The Roles of K5 and K14 Head, Tail, and R/K L L E G E Domains in Keratin Filament Assembly in Vitro

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Abstract. Type I and type II keratins form obligatory heterodimers, which self-assemble into 10-nm intermediate filaments (IFs). Like all IF proteins, they have a central α-helical rod domain, flanked by nonhelical head and tail domains. The IF rod is more highly conserved than head and tail, and within the rod, the carboxy R/K L L E G E sequence is more highly conserved than most other regions. Mutagenesis studies have shed some light on the roles of the head, tail, and R/K L L E G E sequence in 10-nm filament structure. However, interpretations have often been complicated in part because many of these studies have focused on transfected cells, where filament structure cannot be evaluated. Of the few in vitro assembly studies thus far conducted, comparison of keratin mutants with other IF mutants have often been difficult, due to the obligatory heteropolymeric nature of keratin IFs. In this report, we describe in vitro filament assembly studies on headless, tailless, headless/tailless, and R/K L L E G E truncated mutants of keratin 5 and its partner keratin 14. Using varying conditions of ionic strength and pH, we examine effects of analogous K5 and K14 mutations on the stability of 10-nm filament structure. Using EM, we examine effects of mutations on the ability of subunits/protofibrils to (*a*) elongate and (*b*) laterally associate. Our results demonstrate that (*a*) tails of K5 and K14 are required for filament stabilization; (*b*) the head of K5, but not of K14, is required for filament elongation and lateral alignments; and (*c*) the R/K L L E G E domains are required for lateral alignments, but not for filament elongation.

INTERMEDIATE filaments (IFs)[1] are composed of proteins that have a central 310–350 amino acid rod domain, predicted to be largely α-helical (for reviews see 2, 10). IFs have been subdivided into six different types, based on their sequence homology within the relatively conserved rod (19, 20, 30, 66). The rod is flanked on either end with nonhelical head and tail domains that are highly variable in size and sequence, even for members within a single IF subtype. IF assembly begins with the formation of a coiled-coil dimer, which occurs through intertwining of parallel rod domains in register (1, 55). The periodic stabilizing forces behind these coiled-coil interactions are largely hydrophobic, with some contribution by ionic interactions (40, 53, 55). Most IF subtypes can form homopolymers, but keratin IFs are unusual in that they are obligatory heteropolymers, composed of type I and type II polypeptides (31, 65). The heteropolymeric nature of keratin IFs is at the level of the coiled-coil dimer (11, 32, 62).

Dimers readily interact with each other in an antiparallel fashion to form stable tetramers (58, 71). At least for keratins, tetramers exist in both staggered and in register conformations in vitro (11, 63, see also 2), and both of these may be functional in 10-nm filament assembly (14, 63). Delineating the precise steps in the filament assembly process has been difficult, due to the fact that lateral and end-to-end associations of subunits often take place simultaneously under similar conditions, yielding a number of polymorphic supramolecular structures under partially denaturing or preassembly conditions (11, 64). However, partially unraveled IFs reveal an underlying structure of protofibrils (4–5 nm), which have a diameter equivalent to an octamer, and protofilaments (2–3 nm), which have a diameter equivalent to a tetramer (2, and references therein; see also 38). The entire IF assembly process can take place in vitro, in the absence of auxiliary proteins (18, 65).

Mutagenesis of IF cDNAs, coupled with transfection studies, have demonstrated the importance of the rod domain in filament network formation in vivo (3, 4, 28, 37, 50, 51, 60, 68, 70). However, it is not yet clear whether the entire rod is necessary for IF structure. In one study, a truncated keratin 14, missing the highly conserved R L L E G E consensus sequence as well as the tail domain, associated with its K5 partner to form filaments which seemed to be of appropriate width and length, but which tended to aggregate (12). Other IF subtypes appeared to be more compromised by such truncations (28, 37, 68, 70). However, in vitro filament assembly studies involving either truncations or dele-

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1. *Abbreviation used in this paper*: IF, intermediate filament.

tions of the R/K L L E G E sequences of both members of the keratin heterodimer have not yet been conducted, and in the absence of these studies, it is not clear whether these differences are rooted in IF stabilities or in IF protein composition.

The roles of the end domains in IF assembly and organization are even less clear than the role of the rod. Some studies have suggested that the ends may be important for such diverse functions as IF network assembly (28, 37, 39, 51, 54, 60, 70), end-to-end elongation of lamin subunits (head domain; 39), nuclear (tail) or plasma membrane (head) interactions with desmin and vimentin IFs (24–26), cytoplasmic localization of keratin IFs (tail; 5), and calibration of the thickness of vimentin IFs (tail; 46). In addition, both the head and the tail domains of type III and type V IF proteins contain specific phosphorylation sites which play a role in mitosis-mediated reorganization or disassembly of these IFs in some cell types (9, 16, 17, 21, 23, 37, 38, 43, 45, 50, 56, 57, 69). Finally, recent studies have suggested that specific sites in the head and/or tail domains may interact with the rod, thereby influencing filament formation (7, 41, 42, 46, 67).

Despite many findings supporting the involvement of the head and tail domains in IF organization and assembly, the role of the end domains in IF structure is still controversial. One IF protein, keratin 19, is nearly tailless, and it has been suggested that on this basis, the tail domain may not be necessary for IF structure (5). Additional in vivo and in vitro studies have also indicated that portions of tail domains are dispensable for the structure of at least some IFs (3, 12, 33, 44, 51, 68). This said, completely tailless glial filament protein does not seem able to assemble into 10-nm filaments (59). Similarly, while several studies have suggested that segments of the head domains of some keratin IF proteins can be removed without blocking their ability to form filament networks in vivo (4, 6, 51) or 10-nm filaments in vitro (12), other studies have indicated that at least a portion of the heads of some IFs are required for IF structure (41, 42, 44, 61, 67). Whether these differences arise from the diversity among IF head and tail sequences, from the precise locations of the truncation sites relative to the rod domain, or from the position and sequences of various polypeptide tags used in many of the analyses is not yet clear.

Another difficulty unique to relating studies of keratins to those of other IF subtypes stems from their obligatory heteropolymeric nature. It seems likely that the principles governing the roles of the rod, tail, and head domains in IF assembly and structure may be tempered by the heteropolymeric nature of these IFs. However, this has not yet been extensively tested, nor is it known whether type I and type II keratins contribute equally to filament assembly and structure. Moreover, sequences of the tail and head domains are remarkably diverse even among different keratin pairs, and such differences have exacerbated the problems in comparing data obtained for headless and tailless IFs even among different keratin pairs. To begin to examine these issues and to more generally decipher the possible roles of the head, tail, and rod domains in keratin structure and assembly, we have engineered complementary headless, tailless, headless/tailless, and truncated R/K L L E G E rod mutations in a type I (K14) and type II (K5) keratin pair. We have conducted in vitro filament assembly studies using different combinations of these mutant and wild-type K5 and K14 keratins. In addition, we have explored the effects of pH and/or ionic strength on the stability of these filaments. Our results have yielded some intriguing new insights into the roles of the type I and type II keratin head, tail, and rod domains on IF structure and stability.

Materials and Methods

Construction of pETK5, pETK14, and Deletion Mutants

Constructions of pETK14 and pETK5 have been described (11, 12; for K5 sequence, see 48). Deletion mutants K5NΔ71, K5NΔ157, K5NΔ164, K5CΔ111, K5CΔ119, K5NΔ157/CΔ111, and K5NΔ164/CΔ111 were subcloned from pETK5. Deletion mutants were designated as K5NΔX or K5CΔX, where X denotes the number of encoded amino acids missing, from the head (N) or tail (C). All constructs were confirmed by DNA sequencing across all ligation junctions and throughout all segments generated by PCR. Where indicated, clones were sequenced in their entirety.

K5NΔ71. pETK5 was partially digested with NcoI/SpeI to remove the sequence encoding the first 71 amino acids of the head. The ends were bridged with an oligomer that recreated the ATG start codon. Due to the cloning method employed, residues 72 and 74 were changed from Arg and Ser to Gly and Cys, respectively.

K5NΔ157. pETK5 was digested with NcoI to remove sequences from the start codon (within the NcoI site) to the linker between helix 1B and 2A. PCR was used to generate a replacement fragment from an artificial NcoI site, 21 nucleotides 5′ to helix 1A, to a natural NcoI site in the linker between helix 1B and 2A. This fragment was then cloned into the NcoI digested pETK5 plasmid. The entire K5NΔ157 coding segment was sequenced and verified.

K5NΔ164. A BspEI site was created just 5′ to helix 1A by PCR site-directed mutagenesis. An 1,135-bp SacI–SpeI fragment of the PCR generated product was used to replace the wild-type sequence in pETK5. Nucleotides encoding 155 amino acids of the head were excised by digesting the new pETK5 with BspEI, followed by ligation of the purified plasmid. By virtue of the BspEI site 27 nucleotides 3′ of the ATG start codon, K5NΔ164 retained the initial nine amino acids of the head. The entire K5NΔ164 coding segment was sequenced and verified.

