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Preferential Sites in Keratin 10 That Are Mutated in Epidermolytic Hyperkeratosis

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Summary

Epidermolytic hyperkeratosis (EH) is a rare autosomal dominant skin disease. Recent studies in our laboratory established genetic linkage to the type II keratin gene locus on chromosome 12q in one family with EH and identified a single amino acid mutation in keratin 1 that is responsible for the disease. Other point mutations in the keratin 1 or keratin 10 genes have now been reported in other patients with EH. We have examined a series of probands with EH in order to develop a catalog of mutations in keratin 10. Using direct sequencing of PCR-amplified genomic DNA, we have identified mutations in six families, in which five mutations occur in the beginning of the 1A rod domain of keratin 10–namely, two Arg10 to His, one Arg10 to Cys, an Asn8 to His, and a Tyr14 to Asp. This region contains highly conserved residues among all keratins. An additional mutation (Leu103 to Gln) was found in the conserved region late in the 2B rod domain in keratin 10. We developed several allele-specific assays to assess the frequency of these mutations in the general population. No evidence was found for the presence of such changes in unaffected individuals. In vitro functional assays performed with peptides corresponding to the 1A mutations in these families show severely diminished capacity to disaggregate preformed keratin intermediate filaments, in comparison with a wild-type control peptide. Results from this work support the hypothesis that the beginning of the 1A rod domain segment in keratin 10 contains preferential sites for disease-causing mutation in EH. This should be of considerable use when developing prenatal diagnostic tests and biologically based therapies for this disease.

Introduction

Epidermolytic hyperkeratosis (EH) is a rare, autosomal dominant type of ichthyosis, although half of the cases occur sporadically as the result of a new mutation (Goldsmith 1976). EH often presents neonatally with blistering and redness, but the primary feature of the disease is hyperkeratosis (thickening of the uppermost layer of the epidermis, the stratum corneum) and blistering. The disorder has a characteristic appearance on histopathologic examination, consisting of hyperkeratosis and ballooning degeneration of the cells of the granular and spinous layers of the skin. Electron microscopic examination of EH skin has revealed clumps of keratin intermediate filaments (KIF) in the suprabasal epidermal cells (Anton-Lamprecht 1983; Williams and Elias 1987), which were shown to contain the keratins 1 and 10 (Cheng et al. 1992; Ishida-Yamamoto et al. 1992). These observations suggested that EH was related to abnormalities of keratins 1 or 10, the keratin pair that forms heterodimers, and is known to comprise the KIF in the cells of the upper epidermis.

Linkage studies in a large multiplex EH family demonstrated conclusively that a disease locus was present on chromosome 12, and no recombination with the keratin 1 locus at 12q11-q13 was observed (Compton et al. 1992). Sequencing of the keratin 1 gene revealed that a point mutation in the evolutionarily conserved codon 160, resulting in a Leu-to-Pro substitution, was

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the cause of disease in this family (Chipev et al. 1992). A different keratin 1 mutation was identified in another proband with the disorder (Rothnagel et al. 1992), and, subsequently, linkage analysis in other families confirmed the chromosome 12 location (Bonifas et al. 1992). However, since KIF in the suprabasal epidermal cells are assembled from heterodimers of keratin 1 and keratin 10, it was likely that mutations in keratin 10 could also cause EH. This was suggested by Fuchs et al. (1992) when an EH-like phenotype was produced in a transgenic mouse bearing a hybrid keratin chain coupled to a keratin 10 promoter. Definitive evidence of keratin 10 involvement in EH pathology was provided when three probands from different families were shown to have an Arg10-to-His mutation in the 1A rod domain (see fig. 1) of keratin 10 (Cheng et al. 1992; Rothnagel et al. 1992) and when a Leu-to-Ser substitution of codon 15 of the 1A keratin 10 rod domain was found in another (Rothnagel et al. 1992). Mutations in EH in the conserved residues of either the keratin 1 or keratin 10 chains extend the paradigm of keratin 5/keratin 14 mutations in another disease of the epidermis, epidermolysis bullosa simplex (EBS) (for review, see Epstein 1992). It is interesting that mutations in both EH and EBS have been found in the analogous segments, and even in the corresponding codons, of the respective keratin genes (i.e., Arg10 of keratin 10 and keratin 14).

In the present paper we describe keratin 10 mutations in six families with EH and show that these mutations occur in or near the highly conserved ends of the rod domain. This information (*a*) furthers the effort to develop a catalog of EH mutations for the purposes of prenatal diagnosis, genetic counseling, and genotype-phenotype correlation, and (*b*) provides additional insight into the structural alignment of the keratin molecules and their interactions in KIF in the epidermis.

Subjects, Material, and Methods

Description of Patients and Families

Patients with a diagnosis of EH were referred to the National Institutes of Health. After they granted informed consent, a detailed medical history and skin examination were obtained from each subject. A punch biopsy from at least one affected member of each family was fixed in formalin and stained with hematoxylin and eosin and was examined by light microscopy. All biopsies were consistent with the histologic appearance of EH. Blood was drawn in acid citrate dextrose tubes for direct extraction of DNA and establishment of lymphoblastoid cell lines.

Family I001.—This family consisted of a 43-year-old woman and her two unaffected parents. She had generalized, relatively mild disease. The most pronounced involvement was over the joints, with linear hyperkeratosis in the flexures. She was not erythrodermic, and her palms and soles were completely spared.

