An Alanine to Proline Mutation in the 1A Rod Domain of the Keratin 10 Chain in Epidermolytic Hyperkeratosis

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We report a mutation in a case of epidermolytic hyperkeratosis that results in a proline for alanine substitution in the residue position 12 of the 1A subdomain of the keratin 10 chain (codon 158). The disease phenotype is consistent with the inappropriate substitution of a proline near the beginning of the rod domain, because it is likely to seriously disrupt the structural organization of coiled-coil molecules within keratin intermediate filaments. Mutations/substitutions in this position have not been reported in any keratin disease. Position 12 is an alanine in all intermediate filament chains, and lies in the outer *b* heptad position of the coiled-coil. *In vitro* peptide interference assembly assays revealed that substitutions that alter residue size or charge at this position primarily interfere with keratin filament elongation. ***Key words: keratin intermediate filament structure/type I keratin. J Invest Dermatol 109:692–694, 1997***

Epidermolytic hyperkeratosis (EHK) is a rare autosomal dominant disorder affecting the structural integrity of the suprabasal layers of human epidermis. EHK is characterized by abnormal keratin intermediate filament (KIF) clumping, cell lysis of the spinous and especially the granular layers, hyperproliferation, and a defective stratum corneum (Ishida-Yamamoto *et al*, 1992; DiGiovanna and Bale, 1994a, b; Huber *et al*, 1994; Chipev *et al*, 1996). Recent studies have established that EHK is caused by mutations in either the keratin 1 or the 10 gene, which result in inappropriate amino acid substitutions in the K1 or K10 proteins (Cheng *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992; Bale *et al*, 1993; Steinert, 1993; Compton, 1994; Fuchs and Weber, 1994; McLean and Lane, 1995; Parry and Steinert, 1995; Fuchs, 1996; Korge and Krieg, 1996; Steinert *et al*, 1997). Most of the deleterious substitutions reside in the beginning or end of the rod domain segments of these chains. Clinically, EHK has been classified into two types depending on the presence or absence of palmoplantar involvement, and each type has been subdivided into several subtypes according to the nature and severity of scaling (DiGiovanna and Bale, 1994a, b). Mutation/clinical phenotype correlations available to date suggest that the palm-sole subtypes are due primarily to K1 gene defects and the nonpalm-sole subtypes are due to K10 gene defects (DiGiovanna and Bale, 1994a, b). In this paper, we describe a case of EHK, classified as nonpalm-sole-2, caused by a novel substitution A158P located in the beginning of the 1A domain segment (A12P).

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Abbreviations: EHK, epidermolytic hyperkeratosis; K, keratin as in keratin 1, K1; IF, intermediate filaments.

MATERIALS AND METHODS

Source of DNA DNA was isolated as described (Chipev *et al*, 1994; Yang *et al*, 1994) from freshly drawn blood obtained from both affected and unaffected family members. Genomic DNA was also extracted from 50 unrelated individuals from the normal population and used as controls.

Direct polymerase chain reaction amplification and sequencing of the keratin 10 gene The oligonucleotide primers used for the amplifying exon 1 of K10 were: H(+), 5′-TTAGGAGGTTTTGGTGGAGGTAGCTTTCGT-3′; H(+ +) 5′-CGTGGAAGCTATGGAAGTAGCTTT-3′; H(–), 5′-CATG-GACAAGATACT TAAAGCTGGAT-3′. Reamplification was carried out with the primers H(+ +)/H(–). Primer H(–) was also used as a sequencing primer, using the single-stranded binding protein (United States Biochemical, Cleveland, OH) (Chipev *et al*, 1994).

KIF assembly assay with synthetic peptides A peptide corresponding to the first 18 residues of the 1A segment of the wild-type human K10 chain (amino acids 147–164, of sequence RVTQMNLNDRLASYLDKV), and substituted peptides of alanine12threonine (A12T) and A12D have been made previously (Steinert *et al*, 1993b). Additional new peptides with the following substitutions were made and purified: A12P, A12G, A12 L, A12 V, A12I, A12Y, A12S, and A12K. These were added in a 2-fold molar excess during the assembly reaction of K1/K10 KIF (0.8 mg per ml) (Steinert *et al*, 1993a, b). The reverse disassembly assay was carried out by adding the peptides to preformed K1/K10 KIF and incubating for 60 min at 37°C. The results on KIF integrity were examined by electron microscopy following negative staining as described previously (Chipev *et al*, 1992; Steinert *et al*, 1993a, b). Statistical data on the lengths of 100–125 individual KIF were measured using a map measuring device (Steinert *et al*, 1976).