K5CΔ111. The BsmI and BamHI sites located just 3′ to helix 2B of pETK5 were used to insert an oligomer containing an in-frame TGA stop codon, resulting in a tailless K5.

K5CΔ119. K5CΔ111 was digested with SacI/SacII to remove additional nucleotides encoding the highly conserved KLLEGEE sequence at the end of helix 2B. The unique ends were bridged with an oligomer containing a TGA stop codon.

K5NΔ157/CΔ111 and K5NΔ164/CΔ111. Existing BglII sites, located just 5′ to the T7 promoter sequence and at the beginning of helix 2B, facilitated the exchange of the 5′ coding segment of K5CΔ111 for the headless 5′ coding segment of either K5NΔ157 or K5NΔ164.

K14 mutants. Headless and headless/tailless deletion mutants of K14 (K14NΔ107 and K14NΔ115, and K14NΔ107/CΔ42, respectively) contained a sequence encoding the last five carboxy-terminal residues of the neuropeptide substance P. It was previously demonstrated that the substance P-tag had no apparent effect on in vitro filament reconstitution (11, 12). For the studies described here, we made the P-tag-less version of K14CΔ50 and K14CΔ42.

Expression of Keratins in Bacteria and Purification

Keratins were expressed in bacteria, isolated from inclusion bodies, and purified by anion exchange chromatography in 6 M urea buffer as described (11). Peak fractions were pooled and stored at −70°C. Before use, keratins were subjected to SDS-PAGE and immunoblot analysis to verify their purity and quality.

Keratin Complex Formation, Isolation, and In Vitro Reconstitution

Pure preparations of K5 and K14 proteins were mixed in an ~1:1 molar ratio (~1 mg/ml; slight excess of K5) at room temperature and then desalted at 4°C by ultracentrifugation through Centricon-10 or -30 units as specified by the manufacturer (Amicon, W. R. Grace and Co., Boston, MA). K5–K14

complexes formed in 6 M urea buffer were separated from uncomplexed protein by anion-exchange chromatography. Peak fractions were pooled, adjusted to 200 μg/ml protein, and stored at 4°C for future use.

K5-K14 complexes were equilibrated by dialysis against a 9.5 M urea buffer containing 10 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 7.5 for 4 h at room temperature. Additional dialyses were performed against a low ionic strength buffer (2.5 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 6.0, 7.0, 7.5, 8.0, or 9.0) or, where indicated, against a high ionic strength buffer (50 mM Tris, 5 mM EDTA, pH 6.0, 7.0, 7.5, 8.0, or 9.0) for ~16 h at 4°C with two changes of buffer. In one experiment, 47.5 mM NaCl or KCl was added to 2.5 mM Tris to create more physiological reconstitution conditions. In another experiment, after dialyzing against 50 mM Tris, pH 7.0, 10 mM sodium phosphate buffer was added for 10 minutes prior to fixing for EM (see 2, 15, and references therein). Since slight variations in assembly conditions can sometimes lead to differences in the overall quality of resulting filaments and/or their degree of aggregation, control reactions containing wild-type K5 and K14 were always run in parallel with all reactions involving mutants, and reactions were always repeated in triplicate and with at least two different preparations of purified protein.

EM of Keratin Filaments and Measurements of Filament Forming Efficiency and Filament Dimensions

Reconstituted filaments were negatively stained with 0.75–1.0% uranyl acetate as described (12). Specimens were examined in a Philips CM-10 electron microscope operated at an acceleration voltage of 80 kV. Magnification was calibrated using a diffraction grating replica (No. 10021; Ernest Fulham, Inc., New York, NY).

An aliquot of the final dialysate was subjected to centrifugation at 27,000 *g* for 2 h, and the relative amounts of protein in pellet and supernatant fractions were determined by SDS-PAGE. EM verified that under these conditions, the pellet contained filamentous keratin and the supernatant contained incompletely polymerized keratins. Filament-forming efficiency was defined as the percentage of total protein contained in the pellet.

To quantitate filament width and length, random series of three or more micrograph negatives from each experimental condition were examined under a Nippon Kogaku shadowgraph. Widths were measured from 18 randomly selected filaments. Quantitative analyses were made using the Macintosh StatView program. The variation in filament width among a given population was indicated by the coefficient of variance. The width measurements reported in Table I represent relative, not absolute values, due to variations in negative staining and the degree of filament flattening during fixation on the grid. Because of the aberrant associations of many mutant keratin filaments, the true length of keratin filaments was difficult to assess. Therefore, lengths were estimated from five representative filaments and expressed as the range of this population. Where the average filament length was especially long, it was then expressed as greater than the length which could be accurately measured.

Results

Previously, bacterial expression vectors were engineered encoding wild-type, headless, tailless, headless/tailless, and truncated rod forms of human keratin 14 (11, 12). A bacterial expression vector was also engineered encoding the wild-type human keratin 5, the natural partner of K14 in basal epidermal IFs. We now constructed the corresponding headless, tailless, headless/tailless, and truncated COOH-terminal rod mutations in the human K5 cDNA (Fig. 1 *A*). Mutants of K14 or K5 have been designated as NΔX or CΔX, where X denotes the number of encoded NH_2- or COOH-terminal amino acids deleted from the protein.

The rod is composed of four α-helical segments, demarcated by short helix-perturbing spacers (10, 29). Sequences extending ~10–20 residues on either side of the K14 and K5 rods can be aligned nearly perfectly (48, 52). The tailless deletion mutants K5CΔ111 and K14CΔ42 retain the highly conserved consensus sequence. TYR(R/K)LLEGE, at the carboxy end of the rod (Fig. 1 *B*). The truncated COOH-terminal rod mutants K5CΔ119 and K14CΔ50 are missing the R/KLLEGE sequence at the end of the helix 2B in addition to the tail. The headless deletion mutants used for most of our studies, K5NΔ157 and K14NΔ107, retain several residues prior to the predicted start of helix 1A.

Wild-type and mutant keratins were expressed in bacteria and purified by ion exchange chromatography in 6 M urea buffer (11). Fig. 2 illustrates the purity of proteins used for these studies. Each protein migrated on SDS polyacrylamide gels as a single species.

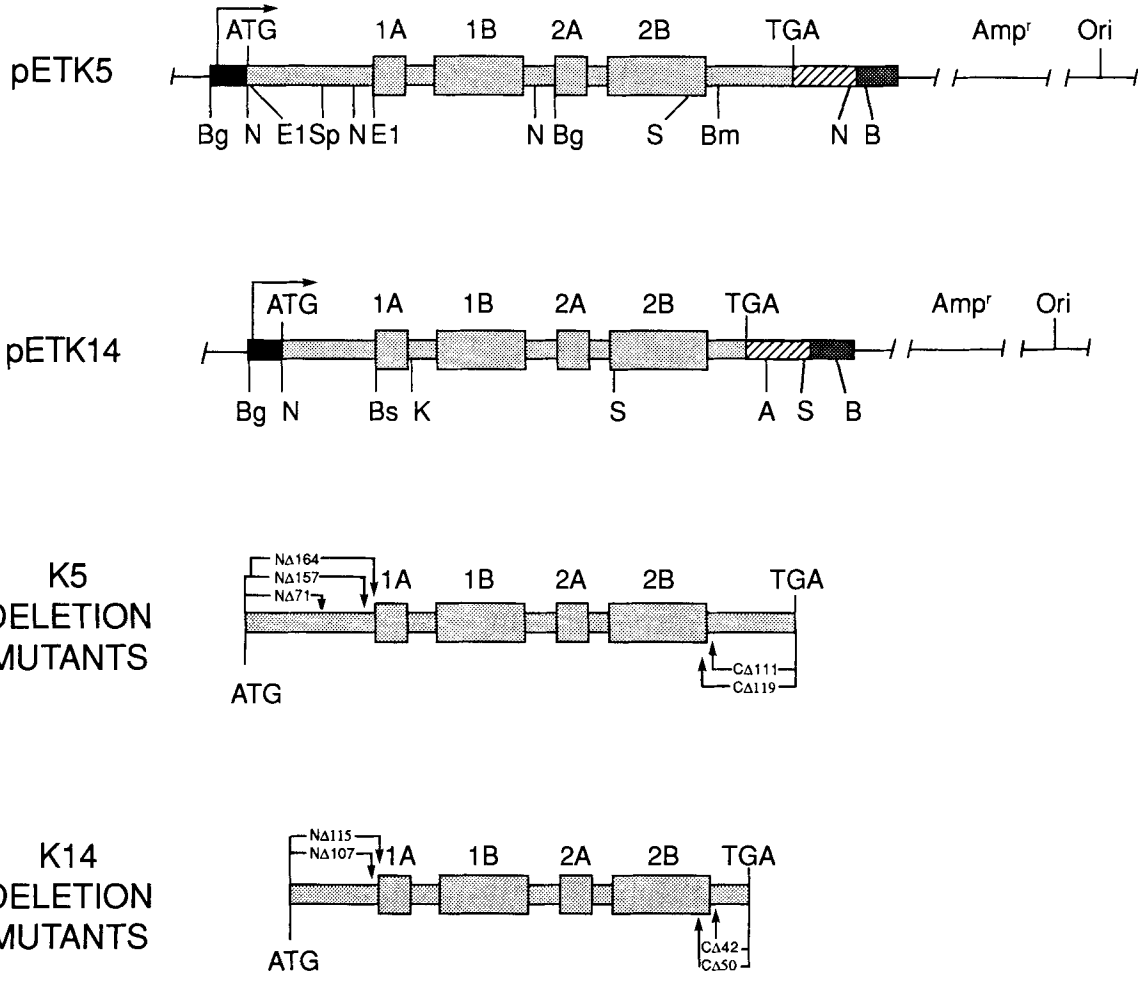
Tailless K14 and K5 Filaments Are Less Stable than Wild-Type Keratin Filaments

