Molecular Mechanisms of Junctional Epidermolysis Bullosa: Col15 Domain Mutations Decrease the Thermal Stability of Collagen XVII

Laura Väisänen,*† Cristina Has,‡ Claus Franzke,‡ Tiina Hurskainen,*† Marja-Leena Tuomi,§ Leena Bruckner-Tuderman,‡ and Kaisa Tasanen*†

*Department of Dermatology, University of Oulu, Oulu, Finland; †Clinical Research Center, Oulu University Hospital, Oulu, Finland; ‡Department of Dermatology, University of Freiburg, Freiburg, Germany; §Department of Dermatology, University of Tampere, Tampere, Finland

Mutations in the collagen XVII gene, *COL17A1,* are associated with junctional epidermolysis bullosa. Most *COL17A1* mutations lead to a premature termination codon (PTC), whereas only a few mutations result in amino acid substitutions or deletions. We describe here two novel glycine substitutions, G609D and G612R, and a splice site mutation resulting in a deletion of three Gly–X–Y amino acid triplets. In order to investigate the molecular pathomechanisms of non-PTC mutations, G609D and G612R and two previously known substitutions, G627V and G633, and deletion of the amino acids 779–787 were introduced into recombinant collagen XVII. The thermal stability of the mutated collagens was assessed using trypsin digestions at incremental temperatures. All the four glycine substitutions significantly destabilized the ectodomain of collagen XVII, which manifested as 16°C–20°C lower T_m (midpoint of the helix-to-coil transition). These results were supported by secondary structure predictions, which suggested interruptions of the collagenous triple helix within the largest collagenous domain, Col15. In contrast, deletion of the three full Gly–X–Y triplets, amino acids 779–787, had no overall effect on the stability of the ectodomain, as the deletion was in register with the triplet structure and also generated compensatory changes in the NC15 domain.

Key words: basement membrane/BP180/collagen/epidermolysis bullosa/hemidesmosome
J Invest Dermatol 125:1112–1118, 2005

Collagen XVII belongs to the family of collagenous transmembrane proteins, which share a number of structural features and are involved in a broad spectrum of biological functions, ranging from cell adhesion to immunological defence (for a review, see Franzke *et al*, 2005). Collagen XVII, also referred to as the 180-kDa bullous pemphigoid antigen or BP180, is a structural component of hemidesmosomes, i.e., multiprotein complexes that anchor epithelial cells, such as the basal keratinocytes, to the underlying basement membrane (Borradori and Sonnenberg, 1999). The molecule has a type II orientation in the plasma membrane and it is involved in multiple intra- and extracellular protein–protein interactions (Giudice *et al*, 1992; Schäcke *et al*, 1998; Tasanen *et al*, 2000a, 2004, see Van den Bergh and Giudice, 2003). The globular intracellular domain resides in the cytoplasmic plaque of hemidesmosomes, and the large, extracellular collagenous domain is located in the region of anchoring filaments in the basement membrane. The ectodomain has significant thermal stability and is proteolytically released from the cell surface by ADAMs (a disintegrin and metalloproteinase; Tasanen *et al*, 2000a; Areida *et al*, 2001; Franzke *et al*, 2002, 2004).

Abbreviations: Δ779–787, deletion of amino acids 779–787; JEB, junctional epidermolysis bullosa; NC, non-collagenous sequence; PTC, premature termination codon

Junctional epidermolysis bullosa (JEB) consists of a group of autosomal recessive bullous disorders caused by mutations in the genes for laminin 5, α6β4 integrin, and collagen XVII (Fine *et al*, 2000; Bruckner-Tuderman, 2002). Characteristic morphological abnormalities caused by mutations in *COL17A1* include rudimentary hemidesmosomes and separation of the basal keratinocyte from the underlying basement membrane (Fine *et al*, 2000; Bauer and Lanschuetzer, 2003). The majority of *COL17A1* defects are nonsense mutations, or small insertions or deletions. They result in a premature termination codon (PTC), absence of collagen XVII in the skin and phenotypic features, such as generalized, life-long blistering of skin and mucous membranes, skin atrophy, total alopecia, nail dystrophy, and dental abnormalities (McGrath *et al*, 1995; Floeth *et al*, 1998; Pulkkinen *et al*, 2002 and references therein). In contrast, missense mutations or amino acid deletions are more rare (see Bauer and Lanschuetzer, 2003; Van den Bergh and Giudice, 2003). The mutations G627V (McGrath *et al*, 1996) and G633D (Tasanen *et al*, 2000b) and an in-frame deletion of the amino acids 779–787 (Chavanas *et al*, 1997) affect the largest collagenous domain, Col15, of collagen XVII and cause skin fragility and dystrophies in nails and teeth.

We describe two new glycine substitutions, G609D and G612R, which also target the Col15 domain of collagen XVII. In addition, we report a novel carrier of the in-frame

deletion of the amino acids 779–787. To investigate the consequences of these non-PTC mutations at the molecular level, we used recombinant expression of full-length collagen XVII and analyzed the effects of the mutations on the thermal stability of the ectodomain.

