Dominant and Recessive Compound Heterozygous Mutations in Epidermolysis Bullosa Simplex Demonstrate the Role of the Stutter Region in Keratin Intermediate Filament Assembly*

Received for publication, January 30, 2002, and in revised form, April 19, 2002
Published, JBC Papers in Press, April 24, 2002, DOI 10.1074/jbc.M200974200

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Keratin intermediate filaments are important cytoskeletal structural proteins involved in maintaining cell shape and function. Mutations in the epidermal keratin genes, keratin 5 or keratin 14 lead to the disruption of keratin filament assembly, resulting in an autosomal dominant inherited blistering skin disease, epidermolysis bullosa simplex (EBS). We investigated a large EBS kindred who exhibited a markedly heterogeneous clinical presentation and detected two distinct keratin 5 mutations in the proband, the most severely affected. One missense mutation (E170K) in the highly conserved helix initiation peptide sequence of the 1A rod domain was found in all the affected family members. In contrast, the other missense mutation (E418K) was found only in the proband. The E418K mutation was located in the stutter region, an interruption in the heptad repeat regularity, whose function as yet remains unclear. We hypothesized that this mutated stutter allele was clinically silent when combined with the wild type allele but aggravates the clinical severity of EBS caused by the E170K mutation on the other allele. To confirm this in vitro, we transfected mutant keratin 5 cDNA into cultured cells. Although only 12.7% of the cells transfected with the E170K mutation alone showed disrupted keratin filament aggregations, significantly more cells (30.0%) cotransfected with both E170K and E418K mutations demonstrated keratin aggregation (p < 0.05). These transfection assay results corresponded to the heterogeneous clinical findings of the EBS patient in this kindred. We have identified the first case of both compound heterozygous dominant (E170K) and recessive (E418K) mutations in any keratin gene and confirmed the significant involvement of the stutter region in the assembly and organization of the keratin intermediate filament network in vitro.

The epidermis plays an important role in the protection from environmental insult by forming an extensive cytoskeletal network within keratinocytes, comprising of keratin intermediate filaments (KIF) belonging to the intermediate filaments (IF) superfamily (1, 2). Keratins can be subdivided into two separate subfamilies, type I, the acidic keratins, and type II, the neutral-basic keratins (3–5). They have a central 310-amino acid α-helical rod domain composed largely of seven residue heptad repeats. This domain exists in four segments (1A, 1B, 2A, and 2B) interrupted by three non-helical linkers (L1, L12, and L2) and a so-called, stutter region (6, 7). The stutter region is an interruption in the regularity of the heptad repeat substructure, which generates a helix phase reversal near the middle of the 2B rod domain segment and is considered to be the result of a deletion of three residues at the junction of two heptads (8). Although the stutter region is highly conserved among all IF (7), its molecular functions in KIF assembly remain unknown.

Mutations in either the KRT5 or KRT14 gene, which encode the epidermal keratins K5 and K14, respectively, lead to the epidermolysis bullosa simplex (EBS), the majority of which are inherited in an autosomal dominant manner and are characterized by intraepidermal blister formation (9). According to the clinical severity of blister formation, EBS can be subdivided into three major subtypes (10). The mildest variant is the Weber-Cockayne type EBS (EBS-WC) with blistering restricted to the hands and feet, the more moderate variant is the Koebner type (EBS-K) with generalized blister formation, and the most severe variant (Dowling-Meara type; EBS-DM) is characterized by severe herpetiform blistering (10).

EBS was the first human keratin disease to be identified (11–13) that came from the ultrastructural findings demonstrating the clumping of KIFs in the basal keratinocytes of patients with EBS-DM (14, 15). Transgenic mice engineered to express the mutant human K14 exhibited similar phenotypic and morphological characteristics to EBS-DM, which suggested that keratin mutations disrupt KIF assembly in a dominant negative fashion (16, 17). It showed that if even a small amount of copolymerizing keratin protein is defective, this is sufficient to disrupt the whole KIF network. The severity of EBS has been shown to be dependent upon the structural implications for each mutation, including its location within the protein and its effect upon KIF formation (18, 19). The mutations responsible for EBS-DM lie within the highly conserved ends of the rod domain, which are critical for proper K5 and K14 filament assembly (11, 20, 21). In contrast, EBS-K

* This work was supported by Grants-in-aid for Scientific Research (A, 13357008) and (B, 12470175) (to H. S.) from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) NM000429, AF057621, AF071984, X05418, X05421, X14028, L25583, BC000163, Y00067, and M13451.

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1 The abbreviations used are: KIF, keratin intermediate filament; EBS, epidermolysis bullosa simplex; EBS-DM, Dowling-Meara type EBS; EBS-K, Koebner type EBS; EBS-WC, Weber-Cockayne type EBS; HIP, helix initiation peptide; HTP, helix termination peptide; IF, intermediate filament; K5, keratin 5; K14, keratin 14; MDCK, Madin-Darby canine kidney.
mutations are also located within the rod domain but are more centrally located (12, 13) and EBS-WC mutations are mostly found in the non-helical regions (22-24).

