A Novel Mutation in the L12 Domain of Keratin 5 in the Köbner Variant of Epidermolysis Bullosa Simplex

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We have identified a novel mutation within the linker L12 region of keratin 5 (K5) in a family with the Köbner variant of epidermolysis bullosa simplex. The pattern of inheritance of the disorder in this family is consistent with an autosomal dominant mode of transmission. Affected individuals develop extensive and generalized blistering at birth or early infancy but in later years clinical manifestations are largely confined to palmoplantar surfaces. Direct sequencing of polymerase chain reaction products revealed a T to C transition within codon 323 of K5 in affected individuals, resulting in a valine to alanine substitution of the seventh residue within the L12 linker domain. This mutation was not observed in unaffected family members or in 100 K5 alleles of unrelated individuals with normal skin. The other critical regions of K5 and K14 were unremarkable in this family except for common polymorphisms that have been previously described. The valine at position 7 of the L12 domain is absolutely conserved in all type II keratins, and in other intermediate filament subunits as well, which suggests that this residue makes an important contribution to filament integrity. Secondary structure analysis revealed that alanine at this position markedly reduces both the hydrophobicity and the β-sheet nature of the L12 domain. This is the first report of a mutation at this position in an intermediate filament subunit and reinforces the importance of this region to filament biology. ***Key words: disease/genetics/intermediate filaments.*** ***J Invest Dermatol 111:524–527, 1998***

Several genetic disorders of the integument, characterized by cell cytolysis as a result of physical trauma, have been shown to be associated with keratin mutations (reviewed in Rothnagel, 1996). Electronmicroscopy of these disorders invariably reveals abnormal keratin filaments with tonofilament clumping and a collapsed keratin filament cytoskeleton, often with a perinuclear distribution (Anton-Lamprecht, 1994). Keratins are intermediate filament proteins that are expressed as coordinate pairs of type I and type II subunits in a tissue and differentiation-state specific manner (Steinert, 1993). Keratins have a conserved domain structure, which is common to all intermediate filament proteins, consisting of a central α-helical rod domain flanked by nonhelical N- and C-terminal domains (Steinert and Roop, 1988). The α-helical domain is interrupted by three nonhelical linker peptides denoted as L1, L12, and L2, which separate the rod domain into the 1A, 1B, 2A, and 2B helical segments. Mature keratin filaments are assembled from coiled-coil heterodimers, each consisting of a type I (keratins K9–K21) and a type II (K1–K8) subunit held together through interactions between the α-helical domains (Hatzfeld and Weber, 1990; Steinert, 1990). The dimers in turn polymerise both laterally and longitudinally to form the 10 nm filaments. Mutations in keratin subunits generally do not interfere with dimer formation or polymerization but instead act dominantly to reduce the tensile strength of the mature filament.

Epidermolysis bullosa simplex (EBS) is a rare genetic disease characterized by blistering of the skin and internal epithelia (Fine *et al*, 1991; Epstein, 1992). Blistering occurs as a result of cytolysis of basal layer keratinocytes that have a reduced capacity to withstand environmental insults such as mild mechanical trauma, increased temperature, sweating, or stress. The disorder is usually autosomal dominant but some recessive cases have been noted (Fine *et al*, 1991). Three clinical subtypes of EBS have been defined on the basis of phenotypic severity; Dowling–Meara, Köbner, and Weber–Cockayne (Fine *et al*, 1991). The Dowling–Meara type represents the severest form of the disease, the Köbner type an intermediate form, and the Weber–Cockayne variant the mildest, with blisters limited to surfaces that receive the most insults, such as the palms and soles. Other EBS subtypes have been defined on the basis of associated pathologies such as EBS with mottled pigmentation and EBS with muscular distrophy (Fine *et al*, 1991).

