Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain

MECHTHILD HATZFELD and KLAUS WEBER

Max Planck Institute for Biophysical Chemistry, Department of Biochemistry, P.O. Box 2841, D-3400 Goettingen, FRG

Summary

All known intermediate filament (IF) proteins display a consensus sequence TYRKLLGE at the carboxyl end of the rod domain. To analyse the contribution of this sequence to the formation of IF we have changed two of the invariant positions of this motif by site-directed mutagenesis. We produced three mutant keratins, each containing a single point mutation. Tyrosine at position —8 was changed to alanine in keratin K8 (K8Y—A—8) and keratin K18 (K18Y—A—8) and leucine at position —4 was changed to glycine in keratin K18 (K18L—G—4). Mutant keratins were expressed in *Escherichia coli*, purified and analysed for their filament-forming capacity *in vitro* using either the complementary wild-type keratin or the corresponding mixture of mutant keratins.

In standard filament buffer (50 mM Tris-HCl, pH 7.5), assembly involving any of the mutants leads to large electron-dense aggregates instead of normal IF. In order to explain this effect, we studied the process of filament formation in more detail.

Whereas the formation of tetramers in buffers containing 4M urea is unaffected, the elongation process seems slowed down. In buffer of lower ionic strength (10 mM Tris-HCl, pH 7.5) mutant keratins K8Y—A—8 plus K18Y—A—8 become able to form long filaments, although short filaments and protofilamentous material are still detected. The filaments formed differ from normal keratin IF by their remarkable tendency to aggregate into thick cables. Assemblies involving K18L—G—4 can only form short IF lengths. The dense aggregates formed in standard filament buffer are able to dissociate into IF and their fragments upon dialysis into 10 mM Tris-HCl, pH 7.5. The results show that the consensus sequence is needed for IF formation under normal conditions and that already one mutation per heterodimer affects the assembly.

Key words: keratins, intermediate filaments, point mutations, consensus sequence.

Introduction

Intermediate filaments (IF) are almost ubiquitous cytoskeletal structures. Vertebrate IF are composed of different but related proteins that show cell-type specific expression patterns (Quinlan et al. 1985; Moll et al. 1982; Fuchs et al. 1984). All IF proteins share a common molecular architecture: each polypeptide chain consists of non-a-helical head and tail domains that vary in size and amino acid composition, and a central a-helical coiled coil domain of ~310 amino acids. This domain contains two highly conserved stretches of amino acids at its ends. The most prominent is the EIATYRKLLGE consensus sequence at the C-terminal end of the rod domain (Geisler and Weber, 1982; Geisler et al. 1982; Steinert et al. 1983, 1985; Hanukoglu and Fuchs, 1983; Weber and Geisler, 1984).

Keratins, specifically expressed in epithelial cells, differ from other IF proteins in that they are obligatory heteropolymers consisting of equal numbers of the smaller acidic type I keratins and the larger neutral to basic type II keratins (Fuchs et al. 1981; Schiller et al. 1982; Hatzfeld and Franke, 1985; Eichner et al. 1986). The first step of keratin IF assembly *in vitro* is the formation of the double-stranded coiled-coil-molecule containing one type I and one type II keratin aligned parallel and in register (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990a; Steinert, 1990). The coiled-coil dimers have a strong tendency to assemble into tetramers (double coiled coils) which seem the predominant soluble intermediates of cytosolic IF *in vivo* and *in vitro* (Ahmadi and Speakman, 1978; Geisler and Weber, 1982; Quinlan et al. 1984; Ip et al. 1985; Parry et al. 1985; Soellner et al. 1985). Several studies have suggested that the coiled-coil molecules in the tetramer are arranged antiparallel and staggered (Geisler et al. 1985; Parry et al. 1985; Fraser et al. 1985; Stewart et al. 1989), but a recent report proposed that antiparallelity and stagger are imposed only in the octamer stage (Hisanaga et al. 1990). Tetramers assemble into a series of higher-order structures: the protofilaments of 2–3 nm diameter, the 4.5 nm protofibrils and finally the 8–10 nm filaments, which seem to be composed of four protofibrils (Aebi et al. 1983).

