cDNA Cloning, Expression, and Assembly Characteristics of Mouse Keratin 16*

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There has been speculation as to the existence of the mouse equivalent of human type I keratin 16 (K16). The function of this keratin is particularly intriguing because, in normal epidermis, it is usually confined to hair follicles and only becomes expressed in the suprabasal intrafollicular regions when the epidermis is traumatized. Previous studies suggested that K16 is highly expressed in the skin of mice carrying a truncated K10 gene. We therefore used the skin of heterozygous and homozygous mice to create a cDNA library, and we report here the successful cloning and sequencing of mouse K16. Recent in vitro studies suggested that filaments formed by human K16 are shorter than those formed by other type I keratins. One hypothesis put forward was that a proline residue in the IB subdomain of the helical domain was responsible. The data presented here demonstrate that this proline is not conserved between mouse and human, casting doubt on the proposed function of this proline residue in filament assembly. In vitro assembly studies showed that mouse K16 produced long filaments in vitro. Also, in contrast to previous observations, transfection studies of PtK2 cells showed that mouse K16 (without the proline) and also human K16 (with the proline) can incorporate into the endogenous K8/K18 network without detrimental effect. In addition, K16 from both species can form filaments de novo when transfected with human K5 into immortalized human lens epithelial cells, which do not express keratins. These results suggest that reduced assembly capabilities due to unusual sequence characteristics in helix 1B are not the key to the unique function of K16. Rather, these data implicate the tail domain of K16 as the more likely protein domain that determines the unique functions.

Keratins are a family of proteins that form the intermediate filament cytoskeleton in epithelial cells. They are divided into two groups, the type I acidic keratin and the type II basic keratins (1). The first step in keratin intermediate filament (KIF) assembly is the formation of heterodimers between a type I and a type II keratin (2, 3). Although any type I keratin can bind with any type II keratin, only certain pairs of keratins exist in vivo (4). These pairs are expressed in a tissue-type and developmentally regulated manner (5). However, for some keratins, such as K17, a specific/unique partner has not been found.

K16 and its partner, K6, are expressed in various stratified epithelia including those of the oral cavity, esophagus, genital tract, and epidermis (6). In the epidermis, they are normally confined to the hair follicles, sweat and sebaceous glands, although they are also expressed in the suprabasal layer of palmpoplantar epidermis (6, 7). The expression of these two keratins is of particular interest since, together with K17, they are expressed in hyperproliferative situations such as benign and malignant tumors, keratinocytes in culture, hyperproliferative skin diseases such as psoriasis, and wound healing (8–11). K6, K16, and K17 are therefore sometimes referred to as the “hyperproliferation-associated” keratins. Often associated with the expression of these keratins is the down-regulation of the differentiation-specific keratins 1 and 10, which normally predominate in the suprabasal epidermis (12). In vitro studies suggest that K16 has unique properties that could affect the structural organization of KIFs (13). Specifically, K16 appears to form shorter filaments than K14 with the type II keratin K5 and K6a. If this property of making shorter filaments is important for the modulation of keratinocyte behavior via alterations in the cytoskeleton during wound healing and in hyperproliferating keratinocytes, then the K16 sequence would be expected to be highly conserved among mammalian species.

Mutations in keratin genes, including K16, have been identified as the underlying cause of several inherited epithelial disorders (14). Mutations in human K16 (hK16) result in pachyonychia congenita type I, which is characterized by nail dystrophy, focal palmpoplantar keratoderma, and oral keratoses reflecting the expression pattern of this keratin (15). Despite the apparently important functions of K16 in humans, there has been some speculation as to the existence of a K16 gene in mice (16). Using a mouse model for the inherited skin disease bullous congenital ichthyosiform erythroderma, our previous studies suggested that K16 is highly expressed in the anacanthotic epidermis of these mice (17). Although there is some evidence to suggest that the hyperproliferation-associated keratins are controlled, at least in part, post-transcriptionally (18), we observed a significant increase in the putative mRNA levels of K16 in K10 mutant mice. It therefore seemed logical to assume that a cDNA library generated from the skin of these mutant mice would facilitate the isolation of the gene for mouse K16 (mK16). Here we describe the cloning and sequencing of a rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis.
cDNA Cloning of mK16