RESULTS AND DISCUSSION

Clinical description of family EHK-KZ This three-generation Japanese family consisted of two patients and five unaffected individuals (**Fig 1*a***). The diagnosis of EHK was confirmed by histopathologic analyses of skin biopsy specimens of patient III-2. This 17-y-old boy had brownish wrinkled, encrusted hyperkeratosis and erosions throughout his trunk and extremities, especially on flexural areas, from birth. The lesions often give unpleasant odors. His mother has the

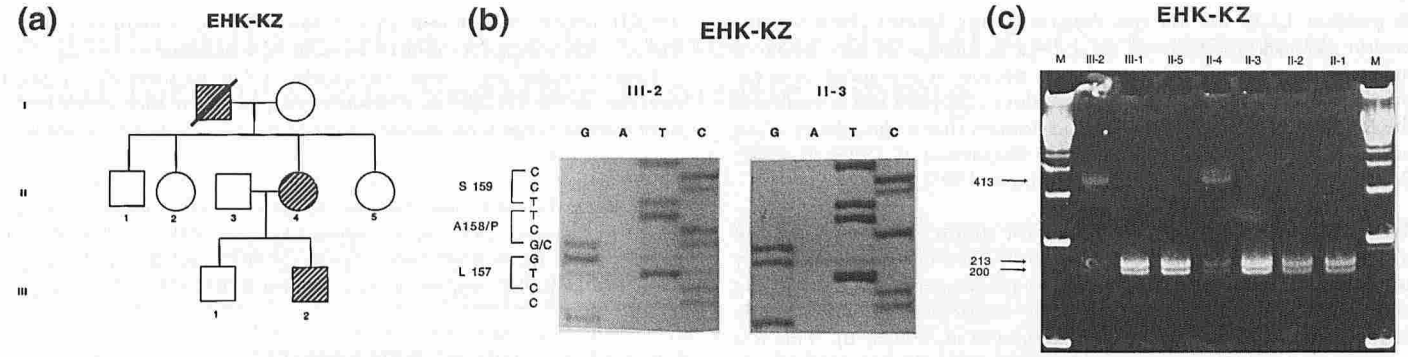

Figure 1. Characteristics of family EHK-KZ. (*a*) Pedigree of the EHK-KZ. (*b*) DNA sequences showing G to C change, which introduces an A12P amino acid substitution in codon 158. (*c*) ScrF1 digestion of polymerase chain reaction-amplified DNA of the family members. This mutation abolishes a ScrF1 site in the patients. Only the affected individuals show the undigested 413-bp fragment.