When equimolar amounts of wild-type K5 and K14 proteins in 9.5 M urea buffer were dialyzed against low ionic strength buffer, pH 7.5 (see Materials and Methods), long (>1 μm) 10-nm filaments were formed, as judged by negative staining and transmission EM (Fig. 3 *A*; *inset* is at higher magnification). In addition, when paired with wild-type K5, a tailless K14 (K14CΔ42) assembled into filaments, nearly indistinguishable from wild-type (Fig. 3 *B*; see also reference 12). In contrast, when tailless K5 (K5CΔ111) was paired with wild-type K14 under standard dialysis conditions, filaments formed that appeared somewhat aberrant (Fig. 3 *C*). Most notably, these filaments had a tendency to associate laterally for hundreds of nanometers. This gave the appearance of cables of two to three intertwined filaments (arrows denote branchpoints where cables separated into individual or smaller bundles of filaments). In addition, there were more filament ends per unit area, indicating that filaments were somewhat shorter than normal. Since branchpoints were sometimes indicative of an interaction between the lateral surface of one IF and the free end of another IF, this may have obscured an even greater negative effect on filament length. Collectively, these findings suggested that as defined here, the K5 tail may play a greater role in IF assembly or stabilization than the K14 tail.

The features noted in the K5 tailless assembly mixture were significantly more exaggerated when both tailless keratins (K5CΔ111 + K14CΔ42) were combined (Fig. 3 *D*). Thus, filaments were shorter, and cabling and branching were prevalent. Upon close inspection, short filaments laterally associated in an overlapping fashion to form longer and wider "pseudofilaments." Since the effects of the two tailless mutants combined were more severe than either alone, each tail must contribute to IF formation.

Several prior studies indicated that conditions for optimal IF assembly can vary for each specific pair of keratins (15, 31). Given the wide variation in head and tail sequences among keratin pairs, it seemed likely that these domains could be responsible for the observed differences in assembly conditions. If true, then the optimal conditions for assembling tailless K5 and K14 keratins might differ from wild-type, a phenomenon already demonstrated by Hatzfeld and Weber (33) for K8 and K18. To investigate this possibility, we compared the behavior of wild-type K5/K14 and tailless K5/K14 in assembly buffers of varying pH and ionic strength. Interestingly, for wild-type K5/K14, reduction of the pH from 7.5 to 7.0 led to filament bundling (Fig. 3 *E*), whereas a further decrease to pH 6.0 resulted in electron dense aggregations of filaments (not shown, but similar to

A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1B
2A
2B
TGA
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Ori
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pETK14
ATG
1A
1B
2A
2B
TGA
Amp^r
Ori
Bg N
Bs K
S
A S B
K5 DELETION MUTANTS
NΔ164
NΔ157
NΔ71
1A
1B
2A
2B
TGA
CΔ111
CΔ119
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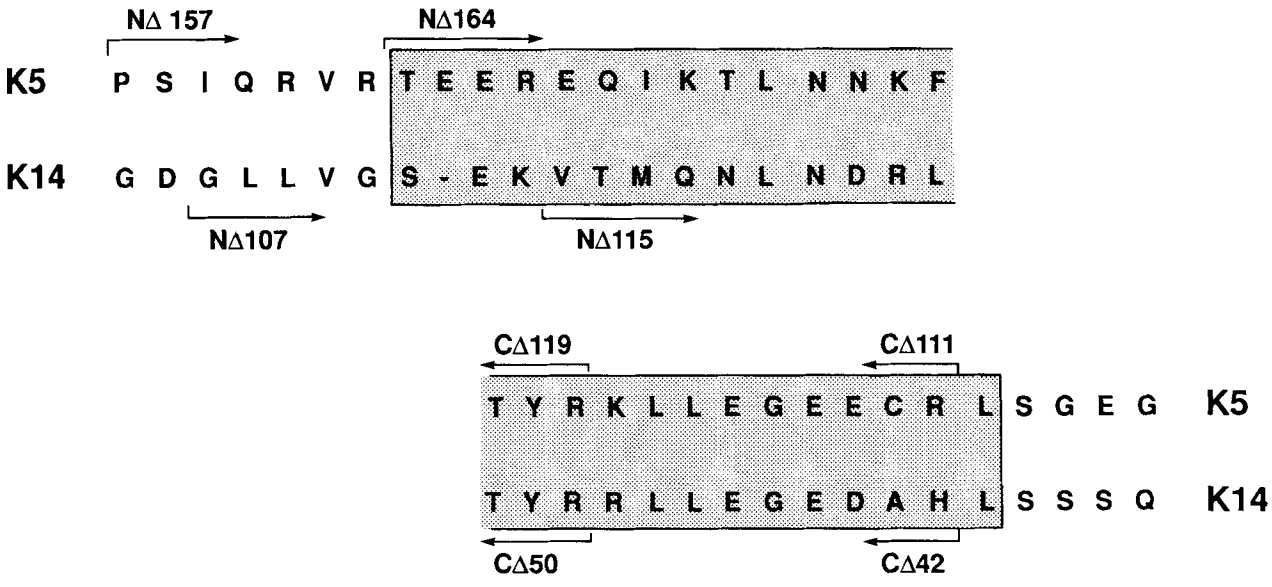
Helix 1A
NΔ 157
NΔ164
K5 P S I Q R V R T E E R E Q I K T L N N K F
K14 G D G L L V G S - E K V T M Q N L N D R L
NΔ107
NΔ115
CΔ119
CΔ111
T Y R K L L E G E E C R L S G E G K5
T Y R R L L E G E D A H L S S S Q K14
CΔ50
CΔ42
Helix 2B

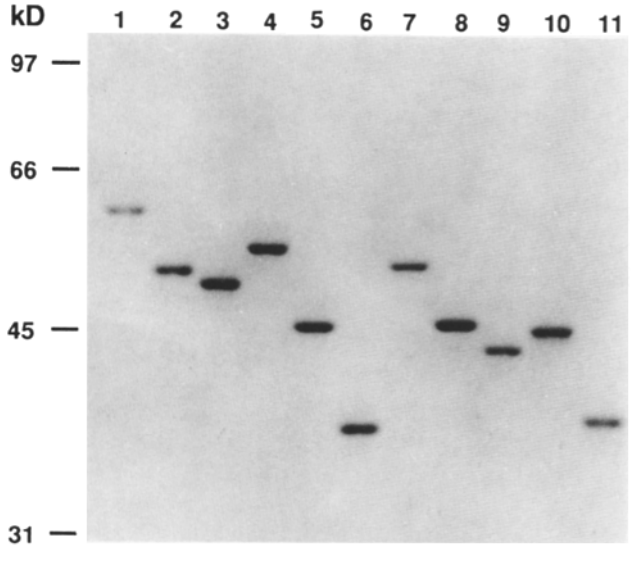

Figure 2. SDS-PAGE analysis of bacterially expressed wild-type and truncated keratins. Purified keratins were analyzed by electrophoresis through an 8.5% SDS-polyacrylamide gel and visualized by staining with Coomassie blue. (Lane *1*) Wild-type K5; (lane *2*) K5CΔ111; (lane *3*) K5CΔ119; (lane *4*) K5NΔ71; (lane *5*) K5NΔ157; (lane *6*) K5NΔ157/CΔ111; lane *7*) wild-type K14; (lane *8*) K14CΔ42; (lane *9*) K14CΔ50; (lane *10*) K14NΔ107; (lane *11*) K14NΔ107/CΔ42.

Fig. 3 *G*). Reducing the pH caused tailless keratins to assemble into increasingly longer filaments which had a tendency to bundle (Fig. 3 *F*). While decreasing pH generally promoted filament elongation, increasing pH resulted in progressively weaker lateral and end-to-end interactions until only short protofilamentous structures were present at pH 9.0 (data not shown).