Early ultrastructural analyses first implicated the keratins as the site of the defect in EBS (Anton-Lamprecht, 1983), and later immunoelectron microscopy revealed that the aggregated filaments were composed of the basal cell keratins K5 and K14 (Ishida-Yamamoto *et al*, 1991). Experimental studies demonstrated that mutant forms of K14 could induce filament clumping, similar to that observed in EBS patients, when expressed in cultured cells or in the epidermis of transgenic mice (Albers and Fuchs, 1989; Vassar *et al*, 1991). Gene linkage data showed that EBS mapped to the keratin gene clusters on chromosomes 12 and 17 and mutations were subsequently identified in K5 and K14 in affected individuals (Epstein, 1992). Both patient studies and experimental data have shown a striking correlation between the position of the mutation and the severity of the clinical phenotype (Letai *et al*, 1993; Rothnagel, 1996), with mutations in the highly conserved motifs at the ends of the rod domains producing severe blistering as seen in Dowling–Meara patients, in contrast to mutations within the H1 or L12 regions, which are usually associated with the milder forms of disease such as the

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Abbreviation: EBS-K, epidermolysis bullosa simplex-Köbner.

Weber–Cockayne variant of EBS. Here we report a family diagnosed with the more severe Köbner form of EBS (EBS-K) and demonstrate the presence of a novel mutation in the L12 region of K5 in affected individuals.

MATERIALS AND METHODS

Polymerase chain reaction and DNA sequencing Genomic DNA was extracted and purified from whole blood using the QIAamp blood kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The L12 region of K5 was amplified from genomic DNA using specific oligonuleotides (5′-CACAACACAGAACCAGATGAC-3′ and 5′-CATTCTTAGTGTCGT-CATGGC-3′) corresponding to bp 101–121 of intron 4 and to 59–79 of intron 5 of the sequences determined by Stephens *et al* (1997). The sequencing primer (5′-TGACCGACTCCAAATCTC-3′) corresponds to bp 118–135 of the published intron 4 sequence (Stephens *et al*, 1997). The polymerase chain reaction reactions were preheated (95°C, 5 min), 2.5 units of Taq polymerase added, and cycled 35 times (94°C, 45 s; 56°C, 30 s; 72°C, 45 s) with a final extension at 72°C for 10 min. Polymerase chain reaction products were purified using QIAEX II (QIAGEN) and directly sequenced using the Thermo Sequenase dye terminator cycle sequencing system (Amersham Life Science, Cleveland, OH). Sequencing products were analyzed on an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA).

Mutation analysis by restriction enzyme digestion The L12 region of K5 was amplified using the oligonucleotides and polymerase chain reaction conditions listed above and the purified DNA digested with *Msp*A1 I (New England Biolabs, Beverly, MA). The digest products were separated on a 2% agarose TAE gel (Sambrook *et al*, 1989) in the presence of ethidium bromide and visualized by UV light.

RESULTS

Clinical description of the EBS family We studied a multigeneration family in which a diagnosis of EBS-K was made on the basis of a clinical history of generalized blistering with minimal oral involvement developing at birth or early in infancy and associated with a histopathology of basal layer degeneration. The index case, an 18 y old male, also suffered from microcytosis (cause not identified) and cystic acne. His treatment included oral retinoids (Roaccutane), which was complicated by increasing epidermal fragility requiring dosages to be maintained below 0.5 mg per kg per d. There was significant variation in the severity of symptoms exhibited by affected family members. Although the proband and his mother still suffer from generalized blistering, other family members reported that blistering rarely occurred outside of palmoplantar surfaces. All reported that blistering was more severe and generalized during childhood.

Identification of a valine to alanine substitution in the L12 region of K5 We sequenced the coding regions of both K5 and K14 of affected and unaffected family members. This analysis revealed a number of base changes from the published sequences that were present in both affected and unaffected individuals and represent allelic differences in the population (Wanner *et al*, 1993); however, we noted a single base substitution (G*T*G→G*C*G) in codon 323 of K5 that was only present in affected individuals (**Fig 1**). The T to C transition results in the expression of an alanine residue instead of valine at position 7 (V7A) of the L12 domain. The base change also creates a restriction enzyme site recognized by *Msp*A1 I and this was exploited to rapidly screen for the mutation in the EBS-K family (**Fig 2**) and in the normal population (data not shown). Notably, this change was not observed in unaffected family members or in the DNA from 50 unrelated and unaffected control individuals.