Studies using proteolytic fragments of keratins have shown that the rod domain is sufficient for specific heterotypic binding and tetramer formation (Hatzfeld et
Filament formation, however, seems to depend on the additional presence of the head domains, while tail domains are not an obligatory requirement for IF formation in vitro, although they may have specific functions in vivo (Sauk et al. 1984; Kaufmann et al. 1985; Hatzfeld and Weber, 1990a; Lu and Lane, 1990). Genetic introduction of a large terminal deletion into a type I keratin cDNA (K14) and transfection into keratin-introduction of a large terminal deletion into a type I keratin cDNA (K14) and transfection into keratin-expressing cells lines indicated that the head and tail domains are not essential for copolymerization with the endogenous IF system (Albers and Fuchs, 1987, 1989). However, transfection of deletion mutants missing parts of the highly conserved sequence motif at the C-terminal end of the rod domain resulted in disruption of the endogenous IF network (Albers and Fuchs, 1987). In agreement, deletion mutants of neurofilament (NF) proteins L and M that lack parts of the conserved sequence motif caused the disruption of endogenous vimentin and NF-L networks, although in the case of NF-L mutants certain deletions involving only the tail domain had also a severe effect on the endogenous IF (Wong and Cleveland, 1990; Gill et al. 1990). Taken together, these studies suggest an important role of the consensus sequence at the end of the rod domain in filament assembly, although the process of disruption in copolymerisation experiments under in vitro conditions is not yet understood. We have studied the function of the consensus sequence using single amino acid substitutions in two of the most conserved positions rather than by deletion mutants and have analysed the filament-forming ability of the mutant keratins in vitro. Here we show that the substitutions in a type I or in a type II keratin have a profound effect on IF assembly properties.

Materials and methods

Site-directed mutagenesis and cloning procedures

Site-directed mutagenesis was performed as described by Nakayama and Eckstein (1986) and Escherichia coli strain JM 101 was used for transformation. Mutants were identified by sequence analysis and cloned into the bacterial expression plasmid as previously described (Hatzfeld and Weber, 1990a, b). After cloning into the expression vector all mutants were completely sequenced. All cloning procedures were carried out as previously described.

Purification of mutant keratins

Bacterial cultures expressing wild-type or mutant keratins were grown overnight in LB medium containing 200 μg/ml ampicillin. Bacteria were harvested and inclusion bodies were isolated as described (Nagai and Thogersen, 1987). High molecular weight DNA was destroyed by shearing in a Dounce homogenizer in detergent-containing buffer prior to centrifugation. Purified inclusion bodies were dissolved by addition of 5 M urea, 5 mM EDTA, 10 mM Tris–HCl, pH 8.5, containing the following protease inhibitors: 0.5 μM E64 (L-3-trans-carboxy-oxiran-2-carboxylyl)-1-leu-agmatrn), 100 μg/ml ovomucoid and 2 mM phenylmethylsulfonyl fluoride (PMSF). All inhibitors were from Sigma Chemical Co, Deisenhofer, FRG). Proteins from inclusion body preparations were further purified at room temperature by ion exchange chromatography on Mono Q (Pharmacia Fine Chemicals, Uppsala, Sweden) in 8.5 M urea, 5 mM EDTA, 10 mM Tris–HCl, pH 8.5, containing the following protease inhibitors: 0.5 μM E64 (L-3-trans-carboxy-oxiran-2-carboxylyl)-1-leu-agmatrn). 100 μg/ml ovomucoid and 2 mM phenylmethylsulfonyl fluoride (PMSF). All inhibitors were from Sigma Chemical Co, Deisenhofer, FRG). Proteins from inclusion body preparations were further purified at room temperature by ion exchange chromatography on Mono Q (Pharmacia Fine Chemicals, Uppsala, Sweden) in 8.5 M urea, 5 mM EDTA, 10 mM Tris–HCl, pH 8.5. The preparations were diluted in chromatography buffer, applied to the ion-exchange column using an FPLC facility and eluted with a linear gradient from 0 to 200 mM guanidine-HCl in chromatography buffer. Relevant fractions were pooled, dialysed overnight against 8.5 M urea, 10 mM Tris–HCl, pH 7.5. buffer and applied to a single-stranded DNA affinity column (Gibco/BRL Life Technologies, Eggenstein, FRG) equilibrated in the same buffer. Bound keratin was step eluted by 200 mM guanidine-HCl in equilibration buffer (Hatzfeld and Weber, 1990a, b). Protein concentration was determined according to the method of Bradford (1976). Keratins were stored in aliquots at −20°C in their elution buffer.

Gel electrophoresis and immunoblotting

One-dimensional SDS–PAGE electrophoresis was performed according to Laemmli (1970) using 10% acrylamide gels. Polypeptides were transferred to nitrocellulose according to the method of Khyse-Anderson (1984). Immunoblots with the monoclonal antibody IFA (Pruis et al. 1981) were developed using alkaline phosphatase-coupled anti-mouse Ig as described previously (Achtstätter et al. 1986; Hatzfeld and Weber, 1990a,b).

Chemical cross-linking

Keratin complexes were cross-linked in 4 M urea-containing buffer. Equimolar amounts of wild-type or mutant type I and type II keratins were mixed and dialysed overnight at room temperature against 4 M urea, 50 mM sodium phosphate buffer, pH 8.0, at a protein concentration of ~0.5 mg/ml ‐ 1 . Samples were then diluted to a protein concentration of ~80 μg/ml ‐ 1 with dialysis buffer, and ethylaminecetate (Sigma Chemical Co.) was added to a final concentration of 1 mM. Cross-linking was allowed to proceed for 90 min at room temperature, stopped by addition of 1 M Tris–HCl, pH 7.5, and the protein was collected by precipitation with methanol and chloroform as described by Wessel and Flügge (1984). Cross-linked products were separated on 5% to 10% gradient gels.