Results

Identification and verification of mutations Patient 1 **(**Fig 1*A***)** was known to be compound heterozygous for a c.3781C > T transition, but his maternal mutation remained unknown (Floeth *et al*, 1998). Repeated mutation screening revealed the second mutation, a c.1826G > A transition, which results in a Gly to Asp substitution at the amino acid position 609 and was designated as p.G609D. Verification by *Ita*I endonuclease digestion showed that the patient and his mother were heterozygous for this mutation **(**Fig 1*A***)**. By restriction digestion with *Ita*I, this sequence variant could not be identified in 100 control alleles. Patient 2 **(**Fig 1*B***)** was homozygous for a transition c.1834G > A, which causes a Gly to Arg substitution at the amino acid position 612 and was designated as p.G612R. This sequence variant was not identified in 100 control alleles. Automated

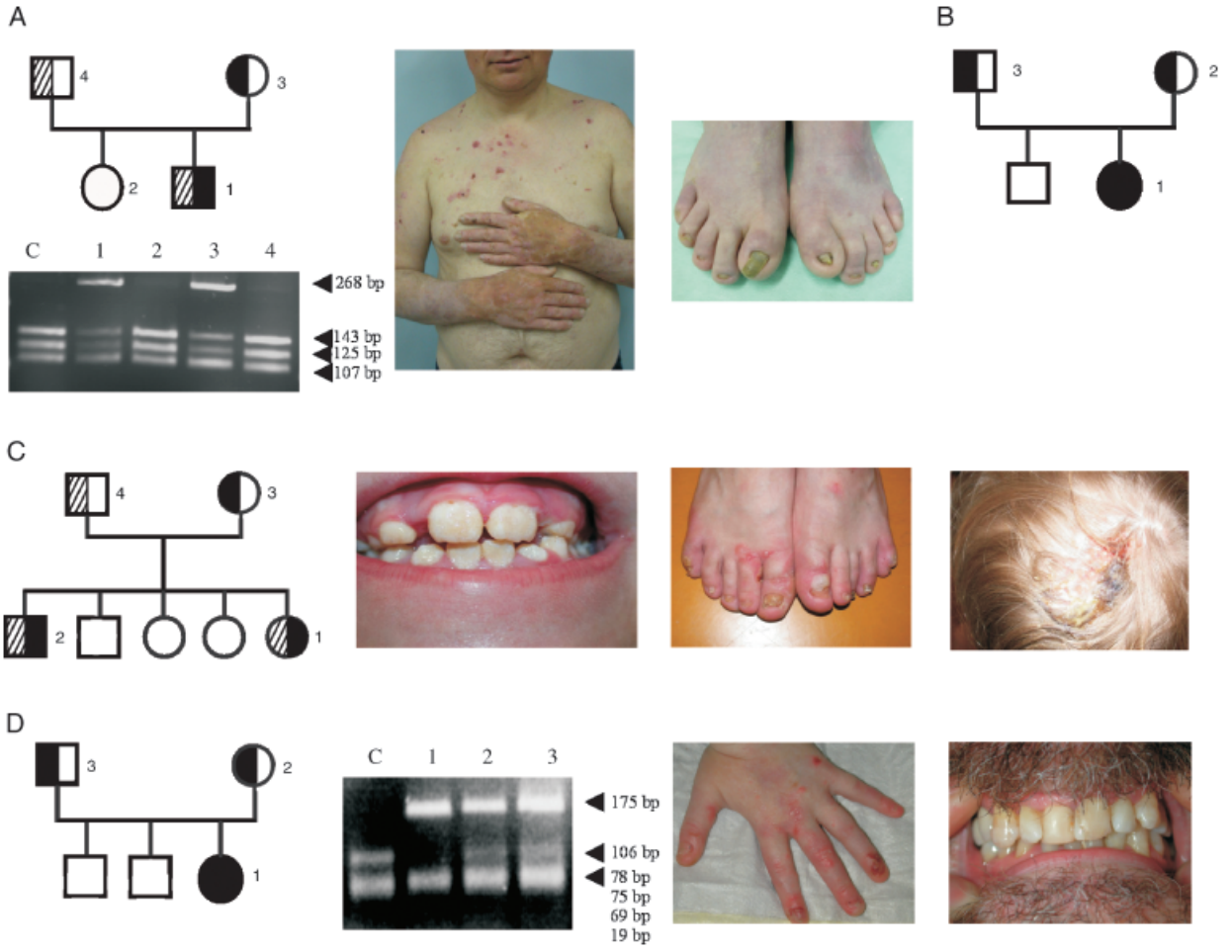

Figure 1
Pedigrees and mutations in the COL17A1 gene. Pedigrees demonstrate recessive inheritance of the mutations. (*A*) Family 1: patient 1 was a compound heterozygous for the mutations c.3781C → T (*striped areas*) and c.1826G > A (*black areas*). Restriction digestion with *Ita*I shows in the sister (2) and the father (4) 143, 125, and 107 bp bands corresponding to the wild-type allele (C). The patient (1) and his mother (3) show 268, 143, 125, and 107 bp bands corresponding to their heterozygous states. The generalized mild blistering and toenail dystrophy of the proband are also illustrated. (*B*) Family 2: patient 2 (1) was homozygous for the transition c.1834G > A in exon 22 (*black areas*). The mutation was present in a heterozygous state in both the mother and the father, but was absent from the controls. (*C*) Family 3: patients 3 (1) and 4 (2) were heterozygous for a duplication of C at the position 3800 in exon 52 (*striped areas*) and the mutation at the position c.1834G > A, at the last exonic position before intron 22 (*black areas*). To verify if the transition c.1834G > A disturbs normal splicing, RT-PCR with total RNA extracted from keratinocytes of patient 3 was performed using the exonic primer spanning the exons 17–23. Direct sequencing indicated normal splicing and homozygocity for c.1834G > A (not shown). These findings show that c.1834G > A is a missense mutation changing the codon 612 GGA to AGA (p.G612R), and that the frameshift mutation in exon 52 leads to nonsense-mediated RNA decay. The mother (3) was heterozygous for the c.1834 G > A transition and the father (4) for c.3800dupC. The dental abnormalities, toenail dystrophy, and a poorly healing wound area on the scalp of patient 3 are also shown. (*D*) Patient 5 was homozygous for a splice site mutation c.2336-2A > G (*black areas*), which results in in-frame skipping of the entire exon 32. This mutation was absent from the control, but the mother (2) and the father (3) were both heterozygous. A restriction site for *Pst*I was eliminated by the mutation c.2336-2A > G. The 347 bp normal control PCR product was cleaved into 106 bp (*middle band*), 78, 75, 69, and 19 bp products (not separated, can be seen as a lower band). The digestions of amplimers of the proband (1), the mother (2), and the father (3) resulted in an additional uncleaved 175 bp product (*upper band*), which reflects the loss of the *Pst*I restriction site because of an A to G mutation. The acral blistering and fingernail dystrophy of the proband as well as the dental anomalies of the father are also shown.

