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15 March 2005

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cDNA Cloning and Bacterial Expression of the Human Type I Keratin 16

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The human type I keratin 16 is constitutively expressed in a number of complex epithelial tissues, including skin, but is better known for its induction under conditions favoring enhanced proliferation or abnormal differentiation, including wound healing, psoriasis, and cancer. We cloned the coding sequence of human K16 by applying a coupled reverse transcription-polymerase chain reaction procedure to mRNAs prepared from cultured human skin keratinocytes. We then expressed the human K16 coding sequence in E. coli and purified the solubilized protein by anion-exchange chromatography. The recombinant protein recovered behaves similarly to human K14 (a related acidic keratin) on the anion-exchanger, co-migrates with native human K16 on SDS-PAGE (M_r 48 kD), and reacts with antisera directed against human K16. Based on the nucleotide sequence obtained and the properties of the corresponding recombinant protein, we conclude that we have cloned the coding portion of the human K16 cDNA. The sequence data obtained in this study is compared to earlier reports of the human K16 sequence, which are conflicting in many respects. The availability of K16 in a purified recombinant form will allow us to study how its properties may relate to its function during wound healing and in skin diseases. © 1995 Academic Press, Inc.

Keratins are Intermediate Filament (IF) proteins encoded by a large multigene family and expressed in epithelial tissues. The >30 known keratins (M_r 40-70 kD) have been subdivided into type I (acidic, K9-K20) and type II (basic, K1-K8) based on DNA sequence homology and gene structure (1). Keratin filament assembly begins with the formation of a type I-type II heterodimer (2), distinguishing them from many other IF proteins, which form homodimers.

Consequently, all epithelial cells must express at least one member of each sequence subtype in order to assemble a keratin filament network in their cytoplasm. Many keratin genes are in fact regulated in a pairwise, differentiation-specific manner, creating patterns that have been well-conserved among mammalian species (3,4). The functional basis underlying these expression patterns remains poorly understood. At least in epidermis, keratin filaments provide the physical strength that is necessary to maintain its cellular integrity in response to normal mechanical stress

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(5). Mutations affecting specific keratins genes underlie several inheritable skin blistering disorders such as epidermolysis bullosa simplex, epidermolytic hyperkeratosis, and palmoplantar keratoderma (see 6,7).

Injury to stratified epithelial tissues significantly alters keratin gene expression. A strong induction of K6, K16 and K17 expression occurs in the postmitotic, differentiating layers of epidermis and other stratified epithelia (8,9). Following injury to human (e.g. 9,10) and mouse epidermis (our unpublished data), this "switch" in keratin gene expression occurs very rapidly, such that keratins K6 and K16 are detected within a few hours at the edges of the wound. In addition to wound healing, these keratin genes are also expressed in stratified epithelia undergoing chronic hyperproliferation or abnormal differentiation, including cancer (8, 11,12). The specific contribution of K6, K16 and K17 to these alterations in keratinocyte differentiation during wound healing and hyperproliferation in stratified epithelia are not yet understood (see 13 for discussion).

There is considerable confusion in the literature regarding the precise sequence of human K16. There are four published reports containing diverging information concerning the precise nucleotide and / or deduced amino acid sequence for this particular keratin (14,15,16,17). We here report on the cloning of the coding sequence for human K16, and show that it encodes an acidic protein with an apparent molecular weight of 48 kD that immunoreacts with antisera directed against K16. The features of the K16 sequence are discussed in light of recent findings concerning its assembly properties.

Materials and Methods

Cell culture: Epidermal keratinocytes were isolated from human foreskin tissue and seeded as primary culture on mitomycin C-treated NIH 3T3 fibroblast feeders as described (18). The cells were maintained in a 3:1 mixture of Dulbecco-modified Eagle's medium and Ham's F12 medium supplemented with 20 % calf serum, 0.4 µg/ml hydrocortisone, 20 pM triiodothyronine, 5 µg/ml transferrin, 5 µg/ml insulin, and 4 ng/ml EGF. For RNA extraction (see below), cells from the second passage were used.