Increasing the ionic strength of the dialysis buffer caused a marked aggregation of the 10-nm filaments formed from either wild-type K5/K14 or from combinations involving one wild-type and one tailless keratin (Fig. 3 *G*). Surprisingly, these conditions seemed to be near optimal for tailless K5/K14. Thus, in the presence of 50 mM Tris, pH 7.0, tailless K5/K14 assembled into filaments that seemed quite similar to wild-type, except for slight irregularities in width (Fig. 3 *H*). Although it was difficult to assess the molecular basis for this variation, it seems most likely that the stretches of thicker regions were due to lateral associations between IFs of different lengths. However, perturbations in protofibrillar interactions could not be ruled out.

Additional reconstitution experiments in 50 mM Tris but at higher or lower pH did not improve the quality of filaments produced with tailless keratins. Interestingly, the aggregation seen with wild-type filaments in 50 mM Tris at pH 7.0 was decreased when the pH was raised to 9.0. However, filaments were also shorter and less uniform than those assembled under standard dialysis conditions (data not shown). Finally, replacing Tris with NaCl or KCl did not improve filament formation either for wild-type or tailless keratins. Collectively, our data suggested that (*a*) optimal conditions for assembly of tailless keratins require higher ionic strength than for wild-type keratins; and (*b*) neither the K5 nor the K14 tails are essential for filament assembly, but both contribute to stabilizing IF structure during filament elongation.

The Head Domains of Type I and Type II Keratins Differ in their Effect on Filament Elongation: Critical Roles for the Type II Head Domain in Protofibril Elongation and in Lateral Alignments

To determine whether the head domains are obligatory for filament assembly and/or structure, we performed in vitro assembly studies on headless K5 and K14 mutants. While the precise boundaries of the amino end of the rod have not been unequivocally established, there are three glycine residues between N105 and N113 of K14 and a proline residue at N157 of K5 (see Fig. 1 *B*). As observed previously, the K14 sequence between N107 and N115, which includes the first three residues of the predicted start of helix 1A, appear to be critical for keratin filament assembly (12). Thus, K14NΔ107 formed long filaments in vitro when paired with wild-type K5 (see Fig. 4 *A*, Table I), whereas K14NΔ115 formed significantly shorter and fewer filaments with wild-type K5 (Fig. 4 *B*). Similar to our studies with K14NΔ115, the analogous K5 mutant (K5NΔ164) formed very short filaments when paired with wild-type K14 (Fig. 4 *C*, see Fig. 1 *B* for linear alignment). Surprisingly however, and in contrast to K14NΔ107, the analogous K5 mutant (K5NΔ157) produced very short and branched filaments when combined with wild-type K14 (Fig. 4 *D*). Filaments also varied widely in diameter, ranging from 5 to 22 nm. Nonfilamentous aggregates were also seen, and filaments were fewer in number. Thus, the head sequences closest to the predicted start of helix 1A seemed more critical for K5 than for K14.

Not unexpectedly, the structures formed from K5NΔ157 + K14NΔ107 were also aberrant (Fig. 4 *E*). In particular, short filaments with irregular widths were often interconnected at their ends (Fig. 4 *E*, *inset*). In contrast to our results with tailless keratins, dialysis of headless keratins under various conditions of ionic strength and pH did not improve filament formation (data not illustrated; see Table I). Thus, when compared to K14 by linear alignment, the amino end of K5 appears to play a more crucial role than that of K14, and is important both for subunit elongation and for proper lateral alignment of subunits.

While we have not yet determined the precise sequences in the K5 head critical for 10-nm filament structure/forma-

Figure 1. Wild-type, headless, tailless, and R/K LLEGE mutants of K5 and K14. Details of plasmid constructions are in Materials and Methods. (*A*) Components are represented as follows. (*Black box*) T7 RNA polymerase promoter; (*light dotted boxes*) complete coding sequences of human K5 and K14 cDNAs; (*diagonal lined boxes*) 3′ noncoding sequences of K5 or K14 genes; (*dark dotted boxes*) transcription termination sequences in pET8C; (*ori*) origin of replication; (*amp^r*) ampicillin resistance gene. The α-helical domain is represented by boxes designated 1A, 1B, 2A, and 2B as predicted by secondary structure methods (29). Restriction endonuclease sites: A, AvrII; B, BamHI; Bg, BglII; Bm, BsmI; Bs, BsteII; E1, BspE1; K, KpnI; N, NcoI; S, SacI; Sp, SpeI. The extent of amino- and carboxy-terminal deletions is indicated. (*B*) K5/K14 sequences at beginning of helix 1A and end of helix 2B indicate precise junctions of the headless, tailless, and R/K LLEGE mutants. Note: with the exception at the beginning of Helix 1A, K5/K14 rod sequences could be aligned without gaps.

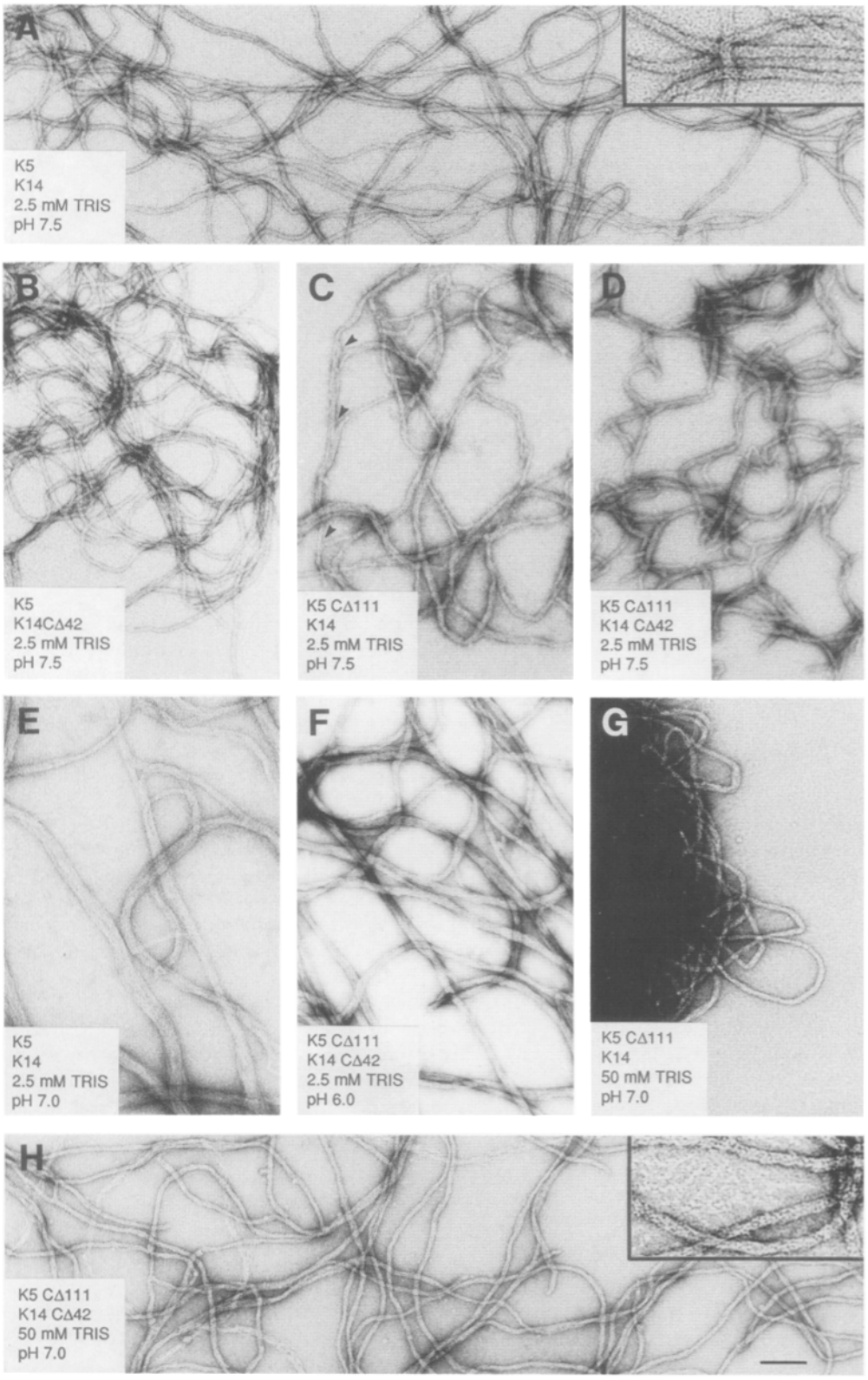

Figure 3. Assembly of tailless keratin filaments. Equimolar amounts of type I and type II keratins were combined in vitro and dialyzed against standard low ionic strength buffer (2.5 mM Tris) at pH 7.5 (*A–D*), pH 7.0 (*E*), pH 6.0 (*F*) or dialyzed against the high ionic strength buffer (50 mM Tris) at pH 7.0 (*G* and *H*) as described in Materials and Methods. Assembled structures were subjected to negative staining and EM. Keratins used in assembly reactions are indicated at lower left of each frame. Arrowheads in *C* denote branchpoints. Note that filaments formed from tailless K5 and tailless K14 were near wild-type at 50 mM Tris, but not at 2.5 mM Tris. Note also that decreasing pH or increasing ionic strength caused aggregation of wild-type filaments. Bar (*main frame*) 100 nm; (*insets*) 39 nm.