sequence analysis demonstrated that the parents were heterozygous. Patients 3 and 4 **(**Fig 1*C***)** were compound heterozygous for the same c.1834G>A transition as patient 2 and for a duplication in exon 52, c.3800dupC, which causes a frameshift, a PTC, and non–sense-mediated RNA decay, as indicated by RT-PCR with total RNA extracted from keratinocytes of patient 3. The mother was heterozygous for c.1834G>A and the father for c.3800dupC. Patient 5 (Fig 1*D*) had a homozygous splice site mutation c.2336-2A>G, which results in an in-frame skipping of the entire exon 32 (Chavanas *et al*, 1997). This leads to deletion of the amino acids 779–787 (Δ779–787). The mutation c.2336–2A>G eliminated the restriction site for *Pst*I, and endonuclease digestions verified the patient to be homozygous and both parents to be heterozygous for this mutation.

Glycine substitution mutations decrease the thermal stability of the collagen XVII ectodomain The two novel glycine substitutions, G609D and G612R, and G627V (McGrath *et al*, 1996) and G633D (Tasanen *et al*, 2000b), and Δ779–787 were introduced into the expression vector coding for full-length collagen XVII (Franzke *et al*, 2004). The mutated recombinant collagens were expressed transiently in COS-7 cells, which have recently been found suitable for studying collagen XVII synthesis and triple-helix formation (Franzke *et al*, 2004). The transmembrane and shed forms of collagen XVII were found in cell extracts and media, respectively, indicating that the mutated variants were expressed and shed from the cell surface (data not shown). After immunoprecipitation of the ectodomain, its triple-helical structure and thermal stability were assessed using limited trypsin digestion as probe. Similar brief protease digestions have been previously used to assay the effects of glycine substitutions on the thermal stability of α-chains of other collagen types (Tromp *et al*, 1989; see Prockop, 1990; Byers, 2001 and references therein). Both wild-type and mutated shed ectodomains of collagen XVII (120 kDa) were degraded into trypsin-resistant intermediates of 90 kDa at 4°C already, but in further digestions, the intermediate fragments lost their stabilities at different temperatures (Fig 2). The 90 kDa fragment was recognized by the NC16A antibody (Fig 2), indicating that the Col15 domain, located C-terminally to the NC16A domain and, at least, a part of the NC16A domain, were present in the intermediate fragment. The mutated ectodomains with the glycine substitutions G609D and G612D started to unfold at 24°C and lost their stability to 50% at 28°C. The mutated ectodomains with G627V and G633D started to unfold at 22°C and lost their stability to 50% at 24°C. Thus, the glycine substitution mutations destabilized significantly the ectodomain of collagen XVII. In contrast, the melting ranges of the wild-type ectodomain, the deletion mutant Δ779–787, and the ectodomain with valine substitution V703M were very similar (Fig 2). These results indicate that the deletion of nine amino acids, or three Gly–X–Y triplets, and the substitution of another amino acid than glycine had no effect on the thermal stability of the collagen XVII ectodomain.

Structural predictions of the consequences of Gly substitutions The substitution of the smallest amino acid, glycine, with other amino acids within a collagenous sequence can interfere with its triple-helical structure, leading to a disturbed, non-functional collagen molecule. Similarly, sequence alterations within the collagenous domain caused by deletions or insertions of amino acids can disrupt its typical Gly–X–Y triplet sequence and damage the triple-helical tertiary structure. The conformational consequences of the glycine substitutions and Δ779–787 were analyzed using secondary structure prediction computer programs PSAAM (University of Illinois), the PredictProtein Server (CUBIC, Biochemistry, Columbia University, New York), and the Ambivalent Structure Predictor (Young *et al*, 1999), in combination with the program SEG (Wootton and Federhen, 1996), which divides sequences into low-complexity ("simple") and high-complexity segments that can be used for the identification of specific structural alterations within the protein sequence.

The collagen XVII stretch selected for analysis spanned the amino acid residues 490–810, which contain the NC16A, the Col15, and the NC15 domains. Wild-type collagen XVII was predicted to have three major α-helical elements in the NC16A domain, an all-over loop (random coil) formed by the Col15 domain, representing the collagenous domain, and the NC15 domain with a short β-sheet portion (Fig 3). The structure predictions of G609D and G612R revealed one disruption of the all-over Col15 loop structure