cDNA cloning and DNA sequencing: The K16 cDNA was cloned from cultured human epidermal keratinocyte RNA using a coupled reverse transcription-polymerase chain reaction (RT-PCR) protocol as described (19). RNA was isolated from near-confluent cultures of keratinocytes according to Chomczynski and Sacchi (20), treated with RNAse free-DNAse (Boehringer Mannheim, Indianapolis, IN), and resuspended at 1 µg/ml in diethyl pyrocarbonate-treated H_2O. Total RNA was primed with random hexamers (Pharmacia Biotech, Piscataway, NJ) and reverse-transcribed into cDNA using Moloney murine leukemia (MMLV) virus reverse transcriptase (BRL, Gaithersburg, MD) in a 40 ul reaction according to Kawasaki (21). PCR was carried out in the same reaction mixes by adding 10x PCR buffer, 40 pmol of primers, and 2.5 U of Amplitaq (Perkin-Elmer Cetus, Norwalk, CT). PCR conditions were 30 cycles of 1 min at 94°C, 45 sec at 59°C, and 1.5 min at 72°C. The oligonucleotide primers used were designed from the published sequence of the human genomic clone GK-3, previously shown to encode K16 (15). They were (forward) 5'-CGCTGACCTCCCTCCTTGGCACCATGG CCA-3', where **ATG** corresponds to the start codon, and (reverse) primer 5'-AGGCCATGGTAGAGGCAGCTCAG TTCTAGGAGC-3', where CTA corresponds to the inverted sequence for the stop codon TAG. In addition, both primers contained the recognition motif for the restriction enzyme Nco I (CCATGG; New England BioLabs (Beverly, MA) to facilitate subcloning of the PCR products into plasmid pET-3d (22) as previously described (23). Candidate recombinant clones were grown, the plasmid DNA isolated (QIAGEN columns, Chatsworth, CA), and the entire PCR-derived insert sequenced according to Sanger et al. (24) using synthetic oligonucleotide primers designed from the published K16 genomic sequence (15).

Keratin expression, purification, and expression system based on the phage T7 RNA recombinant human epidermal keratins as descri E.coli BL21(DE3) strain, grown to O.D.600 o induced by adding IPTG (isopropyl ß-D-thio- hours. Inclusion bodies were isolated from lyse containing 6.5 M urea, 50 mM Tris-HCl, 2 mM sulfonylfluoride), pH 8.1 (Q buffer; see 23). Re by chromatography in Q buffer on a Pharmacia ml·min-1. Proteins of interest were eluted with a 15 ml volume, and 0.5 ml fractions were collect profile was compared to those of the previousl (23). Native human keratins were isolated fron using the high-salt extraction method, and the concentration was determined by the Bradford a For immunoblot analyses, known quantities c electrophoresed, electroblotted to nitrocellulos diluted primary antisera prepared in blocking b and 5 % dry milk). We used a rabbit polyclonal corresponding to the carboxyterminal sequence c recognizes K16, K13 and K15 (clone K8.12; S antibodies were revealed by alkaline phospha manufacturer's intructions (Bio-Rad).

Results

We obtained the entire coding sequence f extracted from cultured primary human epiderm used for PCR generated a reaction product with eletrophoresis; data not shown) which, after re plasmid pET-3d (22), generating pET-K16. T sequenced (Fig. 1), and found to correspond to previously shown to encode human K16 (15) subcloning strategy, from ACC (Thr) to GCC (was shown to have no effect on its assembly be difference was found, 390C->T (codon 130 in code (28), it does not result in an alteration of whether this nucleotide substitution was intro conversely, represents an allelic polymorphism was identical to that of the relevant portion of th

Upon transformation of pET-K16 in recombinant protein expression with IPTG, a n inclusion bodies, as determined by SDS-PAG The solubilized inclusion body fraction was s Mono Q column (Pharmacia) at pH 8.1 and in t the 48 kD protein product eluted with 110 mM for the recombinant form of human K14 (23). purified 48 kD protein co-migrated with native

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24) using synthetic oligonucleotide primers