After removing coverslips from the culture dishes, the remaining cells were scrapped from the dish and solubilized in SDS sample buffer (100 μl). One-dimensional SDS-PAGE and immunoblotting were carried out as described previously (19). Primary antibodies were diluted as follows: L6B5 and LL025 (1:5) and RpMk16 (1:5000) in Tris-buffered saline (TBS) (20). Secondary antibody was goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase diluted 1:1000 (Dako, Bucks, UK). Detection was with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Transfection of Eye Lens Cell Line H35CEI—H36CEI cells are derived from human epithelial cells of the eye lens (20). Subconfluent cells grown on 10-mm glass coverslips were individually transfected in a 0.1% (w/v) calcium chloride (CaCl2) solution containing 0.1 M ethanolamine (EtN) at 37 °C for 30 min, annealing at 55 °C for 30 s, and synthesis at 72 °C for 1 min. Random-primed DNA labeling of the probe with digoxigenin was carried out using a DIG-high prime labeling and detection kit (Boehringer Mannheim). The library was screened with a probe prepared by polymerase chain reaction using a 5'-primer with a 3-base pair extension of CAT. After sequencing to check that no errors had been incorporated, the cDNAs were cloned into pT7-7 (mK16) (22) or pEFT-23b (hK16) and (hK5) (Novagen) vectors. These plasmids were individually transformed into Escherichia coli strain BL21(DE3) λlyS3 and grown to an A600 ~ 0.5 before inducing protein expression with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 4 h. Inclusion body preparation was carried out as described previously (23). Keratins were purified by ion-exchange chromatography using a Merck-Hitachi Biochromatography system with a 10 × 150-mm Fractogel-EMD DEAE-650S column (Merck) equilibrated in 8 M urea, 20 mM Tris-HCl (pH 8), 2 mM EDTA, 1 mM Dithiorethiol. Fractions of 3 ml were eluted with a linear salt gradient of 0–0.4 M NaCl over 40 min at 1 ml/min and analyzed by SDS-PAGE.

In Vitro Filament Assembly and Electron Microscopy—Recombinant type I and II keratins were mixed in a 1:1 molar ratio at a concentration of 0.2 mg/ml. Assembly was achieved either by a rapid dialysis procedure against 2.5 mM Tris-HCl (pH 7.5), 25 mM 2-mercaptoethanol at 4 °C for 16–20 h (13, 24) or by a staged dialysis procedure essentially as described (25). Briefly, samples in 8 M urea-containing buffer were dialyzed in 4 × urea-containing buffer and then in 10 mM Tris-HCl (pH 8.0), 25 mM 2-mercaptoethanol. Assembly was monitored at all stages by electron microscopy. Proteins were diluted to 1:10 and negatively stained using 1% (w/v) uranyl acetate. Grids were examined in a Jeol 1200EX transmission electron microscope using an accelerating voltage of 80 kV.

Sedimentation Assay—The efficiency of filament formation was assessed by sedimentation assay (25, 26). Briefly, the assembly mixture was layered on top of 0.85 M sucrose in the final assembly buffer and centrifuged for 30 min at 80,000 × g at 20 °C in a Beckman TLS-55 rotor using a TL100 bench-top ultracentrifuge. The final pellet was dissolved directly in SDS-PAGE sample buffer, and the remaining protein in the supernatant was precipitated (27) before also being resuspended in sample buffer. Both protein samples were dissolved in volumes directly proportional to the original sample volume for direct comparison by SDS-PAGE.

RESULTS
cDNA Cloning and Sequencing of mK16—We have generated a Zap Express mouse cDNA library from the skin of mice created as a model for the skin disease bullous congenital ichthyosiform erythroderma. Using the hK16 probe, two mK16 clones (4.2 and 8.1) and one K14 clone (5.1) were isolated as pBK phagomides. None of the clones contained full-length keratin cDNAs, so the library was rescreened with mK16 cDNA from the longer of the two clones. Two additional clones containing K16 were obtained (7.31 and 2.11), the largest of which was 50 base pairs short at the 5'-end (clone 7.31). The shared sequence of all four clones was identical. We obtained the 5'-sequence including the start codon and 10 base pairs of untranslated sequence using RACE. The complete cDNA se-
The sequence obtained from sequence analysis of the clones and the RACE product is shown in Fig. 1. When scanned against the database, the sequence is most highly related to hK16 (GenBank™ accession number S79867). After the TAA stop codon, there is a 157-base pair untranslated region including a poly(A) addition signal (AATAAA) and an 18-residue poly(A) tail. The K14 clone 5.1 was identical in sequence to the mK14 clone pkSCC52 reported previously (GenBank™ accession number J02644) (28). Our sequence analysis extends the mK14 sequence by an additional 17 amino acids upstream of QNLNDRLATY. The additional amino acids are identical to mK16.