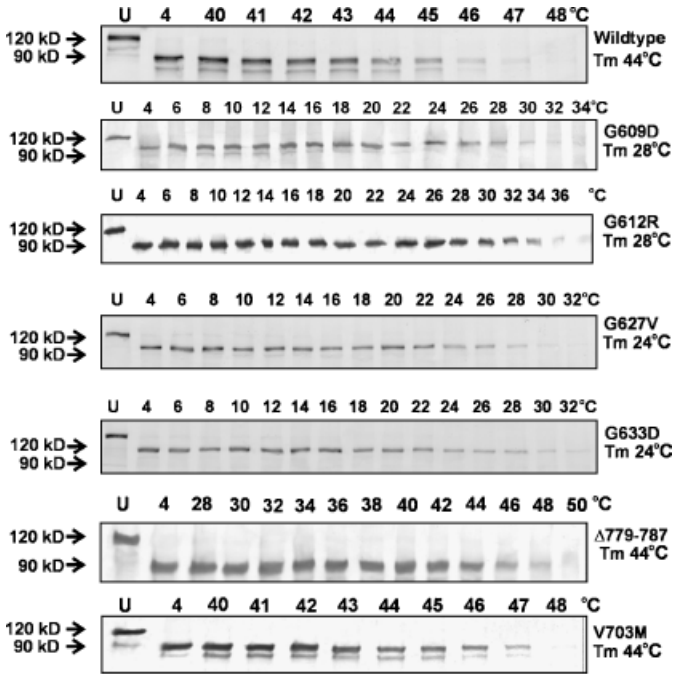

Figure 2
Trypsin digestions of recombinant wild-type and mutated ectodomains. The immunoprecipitated shed ectodomain was treated with trypsin for 2 min at incremental temperatures between 4°C and 50°C. Quantitation of the immunoblot signals obtained with the NC16A antibody showed that the treatment first resulted in a 90 kDa intermediate digestion product, whose stability was reduced by substituting the Gly residue at the positions 609, 612, 627, or 633. The wild-type ectodomain lost its stability by about 50% at 44°C. The ectodomains with the glycine substitutions G609D or G612R lost about 50% of their stability at 28°C, whereas the substitutions G627D or G633D resulted in a T_m of 24°C. The deletion of the amino acids 779–787 (Δ779–787) and the substitution V703M had no detectable effect on the thermal stability of the ectodomain. (U, undigested sample).

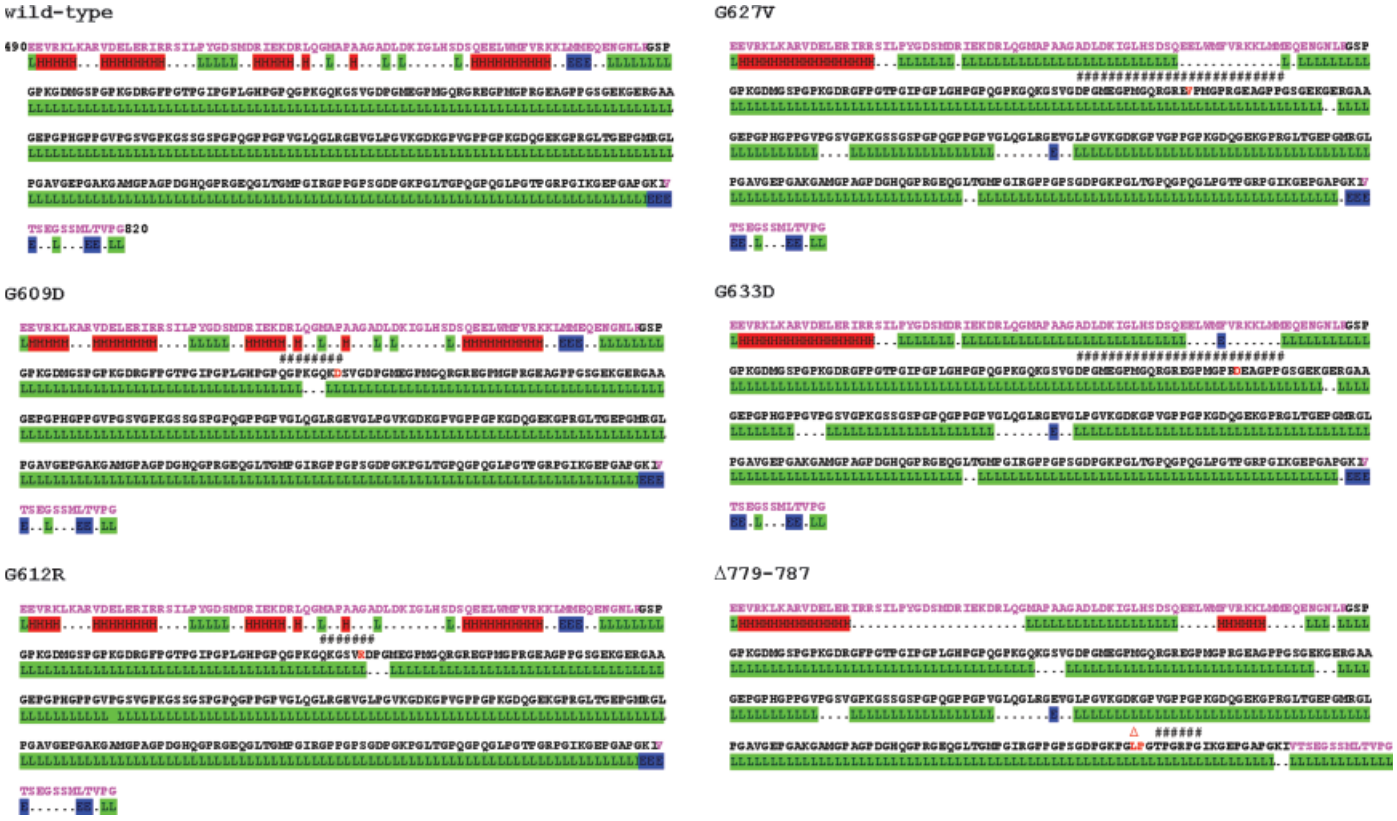

Figure 3
Structural predictions of the extracellular juxtamembranous part of the wild-type and mutated collagen XVII (amino acids 490–810). Secondary protein structure prediction based on the amino acid sequence and the comparison of known protein structures with similar amino acid sequences was performed as described in Results. The collagen XVII stretch (amino acids 490–810) including the NC16A, the Col15, and the NC15 domains was analyzed for wild-type collagen XVII and the mutants. The amino acids are shown in a single-letter code, the magenta characters represent the NC16A and NC15 domains, and the black characters stand for the Col15 domain. The lower row indicates the secondary structure predictions α-helix (*red boxed H*), β-sheet (*blue boxed E*), and loop (*green boxed L*) with an expected average accuracy of > 82%. For residues with secondary structure predictions of lower confidence, no prediction was made, and a dot (.) was used instead. Differences in the distribution of high-complexity regions within the amino acid sequence of the wild-type collagen XVII and the mutated forms are indicated with #. The prediction of the wild-type collagen XVII showed three major α-helical elements in the NC16A domain, an all-over loop formed Col15 domain, representing the collagenous triple helices, and the NC15 domain with a slight β-sheet portion.