In vitro assembly and electron microscopy

Equimolar amounts of type I and type II keratins were mixed in 8.5 M urea buffer at a protein concentration of 0.25 to 0.5 mg/ml ‐ 1 . Samples (30–50 μl) were dialysed at room temperature against standard filament assembly buffer (60 mM Tris–HCl, pH 7.5, 5 mM EDTA) or low salt buffers (10 mM Tris–HCl, pH 7.5, 5 mM EDTA or 2 mM Tris–HCl, pH 8.0, 5 mM EDTA) using microdialysis filters (Millipore GmbH, Eschborn, FRG) as described (Hatzfeld and Weber, 1990b). In Results, these buffers are referred to as Tris–HCl buffers, with the molarity and pH stated. Since the EDTA concentration is the same, it is not specifically mentioned. In some experiments the carbon-coated grids were put onto the dialysis filters to collect precipitates formed during dialysis directly on the grid. Structures formed after dialysis were analysed after negative staining with 2% uranyl acetate and rotary shadowing as described (Quinlan et al. 1984; Hatzfeld and Franke, 1986; Hatzfeld and Weber, 1990a,b). For analyses of tetramers, samples were dialysed from 8.5 M urea buffer to 4 M urea, 10 mM Tris–HCl buffer, pH 8.0.

Reversibility of aggregate formation in standard filament assembly buffer was tested in the following experiment: samples were dialysed overnight at room temperature either against 10 mM Tris–HCl, pH 7.5, 5 mM EDTA or against standard filament assembly buffer. Samples were taken from both filters for negative stain and each of the filters then transferred to the other buffer. After another 6 h of dialysis, samples were again processed for negative stain analysis.

Results

Construction and purification of mutant keratins

In order to analyse the functional role of the consensus sequence at the end of the rod, we have introduced point mutations into K8 and K18 cDNAs in two of the most strongly conserved positions along this sequence. The alignment of the consensus sequences from position −18 to position +4 of various IF proteins indicates that positions −8 and −4 are identical in all IF proteins characterized so far (for reference points of the alignment of the consensus sequences, see Fig. 1 and Franz and Franke, 1986). In the heptad repeat pattern typical of coiled coil molecules, the
tyrosine (position -8) and leucine (position -4) occupy a and d positions, i.e. interior positions along the end of the rod of the putative coiled coil. We have changed the tyrosine to an alanine in keratin 8 (K8Y→A-8) and in keratin 18 (K18Y→A-8) by site-directed in vitro mutagenesis. In addition we have substituted the leucine in position -4 of keratin 18 by a glycine (K18L→G-4). The mutant keratins were cloned into the pINDU expression vector (Magin et al. 1987; Hatzfeld and Weber, 1990a,b) and sequenced completely to ensure that no additional mutations had been introduced during the mutagenesis reaction. In this context we noticed that in the tail domain of K8 wild type, as well as of the K8 mutant protein, a stretch of five amino acids differs from the published sequence (Franz and Franke, 1986). Instead of the nucleotide sequence TAC ATT GGA GAC ACC AGC AGG CGT AGA CTT, corresponding to the amino acid sequence YIGDFTKSKRL (position 470 to 481 of the original sequence), we found the nucleotide sequence TTA CCA TTG GAG ACA TCC AAG ACA AGC AGG CGT AGC ATT, representing amino acids LPLETSTKS. 

Wild-type keratin 8 and 18 and the mutant keratins were expressed in E. coli and purified from inclusion bodies as described (Hatzfeld and Weber, 1990a,b). Fig. 2 shows SDS-PAGE of purified wild-type keratin 8 and 18 and the purified mutant keratins K8Y→A-8, K18Y→A-8 and K18L→G-4. Whereas both wild-type keratins are recognized by the IF-specific monoclonal antibody IFA described by Pruss et al. (1981) (Fig. 3, lanes 1 and 2), all three point mutations abolished the reactivity (Fig. 3, lanes 5–7), probably indicating that the mutated residues and their environment are part of the epitope recognized by the antibody.

Characterization of the soluble keratin complexes formed in 4 M urea

Soluble tetramers are stable intermediates in buffers containing 4 M urea when the homopolymer forming IF proteins vimentin and desmin are studied. Similarly, in the obligatory heteropolymer system of the keratins the same solvent leads again to tetramers (Geisler et al. 1982; Quinlan et al. 1984). The structures formed in the 4 M urea samples were visualized after glycerol spraying by rotary