tion, we have constructed a truncated head mutant, K5NΔ71, retaining approximately half of the nonhelical head domain. This protein was able to coassemble with wild-type K14 into long filaments, which were similar, but not perfectly wild-type (Fig. 4 *F*). Like K14NΔ107-containing filaments, these filaments were slightly thinner than normal (Table I), and slight irregularities in filament width, including beading, were seen (Fig. 4 *F*). Interestingly, and unlike the K5 headless mutants, K5NΔ71 was able to form more wild-type looking filaments under reconstitution conditions of higher

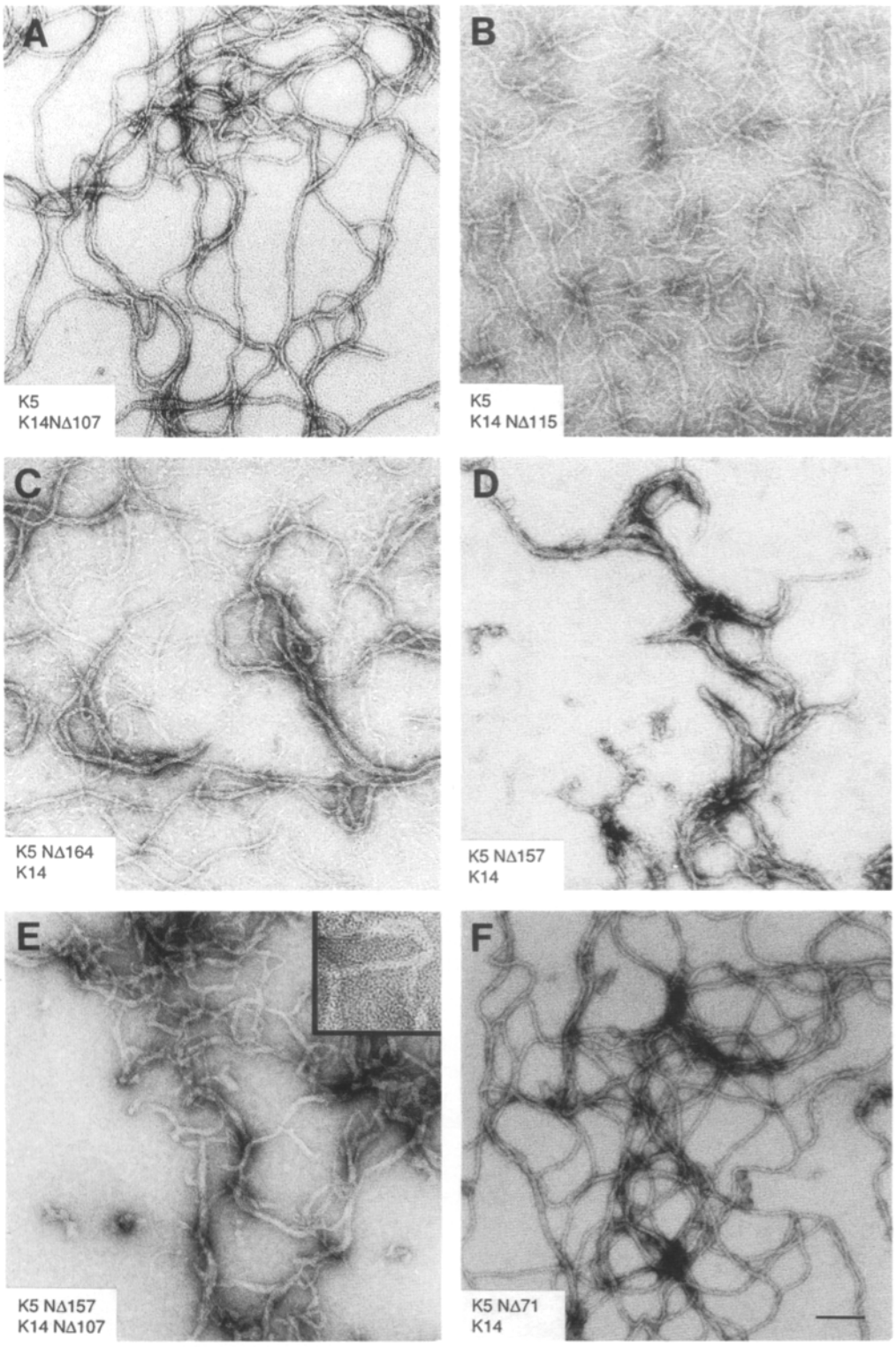

Figure 4. Assembly of headless keratin filaments. Equimolar amounts of type I and type II keratins were combined in vitro and dialyzed under standard conditions at pH 7.5 as described in Materials and Methods. Assembled structures were subjected to negative staining and EM. Keratins used in assembly reactions are indicated at lower left of each frame. Note branchpoints and variability in filament width in inset to frame *E*. Note that headless K5 did not assemble into proper IFs, whereas a partial head mutant, K5NΔ71, assembled into seemingly wild-type IF structures. Bar (*main frames*) 100 nm; (*inset*) 39 nm.

Tris. Dialysis against 7.5–20 mM Tris, pH 7.5, yielded long filaments that were uniform in width (data not shown). However, these filaments had a tendency to form aggregates. In comparing K5NΔ157 and K5NΔ71, the half of the K5 head nearest to the start of helix 1A seemed to be more critical for IF structure than the amino-terminal end. This finding is interesting in light of recent studies on the vimentin head domain, unveiling a nine-amino acid sequence essential for the structure of these IFs (41, 42, 67). While sequences in the head domains of K5, K14, K8, and K18 did not have an obvious relation to this vimentin sequence, the importance of sequences within both the K5 and the vimentin heads is intriguing. As we precisely define those sequences in the K5 head that are critical for IF assembly, it should become more

Table I. Determination of Filament Forming Efficiencies and Width and Length Measurements of Filament-like Structures Obtained with K5 and K14 Mutant Proteins

	Filament forming efficiency*		Width‡ (n = 18)		Length¶ (n = 5)	
	2.5 mM Tris	50 mM Tris	mean ± SEM	CV	2.5 mM Tris	50 mM Tris
			nm	%	*nm*	*nm*
K5 + K14	++++	++++	9.7 ± 0.2	8	>1,000	ag
K5 + K14CΔ42	++++	++++	10.4 ± 0.2	9	>1,000	ag
K5CΔ111 + K14	++	++++	12.9 ± 0.3‖	11	>750	ag
K5CΔ111 + K14CΔ42	+	++++	10.0 ± 0.2§	9	30–250	>1,000
K5 + K14NΔ115	+	ND	8.8 ± 0.5	23	30–250	ND
K5 + K14NΔ107	++++	++++	10.1 ± 0.2	8	>1,000	ag
K5NΔ164 + K14	+	+	10.7 ± 0.8	28	30–750	30–750
K5NΔ157 + K14	+	++	14.8 ± 1.1	32	30–250	30–250
K5NΔ157 + K14NΔ107	+	+	11.7 ± 0.9	34	30–250	30–250
K5NΔ71 + K14	++++	++++	9.1 ± 0.4	15	>1,000	ag
K5 + K14NΔ107CΔ42	++++	++++	9.2 ± 0.2	10	>750	ag
K5CΔ111 + K14Δ107	+	++++	7.7 ± 0.3§	15	30–250	>750
K5CΔ111 + K14NΔ107CΔ42	+	++++	7.8 ± 0.2§	11	30–250	>750
K5NΔ157CΔ111 + K14NΔ107CΔ42	+	+	9.3 ± 0.7§	33	30–250	30–250
K5 + K14CΔ50	++++	++++	11.6 ± 0.3	9	>1,000	ag
K5CΔ119 + K14	+	++++	16.1 ± 1.0	28	30–250	ag
K5CΔ119 + K14CΔ50	+	++++	15.7 ± 0.8§	21	30–250	>500

* Filament forming efficiency was measured after filament reconstitution at 2.5 mM Tris, pH 7.5, or 50 mM Tris, pH 7.0, as described in Materials and Methods. (+ to ++++) denotes the amount of total protein (0–100%) detected in the pellet fraction.
‡ Width measurements were determined as described in Materials and Methods. Single filamentous structures were used for width measurements; obvious bundled or cabled filaments were not included.
§ Widths were measured for filaments formed under optimal conditions, in these cases dialyzed at 50 mM Tris, pH 7.0. The others were dialyzed at 2.5 mM Tris, pH 7.5.
‖ Filaments were measured at the periphery of large aggregates of keratins.
¶ Due to aberrent lateral interactions of the shorter and wider filaments, the lengths of five representative filamentous structures were measured and the approximate range was expressed. Under the magnification conditions used, large filaments were too long and convoluted to obtain an unbiased estimate of their length and therefore were expressed as greater than the shortest representative filament observed. ag, not determined due to filament aggregation; CV, coefficient of variance.

apparent whether a particular secondary or tertiary structure is shared by the vimentin and K5 heads and is involved in IF assembly/structure.