The valine to alanine substitution produces a significant structural change in L12 The affected valine residue at position 7 of the K5 L12 domain occurs at the beginning of a conserved motif of alternating apolar-nonpolar residues (VXLXMD in type II keratins) that has been recognized in all L12 sequences and suggests a β-type structure for this region (North *et al*, 1994). The V7 residue is absolutely conserved in all type II keratins and in many other intermediate filament proteins as well (**Fig 3**). The equivalent residue in the type I keratins is also valine or another apolar residue such as leucine (Conway and Parry, 1988). The strict conservation of valine at this site suggests

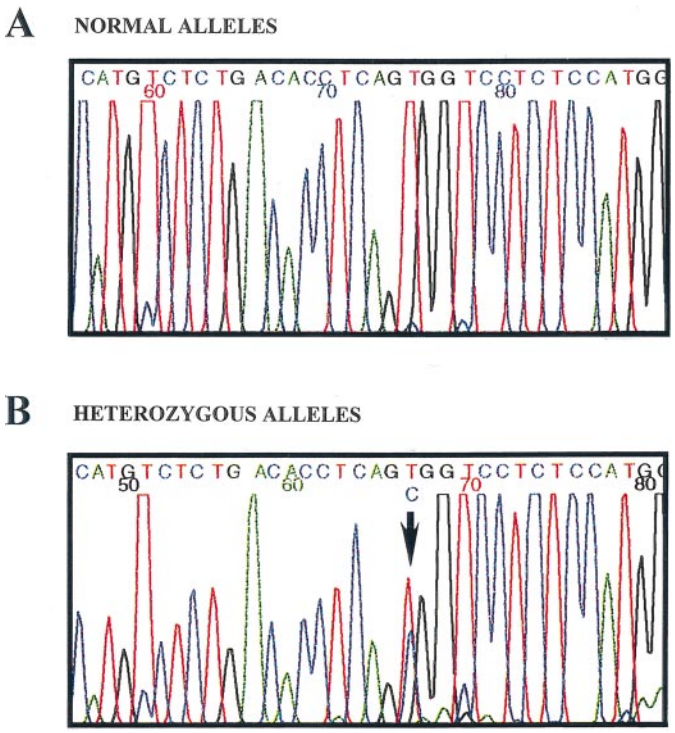

Figure 1. Sequence analysis of the L12 domain of K5 in EBS-K patients and unaffected family members. (*a*) Sequence of the normal K5 alleles obtained from an unaffected family member. (*b*) Direct genome sequencing of the proband (individual III.2 in **Fig 2**) who is heterozygous for the mutant allele. The affected individual contains a T to C mutation (indicated by the arrow) in codon 323, resulting in the expression of alanine at position 7 of the L12 domain. The sequence shown begins at the first codon of the L12 domain.

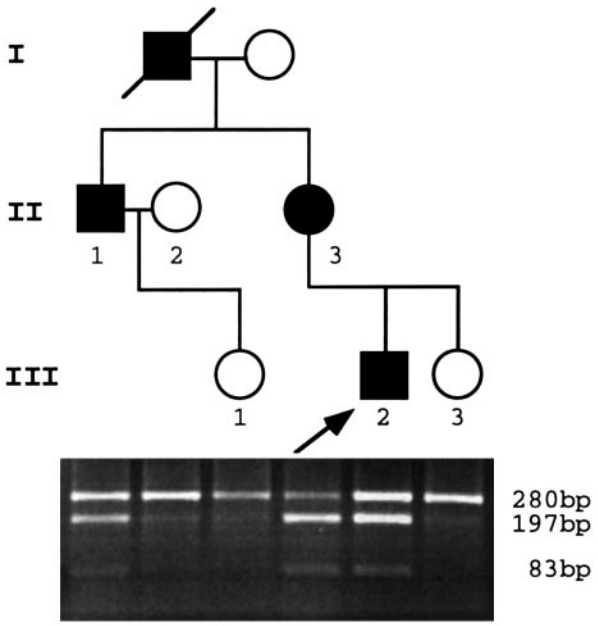

Figure 2. Restriction enzyme analysis of codon 323 showing cosegregation of the mutation with affected individuals. The L12 region of K5 was amplified and the 280 bp product digested with *Msp* A1 I. The T to C transition identified in the proband creates a *Msp* A1 I site in the mutant allele and results in 197 bp and 83 bp fragments after digestion. Wild-type alleles remain uncut. Solid symbols denote affected individuals, open symbols unaffected individuals; males are represented by squares and females by circles. The arrow indicates the proband (individual III.2).