**Table 1. Alignment of the carboxy-terminal sequences of the rod domains of various IF proteins and nuclear lamins.** Only residues exchanged versus the hamster vimentin sequence are given; shared residues are marked by dashes. The presumptive coiled coil is marked by the interior positions a and d of the consecutive heptads (top line). These are also emphasized at the bottom by filled circles. Owing to some irregularities at the very end of the rods of some type I keratins, the consensus sequence is only counted from the arrow (position 0, bottom line) and the four following residues are neglected (positions 1 to 4). The number of IF sequences in the data bank is much larger than the sample given in the table. Those listed here stand also for other sequences that are identical over the consensus region. Sequences not yet entered in the bank are those of rat nestin (Lendahl et al. 1990), Xenopus lamin LIII (Doöring and Stick, 1990) and the three invertebrate IF proteins from Ascaris and Ascaris lumbricoides (Dodemont et al. 1990; Weber et al. 1989). The four strictly invariant residues are enclosed by a square in the master sequence at the top. The term nearly invariant is used for those three residues for which only one strictly homologous replacement is found in the data bank and the table. These positions are marked by a dotted square. Note that all a and d positions of the presumptive coiled coil (residues -18, -16, -11, -8 and -4) except for residue -1 are occupied by invariant or nearly invariant residues. The three sequences at the bottom are the point mutants of keratins used in this study. The replacements marked by brackets involve the change of the invariant Y at position -8 and the change of the invariant L at position -4. Both residues lie in interior position of the presumptive coiled coil.

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Fig. 1. Alignment of the carboxy-terminal sequences of the rod domains of various IF proteins and nuclear lamins. Only residues exchanged versus the hamster vimentin sequence are given; shared residues are marked by dashes. The presumptive coiled coil is marked by the interior positions a and d of the consecutive heptads (top line). These are also emphasized at the bottom by filled circles. Owing to some irregularities at the very end of the rods of some type I keratins, the consensus sequence is only counted from the arrow (position 0, bottom line) and the four following residues are neglected (positions 1 to 4). The number of IF sequences in the data bank is much larger than the sample given in the table. Those listed here stand also for other sequences that are identical over the consensus region. Sequences not yet entered in the bank are those of rat nestin (Lendahl et al. 1990), Xenopus lamin LIII (Döring and Stick, 1990) and the three invertebrate IF proteins from Helix aspersa and Ascaris lumbricoides (Dodemont et al. 1990; Weber et al. 1989). The four strictly invariant residues are enclosed by a square in the master sequence at the top. The term nearly invariant is used for those three residues for which only one strictly homologous replacement is found in the data bank and the table. These positions are marked by a dotted square. Note that all a and d positions of the presumptive coiled coil (residues -18, -15, -11, -8 and -4) except for residue -1 are occupied by invariant or nearly invariant residues. The three sequences at the bottom are the point mutants of keratins used in this study. The replacements marked by brackets involve the change of the invariant Y at position -8 and the change of the invariant L at position -4. Both residues lie in interior position of the presumptive coiled coil.
Fig. 2. SDS-PAGE of purified wild-type and mutant keratins. Reference proteins (R); and (Mr): BSA (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000) and trypsinogen (24,000). Lane 1, purified wild-type K18; lane 2, purified wild-type K8; lane 3, purified mutant keratin K18L—^G—4; lane 4, purified mutant keratin K18Y—A—8; lane 5, purified mutant keratin K8Y—A—8.

shadowing. Complexes formed by wild-type keratins K8 and K18 revealed rod-like structures (Fig. 4A). No difference was observed when one of the wild-type keratins or both keratins were substituted by the analogous mutant keratins (Fig. 4B-F). All complexes were similar in diameter and length to the rod-like tetramers formed by wild-type K8 and K18. These results suggest that the single amino acid exchanges in the consensus sequence do not impair the ability of the mutant keratins to participate in tetramer formation. Since we previously demonstrated that single keratins can also form similar rod-like structures in 4 M urea buffer, which cannot be distinguished from heterotetramers by electron microscopy (Hatzfeld and Franke, 1985), we have further characterized the complexes formed by the keratin mixtures in 4 M urea using chemical cross-linking with ethylacetimidate. Gel electrophoretic separation of the

1 2 3 4 5 R

Fig. 3. SDS-PAGE and immunoblot analysis of mutant keratins using inclusion body preparations. Lane 1, wild-type K8; lane 2, wild-type K18; lane 3, K8/Cys (see Hatzfeld and Weber, 1990a); lane 4, K18/Cys; lane 5 K8Y—A—8; lane 6, K18Y—A—8; lane 7, K8L—G—4. Lanes 1'—7' show the corresponding immunoblot reaction after incubation with the monoclonal antibody IFA (Pruss et al. 1981) and an alkaline phosphatase-coupled second antibody. Whereas wild-type keratins and cysteine-containing mutants show a strong signal, reactivity was abolished in all three consensus sequence mutants.

Fig. 4. Electron microscopy of subunit structures formed in 4 M urea-containing buffer demonstrated by rotary shadowing. Wild-type keratins K8 plus K18 (A), mutant keratins K8Y—A—8 plus wild-type K18 (B), wild-type K8 plus mutant keratin K18Y—A—8 (C), wild-type K8 plus mutant K18L—G—4 (D), mutants K8Y—A—8 plus K18Y—A—8 (E) and mutants K8Y—A—8 plus K18L—G—4 (F) were mixed in equimolar amounts and dialysed into 4 M urea, 10 mM Tris-HCl, pH 8.0, overnight at room temperature. All molecules are very similar and appear as rods that are found to be around 50–70 nm long. Bar, 0.2 μm.

