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22. Suckling mice (2 to 3 weeks old) from ten independent litters were studied. This group comprised CFTR(−/−): 10 ± 1 g, range 9 to 14 g; $n = 7$ (6 females, 1 male); CFTR(+/−) and CFTR(+/−): 11 ± 1 g, range 5 to 18 g, $n = 9$ (6 females, 3 males). Four to eight sections of gut (jejunum, cecum, proximal colon) from each mouse were mounted full thickness (unstripped) in Ussing chambers (aperture = 0.045 or 0.25 cm^2). Tissues were bathed on the luminal and basolateral surfaces with oxygenated Krebs bicarbonate Ringer (KBR) buffer (*33*) containing 5 mM glucose, and bioelectric measurements were made under voltage clamp conditions [R. C. Boucher *et al.*, *J. Clin. Invest.* **78**, 1245 (1986)]. In jejunal preparations, glucose was replaced with 5 mM mannitol in the luminal KBR solution.
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Mutations in the Rod Domains of Keratins 1 and 10 in Epidermolytic Hyperkeratosis

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Epidermolytic hyperkeratosis is a hereditary skin disorder characterized by blistering and a marked thickening of the stratum corneum. In one family, affected individuals exhibited a mutation in the highly conserved carboxyl terminal of the rod domain of keratin 1. In two other families, affected individuals had mutations in the highly conserved amino terminal of the rod domain of keratin 10. Structural analysis of these mutations predicts that heterodimer formation would be unaffected, although filament assembly and elongation would be severely compromised. These data imply that an intact keratin intermediate filament network is required for the maintenance of both cellular and tissue integrity.

Epidermolytic hyperkeratosis (EHK) (bullous congenital ichyosiform erythroderma) is clinically distinct from epidermolysis bullosa simplex (EBS) (*1*) and is characterized by an ichthyotic, rippled hyperkeratosis, reminiscent of corrugated cardboard, particularly around joints and folds (*2*, *3*). At birth and during early life, affected individuals exhibit erythroderma, widespread blistering, and erosions due to continuous lysis of the supra-basal keratinocytes. With age, the erythroderma resolves, blistering diminishes, and hyperkeratotic lesions become the predominant feature. Normal epidermis undergoes continuous renewal as keratinocytes move from the proliferative basal layer to the terminally differentiated squames of the stratum corneum. In patients affected with EHK this process is perturbed (*4*) and the normal epidermal barrier function is lost (*2*).

Expression of keratin proteins is specific for each epithelial cell type and its state of differentiation. Keratins consist of a conserved rod domain with four α-helical regions separated by short non-helical linker sequences and flanked by non-helical, globular sequences of varying size and composition (*5*). The smaller, acidic, type I keratins (keratins 9 through 19) are encoded on chromosome 17, and the larger, more basic, type II keratins (keratins 1 through 8) are encoded on chromosome 12 (*6*). One member from each type is required to form the coiled-coil structure of the heterodimer subunits, which then assemble into tonofilaments (*7*). Basal epidermal cells express keratins 5 and 14, but once cells commit to terminal differentiation and begin to migrate into the spinous layer, expression of keratins 5 and 14 is downregulated and expression of keratins 1 and 10 is induced (*8*).

EHK is characterized by a collapsed keratin filament network with clumping of the tonofilaments around the nucleus in the supra-basal spinous and granular cells (*9*, *10*). Although such collapse suggests a defect in a differentiation-specific keratin (*11*), other possible causes for EHK include abnormalities in filaggrin, a keratin filament-associated protein (*10*), involucrin, a cornified envelope precursor (*12*), or a defect in lysosomal metabolism (*13*). However, several families with EHK show linkage to chromosomal 12 near the gene locus for the type II keratins (*14*). We therefore

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analyzed DNA from patients with EHK for abnormalities in the differentiation-specific keratins, 1 and 10.

We have studied three families in which the clinical diagnosis was confirmed by histopathology (Fig. 1). The affected individuals of the EHK-P family exhibited rare blistering neonatally and during treatment with retinoids. Otherwise, they suffered primarily from disseminated hyperkeratotic lesions over joints, hands, and feet. WL, now age 17, still exhibits frequent blistering in addition to extensive hyperkeratotic lesions on extremities, trunk, and face. Both affected individuals of the EHK-O family show widespread hyperkeratosis and palmo-plantar keratoderma, and have responded to isotretinoin therapy with diminution of hyperkeratosis and blistering. Blistering has not been a significant problem since childhood.