Further Studies to Assess the Relative Importance of the K5 Head and the Dispensability of the K14 Tail in IF Structure

Studies using proteolytic fragments of K8/K18 keratins have indicated that the segments of keratin containing predominantly the α-helical rod can form heterodimers and tetramers (36). Using chemical cross linking, gel filtration analysis, and immunoblotting of SDS-PAGE separated cross-linked products, we have extended these observations to demonstrate that keratin fragments composed exclusively of the rod domains (K5NΔ164/CΔ111 + K14NΔ107/CΔ42) can also form heterodimers and heterotetramers in 6 M urea (data not shown). Moreover, under these conditions, >90% of the complexes were tetramers.

Despite the ability of rod segments to form heterotetramers, assembly of tetramers into 10-nm filaments requires additional nonhelical end domain sequences, as we had shown thus far for K5/K14, and as had been shown previously for K8/K18 (6, 51). To identify the limit sequence necessary for forming K5/K14 filaments, we conducted a series of in vitro assembly studies combining different K5 and K14 tailless, headless, and rod mutants. As demonstrated previously (12), we confirmed that the rod domain of K14 (K14NΔ107/CΔ42) could form long filaments with wild-type K5 (Fig. 5 *A*, see also Table I). As was seen previously (12), slight perturbations were detected, most notably in smoothness and regularity of filaments. In addition, filaments were narrower than normal (see Fig. 5 *A* and Table I).

To determine whether IFs could form when missing the head of K14 and either the K5 or K14 tail, we combined K5CΔ111 with K14NΔ107. Only short, irregular filaments were formed under standard dialysis conditions (data not shown). However, when the ionic strength was increased to 50 mM Tris, filaments of considerable length were formed (Fig. 5 *B*). These filaments were generally narrower than wild-type filaments (Table I), and they often bundled laterally (Fig. 5 *B*). There were also more free ends per unit area, indicative of generally shorter filaments. In addition, even at high ionic strength, filaments were sometimes unraveled, indicating that their stability was less than normal. Overall, these data suggested that while filaments could form with a headless K14 and a tailless K5, their stability, and possibly other (more subtle) aspects of their structure as well, were compromised.

To determine the limit sequences necessary for IF structure, we combined the K14 rod with either tailless K5 (Fig. 5 *C*) or the K5 rod (Fig. 5 *D*). At 50 mM Tris, filaments of appreciable length and regularity were seen only with the K14 rod/tailless K5 combination (Fig. 5 *C*). Collectively, these data demonstrate that of the four end domains as defined here, the K5 head is most essential to IF structure,

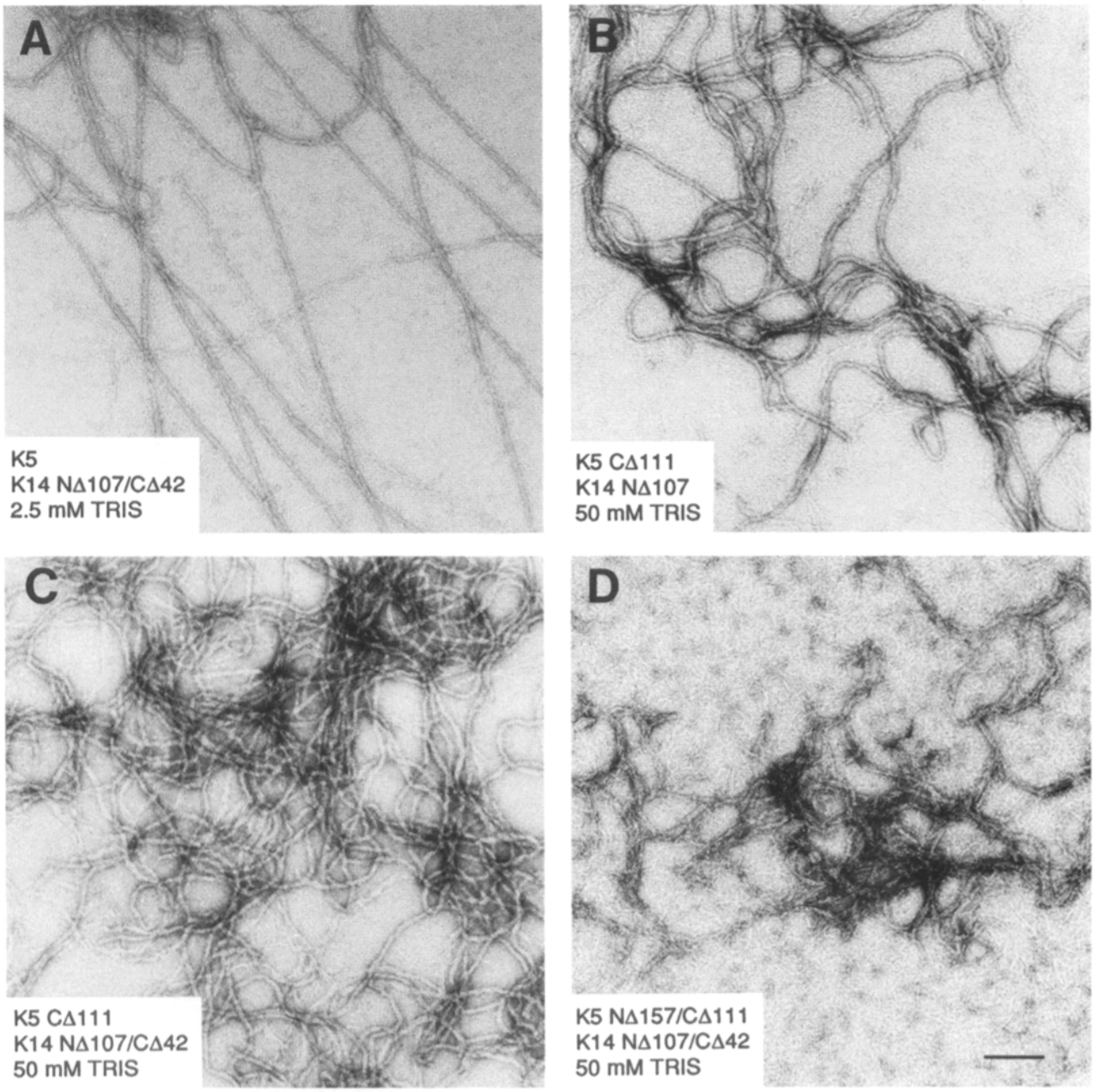

Figure 5. Assembly of rod mutants and heterotypic combinations of headless or tailless keratins. Equimolar amounts of type I and type II keratins were combined in vitro and dialyzed against standard low ionic strength buffer (2.5 mM Tris, pH 7.5) (*A*) or against high ionic strength buffer (50 mM Tris, pH 7.0) (*B–D*) as described in Materials and Methods. Assembled structures were subjected to negative staining and EM. Keratins used in assembly reactions are indicated at the lower left of each frame. Note that any combination involving a headless K5 was unable to assemble into keratin filaments. Bar, 100 nm.

while the other three ends seem to play a role in filament stabilization. These data also suggest that with the exception of the K5 head, interactions between the head and tail domains of neighboring keratins are not essential for filament elongation.

Removal of the R/K L L E G E Consensus Sequences Perturbs Proper Protofibril Alignment to a Greater Extent than Protofibril Elongation

One of the more unexpected findings of IF assembly studies in vitro was the ability of a K14 mutant, missing the R L L E G E consensus sequence at the carboxy end of the rod domain, to assemble into quite long and uniform filaments in vitro under wild-type assembly conditions (Fig. 6 *A* and Table I; see also reference 12). The seemingly greater importance of the K5 tail domain over that of K14 made it likely that the comparable K5 mutant would have a greater importance in filament structure. To test this hypothesis, we combined wild-type K14 with the K L L E G E mutant, K5CΔ119, under standard assembly conditions. As expected, K14 + K5CΔ119 filaments were substantially fewer,

