unexpected biological and clinical phenotypes will be found when new defects in other collagen genes are disclosed.

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