Family I006.—There were three affected individuals in this family. The 60-year-old woman (I-1 in fig. 5) reported that both of her deceased parents were free of skin disease. She was diagnosed with ichthyosis hystrix (localized epidermolytic hyperkeratosis) at age 5 mo. Her son presented with blistering at birth, and her granddaughter was born with a tight, white membrane, probably representing a collodion membrane. At age 3 years, she had blistering and generalized hyperkeratosis, especially over the flexural surfaces. All three affected family members had normal palms and soles.

Family I008.—A 32-year-old woman and her two unaffected parents composed this family. She had generalized involvement with erythroderma. Her scaling consisted of white, peeling flakes, which were most prominent over the trunk. At the flexures, there was hyperkeratosis arrayed in a linear distribution. Her palms were hyperlinear with extremely mild thickening.

Family I013.—A 12-year-old boy and his unaffected parents were examined. The clinical phenotype in the affected patient was similar to that seen in family I008.

Family I014.—This family consisted of a 21-year-old female patient and her unaffected parents. She presented at birth with redness and blistering, which was followed by development of hyperkeratosis. On examination she had severe, generalized, thick, verrucous hyperkeratosis that was accentuated over the flexures. Her palms and soles were normal.

Family I024.—This family consisted of an affected mother and daughter and their parents. Clinical findings were similar to that of the affected individual in family I001.

PCR Conditions and Sequencing

Amplification and sequencing of the keratin 10 gene from genomic DNA was done as described elsewhere (Chipev et al. 1992), unless otherwise stated. The primers used are shown in table 1 (Rieger and Franke 1988).

DNA fragment A contained part of the N-terminal region and the 1A and part of the 1B rod domain of keratin 10 (fig. 1). The primers used were H(+), H(++),

Table 1

Oligonucleotide Primers Used for PCR Amplification, DNA Sequencing, and PCR Allele-specific Assays

	Nucleotide Position[a]	Sequence
DNA Fragment A:		
H(+)	1902/1931	5′-TTAGGAGGTTTTGGTGGAGGTAGCTTTCGT
H(++)	1929/1955	5′-CGTGGAAGCTATGGAAGTAGCTTT
H(−)	2343/2318	5′-CATGGACAAGATACTTAAAGCTGGAT
H(−)ups	2289/2268	5′-GGTCATCGATGGTTTTGTAGTA
DNA Fragment B:		
LE(+)	4478/4505	5′-CTCAACTGAACAAGGAAGTCACTAAGCC
LE(−)	4791/4767	5′-GGAATATTGAATAAAAGAAACTGGG
Allele-specific primers[b]:		
LQwt(−)	4718/4694	5′-CGGTAGGTTTGAATTTCATTCTCCA
LQmu(−)	4718/4694	5′-CGGTAGGTTTGAATTTCATTCTCCT
NHwt(−)	2156/2133	5′-CAAGTAGGAGGCCAAGCGGTCATT
NHmu(−)	2156/2133	5′-CAAGTAGGAGGCCAAGCGGTCATG
*Sfc*I-mismatched primer(+)	4669/4693	5′-CAACTCCTGGATATTAAGATCCTAC
*Tth*111I-mismatched primer(−)	2175/2152	5′-CCAGAGCCCGAACTTTGACCAAGT

NOTE.—A plus sign (+) indicates sense strand; a minus sign (−) indicates antisense strand.
[a] According to Rieger and Franke (1988).
[b] The sites of mutation or mismatch are underlined.

H(−), and H(−)ups from either exon 1 or intron 1. DNA fragment B encompassed the part of the 2B rod domain (fig. 1) contained on exon 6, and was amplified with primers LE(+) and LE(−) in the surrounding introns.

The following thermocycling procedure (Perkin Elmer Cetus) was more efficient than a step-cycle for amplification in some cases: denaturation for 1 min at 94°C, a 90-s transition to annealing temperature (55°C–60°C), then annealing at 55°C–60°C for 30 s, and extension for 90 s at 72°C. These steps were repeated for each cycle. In some cases the ΔTAQ Cycle-sequencing kit (USB) with α-^{33}P-dATP was used instead of the Sequenase 2.0 kit (USB).

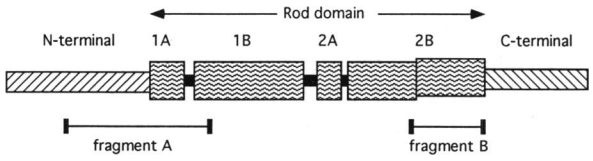

Figure 1 Domain structure of the human keratin 10 protein. Human keratin 10 consists of about 560 amino acid residues. The four designated segments (i.e., 1A, 1B, 2A, and 2B), which are separated by linker regions, form the central α-helical rod domain (Zhou et al. 1988). The rod domain of keratin 10 forms a coiled coil with the rod domain of the keratin 1 molecule. The DNA corresponding to the fragments A and B is analyzed in the present paper.

Alternatives to Sequencing for Detection of Identified Keratin 10 Point Mutations

PCR-based allele-specific assays (PASAs) to detect single-base variants (Sommer et al. 1992) were developed for the mutations that did not involve restriction-enzyme–recognition sequences.

Primers to distinguish the Leu (CTG)-to-Gln (CAG) mutation were LQwt(−) and LQmu(−), respectively, with LE(+) used as the common upstream primer. Amplification was performed for 30 cycles (denaturation for 1 min at 95°C, 1 min cooling down to 55°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C) with 0.1 mM dNTP, 1.5 mM $MgCl_2$, 125 ng DNA/25 μl mix, and 0.1 μM primers.