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ing combinations of wild-type and mutant keratins, and the combinations of two mutant keratins. These experiments strongly suggest that the point mutations in the consensus sequence do not influence the alignment of polypeptide chains at the level of the tetramer.

Fig. 5. Gel electrophoretic characterization of keratin complexes cross-linked in 4 M urea-containing buffer. Reference proteins (Mr) marked by dots are, from the top: myosin (200 000); β-galactosidase (116 000); phosphorylase B (97 400); BSA (66 000); ovalbumin (45 000) and carbonic anhydrase (29 000). Keratins were combined in equimolar amounts and dialysed to 4 M urea-containing buffer. Samples were diluted to a protein concentration of 50 μg ml⁻¹ in 4 M urea buffer and cross-linked with ethylacetimidate at 1 mg ml⁻¹ final concentration at room temperature. Samples were taken at 0 min and 90 min, precipitated and separated by SDS-PAGE. Lanes 1 and 1′, wild-type K8 plus K18 at 0 and 90 min; lanes 2 and 2′, K8Y→A-8 plus wild-type K18 at 0 and 90 min; lanes 3 and 3′, wild-type K8 plus K8Y→A-8 at 0 and 90 min; lanes 4 and 4′, wild-type K8 plus K8L→G-4 at 0 and 90 min; lanes 5 and 5′, K8Y→A-8 plus K8L→G-4 at 0 and 90 min. Brackets denote cross-linked tetramers of apparent Mr ~260 000 and trimers or tetramers of apparent Mr ~210 000. Parentheses denote the dimers. Tetramers and intermediate cross-linked products are the same for wild type, combinations of wild type plus mutant, and combinations of corresponding mutant keratins, indicating that the mutations do not inhibit tetramer formation.

The pattern of dimer and tetramer bands obtained was identical for wild-type keratin tetramers, the correspond-
Assembly in the presence of mutant keratins

The filament-forming capacity of the mutant keratins was analysed by dialysis against standard keratin filament buffer, i.e. 50 mM Tris-HCl, pH 7.5, followed by negative staining. Whereas the mixture of wild-type keratins K8 and K18 assemble into regular IF in this buffer with only a little protofilamentous material left (Fig. 6A), the combinations of mutant keratins K8Y–A–8 plus K18Y–A–8 (Fig. 6B), K8 wild-type plus K18L–G–4 (Fig. 6C) and K8Y–A–8 plus K18L–G–4 (Fig. 6D) yielded predominantly large aggregates of electron-dense material with hardly any visible substructure. Some of the protein was detected in very dense thick bundles of fiber-like structures (Fig. 6B and C). However, this material was so tightly packed that it cannot be decided whether these bundles contain aggregated IF or whether the alignment of subunits is completely different from that seen in normal IF. Combinations of one wild-type keratin with the corresponding mutant keratin and combinations of two mutant keratins showed identical assembly properties, indicating that only one mutated polypeptide chain per heterodimer is sufficient to distort the typical filament assembly properties. There seem to be two possible explanations for this unusual behaviour of mutant keratins: first, mutant keratins assemble into normal IF, which differ, however, from non-mutant IF by a very strong tendency to aggregate. Second, mutant keratins form tetramers that have lost the capacity for specific

Fig. 7. Electron microscopy of a time-course experiment of the assembly process in standard filament buffer. All samples were dialysed against standard filament buffer and negatively stained with 2% uranyl acetate. (A) Protofilaments formed by wild-type keratins 8 plus 18 after 15 min of dialysis. (B) Subunits formed by wild-type K8 plus mutant K18Y–A–8 after 15 min of dialysis. Bars in A and B, 0.1 μm. (C) Filaments and protofilaments assembled from wild-type K8 plus K18 after 30 min of dialysis. (D) Short rod-like fragments formed by mutants K8Y–A–8 plus K18Y–A–8 after 15 min of dialysis. The structures reveal a rough irregular surface. (E) Filaments formed by wild-type K8 plus K18 after 1 h of dialysis. (F) Aggregates formed by mutant keratins K8Y–A–8 and K18Y–A–8 after 1 h of dialysis. Bars in C-F, 0.2 μm. No IF but only short ~100 nm IF-like rods are detected as precursors of the large aggregates formed by mutant keratins in filament buffer.