K5	H	V	S	D	T	S	**V**	V	L	S	M	D	N	N	R	N	L
							↓				↓	↓	↓		↓		
EBS^{mut}							**A**				T	V	K		C		
K1	Q	I	S	E	T	N	**V**	I	L	S	M	D	N	N	R	S	L
K2e	S	V	T	D	T	N	**V**	I	L	S	M	D	N	N	R	S	L
K2p	H	A	S	D	T	S	**V**	V	L	S	M	D	N	N	R	C	L
K3	H	I	S	D	T	S	**V**	V	L	S	M	D	N	N	R	S	L
K4	H	V	S	D	T	S	**V**	V	L	S	M	D	N	N	R	N	L
K6a	H	I	S	D	T	S	**V**	V	L	S	M	D	N	N	R	N	L
K7	Q	I	S	D	T	S	**V**	V	L	S	M	D	N	S	R	S	L
K8	Q	I	S	D	T	S	**V**	V	L	S	M	D	N	S	R	S	L
K14	Q	V	G	G	D	-	**V**	N	V	E	M	D	A	A	P	G	V
XK8	Q	V	T	D	T	S	**V**	V	L	T	M	D	N	N	R	D	L
FK8	Q	I	K	D	T	S	**V**	V	V	E	M	D	N	S	R	N	L
WOOL(7c)	N	I	S	D	T	S	**V**	I	V	K	M	D	N	S	R	D	L
VIMENTIN	Q	I	Q	E	Q	H	**V**	Q	I	D	V	D	V	S	K	P	-
DESMIN	Q	L	Q	E	Q	Q	**V**	Q	V	E	M	D	M	S	K	P	-
GFAP	Q	L	A	Q	Q	Q	**V**	H	V	E	M	D	V	A	K	P	-
HAGFISHγ	G	P	V	Q	T	S	**V**	I	-	E	L	D	N	V	K	S	V

Figure 3. Sequence comparison of the L12 linker peptide showing conservation of the valine residue at position 7. The delineation of the L12 region is as determined by Conway and Parry (1988). EBS^{mut} indicates the disease-associated mutations found in this study (V7A) and in other studies (Rugg *et al*, 1993; Chan *et al*, 1994; Matsuki *et al*, 1995). The SwissProt accession numbers are: K5-P13647; K1-P04264; K2e-P35908; K2p-Q01546; K3-P12035; K4-P19013; K6a-P02538; K7-P08729; K8-P05786; XK8 *Xenopus laevis*-P04266; FK8 *Carassius auratus*-P18520; Wool(7c) *Ovis aries*-P15241; Vimentin-P08670; Desmin-P17661; GFAP-P14136; Hagfishγ polymer-Q90502.

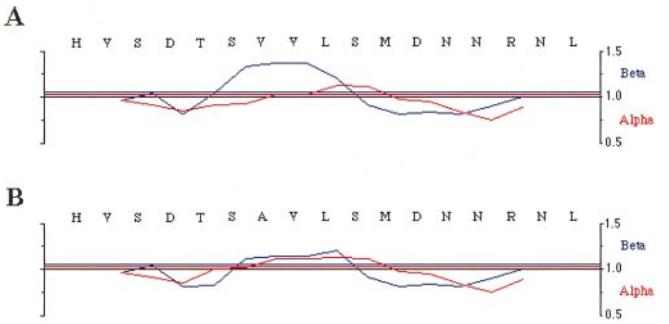

Figure 4. Secondary structure analysis of the L12 linker peptide. The Chou and Fasman algorithm of the peptide analysis tool (PEPPLOT) of ANGIS (http://www.angis.su.oz.au/) was used to calculate the affect of the alanine substitution at position seven. The graphs show the propensity for α-helix (red curve) and β-sheet (blue curve) formation for the normal L12 sequence (*a*) and the mutant L12 sequence (*b*). Both curves are the average of a residue-specific attribute over a window of four residues. The threshold value for the continuation of a given structure is arbitrarily set at 1.0.

that any change is likely to be deleterious although a priori, the alanine substitution at this position might be thought of as semiconservative. We therefore performed secondary structure analyses in an attempt to gauge the effect of the mutation on the L12 domain. A hydropathy plot revealed a marked reduction in hydrophobicity in the L12 peptide when alanine is substituted for valine at position 7 (data not shown) concomitant with a reduction in the β-sheet nature of this domain (**Fig 4**). These results suggest a large departure from the normal secondary structure of L12 in the V7A mutant.