Keratin expression, purification, and immunological analyses: We used an E. coli expression system based on the phage T7 RNA polymerase gene (22) to generate mg quantities of recombinant human epidermal keratins as described (23). Plasmid pET-K16 was transformed into E.coli BL21(DE3) strain, grown to $O.D._{600}$ of ~0.5, and recombinant keratin expression was induced by adding IPTG (isopropyl ß-D-thio-galactopyraoside) to 1 mM and continued for 5 hours. Inclusion bodies were isolated from lysed bacterial pellets (25) and solubilized in a buffer containing 6.5 M urea, 50 mM Tris-HCl, 2 mM DTT, 1 mM EGTA, 1 mM PMSF (phenyl methyl sulfonylfluoride), pH 8.1 (Q buffer; see 23). Recombinant K16 was purified to near-homogeneity by chromatography in Q buffer on a Pharmacia Mono Q anion-exchange column operated at 0.5 $ml \cdot min^{-1}$. Proteins of interest were eluted with a 0-200 mM linear gradient of guanidine-HCl over a 15 ml volume, and 0.5 ml fractions were collected and analysed by 10% SDS-PAGE. This elution profile was compared to those of the previously characterizedrecombinant human K5 and K14 (23). Native human keratins were isolated from cultured human skin keratinocytes (see above) using the high-salt extraction method, and the final pellet was solubilized in Q buffer. Protein concentration was determined by the Bradford assay (27) using reagents purchased from Bio-Rad. For immunoblot analyses, known quantities of recombinant and native human keratins were electrophoresed, electroblotted to nitrocellulose, and the blots incubated with the appropriately diluted primary antisera prepared in blocking buffer (Tris-buffered saline with 0.5 % Tween 20 and 5 % dry milk). We used a rabbit polyclonal antiserum directed against a 22-mer oligopeptide corresponding to the carboxyterminal sequence of K16 (13) and a mouse monoclonal antibody that recognizes K16, K13 and K15 (clone K8.12; Sigma Chem. Co., St-Louis, MI). Bound primary antibodies were revealed by alkaline phosphatase-conjugated secondary antibodies as per the manufacturer's intructions (Bio-Rad).

Results

We obtained the entire coding sequence for human K16 by applying RT-PCR on total RNA extracted from cultured primary human epidermal keratinocytes. The set of oligonucleotide primer used for PCR generated a reaction product with a 1.4 kb size (as determined by 0.8 % agarose gel eletrophoresis; data not shown) which, after restriction digest, was ligated into the NcoI site of plasmid pET-3d (22), generating pET-K16. The entire PCR-derived insert of pET-K16 was sequenced (Fig. 1), and found to correspond to the nucleotide sequence of genomic clone GK-3, previously shown to encode human K16 (15). The first codon was altered as a result of the subcloning strategy, from ACC (Thr) to GCC (Ala). An identical change in the human K14 cDNA was shown to have no effect on its assembly behavior in vitro (23). In addition, a single nucleotide difference was found, ^{390}C->T (codon 130 in Fig. 1), but due to the degeneracy of the genetic code (28), it does not result in an alteration of the amino acid encoded (Ser). We do not know whether this nucleotide substitution was introduced as a result of a Taq polymerase error, or conversely, represents an allelic polymorphism. Otherwise, the nucleotide sequence of pET-K16 was identical to that of the relevant portion of the GK-3 human genomic clone (15).

Upon transformation of pET-K16 in the E. coli BL21(DE3) strain and induction of recombinant protein expression with IPTG, a major protein product of M_r 48 kD accumulated as inclusion bodies, as determined by SDS-PAGE and Coomassie blue staining (data not shown). The solubilized inclusion body fraction was subjected to anion-exchange chromatography on a Mono Q column (Pharmacia) at pH 8.1 and in the presence of 6.5 M urea. Under such conditions, the 48 kD protein product eluted with 110 mM guanidine-HCl, as previously shown to be the case for the recombinant form of human K14 (23), a 50 kD acidic (type I) keratin (pI 5.3; 3). This purified 48 kD protein co-migrated with native human K16 in an extract prepared from cultured

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(M) A  T  C  S  R  Q  F  T  S  S  S  S  M  K  G  S  C  G  I  G  G  G  I  G  G  G  S  S  R  30
ATGGCCACCTGCAGCCGCCAGTTCACCTCCTCCAGCTCCATGAAGGGCTCCTGCGGCATCGGAGGCGGCATCGGGGGCGGCTCCAGCCGC

 I  S  S  V  L  A  G  G  S  C  R  A  P  S  T  Y  G  G  G  L  S  V  S  S  R  F  S  S  G  G  60
ATCTCCTCCGTCCTGGCCGGAGGGTCCTGCCGTGCCCCCAGCACCTACGGGGGCGGCCTGTCTGTCTCCTCTCGCTTCTCCTCTGGGGGA