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Fig. 8. Electron microscopy of structures formed after overnight dialysis against 2 mM Tris–HCl, pH 8.0. (A), (D) and (G) show samples negatively stained with 2% uranyl acetate; (B), (C), (E), (F), (H) and (I) show samples after rotary shadowing. (A)–(C) Protofilaments and short filaments formed by wild-type keratins K8 plus K18. (D)–(F) Structures formed by mutant keratins K8Y→A–8 plus K18Y→A–8. Arrows in E and F denote pairs of tetramers aligned side by side. The arrowhead in E denotes a higher-order oligomer. In F, another higher-order oligomer is seen. (G), (H) and (I) Structures formed by keratin K8 wild-type plus K18L→G–4. Arrow in H denotes a pair of tetramers. Bars in A–I, 0.1 μm. In 2 mM Tris–HCl buffer, pH 8.0, mutant keratins form much smaller oligomers than wild-type keratins, suggesting that the longitudinal association is slowed down.

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interactions leading to normal filament elongation. In order to distinguish between these two possibilities, we followed the assembly process in more detail. Two approaches were chosen in order to visualize intermediate stages during IF formation. In the first set of experiments we tried to visualize intermediate stages at different times during reconstitution. In the second set of experiments we varied the buffer conditions by changing the ionic strength as well as the pH.

**Time course of polymerization in standard filament buffer**

In the time-course experiment we could not detect regular individual filaments in assembly systems containing a mutant keratin. After 15 min of dialysis against standard keratin filament buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA), both the wild-type K8 plus K18 (Fig. 7A) and corresponding combinations of a wild-type and a mutant keratin (Fig. 7B) revealed predominantly small subunits. In addition very loosely packed protofibrils could be detected in the control experiment with wild-type keratins, but not in samples containing mutant keratins. At this time point the only higher-order structures formed in the samples with a mutant keratin were rod-like structures with an approximate diameter of 10 nm and an average length of ~100 nm (Fig. 7D). They typically reveal a rough irregular surface that clearly distinguishes them from short fragments of normal IF. In addition these rod-like structures are characterized by a tendency to accumulate in loose aggregates (Fig. 7D). After a further 15 min of dialysis, typical IF appear only in the control sample (Fig. 7C), and after a total dialysis time of 1 h, filament formation is essentially complete (Fig. 7E).

However, in samples containing mutant keratins, very dense and large aggregates appear after 30 min of dialysis and become even more prominent after 60 min of dialysis (Fig. 7F). This experiment shows that in standard filament buffer, mutant keratins suppress normal IF formation. Thus the short rod-like structures seem to be the precursors of the large aggregates formed by mutant keratins. This suggests interference of the mutations with the elongation reaction leading to IF formation.

**Dialysis against low ionic strength buffers**

Since aggregation of mutant keratins very quickly follows the formation of subunit structures, we tried to enrich for intermediate structures by dialysis against buffers of lower ionic strength and higher pH than those of the standard filament buffer. In 2 mM Tris–HCl, pH 8.0, wild-type keratins assemble into protofilaments (Fig. 8A), which begin to form 10 nm filaments. Rotary shadowing reveals protofilaments, short pieces of IF that already have the typical 21 nm periodicity and normal filaments (Fig. 8B,C).

The combination of the complementary mutant keratins, K8Y→A–8 with K18Y→A–8, protofilamentous material is prominent in the negatively stained samples (Fig. 8D). After rotary shadowing, pairs of tetramers represent a major subunit structure (Fig. 8E, arrows) where tetramers seem aligned side by side. This type of structure is not detected with wild-type keratins under the same conditions. A few pairs of tetramers associate longitudinally to higher oligomeric forms (Fig. 8F). Thus elongation, although still taking place, seems to have slowed down. The combination of K8 wild-type plus K18L→G–4 forms similar structures in 2 mM Tris–HCl, pH 8.0. In the negatively stained sample, protofilaments are easily detected (Fig. 8G). The rotary-shadowed sample again shows single tetramers and association products of tetramers (Fig. 8H and I), which, however, seem arranged in a less regular way than those of mutant keratins K8Y→A–8 plus K18Y→A–8. Both samples containing mutant keratins show only small multimeric association products and not regular IF.

In 10 mM Tris–HCl, pH 7.5, wild-type keratins form a mixture of long IF, short IF and protofilaments (Fig. 9A). After rotary shadowing long and short filaments are detected (Fig. 9B). Most of the short structures contain four 21 nm repeats. The combination of mutant K8Y→A–8 with wild-type K18 reveals protofilamentous material with no IF present (Fig. 9C). The very short filament-like units present reveal two to four 21 nm repeats after rotary shadowing (Fig. 9D). Unexpectedly, mutant keratin K18Y→A–8 assembles with K8 in 10 mM Tris–HCl, pH 7.5, into 10 nm filaments (Fig. 9E and F). However, these filaments differ from those formed by wild type K8 and K18 by their very strong tendency to form very thick and tight IF bundles (Fig. 9E), which possibly represent the precursors of the thick cables detected in standard filament buffer (see above). In addition to these structures, areas of the grid with only protofilamentous material and short IF pieces similar to those detected in the time-course experiment (Fig. 7D) are present (not shown). Mutant K18L→G–4 plus K8 wild-type form pieces of IF that also have a tendency to accumulate, thereby giving the impression of longer filaments (Fig. 9G). Rotary shadowing shows that most of these filament fragments contain three to six 21 nm repeats (Fig. 9H).