in close proximity to the mutated amino acid, which was internally confirmed by the occurrence of new high-complexity regions in the same areas. In contrast, the structure predictions of G627V and G633D showed more severe structural changes within the Col15 domain. Both predictions exhibited four disruptions, which were highly similar, within the loop structure and larger part of a new generated high-complexity region around the mutated amino acid. In addition, structural changes also occurred in the NC16A domain. The structural prediction of Δ779–787 results in four disruptions of the all-over Col15 loop and the generation of a small new high-complexity region situated C-terminally of the deletion. In this case, additional structural changes were also predicted in both the NC16A and the NC15 domain (Fig 3).

Discussion

Mutations in the fibrillar collagen types I, II, III, IX, X, and XI cause a wide spectrum of diseases such as osteogenesis imperfecta, some subtypes of Ehlers–Danlos syndrome, and a variety of chondrodysplasias. In these collagens, substitutions for glycine residues in the triple-helical domains are among the most common effects of mutations, and the substituting residue and its location in the chain contribute to the effect on folding and also on the phenotype (Prockop, 1990; Kuivaniemi *et al*, 1997; Byers, 2001; Persikov *et al*, 2004). Glycine substitutions in dystrophic epidermolysis bullosa are located within the triple helix of the type VII collagen gene *COL7A1* and disturb protein folding by dominant-negative interference (Bruckner-Tuderman, 2002; Mallipeddi *et al*, 2003). In the group of transmembrane collagens, mutations resulting in glycine substitutions have been detected in the human *COL17A1* gene and the human *EDA-1* (ectodysplasin A) gene (see Franzke *et al*, 2005). In *COL17A1*, mutations leading to glycine substitutions are rare, and all the known glycine substitutions affect sequences in the largest collagenous domain, Col15, of collagen XVII (see Table I).

These data indicate that the Col15 domain, which contains 232 amino acids (amino acids 567–808), contributes significantly to the stability of the collagen XVII homotrimer. We have previously shown that this domain alone, when expressed as a eukaryotic recombinant fragment, formed a triple helix with a T_m of 26.5°C (Tasanen *et al*, 2000a). A longer fragment of 281 amino acids, which contained both Col15 and the adjacent 49 amino acids of the NC16A

Table I. Collagen XVII gene (COL17A1) mutations leading to glycine substitutions or in-frame deletions in the largest collagenous domain Col15

Patient	Age/ sex	COL17A1 mutation	Consequences	Skin blistering	Scalp skin/hair	T_m of the mutated ectodomain (°C)	Secondary structure predictions	Reference
1	40/M	1826G>A/3781C>T	G609D/PTC	Mild acral	Normal	28	One Col-15 disruption	Väisänen *et al* (this study)
2	2/M	1834G>A/1834G>A	G612R/G612R	Mild generalized	NA	28	One Col-15 disruption	Väisänen *et al* (this study)
3	8/F	1834G>A/3800dupC	G612R/PTC	Mild generalized	Partial alopecia	28	One Col-15 disruption	Väisänen *et al* (this study)
4	24/M	1834G>A/3800dupC	G612R/PTC	Mild generalized	Partial alopecia	28	One Col-15 disruption	Väisänen *et al* (this study)
5	4/F	2336-2A>G/2336-2A>G	Inf.del/Inf.del	Mild acral	Normal	44	Four Col-15 disruptions NC16A changes NC15 changes	Väisänen *et al* (this study)
6	50/F	3514ins25/1985G>A	PTC/G627V	Moderate generalized	Partial alopecia	24	Four Col-15 disruptions NC16A changes	McGrath *et al* (1996)
7	13/M	538C>A/2003G >A	PTC/G633D	Mild acral/ generalized	Normal	24	Four Col-15 disruptions NC16A changes	Tasanen *et al* (2000b)
8	28/F	2336-2A>G/2336-2A>G	Inf.del/Inf.del	Mild generalized	Total alopecia	44	Four Col-15 disruptions NC16A changes NC15 changes	Chavanas *et al* (1997)

PTC, premature termination codon; Inf. del., in-frame deletion of amino acids 779–787; NA, not available.

domain, formed a triple helix with a melting temperature of 35°C (Areida *et al*, 2001). This difference is feasible, as the NC16A domain contains coiled–coiled structures, which are likely to serve as a nucleus for trimerization and subsequent triple-helix formation (Areida *et al*, 2001). Our recent studies indicated that recombinantly expressed full-length collagen XVII forms a stable triple-helix, with a T_m of 43°C, and that the shed ectodomain serves as a suitable model for studies on protein conformation and stability (Franzke *et al*, 2004). The above observations demonstrate that similar to other collagens, the triple-helix stability of transmembrane collagen XVII depends on the total length of the collagenous domain. For this reason, we opted to engineer mutations into the full-length collagen XVII. This approach allowed us to perform conformational analyses on stable recombinant proteins and to avoid putative complications because of the relative conformational instability of short collagenous fragments.