 A  C  G  L  G  G  G  Y  G  G  G  F  S  S  S  S  S  F  G  S  G  F  G  G  G  Y  G  G  G  L  90
GCCTGCGGGCTGGGGGGCGGCTATGGCGGTGGCTTCAGCAGCAGCAGCAGCTTTGGTAGTGGCTTCGGGGGAGGATATGGTGGTGGCCTT

 G  A  G  F  G  G  G  L  G  A  G  F  G  G  G  F  A  G  G  D  G  L  L  V  G  S  E  K  V  T  120
GGTGCTGGCTTCGGTGGTGGCTTGGGTGCTGGCTTTGGTGGTGGTTTTGCTGGTGGTGATGGGCTTCTGGTGGGCAGTGAGAAGGTGACC

 M  Q  N  L  N  D  R  L  A  S  Y  L  D  K  V  R  A  L  E  E  A  N  A  D  L  E  V  K  I  R  150
ATGCAGAACCTCAATGACCGCCTGGCCTCTTACCTGGACAAGGTGCGTGCTCTGGAGGAGGCCAACGCCGACCTGGAAGTGAAGATCCGT

 D  W  Y  Q  R  Q  R  P  S  E  I  K  D  Y  S  P  Y  F  K  T  I  E  D  L  R  N  K  I  I  A  180
GACTGGTACCAGAGGCAGCGGCCCAGTGAGATCAAAGACTACAGTCCCTACTTCAAGACCATCGAGGACCTGAGGAACAAGATCATTGCG

 A  T  I  E  N  A  Q  P  I  L  Q  I  D  N  A  R  L  A  A  D  D  F  R  T  K  Y  E  H  E  L  210
GCCACCATTGAGAATGCGCAGCCCATTTTGCAGATTGACAATGCCAGGCTGGCAGCCGATGACTTCAGGACCAAGTATGAGCACGAACTG

 A  L  R  Q  T  V  E  A  D  V  N  G  L  R  R  V  L  D  E  L  T  L  A  R  T  D  L  E  M  Q  240
GCCCTGCGGCAGACTGTGGAGGCCGACGTCAATGGCCTGCGCCGGGTGTTGGATGAGCTGACCCTGGCCAGGACTGACCTGGAGATGCAG

 I  E  G  L  K  E  E  L  A  Y  L  R  K  N  H  E  E  E  M  L  A  L  R  G  Q  T  G  G  D  V  270
ATCGAAGGCCTGAAGGAGGAGCTGGCCTACCTGAGGAAGAACCACGAGGAGGAGATGCTTGCTCTGAGAGGTCAGACCGGCGGAGATGTG

 N  V  E  M  D  A  A  P  G  V  D  L  S  R  I  L  N  E  M  R  D  Q  Y  E  Q  M  A  E  K  N  300
AACGTGGAGATGGATGCTGCACCTGGCGTGGACCTGAGCCGCATCCTGAATGAGATGCGTGACCAGTACGAGCAGATGGCAGAGAAAAAC

 R  R  D  A  E  T  W  F  L  S  K  T  E  E  L  N  K  E  V  A  S  N  S  E  L  V  Q  S  S  R  330
CGCAGAGACGCTGAGACCTGGTTCCTGAGCAAGACCGAGGAGCTGAACAAAGAAGTGGCCTCCAACAGCGAACTGGTACAGAGCAGCCGC

 S  E  V  T  E  L  R  R  V  L  Q  G  L  E  I  E  L  Q  S  Q  L  S  M  K  A  S  L  E  N  S  360
AGTGAGGTGACGGAGCTCCGGAGGGTGCTCCAGGGCCTGGAGATTGAGCTGCAGTCCCAGCTCAGCATGAAAGCATCCCTGGAGAACAGC

 L  E  E  T  K  G  R  Y  C  M  Q  L  S  Q  I  Q  G  L  I  G  S  V  E  E  Q  L  A  Q  L  R  390
CTGGAGGAGACCAAAGGCCGCTACTGCATGCAGCTGTCCCAGATCCAGGGACTGATTGGCAGTGTGGAGGAGCAGCTGGCCCAGCTACGC

 C  E  M  E  Q  Q  S  Q  E  Y  Q  I  L  L  D  V  K  T  R  L  E  Q  E  I  A  T  Y  R  R  L  420
TGTGAGATGGAGCAGCAGAGCCAGGAGTACCAGATCTTGCTGGATGTGAAGACGCGGCTGGAGCAGGAGATTGCCACCTACCGCCGCCTG