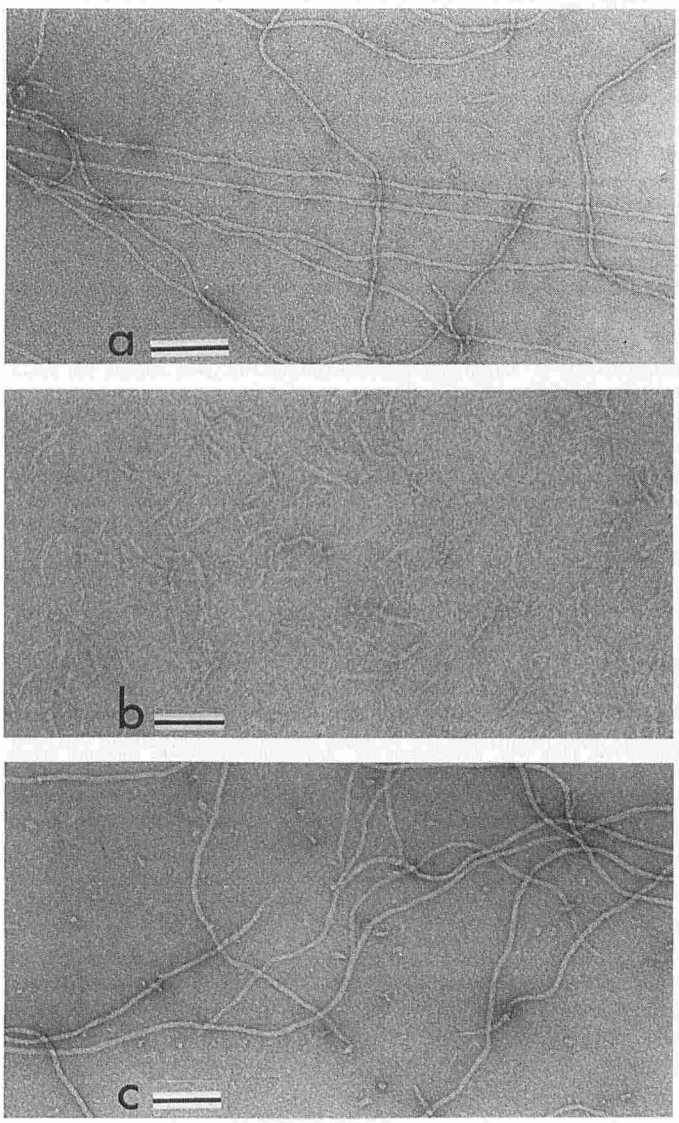

Figure 2. The A12P mutation alters the structural properties of KIF. Using an established *in vitro* assembly assay, a 1:2 molar ratio of an 18-residue K10 peptide of wild-type sequence severely interferes with K1/K10 KIF assembly (*b*), but a peptide bearing the A12P substitution seen in this family does not interfere (*c*). (*a*) Control KIF. *Scale bars*, 100 nm.

same skin lesions; the deceased grandfather-in-law was reported to have had similar clinical features. The patients were not erythrodermic and had no lesions on either palms or soles, and so were classified as the nonpalm-sole-2 type (DiGiovanna and Bale, 1994a). Light

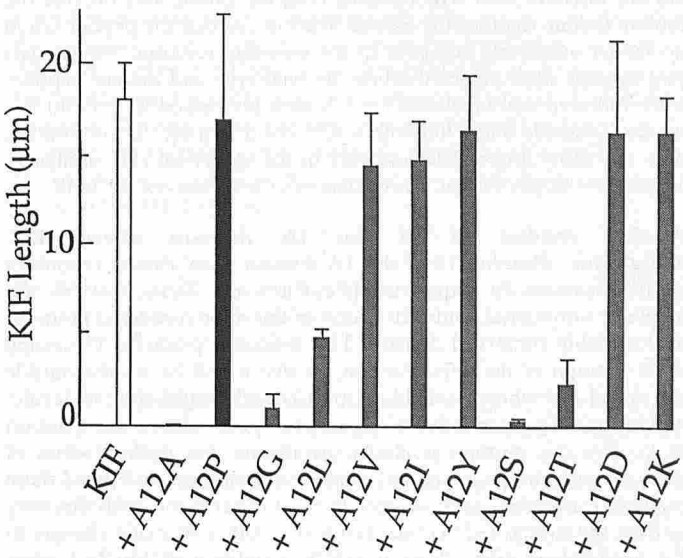

Figure 3. Alanine in residue position 12 of the 1A rod domain is essential for maintenance of KIF length. Peptides of the indicated substitutions were tested in the *in vitro* KIF assembly assay as above. At least 100 KIF particles were measured from negative stained images of each reaction to determine average lengths ± SD. *Open bar*, wild-type K1/K10 KIF assembled in absence of added peptides; *black bar*, KIF assembled in presence of A12P peptide, bearing the mutation reported here; *gray bars*, various other amino acid substitutions as marked. Note that the wild-type peptide A12A did not allow formation of filamentous particles (**Fig 2*b***).

microscopic examination revealed EHK limited to the upper part of the epidermis.