A variation of the PASA technique, which involves a radiolabeled primer, was utilized to detect the Asn (AAT)-to-His (CAT) mutation. Normal and mutant alleles were amplified in the same reaction tube containing unlabeled NHwt(−) and H(+) primers (0.1 μM) and a 20-fold lower concentration of ^{32}P-end-labeled NHmu(−) primer. Amplification was performed for 30 cycles (denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C) with 0.1 mM dNTP, 1.5 mM $MgCl_2$, 125 ng DNA/25 μl mix. After separation in agarose gels (3% Nusieve (3:1); FMC), the product of the normal allele was detected by ethidium bromide staining, since the contribution from the mutant allele was negligible. Autoradiography of

the same gel was used to detect the presence of the mutant allele. This method has the advantage of reducing the number of samples in each experiment.

In two cases mismatch PASAs were used to introduce a restriction site into the product amplified from the mutant allele (Bijvoet and Hayden 1992). The PCR products (50–100 ng) were digested with 2 U of the corresponding restriction enzymes (New England Biolabs) by addition of $MgCl_2$ and the enzymes to the amplification mixes without further purification. In the case of the TAC-to-GAC mutation (Tyr to Asp), a *Tth*111I restriction site was created only in the amplification of the mutant allele, by using the *Tth*111I-mismatch primer ("−") and H(++). The mismatched primer ends before the mutated nucleotide and carries the mismatched T that, together with the mutated G, forms the *Tth*111I site (GACNNNGTC) only for the mutant allele (table 1).

Similarly, an *Sfc*I restriction enzyme site was introduced during amplification of the Leu-to-Gln mutant allele, by using the primer LE(−) with the *Sfc*I mismatched primer ("+"), which contains a mismatched T in place of a G (table 1). This primer will produce an *Sfc*I site (CTACAG) only when an A is incorporated during amplification of the mutant allele (table 1).

In Vitro KIF Disassembly Assay

By using protein prepared from freshly excised human foreskin epidermis, KIF containing keratin 1/keratin 10 chains were assembled in a buffer of 5 mM Tris-HCl (pH 7.6) containing 1 mM EDTA and 1 mM DTT (Steinert and Parry 1993). Synthetic peptides that are based on published sequences of human keratin 10 (Zhou et al. 1988) were prepared and purified by high-pressure liquid chromatography, and their compositions were confirmed and concentrations ascertained by amino acid analysis. The following peptides were utilized in the present study (mutant residues are underlined): (1) wildtype K10—1A, residues 1-18 KVTMQNLNDRLASYLDKV; (2) mutant K10—1A, Arg10 to His KVTMQNLNDHLASYLDKV; (3) mutant K10—1A, Arg10 to Cys KVTMQNLNDCLASYLDKV; (4) mutant K10—1A, Asn8 to His KVTMQNLHDRLASYLDKV; (5) mutant K10—1A, Tyr14 to Asp KVTMQNLNDRLASDLDKV; (6) wildtype K10—2B, residues 77-110 EQLQEIRAETECQNTEYQQLTDIKIRLENEIQTY; and (7) mutant K10—2B, Leu103 to Gln EQLQEIRAETECQNTEYQQLTDIKIRQENEIQTY. Peptides were dissolved, immediately before use, in the assembly buffer at 1–3 mg/ml and were added in 3-μl aliquots to 0.25 ml of KIF, so as to effect a 1× molar excess of peptide over KIF heterodimer (110 kDa). Changes in turbidity were monitored by measuring light scattering at 310 nm in a Beckman DU-65 spectrophotometer at 37°C for 60 min (Chipev et al. 1992; Steinert et al. 1993; Steinert and Parry 1993). It has been determined empirically by electron microscopy (Steinert et al. 1993) that, for KIF 2–5 μm in length, turbidity due to light scattering is approximately proportional to the square of KIF length; that is, minor reductions in KIF length will result in significantly reduced turbidity.

Results

One family (I006) was amenable to limited linkage analysis. An obligate recombinant with D12S14 (a locus that has a recombination distance of .007 [lod score = 27.9] with keratin 1; O. W. McBride, personal communication) strongly suggested that keratin 1 involvement in the disease could be excluded (fig. 5). In each of the other families, all of whom represented new mutations for the disease, DNA was sequenced in selected regions of the keratin 1 and 10 genes (rod domain 1A and the end of rod domain 2B) (fig. 1), where mutations had been reported previously in patients with EH. We report here those mutations found in the keratin 10 gene only. Genomic DNAs from affected patients and their unaffected parents were amplified and compared, in an effort to identify changes that had occurred in proband DNA. The PCR products for DNA fragments A and B (see Subjects, Material, and Methods) were sequenced directly. When a difference was found between a proband and his or her parents, two independent PCR products were analyzed in order to eliminate the possibility that any difference was introduced during PCR amplification. All the mutations found are summarized in table 2.

Mutations in the Arg10 Codon of the 1A Rod Domain

We used *Aci*I digestion as an assay for the presence of mutation at Arg10 in the 1A rod domain (Cheng et al. 1992). Mutation at this site removed an *Aci*I restriction site and resulted in a new, longer, 294-bp fragment, together with the 205-bp product expected for the normal allele. This was seen in the affected individuals in families I013, I014, and I024 (fig. 2*B*). When the PCR products were sequenced directly, a G-to-A substitution was found in the affected members of families I014 and I024 (fig. 2*A*), that was not present in the parental DNAs (fig. 2*C*). DNA from the proband in family I013 revealed a different mutation (C to T) at the same *Aci*I

Table 2

Mutations in the Keratin 10 Gene of EH Patients

Family	Type	Substitution	Amino Acid Change	Position [Rod Subdomain (residue)] of Mutation in K10	Method of Screening
I001	Simplex	AAT→CAT	Asn→His	1A (8)	PASA
I006	Multiplex	CTG→CAG	Leu→Gln	2B (103)	Mismatch PASA
I008	Simplex	TAC→GAC	Tyr→Asp	1A (14)	Mismatch PASA
I013	Simplex	CGC→TGC	Arg→Cys	1A (10)	*Aci*I digestion
I014	Simplex	CGC→CAC	Arg→His	1A (10)	*Aci*I digestion
I024	Multiplex	CGC→CAC	Arg→His	1A (10)	*Aci*I digestion

site (fig. 2*D*). The amino acid encoded by this mutant allele is Cys instead of Arg at position 10 in the beginning of the 1A rod domain. Both unaffected parents had the normal Arg.