**Dialysis of the complementary mutant keratins**

K8Y→A–8 plus K18Y→A–8 against 10 mM Tris–HCl, pH 7.5, leads to filament formation, although the extent of polymerization is small (Fig. 9I). After rotary shadowing filament fragments with two to four 21 nm repeats are detected in addition to short filaments in which the 21 nm repeats seem sometimes irregularly displayed (Fig. 9K). Mutant keratins K8Y→A–8 plus K18Y→G–4 form short filament-like structures (Fig. 9L and M), which after negative staining (Fig. 9L) and rotary shadowing (Fig. 9M) seem very similar to those obtained by K8 wild-type...
type plus K18L→G-4. They share the strong tendency to aggregate into loose clumps and are very rarely detected as single units.

The structures formed by wild-type keratins K8 plus K18 in 10 mM Tris-HCl, pH 7.5, are precursors of those formed in 50 mM Tris-HCl buffer. If this were also the case in the experiments involving mutant keratins, then the large very dense aggregates present in 50 mM Tris-HCl buffer (see e.g. Fig. 6B–D) should contain the IF for Y→A-8 mutants and short IF-like fragments for K18L→G-4 described in 10 mM Tris-HCl buffer (Fig. 9L–M). On the other hand one can easily imagine that a completely different way of polymerization takes place at higher ionic strength. In order to distinguish
between the two possibilities we dialysed the structures obtained in 10 mM Tris–HCl buffer, pH 7.5, to 50 mM Tris–HCl buffer, pH 7.5, and vice versa. Both samples in 50 mM Tris–HCl buffer – the sample dialysed directly overnight into 50 mM Tris–HCl buffer, pH 7.5, and the sample dialysed first overnight into 10 mM Tris–HCl buffer, pH 7.5, and then for 6h against 50 mM Tris–HCl buffer, pH 7.5 – contained almost exclusively very large electron-dense aggregates. However, when these aggregates were dialysed back to 10 mM Tris–HCl buffer, pH 7.5, some normal IF were again detected in both preparations (Fig. 10B). They were indistinguishable from those obtained after direct dialysis into 10 mM Tris–HCl buffer, pH 7.5 (Fig. 10A and B). These results suggest that the dense aggregates obtained in standard filament buffer by the mutant keratins Y→A–8 with their wild-type and their complementary mutant counterpart may contain IF with an unusual tendency to associate into filament cables.

Discussion

We have used in vitro mutagenesis and expression of mutant keratin in E. coli to analyse the importance of individual amino acids within the highly conserved sequence motif TYYKLLEGEE present at the end of the rod domain of all IF proteins. Several previous experiments suggested an important role for this motif in filament formation. Deletion mutants of human keratin K14 lacking parts of the rod domain including the consensus sequence caused endogenous keratin filaments to collapse, upon transfection into keratin-expressing cell lines. In particular the keratin network of cells transfected with a K14 deletion mutant lacking the RRLLEGEDAL sequence of the rod domain and the entire C-terminal tail domain appeared as ‘a single straightened cable of collapsed filaments’ (Albers and Fuchs, 1987). Similarly, deletion mutants of neurofilaments NF-L and NF-M lacking the tail domain and parts of the consensus sequence motif were assembly defective and caused the collapse of internal IF networks when transfected into both vimentin and vimentin plus NF-L-expressing cell lines (Wong and Cleveland, 1990; Gill et al. 1990). However, there are some limitations in interpreting these transfection experiments and these have been reviewed by Wong and Cleveland (1990). First, the resolution of immunofluorescence microscopy is too low to decide at which level the block in assembly occurs (formation of dimers, tetramers or higher oligomers). Thus the effects observed cannot be immediately interpreted at a molecular level. Second, in these experiments rather large deletions were introduced. These included the entire C-terminal tail domain. In addition, for convenience of detection of the mutant protein, an extra sequence recognized by a specific antibody was added as a tag. However, this extra tagging sequence might influence the folding of the protein in a neighboring region independent of the mutation itself, thereby simulating an effect that might not be solely due to the introduced mutation. Furthermore, only in a portion of the transfected cells was an effect of mutant proteins on the endogenous IF network observed, while in other cells the cytoskeleton was unaffected. This particular problem, thought to reflect a quantitative effect of the mutant protein level, has in some studies been overcome by the use of cell lines derived from the transfected cells (Wong and Cleveland, 1990).