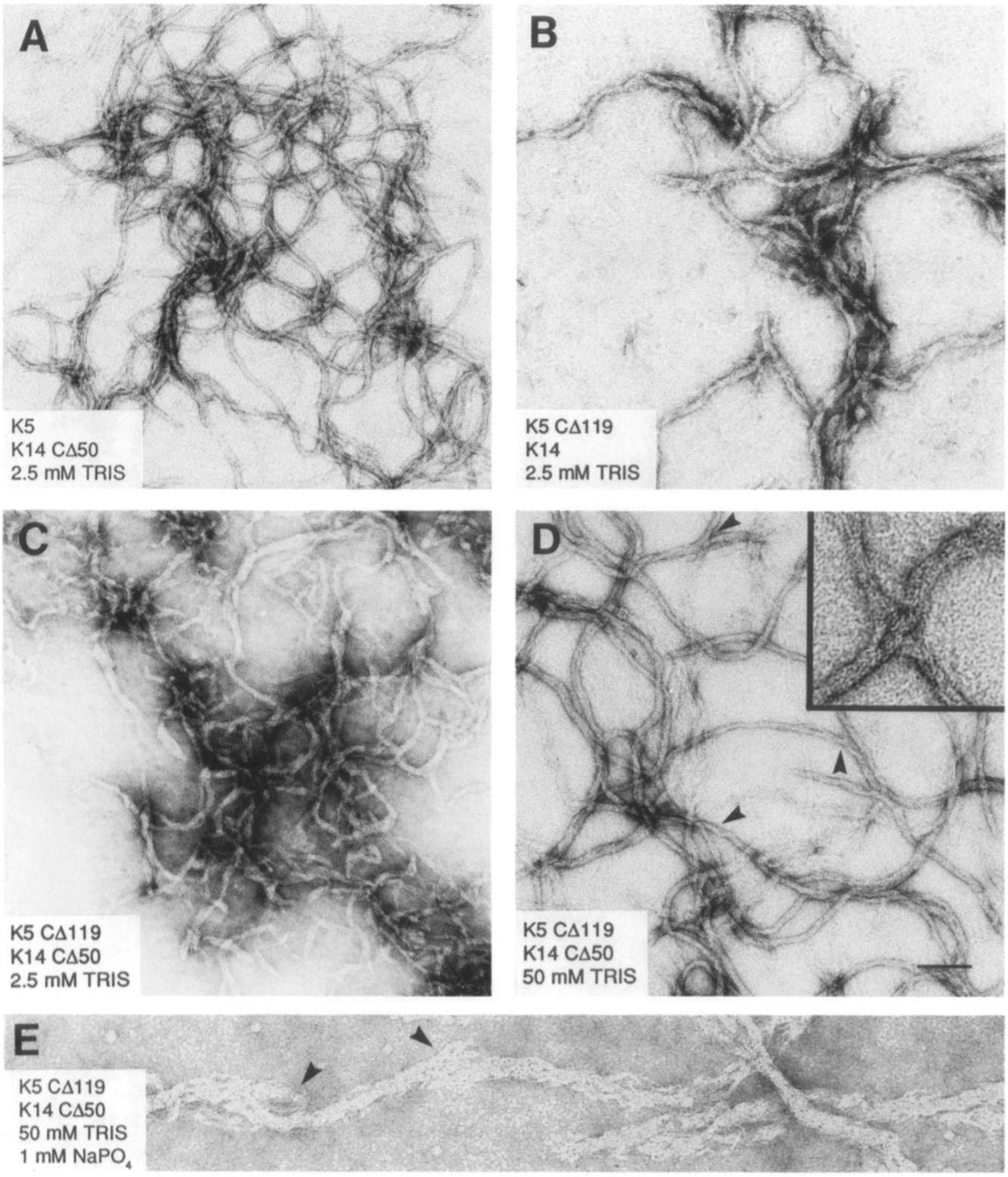

Figure 6. Assembly of R/K L L E G E deletion mutants. Equimolar amounts of type I and type II keratins were combined in vitro and dialyzed against 2.5 mM Tris, pH 7.5 (*A–C*) or 50 mM Tris, pH 7.0 (*D* and *E*). Phosphate buffer was added to *E* as described in Materials and Methods. Assembled structures were subjected to negative staining and EM. Keratins used in assembly reactions are indicated at lower left of each frame. Note knots and twists in filaments assembled from double R/K L L E G E mutants in *D* (see *inset*; *arrowheads* denote twisting). Also note overlapping, shorter filaments in *E* (*arrowheads*). Bar: (*A–D*) 100 nm; (*D, inset*) 39 nm; (*E*) 64 nm.

shorter, and thicker than wild-type (Fig. 6 *B*; see also Table I). It was difficult to assess the ability of increased ionic strength to aid assembly, since under these conditions, large electron dense aggregations of filaments were obtained that were similar to those shown in Fig. 3 *G*. These findings were in agreement with our previous studies, and again emphasized the stabilizing influence of ionic strength on constructs missing a K5 tail.

It seemed likely that the ability to form IFs with R/K L L E G E mutants would be dependent upon the heteropolymeric nature of keratins, with the corresponding wild-type sequence providing a compensatory role to IF structure. To test this hypothesis, we combined the two R/K L L E G E mutants in our standard assembly buffer. At lower ionic strength, the R/K L L E G E mutants formed only short, branched structures that were clearly abnormal (Fig. 6 *C*). Most extraordinarily, however, when the ionic strength was raised, these truncated rod mutants assembled into filaments that were deceptively similar to wild-type (Fig. 6 *D*). However, some filaments were unwound, and overall, the diameter (10–24 nm) was greater than normal. These features suggested that protofibrillar interactions might be altered. At higher magnifications, additional aberrancies in filament structure could be appreciated (Fig. 6 *D, inset*). In particular, filaments were frequently twisted and knotted, and lateral cabling of IFs also occurred.

To investigate in more detail the structure of these R/K L L E G E-less filaments, we repeated the assembly procedure, and then added 10 mM phosphate buffer 10 min before negative staining for EM analysis. Under these conditions, protofibrillar interactions were weakened, and the underlying structure of IFs was unveiled (2, 15 and references therein). The unraveled filaments in Fig. 6 *E* revealed that the larger filaments were composed of both intertwined 10-nm filaments and overlapping, shorter filaments. Because the number of protofibrils cannot be accurately determined from this analysis, isolated protofibril switching from one filament to another cannot be ruled out. Collectively, our findings demonstrate that the R/K L L E G E sequence is not essential for tetramer elongation, but may be required for proper lateral alignment of protofibrils and/or for proper width calibration.

Filament Forming Efficiencies and Width and Length Measurements

While negative staining revealed how our different mutants affected filament structure, the efficiency with which they assembled into higher ordered structures yielded further insights into relative differences in IF stability. Under conditions known to leave incompletely polymerized keratins in the soluble pool (see Materials and Methods), <5% of wild-type assembly mixtures were found in the supernatant (Table I). For wild-type keratins, this was true whether assembly was conducted at low or high ionic strength. Under the higher ionic strength conditions, most mutants assembled efficiently (Table I). Only those assembly mixtures containing the headless K5 protein left a large amount (as much as 75%) of protein in solution. In contrast, at reduced ionic strength (2.5 mM Tris), many mixtures assembled less efficiently than wild-type, including those containing tailless K5 and the K5 K L L E G E rod mutant, as well as those containing headless K5. Collectively, these data provide further confirmation that the tailless and headless mutants of K5 in particular destabilized the normal process of filament assembly.

Discussion

The Tails

K5/K14 heterodimers and heterotetramers form efficiently in 9 and 6 M urea buffers, respectively (11). Moreover, K5/K14 IFs are not grossly affected by variations in pH from 6.5 to 8.0, and formation of long, stable filaments can occur efficiently at ionic strengths as low as 2.5 mM Tris. As such, K5/K14 filaments are more stable than those composed of most other keratins (18), which in turn are generally more stable than most other IFs (for review, see 22). Therefore, K5/K14 are particularly well-suited to elucidating the sequences important for IF structure.

Previously, we showed that the tail domain of K14 can be removed without notable consequence to keratin filament network formation in vivo (3) or to IF structure in vitro (12). We have now shown that the ability to form filaments in vitro with a tailless K14 is not merely due to the compensatory effects of the K5 tail, but rather that neither tail domain of this keratin pair plays a major role in filament elongation. Nevertheless, filaments generated from tailless K14 and K5 were much more sensitive to changes in ionic strength than wild-type filaments, demonstrating that the tails provide increased stability to IF structure/elongation. Both tails contributed to stability, although the contribution of K5 to this process seemed to be greater than that of K14.

Our results were in good agreement with those of Heitlinger et al. (39), who discovered that removing the tail of a lamin did not interfere with the ability of homodimers to elongate in a head-to-tail fashion (see also 27, 54). However, since lamins tend to form paracrystals, rather than IFs in vitro, the effects of tail removal on 10-nm filament formation could not be assessed. In contrast to our studies on K5 and K14, removal of the entire tail of glial fibrillary acidic protein prevented filament formation in vitro (59). Because the tailless keratins were directly analogous to tailless glial fibrillary acidic protein, the most likely explanation for these differences is that K5/K14-containing IFs are significantly more stable than glial filaments, and thus, the destabilizing effects caused by removing the tails were more deleterious to glial filament structure than they were to keratin structure.