We have studied an EBS family in which the affected individuals showed a variable phenotype and have identified two distinct KRT5 mutations in the proband, the most severely affected member. He is compound heterozygous for both a dominant mutation in the helix initiation peptide (HIP) and a recessive mutation in the stuffer region of K5. To confirm whether the stutter mutation exacerbated the clinical severity of EBS caused by the dominant mutation on the other allele, we examined cultured cells transfected with the mutated KRT5 cDNA corresponding to the proband’s mutations. Here we show evidence that the stutter region plays an important role in the organization of the KIF network.

**Experimental Procedures**

**EBS Family with Heterogeneous Clinical Presentation**—The proband (Fig. 1, III:4) was a 22-year-old man with blistering over his entire body from birth and was given a diagnosis of EBS-K. Although the blistering on the trunk improved with age, he continued to get severe blisters and erosions on the hands and feet after minor trauma with a worsening of symptoms during the summer months (Fig. 2). Electron microscopy of the proband showed that cytolysis occurred within the basal keratinocytes consistent with EBS, although apparent clumping of keratin filaments was not observed (data not shown). His family had several affected members (Fig. 1). An older brother (III:6) also presented with blistering over the whole body since birth, with similar clinical findings to the proband, but died in a traffic accident in childhood. In contrast, his deceased father (II:6), deceased grandmother (I:2), and his paternal uncle (II:2) developed few blisters that were restricted to the soles only after extensive walking. They seldom exhibited blisters with increasing age; therefore, they were unaware that they had any disease. All of them were given the diagnosis of EBS-WC, not EBS-K. Thus the markedly heterogeneous clinical presentation in this dominantly inherited EBS family could not be explained by a single dominant keratin mutation (Fig. 1).

**Mutation Analysis**—Genomic DNA extracted from peripheral blood was used as a template for PCR amplification. The KRT5 and KRT14 genes were amplified by the methods previously reported (25, 26). Specifically, for the amplification of the 407-bp DNA fragment comprising the 339-bp exon 1 of KRT5 (nucleotides 216–555 in the cDNA; GenBank™ accession number NM000424) containing the paternal mutation E170K, the following primers were used: 5′-GAGGATATCCATTGAAAGCAC-3′ (forward) and 5′-CCCTCTTCTCATCTCTTGGC-3′ (reverse). For amplification of the 558-bp DNA fragment comprising the 221-bp of exon 7 of KRT5 (nucleotides 1219–1440 in the cDNA; GenBank™ accession number NM000424) containing the maternal mutation E418K, the following primers were used: 5′-GAGGCGGAGATTGACAATG-3′ (forward) and 5′-TAGGACGCTCCTTCCTTATC-3′ (reverse). Fifty-microliter reactions containing 250 ng of DNA, 200 μM of each dNTP, 450 nM of each primer, 2 units of AmpliTaq polymerase, 1.5 mM MgCl2 (PerkinElmer Life Sciences, Foster City, CA), followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. DNA sequencing of the proband and the unaffected members was carried out using a Genetic Analyzer 310A automatic sequencer (PerkinElmer Life Sciences-ABI, Foster City, CA).

**Verification of the Mutations**—Because there was no proper restriction enzyme to verify each mutation, we created restriction enzyme sites designed by BsrBI. The genomic template was amplified by PCR under the same conditions with the following primers: for amplification of the 333-bp DNA fragment containing the E170K mutation and changing the base from A to G at base 512 in exon 1, forward primer 5′-GGTGGTGCCGTTGTTGATT-3′ and reverse primer 5′-GTGGTGCCGTTGTTGATT-3′ were used. For amplification of the 321-bp DNA fragment containing the E418K mutation and changing an A to T at base 1252, forward primer 5′-GAGGACGAGATTGACAATG-3′ and reverse primer 5′-TAGGACGCTCCTTCCTTATC-3′ were used. Both mutations (G to A substitutions) resulted in the loss of the restriction enzyme site for BsrBI. The restriction enzymes used for the verification of each mutation were generated with the use of the site-directed mutagenesis system Mutan-Super Express kit (Takara, Japan) and subcloned into the same vectors (pK5E170KF and pK5E418KF). Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 5% CO2. The results confirm heterozygous E170K mutation in the affected family members (II:2, III:4) and heterozygous E418K mutation in the proband and the unaffected members (II:7, III:4, III:7).

![Fig. 1](http://example.com/fig1.png)
the mutated KRT5 cDNA (pK5F/pK5E170KF or pK5F/pK5E418KF), and the combinations with an equimolar ratio of the two mutated KRT5 cDNA (pK5E170KF/pK5E418KF) were performed using LipofectAMINE reagent (Invitrogen-BRI, Bethesda, MD) according to the distributor’s recommendation.