 L  E  G  E  D  A  H  L  S  S  Q  Q  A  S  G  Q  S  Y  S  S  R  E  V  F  T  S  S  S  S  S  450
CTGGAGGGCGAGGATGCCCACCTTTCCTCCCAGCAAGCATCTGGCCAATCCTATTCTTCCCGCGAGGTCTTCACCTCCTCCTCGTCCTCT

                                                                       473 (A.A.)
 S  S  R  Q  T  R  P  I  L  K  E  Q  S  S  S  S  F  S  Q  G  Q  S  S  *
TCGAGCCGTCAGACCCGGCCCATCCTCAAGGAGCAGAGCTCATCCAGCTTCAGCCAGGGCCAGAGCTCCTAG
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Fig. 1 . Nucleotide and deduced amino acid sequences of human keratin 16 cDNA. The coding portion of the human K16 cDNA is shown in uppercase letter, and the predicted amino acid encoded (one-letter code) is indicated directly above. Amino acid residues are numbered 1-473 starting with the initial methionine (M).

human skin keratinocytes (Fig. 2, compare lanes A and B). Moreover, this 48 kD recombinant protein reacted specifically with a previously characterized polyclonal antiserum monospecific for K16 (13; see Fig. 2, lane C) and with monoclonal antibody K8.12 from Sigma, whose epitope is distinct from those recognized by the polyclonal antiserum (P.A.C., unpublished observations). As a final test of the functional character of the protein product encoded by the K16 coding sequence, we expressed it under the control of the cytomegalovirus promoter in PtK2 cells, a rat kangoroo kidney epithelial cell line (29), and observed that a K16 immunoreactive antigen incorporated within the endogenous keratin filament network (these data, which address the

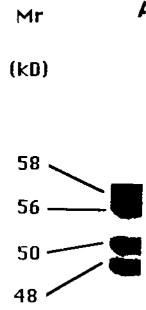

Fig. 2. Purification and characterization Lanes A,B: 8.5 % SDS-polyacrylamide gel stair recombinant human K16 (lane B) co-migrates intermediate filament extract prepared from cu indicated at left correspond to : K5 (58 kD); K note that these two proteins are not resolved ur recombinant protein reacts by Western immuno against a 22-mer peptide corresponding to the C

functional properties of the K16 protein, will be sequence, and the properties of the correspon(immunoreactivity), we conclude that the cDNA

Discussion

The human type I keratin K14 (50 kD predicted to be highly similar at the amino ac identical physicochemical properties (e.g. 3) ar 31). Not surprisingly, there is some confusion human K16 sequence. A human genomic clon as encoding K17 (14), but subsequent work f GK-3 encoded K16 (15). Moreover, in their 1 errors in the GK-3 sequence originally publish acid sequences at codons 187-189 and 449-‹ agreement with the corrected GK-3 sequence ı deduced from a recently reported partial K16 ‹ isolated from cultured human tracheobronchi:

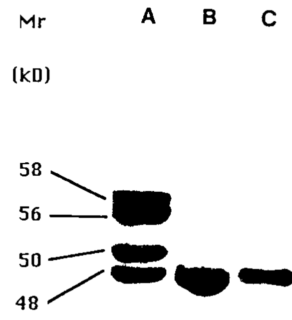

Fig. 2. Purification and characterization of human recombinant K16. Lanes A,B: 8.5 % SDS-polyacrylamide gel stained with coomassie blue. Anion-exchange purified recombinant human K16 (lane B) co-migrates with native human K16 (46 kD in lane A) in an intermediate filament extract prepared from cultured human skin keratinocytes. The Mr values indicated at left correspond to : K5 (58 kD); K6 (56 kD); K14 (50 kDa); K16 and K17 (48 kD; note that these two proteins are not resolved under these conditions). Lane C: the purified 48 kD recombinant protein reacts by Western immunoblotting with a rabbit polyclonal antiserum raised against a 22-mer peptide corresponding to the C-terminus of human K16 (see ref. 13).

functional properties of the K16 protein, will be reported elsewhere). On the basis of its nucleotide sequence, and the properties of the corresponding recombinant protein (solubility, charge, size, immunoreactivity), we conclude that the cDNA clone obtained encodes human K16.