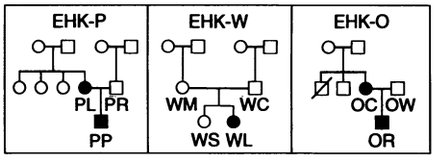

Fig. 1. Pedigrees of the families affected with EHK. Males are represented by squares, females by circles. Affected individuals are represented by solid symbols. DNA was sequenced from individuals indicated by initials. PL (44 years), PR (46 years), PP (22 years), WM (48 years), WC (50 years), WS (20 years), WL (17 years), OC (41 years), OW (44 years), and OR (17 years).

We sequenced the α-helical rod domains of keratins 1 and 10 from the affected individuals (*15*). In the EHK-P family (Fig. 2A), both affected individuals had a mutation within the conserved helix-termination motif at the end of the 2B segment of keratin 1 (Fig. 3). Affected individuals of the EHK-O family had a mutation at the junction of the helix-initiation motif and the 1A segment of the keratin-10 rod domain (Figs. 2B and 3). The affected individual of the EHK-W family had a transition in the keratin-10 rod domain within the highly conserved helix-initiation motif (Figs. 2C and 3). Sequence of the rod domains of keratins 1 and 10 from ten normal individuals was invariant except for two polymorphisms in the 2B segment of keratin 1 and one polymorphism in the 2B segment of keratin 10. These polymorphisms resulted in the expression of the normal amino acid. No sequence changes were observed in the helix initiation and termination motifs from 16 unrelated control individuals and 5 unaffected family members. To confirm that mutations were not introduced by the polymerase chain reaction (PCR), each reaction was repeated at least twice and the products sequenced independently. Keratinocytes cultured after biopsy of affected individuals from the EHK-O and EHK-W families (*16*) expressed mRNA with the same mutations. These cells had a normal keratin filament network when grown in low calcium medium, an environment similar to that experienced by basal cells, in which the cells express only keratins 5 and 14. When differentiation was induced by increasing the extracellular calcium concentration (*17*), the filament network collapsed around the nucleus and the cells expressed the mutant supra-basal keratins (*16*).

Table 1. Ionic interactions between keratin 1 and keratin 10 and their mutant counterparts. Ionic interaction score was calculated for a parallel, in-register chain arrangement (*20, 27*). HK1, human keratin 1; HK10, human keratin 10.

Type II keratin	Type I keratin	Score
HK1	HK10	+9
HK1	HK10(WL)	+9
HK1	HK10(OR)	+9
HK1(PP)	HK10	+10

The helix-initiation motif of the 1A segment and the helix-termination motif at the end of the 2B segment (Fig. 3) are highly conserved among intermediate filament chains (types I through VI) and may affect molecular assembly (*18–20*). Analysis of the interchain ionic interactions between keratins 1 and 10 and the mutant proteins showed that none of the mutant proteins had fewer of the ionic interactions that specify and stabilize the parallel in-register chain arrangement in the multimerized molecule (Table 1). Indeed, the E/Q mutant protein may have an even greater avidity for its partner than the wild-type keratin 1 protein (Table 1). Therefore, these mutations probably do not disrupt heterodimer formation, but may destabilize the head-to-tail overlap between parallel molecules (*20*), and hence cause the malformation and potential collapse of the intermediate filament network.

Analysis of patients with EBS and in vitro mutagenesis have shown that the conserved termini of the α-helical rod domain are essential for filament assembly (*1, 19, 21–24*). The R-to-H (*25*) mutation at position 10 of keratin 10 (patient WL) is analogous to a mutation observed in the keratin 14 gene of a patient with EBS (*22*), and is at a site essential for stability of the nuclear lamin filament network (*23*) and of the keratin 5/14 filament network (*22*). Similarly, the E-to-Q substitution in the helix-termination motif of keratin 1 (patients PL and PP) occurs at a position that is necessary to maintain the integrity of the nuclear lamin filament network (*23*). A mutation in the helix-termination motif of

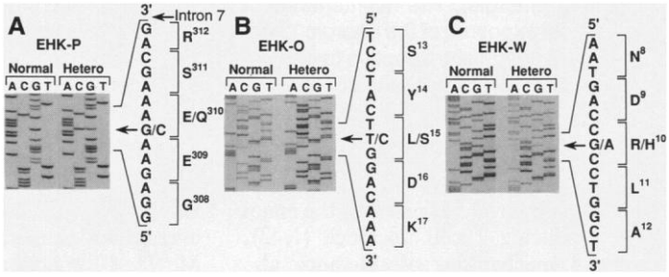

Fig. 2. Sequence analysis of the differentiation-specific keratin genes from families affected with EHK. Normal, sequence obtained from an unaffected family member. Hetero, sequence from an affected individual who is heterozygous for the mutant allele. The amino acid (*25*) and nucleotide sequences have been numbered with respect to the rod domain (see Fig. 3) rather than the start of translation, as extensive size polymorphisms have been observed in keratin globular-end domains (*28*). (**A**) Sequence of keratin 1 near bp 928 from the EHK-P family. Affected individuals show a G-to-C substitution. (**B**) Sequence of keratin 10, near bp 44, for the EHK-O family. Affected individuals show a T-to-C substitution. (**C**) Sequence of keratin 10 near bp 29 from the EHK-W family. Affected individual shows a G-to-A substitution.