Another evidence of the importance of the Col15 domain for the stability of the collagen XVII comes from mutation studies. Two previously described glycine substitution mutations, G627V (McGrath *et al*, 1996; Table I, patient 6) and G633D (Tasanen *et al*, 2000b; Table I, patient 7), occurred in combination with null alleles in compound heterozygous patients. Therefore, they were functionally homozygous and resulted in the production of homotrimeric mutated collagen XVII molecules *in vivo*. The hypothesis is that the mutations may lead to inhibition of physiological ligand binding or proteolytic degradation of the mutant protein and to skin blistering. When these glycine substitutions were generated into a recombinantly expressed Col15 or full-length ectodomain, the thermal stability of the Col15 domain was significantly decreased (Olague-Marchan *et al*, 2000; Tasanen *et al*, 2000a, b).

Also, the two novel glycine substitutions of this study, G609D and G612R, occurred either in a homozygous state (patient 2) or in combination with a PTC (patients 1, 3, and 4). Thus, all carriers known so far are either homozygous or functionally hemizygous for glycine substitutions. This allowed us to utilize homotrimers of the mutated recombinant collagen XVII to study the molecular abnormalities related to these mutations. An examination of the four recombinants with glycine substitutions showed their thermal stability to be significantly reduced, whereas the valine substitution V703M had no effect on the T_m of the ectodomain. The glycine susbtitutions located more aminoterminally, G609D and G612R, reduced T_m slightly less than the mutations G627V and G633D, which are situated more centrally in the Col15 domain. These results are in good accordance with the secondary structure predictions, which revealed that G609D and G612R caused only one interception within the Col15 domain, but no variances in the non-collagenous domains NC16A and NC15, whereas G627V and G633D resulted in four disruptions within the Col15 loop structure and additional changes in NC16A domain. In accordance with previous studies by us (Schäcke *et al*, 1998; Franzke *et al*, 2004) and others (Areida *et al*, 2001), short protease treatment of triple-helical collagen XVII led to its C-terminal truncation, whereas at least part of the NC16A domain remained uncleaved. Possible reasons of this cleavage protection may be because of the structural features of the

ternary NC16A structure, which is influenced by the triple-helical collagenous Col15 domain.

The consequences of the "in-register" deletion Δ779–787 are particularly interesting. Unexpectedly, this had no effect on thermal stability of its molecule. The structural predictions indicated on the one hand changes similar to those caused by G627V and G633D. On the other hand, Δ779–787 caused additional structural variance within the non-collagenous NC15 domain, i.e., formation of predominantly all-over loop structure, instead of the β-sheet structure in the wild type (Fig 3). Therefore, it is likely that the altered NC15 domain compensates deleterious effects in the Col15 domain and thereby contributes to a normal thermal stability. In this case, it seems feasible that the effects of the deletion, which are observed in JEB, involve perturbed ligand interactions rather than triple helix destabilization.

The mutations causing alterations in the Col15 domain of collagen XVII, the clinical presentation of eight probands and results from trypsin digestions and secondary structure predictions are summarized in Table I. Compared with the *COL17A1* null mutations, the phenotypes associated with the Col15 domain mutations are milder, with acral or generalized mild/moderate blistering of the skin, but without major mucosal involvement. In three of the patients the blistering of the skin was localized mainly to acral body parts like hands and feet, which are usually more prone to mechanical traumas than central parts of the body. Similar to null mutations, all carriers of Col15 domain mutations had nail dystrophy and dental abnormalities. Hair involvement was observed in four patients, but only one proband had total alopecia, which is typical of *COL17A1* null mutations. In summary, mutations affecting the Col15 domain are associated with milder phenotypic changes, but an unequivocal correlation between the type or localization of the mutation and the clinical phenotype remains elusive. Further analysis of mutations and their biological consequences are required to determine whether only *COL17A1* mutations, or also putative modifying genes, are involved in determination of the phenotype.

Materials and Methods

Patients

Patient 1 The 40-y-old male was earlier found to be compound heterozygous for a c.3781C → T transition designated as p.R1226X (Floeth *et al*, 1998). At that time, the second mutation was not found. The proband has had non-scarring, generalized, trauma-induced skin blistering since birth, and in the course of the disease, hyper- and hypopigmentation of skin and nail dystrophy have developed (Fig 1*A*).

Patient 2 The newborn male is the second child of consanguineous Turkish parents. The family history includes a great aunt with skin blisters in childhood. The proband had blisters and erosions on his hands and feet at birth and later also on his trunk and ears. Electron microscopy revealed junctional blistering with intact keratinocytes in the blister roof and normal lamina densa and anchoring filaments in the blister base. Antigen mapping of spontaneously blistered skin showed binding of antibodies to cytokeratins, α6β4 integrin to the blister roof, and laminin 5 and collagens IV and VII to the blister base. The stainings for collagen XVII and plectin were reduced and fragmented. For antigen mapping, the rabbit anti-human collagen XVII antibody directed against the NC16A domain (Schumann *et al*, 2000) and the monoclonal mouse BP180/collagen XVII antibody MoAb 123 (kind gift from Dr P. Marinkovich, Stanford) were used. The other antibodies for antigen mapping were described elsewhere (Schumann *et al*, 2000).

Patients 3 and 4 Patient 3 is an 8-y-old girl, the youngest child of healthy, non-consanguineous Finnish parents. She had rather severe blistering at birth already. Generalized moderate blistering continues, and she also has nail dystrophy, dental problems, poorly healing wounds, and partial alopecia of the scalp (Fig 1*C*). Immunohistochemical staining with basement membrane zone antibodies showed junctional blistering with reduced collagen XVII staining. Her 22-y-old brother, who is our patient 4, has had blisters since the neonatal period. His disease is milder than his sister's, but he also has partial alopecia and tooth and nail dystrophy. Based on clinical data and electron microscopic analysis in the early 1980s, he was thought to have epidermolysis bullosa simplex. Otherwise, the family history was negative for blistering skin diseases. The parents have no dental anomalies.