Discussion

The human type I keratin K14 (50 kD), K16 (48 kD), and K17 (47 kD) sequences are predicted to be highly similar at the amino acid level (15,16,30). As a result they display near-identical physicochemical properties (e.g. 3) and are difficult to distinguish immunologically (e.g. 31). Not surprisingly, there is some confusion in the literature regarding the coding portion of the human K16 sequence. A human genomic clone, known as GK-3, was initially described in 1986 as encoding K17 (14), but subsequent work from the same laboratory led to the realization that GK-3 encoded K16 (15). Moreover, in their 1988 report, Rosenberg et al. (15) corrected reading errors in the GK-3 sequence originally published, which resulted in changes in the deduced amino acid sequences at codons 187-189 and 449-458. Our K16 sequence (Figure 1) is in complete agreement with the corrected GK-3 sequence reported in 1988, and with the amino acid sequence deduced from a recently reported partial K16 cDNA (extending from codons 231 to 473-stop and isolated from cultured human tracheobronchial cell mRNAs; 17). On the other hand, it differs at

several locations with the deduced amino acid sequence for human K16 reported by Troyanovsky et al. (16), which, as it turns out, is intriguingly similar to that reported by Raychaudury and colleagues in 1986 (14). The reasons for these discrepancies are not clear. A reasonable possibility is that there would be more that one functional human gene encoding highly-related K16 isoforms, as is it the case for K6 (32). This possibility was effectively raised in a recent report of a point mutation affecting the K16 gene in a patient suffering from pachyonychia congenita (33), in which several alleles were sequenced. Additional studies will be required to resolve this issue.

Ongoing studies in our laboratory suggest that human K16 has peculiar properties in that it promotes the formation of relatively short 10 nm filaments that show a tendency to aggregate near the nucleus (R.P., K.T. and P.A.C. manuscript in preparation; see 13). The molecular basis for this assembly behavior is not yet known, and its characterization should begin with a careful analysis of the amino acid sequence of K16 relating it to that of other type I keratins such as K14 and K17. Examination of the sequence deduced from both the K16 genomic clone (15) and cDNA (this study) reveals the unusual presence of a proline residue within the α-helical coil 1B segment (codon 188). This proline occurs at the "d" position of the third heptad repeat of coil 1B, i.e., it is positioned within the hydrophobic interface of the coiled-coil dimer (34). Of note, proline mutations affecting residues located within the α-helical segments in many epidermal keratins, including K16, are responsible for several genetic skin diseases (6,7), such that it will be interesting to assess the contribution of ^{188}Pro to the assembly properties of K16. Also of note, the carboxy-terminal half of the non-helical tail domain (25 residues out of 47-48) differs significantly in K14 and K16 (15,30). In particular, K16 completely lacks the "$DGK^{I}/_{V}VS^{T}/_{E}$" motif that is conserved among several human type I keratins (35) and some type III IF chains. For many IF proteins, including keratins, vimentin, and NF-L, deletion of the tail domain does not significantly interfere with 10 nm filament formation but causes significant changes in filament-filament interactions in vitro or filament organization in vivo (e.g. 36-43). Further studies will be necessary to delineate the role of the carboxy-terminal sequence in specifying K16 properties.

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nce for human K16 reported by Troyanovsky
imilar to that reported by Raychaudury and
pancies are not clear. A reasonable possibility
n gene encoding highly-related K16 isoforms,
ffectively raised in a recent report of a point
; from pachyonychia congenita (33), in which
ll be required to resolve this issue.

at human K16 has peculiar properties in that it
ments that show a tendency to aggregate near
reparation; see 13). The molecular basis for
characterization should begin with a careful
it to that of other type I keratins such as K14
both the K16 genomic clone (15) and cDNA
residue within the α-helical coil 1B segment
of the third heptad repeat of coil 1B, i.e., it is
ie coiled-coil dimer (34). Of note, proline
elical segments in many epidermal keratins,
ic skin diseases (6,7), such that it will be
assembly properties of K16. Also of note, the
(25 residues out of 47-48) differs significantly
tely lacks the "DGK$^{I}/_{V}$VS$^{T}/_{E}$" motif that is
5) and some type III IF chains. For many IF
etion of the tail domain does not significantly
es significant changes in filament-filament
e.g. 36-43). Further studies will be necessary
in specifying K16 properties.

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