An Asn-to-His Mutation

The sequence of the keratin 10 alleles of the proband DNA from I001 (fig. 3*A*) showed an A-to-C transversion (fig. 3*B*). The mutated allele was present only in the affected person and not in her unaffected parents. This substitution led to a change from Asn to His at position 8 in the beginning of the 1A rod domain. The frequency of this mutation was evaluated by PASA (fig. 3C) in 52 normal individuals. Only lane 1, which contains the product from the affected proband, gave a band both with ethidium bromide staining and on the autoradiogram, indicating the presence of both the normal and mutant alleles. All other DNAs tested were negative for the mutant allele.

A Tyr-to-Asp Mutation

A T-to-G transversion was present in the keratin 10 gene of the proband from family I008 but not in the unaffected parents (fig. 4*B*). This resulted in an Asp in place of Tyr at position 14 in the rod domain 1A. A mismatch PASA was developed to evaluate the frequency of this change in the general population. Only the PCR product of the affected proband was digested by *Tth*111I, and the resulting fragment is 23 bp shorter than the intact PCR band (e.g., see fig. 4*B*). None of 42 other individuals tested showed this band.

A Leu-to-Gln Mutation

The affected members in family I006 (fig. 5*A*) had a mutated keratin 10 allele that carried the nucleotide substitution T to A (fig. 5*B*). This corresponds to a change from Leu103 to Gln, near the end of rod domain 2B. One of the grandparents (I-1) was also affected and had the mutation, although the band in lane A of the sequencing gel did not appear to represent 50% of the total DNA at that position (fig. 5*B*). When mismatch PASA was used, the *Sfc*I digests of the PCR products showed doublets in the lane for affected persons (fig. 5C). Figure 5*D* shows the results of the standard PASA for this family. The mutant and normal alleles give PCR product in neighboring lanes only for affected individuals. Both assays were used to screen for this mutation in unaffected family members and in 48 normal unrelated individuals. None showed evidence of this change.

Functional Assays for Keratin 10 Mutations

The wild-type keratin 10 peptide containing residues 1–18 of the 1A region was highly efficient in disassembling preformed KIF (table 3). In contrast, peptides containing the sequence of each of the 1A mutations found in these families had severely impaired ability to disassemble KIF, as measured by light scattering. The wild-type 2B peptide had no significant effect on preformed filaments; therefore, the peptide competition method could not be used to demonstrate a functional effect in vitro for the Leu103-to-Gln mutation.

Discussion

The evidence that the mutations in the keratin genes identified in this paper are etiologically responsible for EH in the patients comes from several arguments. With one exception (I006), all of the probands represent new mutation events. In each case, both unaffected parents were tested and found not to have the mutated keratin gene. Second, none of these putative mutations were found in a large series of control individuals with normal skin, which supports the fact that they were not