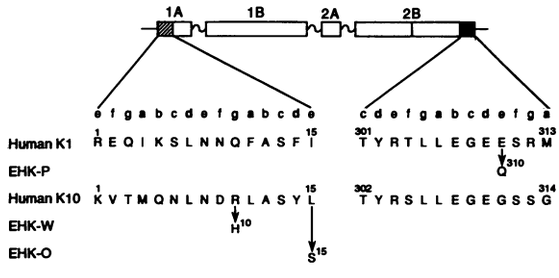

Fig. 3. Organization of the keratin filament rod domain. The α-helical segments, 1A, 1B, 2A, and 2B, are separated by linker sequences (wavy lines). The vertical line in the middle of the 2B segment represents the "stutter"—a discontinuity in the heptad repeat that is conserved in all intermediate filaments. Helix initiation and termination motifs are denoted by hatched and solid boxes, respectively. Amino acid sequences (*25*) for these motifs are shown for keratin 1 (K1) and keratin 10 (K10). The relative position of a given amino acid within the heptad repeat is designated (a through e). Positions a and d are occupied by apolar residues (often leucine) and lie on the inner face of the coiled-coil (*5*).

keratin 5 has also been implicated as the genetic defect in another family affected with EBS (24).

The mutations described in these patients suggest that defects in keratins not only cause cytolysis, as observed in EBS and EHK, but also acanthosis and hyperkeratosis, as observed in EHK. Thus, the integrity of the keratin intermediate filament network contributes to mechanical stability of the keratinocyte and to the maintenance of a functional epidermis. EHK may be heterogenous with respect to the underlying defect, as is EBS, and it is likely that mutations will be discovered in other conserved regions of these keratins (26).

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Maintenance of in Vivo Tolerance by Persistence of Antigen

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T cells of the immune system respond only to foreign antigens because those cells with reactivity for self proteins are either deleted during their development or rendered nonresponsive (anergic). The maintenance of the nonresponsive state was found to require the continual exposure of the anergic T cells to antigen. When anergic T cells were removed from the self antigen by adoptive transfer to a mouse strain lacking the antigen or by in vitro culture, nonresponsiveness was reversed and the anergic cells returned to normal functional status.

Although the thymus is probably the major site for inducing T cell tolerance (*1*, *2*), peripheral mechanisms of tolerance also exist (*3*, *4*). Data from several of these systems indicate that peripheral tolerance may result in the generation of clonal anergy rather than clonal deletion. A nondeletional form of developmental tolerance is induced in radiated bone marrow chimeras (*5*, *6*). This model system takes advantage of the fact that T cells bearing particular T cell receptor (TCR) V_β chains react with minor lymphocyte stimulatory (Mls) or major histocompatibility complex (MHC) antigens. In certain strains that possess specific self antigens, this reactivity results in the deletion of entire subsets of T cells that bear a particular TCR V_β chain. For example, T cells bearing $V_\beta 6$ and $V_\beta 17$ are specific for Mls-1^a and I-E, respectively (*1*, *7*). However, when chimeras are constructed with *Mls-1*^{b}, I-E$^-$ bone marrow cells that are injected into mice that are heavily irradiated (1000 R) and are *Mls-1*^{a}, I-E$^+$, the majority of T cells bearing these receptors are not clonally deleted.

Despite the lack of deletion in such chimeras, no T cell reactivity toward host antigens in mixed lymphocyte reactions in vitro or in graft-versus-host reactions in vivo can be detected (*5*, *6*). The proliferative response to stimulation by monoclonal antibodies to $V_\beta 6$ and $V_\beta 17$ is decreased by 80 to 99% (*5*). This effect can be overcome in the CD8 subset, but not in the CD4 subset, by the addition of exogenous interleukin-2 (IL-2) (*8*). In order to determine whether the nonresponsive state in these chimeric animals was reversible, we adoptively transferred lymphocytes from chimeric and control mice into irradiated host animals that lacked the Mls-1^a and I-E antigens and then assessed the phenotypic and functional status of the transferred cells.

For the purposes of this study, two types of

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