Patient 5 The proband, a 4-y-old German female, is the third child of non-consanguineous parents. The family history is negative for skin diseases. Immediately after birth, she developed blisters on hands. Blistering continued, especially on the extremities. She has both nail and dental dystrophies, and her parents also have dental anomalies (Fig 1*D*). Electron microscopy revealed junctional blistering with intact keratinocytes in the blister roof and normal lamina densa and anchoring filaments in the blister base. Immunohistochemical staining with basement membrane zone antibodies showed junctional blistering with negative collagen XVII staining.

Mutation detection and RT-PCR The study was approved by the ethical committee of the University of Freiburg and conducted according to the declaration of Helsinki principles. After informed consent, genomic DNA was isolated from the patients' peripheral blood using the QIAamp Blood Kit (Qiagen, Hilden, Germany). PCR amplification of all *COL17A1* exons and exon–intron boundaries was performed as described by Gatalica *et al* (1997; Genbank Nr. M91669); the primers used for the amplification of exons 21, 22, and 32 are shown in Table S1. Purified PCR products were sequenced in both directions by using standard methods and an ABI310 sequencer (Applied Biosystems, Foster City, California). The nucleotide positions of the detected mutations were numbered by setting the first nucleotide of the starting methionine codon as 1. To verify if the mutation c.1834G > A disturbs the normal splice site, total RNA was extracted from the keratinocytes of patient 3 and RT-PCR for exons 17–23 was performed using primers shown in Table S1. Excepting the mentioned mutations, some other sequence variants were found in the patients, all of them being known polymorphisms (www.ncbi.nlm.nih.gov/SNP/) like the G > A transition at nucleotide position 2212 resulting in p.V703M in the middle of Col15 domain (Schumann *et al*, 1997). The other clinically observed Col15 domain substitutions and deletions were obtained from the online mutation databases (Human Gene Mutation Database; www.hgmd.org) for the *COL17A1* gene in December 2004.

Generation, expression, and analysis of recombinant mutated collagen XVII The glycine substitution mutations G612R, G627V, and G633D, and the deletion of the amino acids 779–787 were accomplished to the full-length human collagen XVII cDNA (GenBank accession number M911669, see Franzke *et al*, 2004) by using the QuikChange Site-Directed Mutagenesis kit (Stratagene Europe, Amsterdam, the Netherlands) and the sense and antisense primers containing the desired mutations shown in Table S2. The G609D and V703M (Schumann *et al*, 1997) were generated by using The GeneTailor Site-Directed Mutagenesis System (InVitrogen Europe, Leek, the Netherlands) and the overlapping primers shown in Table S2. The generation of the desired point mutations and the deletion were confirmed by DNA sequencing.

The wild-type and mutated constructs were expressed in COS-7 cells in the presence of 50 μg per mL ascorbic acid as described earlier (Franzke *et al,* 2004). After immunoprecipitations of cell culture medium, the triple-helical conformation of the ectodomains was tested using trypsin as probe (see Franzke *et al*, 2004). After immunoblotting with the NC16A antibody (Schumann *et al*, 2000), the scanning and the quantitation of the digestion products were performed by Quantity One software (Bio-Rad, Hercules, California).

We are grateful to Luca Borradori (Department of Dermatology, University Hospital Geneva, Switzerland) for collagen XVII cDNA and to Margit Schubert, Michaela Daamen, Gabriele Grüninger, Seija Leskelä, and Anja Mattila for excellent technical assistance. This work was supported in part by grants from the Kainuu branch of Finnish Cultural Foundation to L. V., from the Commission of the European Communities (Contract Nr. QLG1-CT-2001-02007), from DFG (German Research Council, grants SFB 620-C5 and Br 1475/6–3) to L. B.-T., and from the Academy of Finland, the Alexander von Humboldt Foundation, and Oulu University Hospital to K. T.

Supplementary Material

The following material is available online for this article.
Table S1. Primers used for mutation screening
Table S2. Primers used for site-directed mutagenesis

DOI: 10.1111/j.0022-202X.2005.23943.x

Manuscript received January 19, 2005; revised June 29, 2005; accepted for publication July 22, 2005

Address correspondence to: Kaisa Tasanen, Department of Dermatology, University of Oulu, Aapistie 5 A FIN-90230, Oulu, Finland. Email: kaisa.tasanen@oulu.fi

References

Areida SK, Reinhardt DP, Müller PK, Fietzek PP, Kowitz J, Marinkovich MP, Notbohm H: Properties of the collagen type XVII ectodomain. Evidence for N- to C-terminal triple helix folding. J Biol Chem 276:1594–1601, 2001

Bauer JW, Lanschuetzer C: Type XVII collagen gene mutations in junctional epidermolysis bullosa and prospects for gene therapy. Clin Exp Dermatol 28:53–60, 2003

Borradori L, Sonnenberg A: Structure and function of hemidesmosomes: More than simple adhesion complexes. J Invest Dermatol 112:411–418, 1999

Bruckner-Tuderman L: Epidermolysis bullosa. In: Royce P, Steinmann B (eds). *Connective Tissue and its Heritable Disorders. Molecular, Genetic, and Medical Aspects*. New York: John Wiley and Sons, 2002; p 687–725

Byers PH: Folding defects in fibrillar collagens. Philos Trans R Soc Lond B 356:151–158, 2001

Chavanas S, Gache Y, Tadini GL, Pulkkinen L, Uitto J, Ortonne JP, Meneguzzi G: A homozygous in-frame deletion in the collagenous domain of bullous pemphigoid antigen BP180 (type XVII collagen) causes generalized atrophic benign epidermolysis bullosa. J Invest Dermatol 109:74–78, 1997