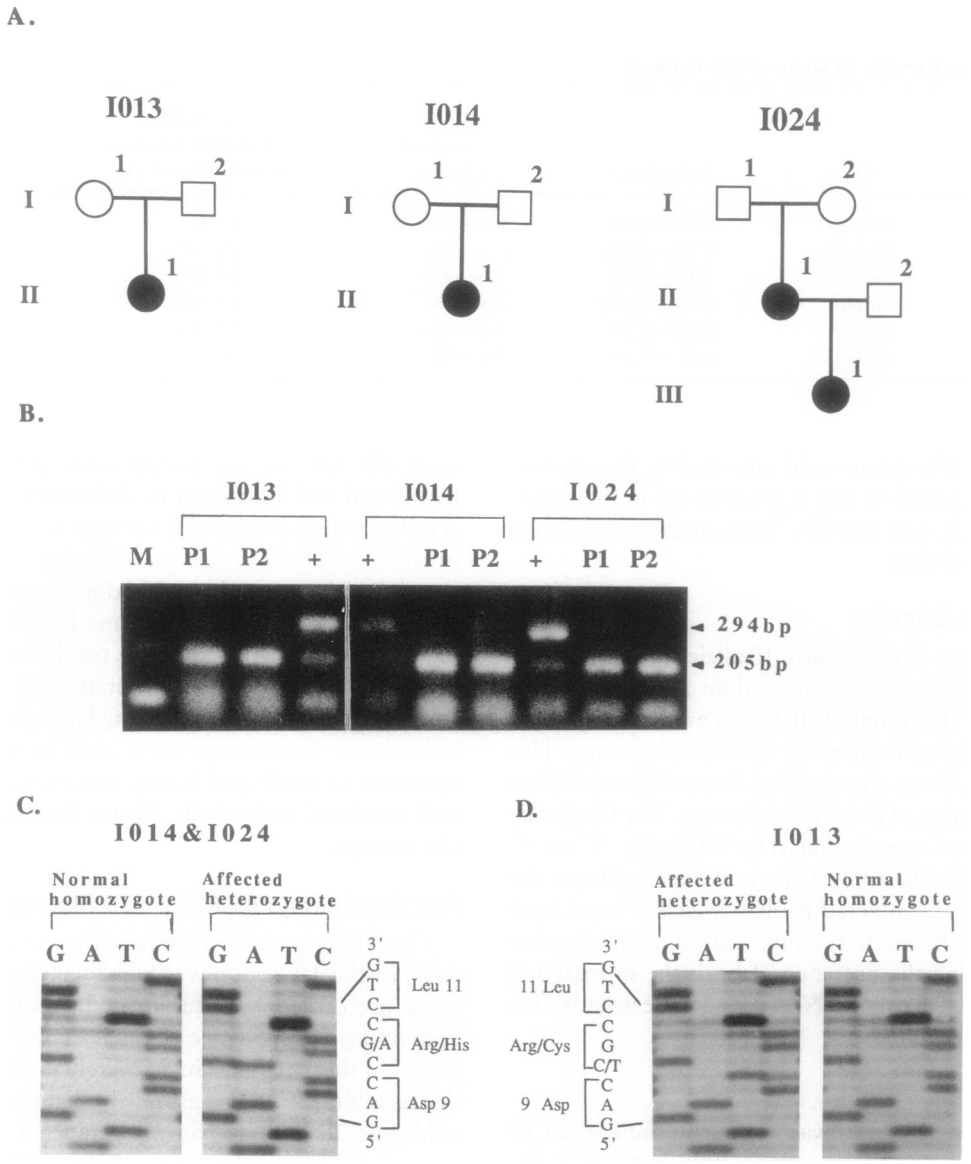

Figure 2 Mutations in the Arg10 codon of the 1A rod domain of K10 in families I013, I014, and I024. *A*, Pedigrees. *B*, *Aci*I-digestion pattern of DNA fragment A, amplified from members of I013, I014, and I024. Lanes P1 and P2, Unaffected parents. Lane +, Affected proband. Lane M, DNA size marker (123-bp ladder; BRL). Ethidium bromide staining of a 1.5% NuSieve 3:1 gel. *C* and *D*, Autoradiograms of direct sequencing of DNA fragment A, showing sequence heterogeneity at a single position in the keratin 10 gene of affected individuals. The same codon is changed from Arg to His or from Arg to Cys in the mutated alleles of the EH patients. Sequence results for families I014 and I024 were the same. The sequence and codons around the mutation are indicated.

simply keratin polymorphisms. Third, previous linkage and mutation studies have implicated these keratin loci in EH in other families so that there was a strong prior hypothesis of keratin involvement.

Results from functional assays of synthetic peptides bearing the observed amino acid substitutions can also be used to imply causality. It has previously been shown that synthetic peptides with amino acid sequence corresponding to the H1, 1A, and end of 2B sequences, which involve the principal overlap regions of keratin 1/keratin 10 molecules in KIF, can disassemble preformed KIF in an in vitro assay (Hatzfeld and Weber

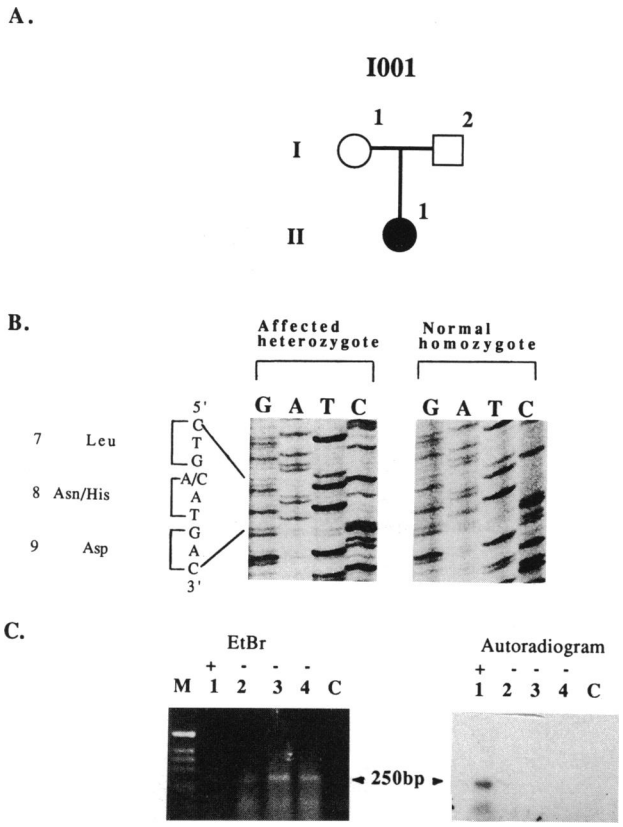

Figure 3 Mutated codon in I001, which encodes a His instead of the normal Asn at position 8 of the 1A rod domain in K10. *A*, Pedigree. *B*, Autoradiogram of a sequencing gel using ΔTAQ Cycle-sequencing (USB) of the PCR products of DNA fragment A showing the presence of both A and C at a single position in the keratin 10 gene of the affected proband. *C*, PASA assay for this mutation. The ethidium bromide–stained 1.5% agarose gel shows the PCR products from the normal allele, and the autoradiogram of the same gel shows the presence of the mutant allele only (for details, see Subjects, Material, and Methods). Lane +, EH-affected. Lane −, Unaffected individual. Lanes 2 and 3, Products from the unaffected parents. Lane 4, Unrelated person. Lane M, DNA size marker (1-Kb DNA ladder; BRL). Lane C, Control PCR reaction with DNA omitted.