Confocal Laser Analysis—At 24 h after transfection, MDCK cells were washed with phosphate-buffered saline and fixed in 2% paraformaldehyde for 10 min and permeabilized by incubation with 0.5% Triton-X for 10 min. To detect transfected cells, an anti-FLAG antibody (Stratagene, La Jolla, CA) was used to recognize the FLAG tag as a secondary antibody. All cells were observed using a confocal laser scanning microscope (Olympus Fluoview FV3000). The cells with keratin aggregates were counted in three different areas, two from each experimental replicate, and the results obtained from six counts were expressed as the mean ± S.D.

RESULTS

A Patient with EBS Compound Heterozygous for a Dominant and a Recessive Mutation in KRT5—KRT5 and KRT14, including all the exons and exon-intron borders, were amplified using the proband’s genomic DNA and PCR primers and directly sequenced. There was no mutation in KRT5. One mutation was a G to A transversion at base 508, leading to the exchange of a glutamic acid to lysine at residue 170 (E170K) in the 1A domain (Fig. 1A). Another mutation was a G to A transversion at base 1252, leading to the same amino acid substitution at residue 418 (E418K) in the 2B domain (Fig. 1B). To verify each mutation, KRT5 was amplified by PCR with primers generating new restriction enzyme sites recognized by BsrBI (Fig. 1, D and E). Both mutations (G → A substitutions) resulted in the loss of the restriction enzyme site for BsrBI. These mutations were not found among 100 normal alleles, suggesting that they are not polymorphisms within the normal Japanese population. The altered glutamic acid at residue 170 is within the second residue of the HIP, a well conserved motif among all IF types. Notably, another mutated glutamic acid at residue 418 in the stutter region of the 2B domain was also highly conserved among all types of IF (Fig. 3).

Analysis of KRT5 of the proband’s relatives (II:2, II:7, III:5, III:7) revealed that the E170K mutation was on the paternal allele and the E418K mutation on the maternal allele. His affected uncle (II:2), who showed a milder clinical phenotype (EBS-WC) than the proband’s (EBS-K), had only the E170K mutation, while his mother (II:7) and his brother (III:7) with the E418K mutation were clinically normal (Fig. 1C). We therefore hypothesized that the E170K mutation caused the EBS-WC phenotype in the family, and we suggest that the proband’s clinical phenotype was aggravated by the E418K mutation consistent with the proband being a compound heterozygote for dominant and recessive mutations. Although we could not perform mutational analysis with DNA from his deceased older brother (III:6), the similar clinical severity of EBS suggests that he might also be a compound heterozygote for dominant (E170K) and recessive (E418K) mutations.

The Mutated K5 Protein Disrupts the KIF Network in Vitro—We examined whether the clinical heterogeneity of the EBS family could be demonstrated in a cell culture system. Three mammalian expression vectors containing the wild type KRT5 (pK5F), the mutated KRT5 correspondent with the E170K mutation (pK5E170KF), and the E418K mutation (pK5E418KF) were constructed. Four distinct gene transfections using the MDCK cells were performed, including the pK5F alone, a combination with equimolar ratio of pK5F and either of pK5E170KF or pK5E418KF, and a combination with equimolar ratio of the two mutated KRT5 cDNA (pK5E170KF and pK5E418KF). We chose MDCK cells for the transfection study, because they do not express endogenous K5 and K14 (27), and previous studies have demonstrated that MDCK cells transfected with the human KRT5 gene could readily incorporate K9 into their endogenous KIF network (28).

A monoclonal antibody against the tag sequence, FLAG, was used to detect the KIF network derived from transfected KRT5. The cells transfected with pK5F alone showed fine bundles of keratin filaments extended throughout the cytoplasm without disturbing the cells’ morphology (Fig. 4, A–C), whereas cells cotransfected with the wild type and mutated KRT5 gene (pK5E170KF/pK5F), (pK5E418KF/pK5F), or both mutated KRT5 (pK5E170KF/pK5E418KF) exhibited small ball-like filament aggregates indicating a disruption in the keratin network (Fig. 4, D–I).

FIG. 2. Clinical features of the proband. The proband continues to get occasional blisters and erosion on the soles and palms, particularly in areas of trauma. Old and new blisters and erosion with crusting are seen on the soles, heels, and dorsum of toes. The other affected family members have never presented any similar clinical findings.
The mutated K5 protein disrupts the KIFs network in vitro. MDCK cells were transfected with plasmid pK5F (A–C) or cotransfected with pK5E170KF/pK5F (D–F) or pK5E170KF/pK5E418KF (G–I). To visualize the transfected gene product, cells were stained with a rat monoclonal antibody recognizing the C-terminal sequence of FLAG. Antibody staining was followed by fluorescein isothiocyanate-conjugated goat anti-rat IgG. Cells transfected with pK5F alone had a normal keratin filament network (A–C), whereas significantly more cells transfected with pK5E170KF/pK5F (D–F), pK5E170KF/pK5E418KF (G–I), and a few of the cells transfected with pK5E418KF/pK5F (data not shown) exhibited small ball-like clump formation (arrows).