Fine J-D, Eady RAJ, Bauer RR, *et al*: Revised classification system for inherited epidermolysis bullosa: Report of a second international consensus meeting on diagnosis and classification of epidermolysis bullosa. J Am Acad Dermatol 42:1051–1066, 2000

Floeth M, Fiedorowicz J, Schäcke H, Hammani-Hauasli N, Owaribe K, Trüeb RM, Bruckner-Tuderman L: Novel homozygous and compound heterozygous mutations associated with junctional epidermolysis bullosa. J Invest Dermatol 111:528–533, 1998

Franzke CW, Bruckner P, Bruckner-Tuderman L: Collagenous transmembrane proteins. Recent insights into biology and pathology. J Biol Chem 280: 4005–4008, 2005

Franzke CW, Tasanen K, Borradori L, Huotari V, Bruckner-Tuderman L: Shedding of collagen XVII/BP180: Structural motifs influence cleavage from cell surface. J Biol Chem 279:24521–24529, 2004

Franzke C, Tasanen K, Schäcke H, *et al*: Transmembrane collagen XVII, an epithelial adhesion protein, is shed from the cell surface by ADAMs. EMBO J 21:5026–5035, 2002

Gatalica B, Pulkkinen L, Li K, Kuokkanen K, Ryynänen M, McGrath J, Uitto J: Cloning of the human type XVII collagen gene (COL17A1), and detection of novel mutations in generalized atrophic benign epidermolysis bullosa. Am J Hum Genet 60:352–365, 1997

Giudice GJ, Emery DJ, Diaz LA: Cloning and primary structural analysis of the bullous pemphigoid antigen BP180. J Invest Dermatol 99:243–250, 1992

Kuivaniemi H, Tromp G, Prockop DJ: Mutations in fibrillar collagens (types I, II, III, and XI), fibril associated collagen (type IX), and network-forming collagen (type X) cause a spectrum of diseases of bone, cartilage, and blood vessels. Hum Mutat 9:300–315, 1997

Mallipeddi R, Bleck O, Mellerio JE, Ashton GHS, Eady RAJ, McGrath JA: Dilemmas in distinguishing between dominant and recessive forms of dystrophic epidermolysis bullosa. Br J Dermatol 149:810–818, 2003

McGrath JA, Gatalica B, Christiano AM, *et al*: Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa. Nat Genet 11:83–86, 1995

McGrath JA, Gatalica B, Li K, *et al*: Compound heterozygosity for a dominant glycine substitution and a recessive internal duplication mutation in type XVII collagen gene results in junctional epidermolysis bullosa and abnormal dentition. Am J Pathol 148:1787–1796, 1996

Olague-Marchan M, Twining SS, Hacker MK, McGrath JA, Diaz LA, Giudice GJ: A disease associated glycine substitution in BP180 (type XVII collagen) leads to a local destabilization of the major collagen triple helix. Matrix Biol 19:223–233, 2000

Persikov AV, Pillitteri RJ, Amin P, Schwarze U, Byers PH, Brodsky B: Stability related bias in residues replacing glycines within the collagen triple helix (Gly–Xaa–Yaa) in inherited connective tissue disorders. Hum Mutat 24:330–337, 2004

Prockop DJ: Mutations that alter the primary structure of type I collagen. J Biol Chem 265:15349–15352, 1990

Pulkkinen L, Ringpfeil F, Uitto J: Progress in heritable skin diseases: Molecular bases and clinical implications. J Am Acad Dermatol 47:91–104, 2002

Schumann H, Hammani-Hauasli M, Pulkkinen L, *et al*: Three novel homozygous point mutations and a new polymorphism of the COL17A1 gene: Relation to biological and clinical phenotypes of junctional epidermolysis bullosa. *AMJ Hum Genet* 60:1344–1353, 1997

Schäcke H, Schumann H, Hammami-Hauasli N, Raghunath M, Bruckner-Tuderman L: Two forms of collagen XVII in keratinocytes: A full-length transmembrane protein and a soluble ectodomain. J Biol Chem 273:25937–25943, 1998

Schumann H, Baetge J, Tasanen K, Wojnarowska F, Schäcke H, Zillikens D, Bruckner-Tuderman L: The shed ectodomain of collagen XVII/BP180 is targeted by autoantibodies in different blistering skin diseases. Am J Pathol 156:685–695, 2000

Tasanen K, Eble JA, Aumailley M, *et al*: Collagen XVII is destabilized by a glycine substitution mutation in the cell adhesion domain Col15. J Biol Chem 275:3093–3099, 2000a

Tasanen K, Floeth M, Schumann H, Bruckner-Tuderman L: Hemizygosity for a glycine substitution in collagen XVII: Unfolding and degradation of the ectodomain. J Invest Dermatol 115:207–212, 2000b

Tasanen K, Tunggal L, Chometon G, Bruckner-Tuderman L, Aumailley M: Keratinocytes from patients lacking collagen XVII display a migratory phenotype. Am J Pathol 164:2027–2038, 2004

Tromp G, Kuivaniemi H, Stolle C, Pope FM, Prockop DJ: Single base mutation in the type III procollagen gene that converts the codon for glycine 883 to aspartate in a mild variant of Ehlers–Danlos syndrome IV. J Biol Chem 264:19313–19317

Van der Bergh F, Giudice GJ.: BP180 (type XVII collagen) and its role in cutaneous biology and disease. Adv Dermatol 19:37–71, 2003

Wootton JC, Federhen S: Analysis of compositionally biased regions in sequence databases. Methods Enzymol 266:554–571, 1996

Young MM, Kirshenbaum K, Dill KA, Highsmith S: Predicting conformational switches in proteins. Protein Sci 8:1752–1764, 1999