1992; Steinert et al. 1993; Steinert and Parry 1993). This is presumed to be due to competition with the similar sequence of the chains that form the intact KIF. Peptides created to correspond to other portions of the keratin chains or those that have specific mutated sequences either lack this ability or possess only limited capacity for disassembly (Chipev et al. 1992). Mutant peptides containing the four single amino acid substitutions in 1A reported here (i.e., Asn8 to His, Arg10 to His, Arg10 to Cys, and Tyr14 to Asp) have severely diminished ability to disassemble preformed KIF as compared with the wild-type peptide sequence (table 3). This may mean that the amino acid substitutions have significantly altered the structure and properties of the 1A region. This implies that residues in this 1A region are indispensable for KIF structural integrity. Furthermore, it implies that full-length keratin 10 chains possessing these amino acid substitutions form

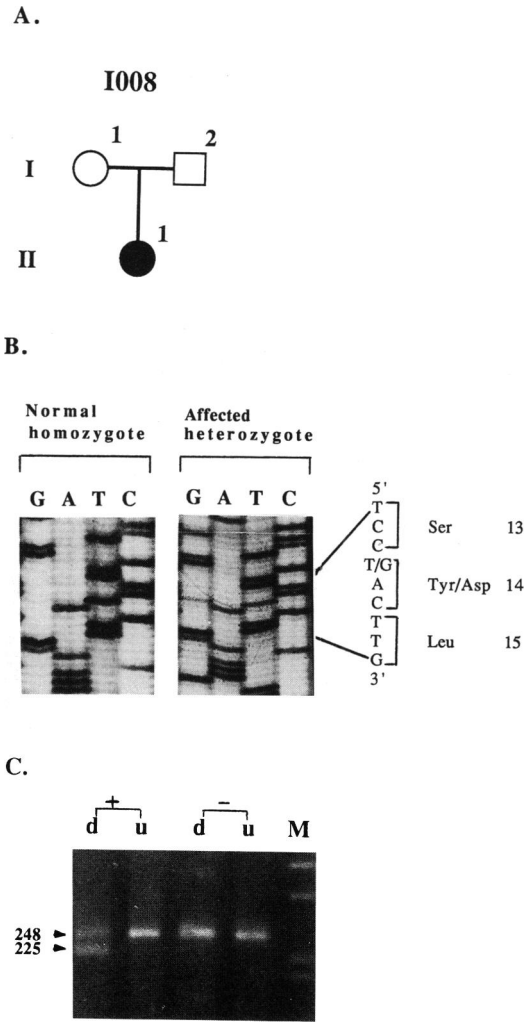

Figure 4 Proband from family I008, who is heterozygous and has alleles containing either Asp or Tyr at position 14 in the 1A rod domain. *A*, Pedigree. *B*, Autoradiogram of direct-sequence analysis of the affected proband and one unaffected parent, showing the sequence heterogeneity. *C*, *Tth*111I-mismatch PASA assay for the T-to-G point mutation of codon 14 in family I008. The allele-specific PCR amplification incorporates a *Tth*111I site into the product from the mutated allele. Shown are results of untreated and *Tth*111I-digested DNA analyzed on a 3% NuSieve 3:1 agarose gel stained with ethidium bromide. Lanes +, Affected proband. Lanes −, Unaffected parent. Lane u, Undigested. Lane d, Digested. Lane M, DNA size marker (1-Kb DNA ladder; BRL).