The Stutter Mutation E418K Exacerbated the KIF Network Disturbance—The percentage of the cells with keratin clumping in each transfection assay was as follows, pK5F (1.5 ± 1.8), pK5F/pK5E1418KF (4.4 ± 1.7), pK5F/pK5E170KF (12.7 ± 4.3), and pK5E170KF/pK5E418KF (30.0 ± 3.1) (Fig. 5). Statistical analysis showed that significantly more clumped cells were observed in cells cotransfected with pK5E170KF/pK5E418KF than those with pK5F/pK5E170KF (p < 0.05). These results suggested that the compound heterozygous mutations (E170K and E418K) within the keratin genes resulted in a more severe keratin network disturbance than the heterozygote for dominant mutation (E170K) alone in vitro. This is concordant with the markedly variable severity of EBS phenotype in this family.

DISCUSSION

We have presented the first case of a compound heterozygote with both dominant and recessive mutations, and have demonstrated that the recessive stutter region mutation (E418K) in KRT5 exacerbated the disruption in the KIF network caused by the dominant mutation (E170K). Although most cases of EBS are autosomal dominant and are caused by missense mutations (9), whether they are fully or partially dominant is still not clear. For example, a partial dominant KRT14 mutation was found in an EBS family with consanguineous marriage, in which homozygotes had a more severe clinical phenotype than heterozygotes (29).

Usually, compound heterozygous genotypes involve different recessive alleles at the same locus; nevertheless, in some diseases compound heterozygotes with one dominant allele and one recessive allele are known (30). In these cases, although the heterozygous individuals with a recessive allele are phenotypically normal, heterozygotes for the dominant allele develop the disease, and compound heterozygotes usually manifest more severe symptoms than heterozygotes due to the dominant allele (30). For instance, we recently reported such cases of dystrophic epidermolysis bullosa in which type VII collagen was the defective gene (31). Heterozygotes with a dominant glycine substitution mutation in COL7A1 presented with only toe nail dystrophy without skin fragility, whereas compound heterozygotes for this dominant and recessive mutation led to dystrophic epidermolysis bullosa with marked skin fragility (32–33).

In this study, the mother and brother who were heterozygous for the E418K mutation were clinically normal, however, it was difficult to demonstrate such a subclinical abnormality in vivo. We examined how the E418K mutation would influence KIF formation in vitro using a transfection assay, for which several studies have been done to examine how EBS mutations perturb the KIF assembly (27, 34, 35). In cultured EBS keratinocytes, keratin aggregates were observed in the majority of EBS-DM keratinocytes but in only a few of the EBS-K cultures (19). Cells transfected with a mutated keratin gene corresponding to each type of EBS showed similar results (19), suggesting that the transfection assay correlated well with the clinical severity of EBS and to the degree to which a particular mutation perturbed filament assembly. In our study, the keratin aggregate formation was observed in significantly more cells when cotransfected with E170K and E418K mutations, which was concordant with the clinical findings. This indicates that the E418K mutation exacerbates KIF disruption, similar to the proband phenotype.

The locations of these EBS mutations in the keratin gene
structure are known to correlate with the disease severity. We know that the ends of the rod domain, HIP and helix termination peptide (HTP), where most EBS-DM mutations are situated, are important areas in which K5 and K14 assemble into filaments (11, 20, 21). Recent research has suggested a role for each region of keratin proteins in heterodimer formation, helix stabilization, and filament assembly (36–38), nevertheless, much about IF structure and function remains unknown.

Particularly, the functions of the IF stutter region have yet to be fully elucidated. The fact that the stutter region is highly conserved among all IFs (7) indicates its importance in IF structure and function. Two computer-based molecular models of the stutter region have been proposed. Steinert and North et al. (39, 40) concluded that the stutter was limited to a very short distance and, therefore, did not cause a significant interruption or kink in the coiled-coil α helix structure. Conversely, Brown et al. (41) proposed that the unwound coiled-coil model, in which the stutter region exacerbated this KIF disturbance. These results and the recessive mutation found in the stutter region of the K5 molecule exacerbated this KIF disturbance. These results and transfection assay data emphasize the sensitivity and importance of the stutter region in the structural alterations and organization of the KIF network.

Acknowledgments—We thank the members of the family described in this report. We thank Dr. Haruki Nakamura for helpful advice of keratin molecules and Maki Goto for excellent technical assistance.

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