Identification of an alanine to proline substitution in the beginning of the 1A rod domain segment We found no sequence variations from normal in all exons of the K1 gene in the affected persons II-4 and III-2; however, we found in the patients a single nucleotide substitution in one allele of codon 158 in exon 1 of the K10 gene (GCT to CCT) so that the sequence gel of **Fig 1*b*** shows both a wild-type G and a mutant C in position 1 of this codon. The mutant allele encodes a proline residue instead of a wild-type alanine residue in position 12 of the 1A rod domain segment. This nucleotide substitution destroys a ScrF1 restriction enzyme site (CCNGG), which was then used as a test for the presence of the mutation in polymerase chain reaction-amplified DNA. In this analysis, the normal alleles yield fragments of 213 bp and 200 bp, but the affected allele yields a fragment of 413 bp, so that only patients II-4 and III-2 yield bands at 413 as well as at 213 and 200 bp (**Fig 1*c***). Because none of the other unaffected family members and none of the 50 other unrelated unaffected persons display the ScrF1 undigested fragment, the G to C substitution in the affected persons represents the mutation.

No previous reports of mutations/substitutions have been reported

in position 12 of the 1A rod domain in any keratin chain for any keratin disorder (Steinert *et al*, 1997). A change in this position, however, is expected to cause disease because it is located near the overlap window of the first several residues of the 1A rod domain and the last several residues of the 2B rod domain that is thought to occur between in-row molecules within KIF (Steinert *et al*, 1993a, b, 1997; Fuchs and Weber, 1994; Parry and Steinert, 1995).

The proline substitution introduces a major structural change to the keratin 10 chain Using an established *in vitro* KIF assembly assay (Chipev *et al*, 1992), an 18-residue synthetic peptide corresponding to the wild-type K10 sequence severely interfered with the assembly of K1/K10 KIF *in vitro* (**Fig 2*b***) (Steinert *et al*, 1993a, b). This is a negative assay for KIF structure: this result is thought to occur because the peptide competes with the full-length K10 chain, thereby interfering with and preventing efficient KIF assembly; however, a synthetic peptide containing an A12P substitution, as identified in this family, did not interfere with KIF assembly (**Fig 2*c***), presumably because the proline residue significantly altered structure, so that the peptide could no longer effectively compete in the assembly reaction. Similar data (not shown) were obtained when the wild-type and mutant peptides were added to and incubated for 1 h with preformed KIF. From this we can conclude that a full-length K10 chain bearing this substitution does not allow proper KIF assembly in the epidermal cell, ultimately leading to the phenotypic consequences of nonpalm-sole-2 EHK.

Alanine residue 12 of the 1A domain effects KIF elongation Residue 12 of the 1A domain is an alanine residue in all IF chains so far sequenced [see Parry and Steinert (1995) for sequence summaries], and thus is one of the most conserved positions in this highly conserved domain. This residue is predicted to occupy the *b* position of the heptad repeat, so that it will lie on the outside of a coiled-coil where it will likely interact with neighboring molecules within the KIF superstructure. In an attempt to address the question as to why this position is always an alanine, we made a series of synthetic peptides with a range of substituted residues and tested them in the KIF assembly reaction assay. In most experiments with this assay to date, the substituted peptides resulted in rather dramatic changes in KIF morphology, often allowing no KIF assembly at all (**Fig 2*c***; Chipev *et al*, 1992; Yang *et al*, 1996), so that a simple light-scattering test could be utilized (Steinert *et al*, 1993b). In most of the peptides used in this study, however, changes in light scattering were minimal, because KIF of length ≥ 3 μm scatter light efficiently and equally (Steinert *et al*, 1993a). Using a more sensitive electron microscopy assay, we found that there were marked changes in the average lengths of the assembled KIF, which seemed to correlate with residue size and charge (**Fig 3**). Peptides containing residues such as glycine or serine that are of a comparable or lesser size to alanine, behaved more like the wild-type peptide in that they severely interfered with KIF assembly because only short particles formed. Peptides containing substituted residues with longer hydrophobic or charged side-chains interfered less, so that much longer KIF were allowed to assemble. That these substituted peptides with a too large or charged side-chain in the *b* heptad position 12 of the 1A rod domain segment cannot interfere with KIF assembly *in vitro* is consistent with the concept that such residues in the intact K10 chain will interfere with the packing of neighboring molecules within the KIF. Our data suggest that the affect will be primarily upon net KIF length. A proline residue in this position, as identified in this study, is likely to severely interfere with KIF structure.

Further studies now seem warranted to solve the three-dimensional structure of the 1A/2B rod domain overlap window that serves as the most sensitive region for disease-causing mutations in keratin disorders.

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