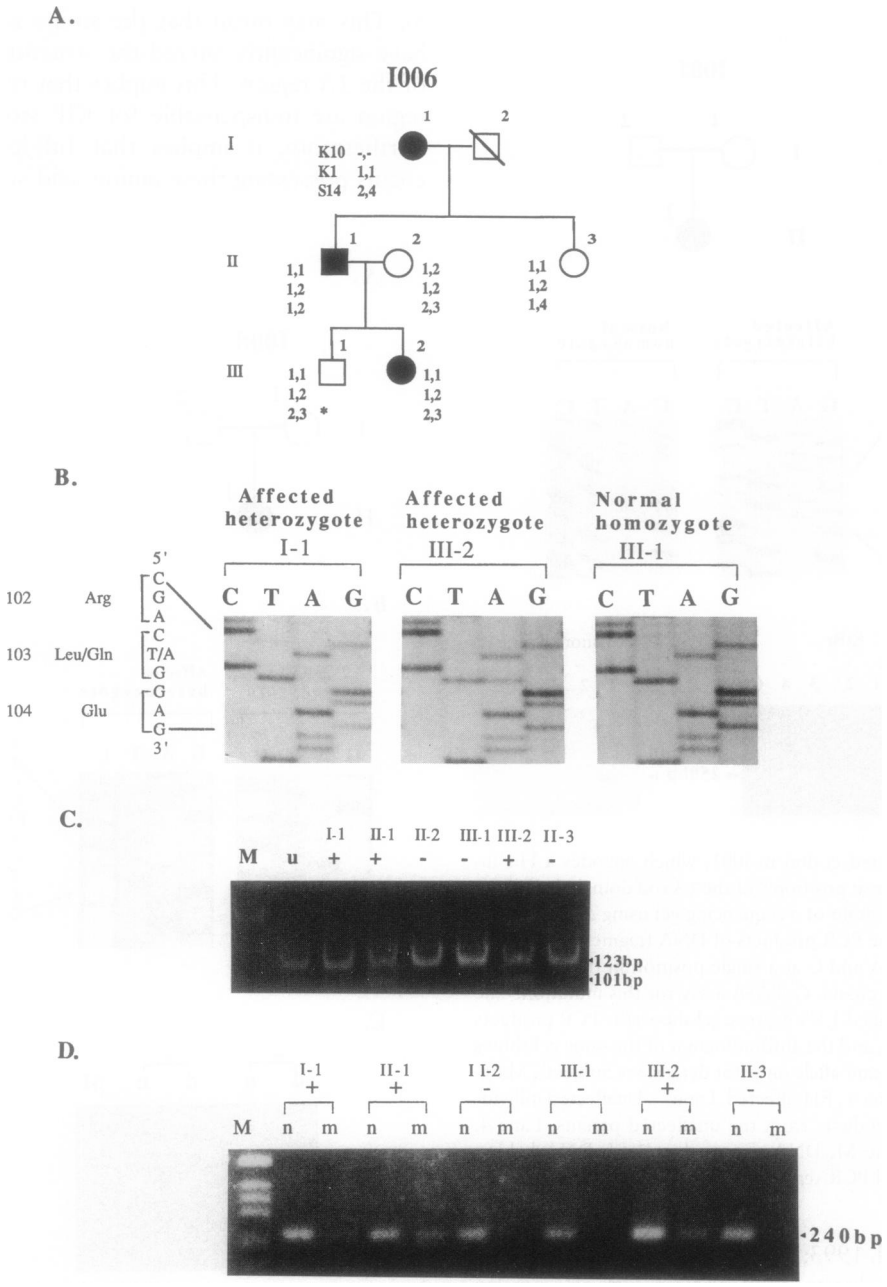

Figure 5 Nucleotide substitution in one of the alleles in I006, which causes a Leu-to-Gln change near the end of the 2B rod domain of K10. *A*, I006 pedigree with results from the typing with three polymorphic markers (*top to bottom*, K1 and K10 length polymorphisms [Korge et al. 1992*a*, 1992*b*] and D12S14). An asterisk (*) identifies the individual recombinant for D12S14. A minus sign (−) indicates not determined. *B*, Direct sequencing of the PCR product for DNA fragment B for members of I006. Autoradiograms are of the region around the position containing both T and A in the indicated affected and unaffected individuals. *C*, *Sfc*I-mismatch PASA–ethidium bromide staining of a 6% native polyacrylamide gel (Novex). Only the *Sfc*I-digested PCR products from the affected family members appear as doublets (labeled "123 bp" and "101 bp"). *D*, PASA–ethidium bromide staining of a 3% NuSieve 3:1 gel with the PCR products from the normal (lanes n) and mutant (lanes m) alleles. Only the affected probands show 240-bp products in both "n" and "m" lanes. Lanes +, Affected proband. Lanes −, Unaffected individuals. Lane M, 1-kb DNA ladder. Patient I-1 shows a smaller amount of the mutated allele.

Table 3

Stability of Preformed KIF, after Treatment with Synthetic Peptides

Peptide No.	Experiment[a]	% Light Scattering Remaining after Peptide Addition[b]
Control	KIF alone	100
1	Wild-type K10 1A residues 1–18	6 ± 2
2	Mutant K10 1A residues 1–18 (Asn8 to His)	87 ± 3
3	Mutant K10 1A residues 1–18 (Arg10 to His)	59 ± 2
4	Mutant K10 1A residues 1–18 (Arg10 to Cys)	55
5	Mutant K10 1A residues 1–18 (Tyr14 to Asp)	85 ± 2
6	Wild-type K10 2B residues 77–110	92 ± 1
7	Mutant K10 2B residues 77–110 (Leu103 to Gln)	94 ± 2

[a] Wild-type peptides were designed from the published K10 sequence (Zhou et al. 1988). When a wild-type peptide destroys the KIF, the light scattering is considerably reduced. When a mutant peptide carrying a single amino acid substitution is unable to cause the same reduction in the scattering of the KIF solution, this position in the keratin 10 chain is presumed to be crucial for the KIF stability. When a wild-type peptide does not cause dramatic reduction in light scattering, this assay is not informative (e.g., see peptide no. 6).

[b] The percentage of light scattering remaining is an average ± SD of two or three measurements on one batch of human K1/K10 KIF.

less stable KIF in vivo, leading to the pathology of EH. While it has been shown elsewhere that synthetic peptides containing sequences at the end of the 2B rod domain of keratin chains are also effective in disassembly of preformed KIF (Hatzfeld and Weber 1992; Steinert et al. 1993), we have found that this disassembly paradigm does not work for substitutions that are farther than approximately 15 residues from the end of the 2B segment; thus, there is no comparable assay to test the structural significance of the Leu103-to-Gln mutation.

The outermost layer of the skin provides a barrier that protects the internal structures of the body from the environment. The epidermis derives this defensive ability in part from its cytoskeletal structure, which is composed of KIF. In the basal layers of the epidermis, keratin 5 (a type II, basic keratin) and keratin 14 (a type I, acidic keratin) pair to form KIF. In the terminally differentiating suprabasal epidermis, keratin 1 (type II) and keratin 10 (type I) are the predominant cytoskeletal components. There is striking evolutionary conservation of amino acid sequence in certain regions of the keratin proteins, both within and across the type I and II family, as well as across the other intermediate filament types (i.e., type III vimentin, type IV neurofilament-L, type V lamin A, and type VI nestin) and among vertebrates. This conservation has probably persisted throughout evolution to allow for the proper assembly of the KIF. In fact, several lines of experimental evidence support the importance of the ends of the rod domains in filament assembly, including in vitro KIF disassembly studies using synthetic peptides (Chipev et al. 1992; Hatzfeld and Weber 1992), the introduction of point mutations (Letai et al. 1992, 1993), and cross-linking studies (Steinert et al. 1993). One model for alignment provides for the last 10–11 residues of the 2B segment of one keratin heterodimer to overlap the first 10–11 residues of the 1A segment of its neighbor (Steinert et al. 1993).