Comparison of mK16 Amino Acid Sequence with Other Keratin Sequences—The sequence codes for a protein of 469 amino acids with a calculated Mr of 51,514. All keratins have structural similarities, including an \( \alpha \)-helical rod domain interrupted by short non-\( \alpha \)-helical linker regions and non-\( \alpha \)-helical head and tail domains. The rod domain is bounded by the highly conserved helix initiation and helix termination motifs and consists of four segments (1A, 1B, 2A, and 2B) of conserved length (29). The sequence of mK16 is 85% homologous to that of hK16 (Fig. 2), with the central \( \alpha \)-helical rod domain being most highly conserved (89% identical over 308 amino acids starting at KVTMQNL). This region bears no homology to hK14 or hK17 and is likely to make K16 unique.

Mouse-specific K16 Polyclonal Antibody—The C terminus of K16 is needed for producing a polyclonal antibody that recognizes K16. This antibody is useful for identifying K16 in tissues and cells.
mK16 is longer than that of hK16 and contains two additional charged residues. Therefore, it is perhaps of no surprise that a polyclonal antibody (RPmK16) raised against the last 15 amino acid residues has proved to be specific for mK16 and does not recognize hK16. We have tested the antibody on human skin specimens including scalp, axilla, thigh, face, breast, lip, palate, and finger and observed no staining of hair follicles or suprabasal epidermis, as is readily seen with the monoclonal antibody to hK16 (LL025) (33). Similarly, the human keratinocyte cell line HaCaT, which constitutively expresses K16, did not stain with RPmK16 (34). Mouse epidermis, however, was recognized by this antibody. Hair follicles and whisker follicles were positive in normal mouse epidermis (data not shown). Also, positive staining of the suprabasal layer of the acanthotic epidermis of K10 mutant mice was observed (data not shown) and was similar to the results obtained with LL025 (35).

Transfection of PtK2 Cells with mK16 cDNA—Previous studies have reported that forced expression of hK16 in PtK2 cells causes the endogenous K8/K18 network to retract from the cytoplasmic periphery (13, 24). This reorganization was described as increasing with time, and at 72 h post-transfection, 50% of the transfected cells showed a juxtanuclear location of the keratin filaments (13). We therefore chose this time point to examine the effect of transfected mK16 in PtK2 cells. In 95% of the transfected cells showed an extensive network of keratin filaments when stained with BL-18 (Fig. 3a). The filaments formed were comparable to those obtained in double transfections of hK14 or hK16 with hK5 in a similar percentage of transfected cells (Fig. 3, b and c). The remainder of the transfected cells either had not formed filaments or contained very short filaments. Sometimes large clumps of keratins were seen in the cells. No filaments were formed in single transfections.

In Vitro Assembly of mK16—The assembly properties of mK16 were of particular interest due to the report that hK16,
under certain conditions, forms shorter filaments with hK5/hK6 than does hK14 (13). Keratins from human and mouse were expressed in bacteria and purified by ion-exchange chromatography for *in vitro* assembly studies. Two different methods of assembly were performed; one used a rapid dialysis procedure, and the other employed a staged dialysis procedure to remove the urea from the protein solution. In both cases, the type I keratins were mixed in a 1:1 molar ratio with hK5. The rapid dialysis procedure was achieved by overnight dialysis into 2.5 mM Tris (pH 7.5) from 8 M urea-containing buffers. These conditions produced shorter filaments for a K5/K16 pairing than for K5/K14 (13, 24). Under these assembly conditions, hK16 with hK5 formed numerous very short filaments (Fig. 6a), similar to those observed previously (13). In contrast, mK16 and K5 formed an abundance of much longer filaments (Fig. 6c). Using the staged dialysis procedure, both mK16 and hK16 formed long filaments at pH 7.5 (Fig. 6b and d).

To assess the efficiency of the *in vitro* assembly experiment, a sedimentation assay was performed. In agreement with the electron microscopy results, the K5/hK16 filaments in the rapid dialysis procedure were not efficiently sedimented due to their short length (data not shown), unlike the K5/mK16 filaments, which sedimented under the conditions of the assay (Fig. 7). Addition of 50 mM salt to the assembly mixture promoted complete assembly of both keratins. The staged dialysis of keratins also led to complete assembly of keratin filaments (data not shown).