DISCUSSION

The L12 linker peptide demarcates the end of the 1B and the beginning of the 2A α-helical segments and is found in all intermediate filament proteins, but its contribution to filament assembly and/or function has not been unequivocally determined. Current filament assembly models predict that the L12 region is in close proximity to the L2 and H2 regions in the staggered alignment of coil-coiled dimers, or with the L12 region of a neighboring pair of subunits in the nonstaggered antiparallel alignment (Steinert, 1993). In the antiparallel alignment, the four L12 regions are in a position to contribute to tetramer stability and in turn to filament stability through hydrophobic interactions with one another. Prior to this study, four other mutations in the L12 region of K5 had been reported and interestingly, all occur in the C-terminal half of the peptide (**Fig 3**) where they are predicted to promote α-helical formation at the expense of the normal β-sheet structure (Rugg *et al*, 1993; Chan *et al*, 1994; Matsuki *et al*, 1995). It has been postulated that these structural changes would result in a more rigid molecule with less resistance to torsional stress (McLean and Lane, 1995). These mutations may also impair the normal packing of dimers in the filament resulting in diminished interactions between residues critical for maintaining tensile strength. *In vitro* modeling of K5 L12 mutations (M11T and N13K) have shown that these substitutions tend to cause filament unravelling and result in loosely packed, shortened filaments with an apparent increase in diameter (Chan *et al*, 1994). These authors concluded that the L12 region plays a prominent role in stabilizing lateral associations between heterodimers. Given that the V7A mutation found in this study also has a marked affect on the secondary structure of L12, it is likewise postulated to interfere with lateral interactions, leading to a weakening of the keratin filament network and the subsequent clinical manifestations in these patients. Notwithstanding the predicted changes in secondary structure, the substitution of alanine for valine also produces a significant side chain alteration at this position. The absence of the extended branched side chain of valine could result in the loss of critical interactions with residues of proximal subunits and a subsequent reduction in filament stability (Alber, 1989; Zhu *et al*, 1993).

It should be noted that although some of the patients in this study display symptoms consistent with the Köbner form of EBS, a diagnosis of EBS–Weber–Cockayne may be equally appropriate for other members of this family, particularly older individuals. By comparison, the other K5 L12 mutations have all been identified in patients with the classical EBS–Weber–Cockayne symptoms of mild blistering limited to palmoplantar epidermis (Rugg *et al*, 1993; Chan *et al*, 1994; Matsuki *et al*, 1995). This suggests that the V7A mutation has a more profound affect on filament function than the other K5 L12 mutations, and further underscores the importance of this valine residue to the biology of the L12 domain. Similarly, both K14 L12 mutations (V8M and M10R) were also associated with more severe symptoms (Rugg *et al*, 1993; Humphries *et al*, 1993), with the M10R substitution identified in a family diagnosed with EBS-K (Humphries *et al*, 1993). Taken together these studies provide an indication of the relative contribution of each of these L12 residues to filament stability.

In this study we have identified a novel mutation within the L12 region of K5 that is associated with a clinical phenotype. Although we have no functional data proving that this mutation is pathogenic, both genetic evidence and structural analysis data strongly suggest that it is. This mutation was only observed in individuals with clinical symptoms and not in unaffected family members. Moreover, the mutation was not observed in any of the 100 normal K5 alleles that were screened as part of this study, nor have there been any reports of benign polymorphisms occurring at this site. The V7A mutation found in this EBS-K family is predicted to markedly alter the secondary structure of the L12 domain, resulting in a weakening of the cytoskeleton of basal layer keratinocytes. The identification of this substitution adds to the catalog of disease-associated mutations involving residues of the K5 and K14 L12 linker domains.

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