Several recent reports have described consequences of removing portions of head or tail domains of simple epithelial keratins (6, 33, 51). In vitro, the IFs formed from wild-type K8/K18 or K8/K19 are less stable than those assembled from K5/K14, and optimal conditions for assembly require higher ionic strengths (50 mM Tris; 18, 33). Under these conditions, a K8 mutant retaining six residues of the tail assembled into IFs when combined with K19, containing the naturally short (13 aa) tail (33). Intriguingly, these filaments were more stable at pH 7.0 than at pH 7.5, and this increased sensitivity to pH prompted these authors to suggest that the tail domains may play a role in filament stability. In an in vivo study, seemingly shorter filament cables were formed from a K8 mutant containing a hybrid K8/SV-40 T antigen tail and a K18 mutant, missing all but seven residues of the tail (51). It seems likely that the difference obtained in this

in vivo study and the one of Bader et al. (6) reflect either differences in the absolute levels of keratins, or in differences generated from using a completely tailless K8 (6) versus a K8 containing a portion of the K8 tail and a tag sequence (51). Collectively, the explanation that agrees best with all studies on tailless or partially tailless keratins is that the tails are important for IF stability, but they do not seem to be required for filament elongation, nor for other major features of overall IF structure. Given the extraordinary stability of K5/K14 filaments among all IF subgroups, our ability to detect a stabilizing influence in the K5/K14 tails makes it likely that this will apply to IF tails in general.

None of the keratin truncation studies conducted thus far have addressed the possible role of the tails when in the context of the natural epithelial cell host. Thus, for example, we cannot rule out the possibility that the tails play an important regulatory role in IF assembly, as suggested by Kouklis et al. (46). Moreover, since the tail sequences may protrude along the surface of the IF (66), they may be important in intracellular interactions between IFs and other organelles or structures, such as the nucleus (24, 26). A most compelling study regarding a function for the tail domains comes from a recent report by Bader et al. (6), revealing that tailless K8/K19 filaments concentrate in the nucleus of transfected fibroblast cultures. Interestingly, in the present study, we discovered that tailless K5/K14 filaments form a disperse filament array under high ionic conditions in vitro, and yet wild-type filaments aggregate under these conditions. These findings suggest that in vivo, there may be some protein(s) or other factor(s) which interact with the tail domains to keep filaments from undergoing these prominent interfilament interactions inside the cell (see 6, 24, 25 for further discussion). As future studies are conducted, the role of the tails in these higher ordered functions should become more apparent.

The Heads

The importance of the amino-terminal head domain in IF assembly appears to vary widely among IF types, and may even differ among members of a single IF subgroup. Studies using partially headless desmin and vimentin demonstrated that most of the head must be present for homopolymeric filament formation (44), but not for heteropolymer formation between a truncated head mutant of desmin and either wild-type desmin or vimentin (41, 44, 60). This finding with type III IFs had prompted us and others to speculate that the underlying basis for the near wild-type formation of IFs between a wild-type K5 and a headless K14 was compensation due to the presence of either the K5 head or the K5 tail in the resulting IFs (4, 12, 60). This was also suggested by Lu and Lane (1990), who discovered that a K7 with an altered head domain (containing a 17 residue SV-40 T antigen tag and 20 residues of the K7 head) could not assemble with the naturally tailless K19. It was further implied by the in vivo studies of Bader et al. (6), who showed that a headless K8 can (at least in some cells) form an IF network with wild-type K18, but forms only a diffuse cytoplasmic network with the naturally tailless K19.

Given prior studies and speculations, it was surprising to find that a headless K5 mutant exhibited properties distinct from our headless K14 mutant. In contrast to headless K14, the comparable K5 mutant could not efficiently form proper IFs in vitro, even in the presence of wild-type K14, and even when ionic strength and pH conditions were varied. Thus, as we have defined the K5 and K14 head domains in the present study, they were not equal with respect to their contributions to filament structure. Of the four head and tail domains of the K5/K14 pair, the most critical to IF structure seemed to be the K5 head. Moreover, the failure of headless K5 mutants to assemble with K14 into normal filaments even in high ionic strength buffers suggested that the K5 head may be involved in assembly processes that go beyond mere filament stabilization. Indeed, filament elongation appeared to be severely compromised, and although more difficult to assess, lateral alignment of protofibrils may have also been affected. Finally, our data do not exclude the possibility that removal of the K5 head may have influenced proper alignment of two antiparallel dimers, giving rise to a tetramer pool which even though still stable in 6 M urea, may have been incompetent for proper filament assembly. Future studies will be necessary to identify the precise molecular alterations imposed as a consequence of deleting the K5 head domain.

In our studies, a K5 mutant containing half of the head domain was still able to assemble into seemingly normal 10-nm filaments. If the principles governing assembly of K5/K14 apply to all type II and type I keratins, then this might explain why Lu and Lane (51) obtained a seemingly normal IF network in cells containing K7 with the altered head domain and a wild-type K18. This said, it is curious that a completely headless K8 protein formed a fibrous network with K18 in at least some transfected fibroblasts (6). If these fibers were composed of bona fide 10-nm filaments, then perhaps there are stabilizing proteins or factors within a cell that allow efficient packing of subunits containing a headless K8 into 10-nm filaments. Alternatively, sequence differences among different keratin pairs might give rise to specialized requirements of different end domains in filament structure. Further studies will be necessary to distinguish between these possibilities.

The R/K L L E G E Sequence

The role of the carboxy-terminal end of the rod domain has been a subject of considerable interest and speculation. Given its extraordinarily high degree of conservation among all IFs, it has long been postulated to be essential for filament assembly. Indeed, removal or perturbation of this highly conserved sequence from different IF proteins has been found to be deleterious to the overall IF network of transfected cells (3, 28, 37, 49, 60, 70). Point mutagenesis of some of these conserved positions within the type II and type I consensus sequences of keratins (34, 49) can also significantly affect filament formation in vitro. In addition, an interesting recent study by Hatzfeld and Weber (35) revealed that a peptide containing this consensus sequence was able to compete with the endogenous sites on keratins to interfere with filament formation in vitro and to cause disassembly of preformed filaments. These studies suggested that normal binding of this motif sequence to its acceptor may play an important role in the proper alignment of tetramers during elongation and protofibril formation.

Our R/K L L E G E mutant studies led to several interesting findings that extend our understanding of IF structure. First, our studies have shown that this highly conserved consensus sequence is not obligatory for all of the higher or-

dered interactions that occur between IF tetramers. Most notably, providing that high ionic strength conditions were used to compensate for destabilization due to the tailless nature of the mutants, filaments formed from keratins missing this sequence were remarkably long, indicating that end-to-end interactions were not dramatically compromised. Secondly, we have found that filaments missing this sequence were often wider than normal, suggestive that their diameter is either composed of (*a*) more loosely wound protofibrils, (*b*) an increased number of protofilaments or protofibrils, or (*c*) protofilaments or protofibrils that are aligned differently than normal. An increase in the twisting, knotting, and branching of these filaments is suggestive of aberrations in lateral alignments. The branching raised an additional possibility that lateral calibration of filaments may be compromised and protofibrillar switching might occur between filaments formed with R/K L L E G E-less keratins. Our finding that the L L E G E sequence plays a role in lateral alignment is interesting in light of a recent study demonstrating that addition of a 10× and 20× excess of a competing L L E G E consensus peptide resulted in filament unraveling and/or thickening (35).

IF Structure

The picture emerging from our work is that the intermediate filament protein is a highly evolved protein, carefully tailored to self-assemble into a complex, but ordered, 10-nm structure. Our studies clearly show that stabilization and/or packing of subunits into a 10-nm filament is dependent upon sequences present in the nonhelical K5 and K14 tail domains and in the K14 head domain. Perhaps most surprising was the striking importance of a portion of the nonhelical head domain of K5 in producing appreciably elongated, stable filaments. Given the high degree of sequence divergence in IF head domains, this was an unexpected finding, particularly for a heteropolymeric structure. However, given the importance of the head domain in the assembly of other IFs, our findings suggest that a specific tertiary structure within the IF head may play an important role in producing subunits which can properly undergo lateral packing and end-to-end interactions to yield bona fide 10-nm filaments. Finally, our studies demonstrate that the R L L E G E sequence plays a greater role in lateral associations than in end-to-end interactions.

An important finding stemming from our studies is that major deletions in keratins can selectively and sometimes subtly perturb only a subset of inter-keratin interactions, leaving a number of higher-ordered interactions either intact, or only partially compromised or altered. In vitro, this results in filaments that look deceptively like bona fide 10-nm filaments. How do we know that the sometimes subtle differences that we have detected in IF structure are relevant to the proper functioning of the filament network? The most compelling evidence is that even single point mutations in a keratin, creating sometimes only subtle differences in the stability/structure of the IF, can nevertheless compromise the mechanical integrity of a cell and give rise to a blistering human skin disease (8, 13, 47, 49). Thus, the ability of IF sequences to select appropriate interactions over inappropriate ones is critical to the overall strength of the resulting intermediate filament. As further studies are conducted, the precise mechanisms by which these selections are governed should become more apparent.

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