**DISCUSSION**

This study demonstrates unequivocally the existence of a mouse gene for K16. The amino acid sequence of mK16 is 85% homologous to that of hK16. hK16 possesses a proline in the helix 1B segment (13). This is not a unique situation, as another intermediate filament (CP49Ins) possesses a proline in a similar position (32). Proline residues are not usually seen in helical proteins, and they would be expected to disrupt the regular helicity of the structure (39, 40). This proline, it was reasoned, could be responsible for the shorter filaments formed when K16 rather than K14 was assembled *in vitro* or in fibroblasts with either K5 or K6b. In other positions in the α-helix,
a proline can be detrimental to KIF assembly, and in many epidermal keratins including K16, such a proline can lead to skin disease (41). In one study, a proline residue was introduced experimentally into the $\alpha$-helical domain at a number of different points; however, an effect on filament assembly was observed only when it was introduced at the ends of the helical region (42). To support the possibility that the proline could be responsible for the unique properties of K16, Wawersik et al. (24) replaced this residue with valine, the corresponding amino acid in hK14, and showed that hK16 could be made to form more stable tetramers. The mouse sequence for K16 lacks this proline residue, which is replaced by a phenylalanine (see Phe-191 in Fig. 2). As this proline is not conserved between mouse and human, it is unlikely to play a key role in the function of K16.

There are several published sequences for hK16 with and without the proline residue (43–46), and it is possible that several different K16 genes exist, as observed with K6 (47).
FIG. 7. Sedimentation assay of K5/K16 assembly. The rapid dialysis procedure was used to assemble in vitro K5/hK16 or K5/mK16 before (+) and after (−) addition of 50 mM NaCl. Keratins in the supernatant (a), in the pellet (b), and before (c) sedimentation were subjected to SDS-PAGE. Note that in the absence of salt, most of the K5/hK16 protein was in the supernatant, in agreement with the electron microscopy data showing that this keratin combination formed short incomplete filaments using the rapid dialysis procedure.

However, no other evidence suggesting more than one hK16 gene exists at present. In our experience, the human sequence containing the proline (GenBank accession number S79867) is the correct one. All four mK16 clones obtained from the expression library in this study were of identical sequence, so we would expect that the sequence reported here (without the proline in coil 1B) is the predominant form of mK16, if not the only form.

Our data do indicate some differences in the in vitro assembly properties of hK16 and mK16. For instance, mK16 forms long filaments using the rapid dialysis procedure, whereas hK16 produces only short filaments with an identical type II partner. Long filaments can be produced in vitro with hK16 or mK16 with hK5 by the staged dialysis procedure. The results obtained using the two methods suggest that hK16 and mK16 have different assembly properties, which may be due to sequence differences between the two keratins. The proline residue is likely to be at least partly responsible for these differences since it has been shown by mutation analysis to affect tetramer stability (24). The reduced tetramer stability of hK16 may mean that this keratin requires more time to reach an energetically favorable state, as provided by the staged dialysis procedure. The weaker interactions at the tetramer stage do not, however, affect the end point of assembly in vitro where both hK16 and mK16 form long filaments. There are also no apparent differences when filaments assemble in transiently transfected cells. The fact that the mouse protein does not share the in vitro filament assembly properties of the human protein suggests that the assembly pathway is secondary to the unique function of this keratin.

In contrast to previous studies (13, 24), we observed no irregularity of the KIF cytoskeleton in K16 transfection studies. In PtK2 cells, we see that both mouse and human K16 are capable of incorporating into the existing K5/K18 keratin filaments. We only see irregular filament formation when the transfected keratin gene carries a pathogenic point mutation as provided by the staged dialysis procedure. The weaker interactions at the tetramer stage do not, however, affect the end point of assembly in vitro where both hK16 and mK16 form long filaments. There are also no apparent differences when filaments assemble in transiently transfected cells. The fact that the mouse protein does not share the in vitro filament assembly properties of the human protein suggests that the assembly pathway is secondary to the unique function of this keratin.

In conclusion, it would appear there is little unusual about the assembly properties of K16 that are likely to be functionally significant for the following reasons. 1) Our transfection studies show that K16 can not only integrate into an existing keratin network, but can also form filaments de novo in vivo. 2) The decreased assembly capability of hK16 in vitro only occurs under certain conditions and is not apparently shared by mK16. 3) Both hK16 and mK16 are equally capable of forming long filaments in vitro with K5. The restricted distribution of K16 in normal tissue and its altered expression pattern in hyperproliferative states such as wound healing and tumorigenesis suggest a specific and specialized role for this keratin. The existence of a mK16 strengthens the evidence for an essential role for this protein that is not provided by other keratins. The unique sequence of the tail domain of K16 is highly conserved between mouse and human and is a favored candidate to provide clues to the function of this protein. Mutation analysis by genetic manipulation could be useful to resolve this issue.

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