Each of the amino acid changes in EH reported in this study, as well as those reported elsewhere (Cheng et al. 1992; Chipev et al. 1992; Rothnagel et al. 1992), occurred at positions that are highly conserved in intermediate filament proteins (fig. 6). Five cases of EH have now been found in which there is an Arg10-to-His mutation in the 1A rod domain segment. This same codon was mutated to Cys in another family. It is interesting that His and Cys substitutions for Arg10 of keratin 14 have been identified in EBS (Coulombe et al. 1991). Each of the mutations reported here are in, or immediately adjacent to, the proposed region of overlap that occurs when keratin heterodimers assemble into KIF. It

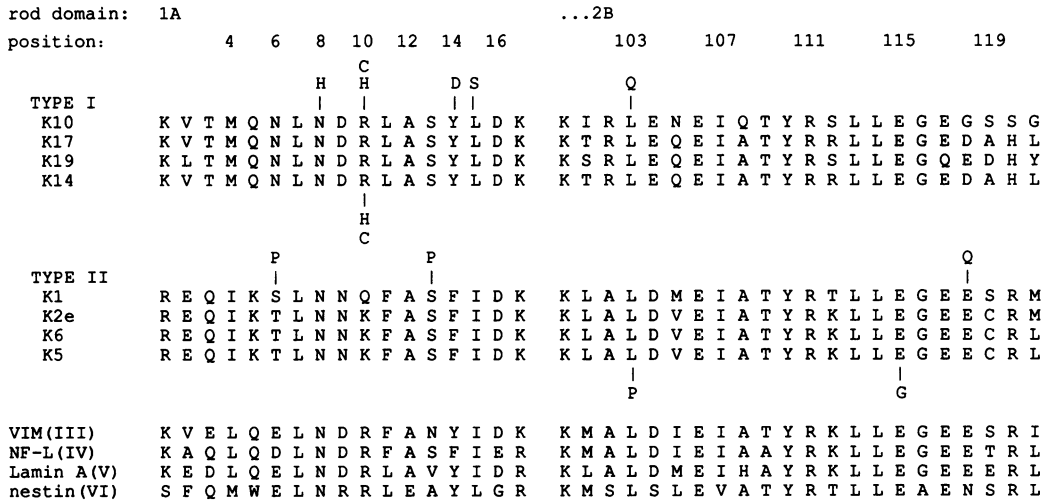

Figure 6 Protein-sequence alignment at the beginning of the 1A rod domain and at the end of the 2B rod domain in all six types (marked with Roman numerals) of intermediate filament proteins, with the keratin mutations in EH and EBS published to date indicated. The sequences are derived from the Swiss-Prot database, release 25, and GenBank, release 76. EBS mutations in keratin 14 and keratin 5 are from Coulombe et al. (1991), Lane et al. (1992), and Dong et al. (1993). The two keratin 1 mutations in EH are from McLean et al. (1993) and Yang et al. (in press). Position numbers are for the 1A and the 2B rod domains, respectively.

can be hypothesized that these mutations prevent or introduce certain hydrogen or ionic bonds which do not allow the normal molecular interactions, and result in defective KIF (e.g., keratin filament clumping and abnormal filament structure) (Steinert et al. 1993). Exactly how these KIF aberrations lead to the histologic and clinical observations in EH remains to be determined.

It is remarkable that the same codon in the 2B rod domain (Leu103), which was mutated to Gln in keratin 10 of family I006, has been found mutated to Pro in the keratin 5 molecule in patients with the Koebner type of EBS (Dong et al. 1993). The evolutionarily conserved residue, Leu103, is outside the end of the 2B region (fig. 6) which is thought to be involved in the overlap according to the model discussed above. Therefore, these mutations must in some other way interfere with molecule interactions in KIF.

Family I006 was also noteworthy for the fact that the abnormal allele did not appear to represent one-half of I-1's complement of keratin 10 DNA, as evidenced on the sequencing gel and in the allele-specific assays (fig. 5). This may be due either to inefficient amplification of the mutant allele, or, alternatively, to somatic mosaicism, with the mutational event occurring postconception. A similar case was reported by Cheng et al. (1992), and both in that case and in I006, the individual of interest was the first case in the family.

There is clear clinical heterogeneity in EH (DiGiovanna and Bale, submitted), and it is interesting to speculate whether the specific clinical phenotype correlates with the member of the keratin pair that is involved, the site on the protein chain in which the mutation has occurred, or with the particular codon substitution. For example, none of the patients reported here with mutations in keratin 10 have severe palmar/plantar hyperkeratosis, whereas all the EH patients we have reported to date in which keratin 1 is implicated have severe palmar/plantar disease (Chipev et al. 1992; Compton et al. 1992; DiGiovanna et al. 1993; Yang et al., in press). Understanding the clinical consequences of the specific mutations should allow for further delineation of the structure/function relationship of keratins and epidermis.

To date, we have searched for mutations in 17 patients with EH and have identified keratin abnormalities in 10 of them (6 described here; 1 published elsewhere [Chipev et al. 1992; Compton et al. 1992], and 3 others [Yang et al., in press]). The remaining families do not have mutations anywhere in the rod domains of either keratin 1 or keratin 10. It is possible that the end domains of these keratins or another keratin gene are responsible. Alternatively, another gene involved in terminally differentiating epidermis may harbor mutations that cause EH in these families.

From our series of EH patients we have added six

new cases of keratin 10 mutations, four of which have not been reported before, to the developing catalog of keratin mutations in EH, bringing the total number of reported unique mutations to six. This information can be used for prenatal diagnosis in affected families and to identify the hot spots for mutation in newly ascertained patients. It is even conceivable that appropriate biologic molecules can be designed (e.g., nucleic acids and peptides) and introduced into the keratinocytes early enough to be of therapeutic value.

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