To overcome some of the problems inherent in these approaches, we have used point mutations rather than large deletions and studied the in vitro assembly process involving mutant keratins directly by electron microscopy. We chose two of the most conserved positions within the consensus sequence for mutagenesis. In our first set of complementary mutants (K8Y→A–8; K18Y→A–8), the invariant Y was substituted by an alanine. Our in vitro reconstitution studies show that such mutants seem unaffected in dimer and tetramer formation when supplemented with the complementary normal keratin or even when used together. In contrast, the regular elongation reaction seems slowed down in comparison to wild-type keratin. Using standard assembly buffer conditions, the mutants aggregate into large clumps instead of normal IF. Formation of such large dense aggregates has two different possible explanations: (1) mutant keratins form rather unspecific aggregates containing denatured or at least partially denatured protein; this could be a consequence of aberrant protein folding of the C-terminal portion of the rod caused by the mutation. (2) Mutant keratins form IF that differ from wild-type IF in their tendency to associate into large bundles and aggregates. Several sets of experiments suggest that mutant keratins are at least partially assembly competent and do not aggregate in their denatured form: (1) in low-salt buffer, mutants become able to assemble into regular IF; (2) Aggregates formed in standard filament buffer dissociate into IF upon dialysis into low salt buffer, suggesting that the aggregates consist of densely packed filaments and not denatured protein.
The observation that IF formed in a mutant assembly differ from wild-type keratin IF in their remarkable tendency to form tight, thick bundles may be relevant to the results of Albers and Fuchs (1987) on a deletion mutant of K14 lacking part of the consensus sequence and the tail domain, showing that upon transfection into epithelial cells the endogenous keratin filaments aggregated into a thick cable. Surprisingly, although our mutations involved only uncharged amino acids, the process of in vitro aggregation is highly dependent on ionic strength. Lowering the ionic strength from 50 to 10 mM Tris–HCl at pH 7.5 results in at least partial disaggregation of the preformed bundles into filaments. Although these two tyrosine to alanine mutants have not entirely lost filament-forming ability, their behavior clearly differs from that of normal keratins, and just one mutant per heterodimer will lead to erratic assembly. This effect is even more pronounced in the mutant K18L→G→4, in which the invariant leucine was replaced. In assemblies involving this mutant, even changes in assembly conditions did not result in regular IF formation and instead only short 'IF-like' pieces were obtained. These structures revealed, however, the 21 nm periodicity typical of IF (Henderson et al. 1982). Under standard assembly conditions (50 mM Tris–HCl, pH 7.5), all three mutants lead to aggregation into large, electron-dense structures. Taken together, a substitution of the invariant tyrosine and leucine residues (positions –8 and –4) of the consensus motif lead to a modulation in the formation of filaments. Since these residues are invariant in all IF, and since the modulation effect occurs with just a single mutant per keratin heterodimer, we expect that the same mutations will also affect IF proteins participating in homopolymer formation.

When this manuscript was completed, an interesting report on keratin assembly appeared. Coulombe et al. (1990) describe a K14 deletion mutant in which the LLEGE sequence from the motif and the entire tail domain, showing that upon transfection into transfected cells, as documented using an antibody against the tag peptide. In 10 mM Tris–HCl, pH 7.2, the only buffer system used in the in vitro studies, the K14 deletion mutant showed with wild-type K5 nearly normal assembly into IF. Our results on K6V→A→8 and K6V→A–8 mutants, where the filament-forming capacity is retained under low salt conditions (10 mM Tris–HCl, pH 7.5) in vitro, agree with these results. Our assembly studies at higher salt conditions (50 mM Tris–HCl, pH 7.5) show a perturbation of assembly that is very similar to the results obtained in vivo (see also Albers and Fuchs, 1987) and therefore possibly offer an explanation for the discrepancy between the in vivo and in vitro analyses of IF formation described by Coulombe et al. (1990). At higher ionic strength in vitro, or at physiological salt conditions in vivo, such mutant keratins seem to form large aggregates, while at low ionic strength, in vitro assembly is nearly normal.

The molecular basis of the mutant's influence raises some interesting questions. In all sequence interpretation the presumptive coiled coil has been extended through the presumptive coiled coil has been extended through the C-terminal part of the rod of mutant keratin K18L→G→4. Thus this mutation could interfere with the coiled coil, one might expect keratins to tolerate any substitution that is compatible with coiled coil formation. However, this is clearly not the case. A possible explanation for the assembly properties of mutant keratins is that the amino acid residues in the a and d positions at the end of the rod may have an additional function requiring precisely tuned side-chains, although such residues are thought to be buried. This is in line with the finding that in the large data base of IF sequences, the tyrosine at position –8 has never been found to be substituted by a phenylalanine. Further experiments must be done to show whether the coiled coil along the motif sequence is a flexible structure in which the residues in the presumptive inner surface participate in rather specific interactions leading to elongation. In the IF model based on paracrystals of the rod domain of GFAP (Stewart et al. 1989), the consensus sequences of neighboring tetramers overlap in such a way that longitudinal association can occur. Our results are compatible with this view and emphasize an obligatory role of the unchanged motif sequence in filament assembly.

In various but still hypothetical views of the filament wall, the elongation step introduces new contacts that would involve several rod segments, including that at the C-terminal end of the rod. This is particularly obvious if the filament wall were to retain those contacts deduced from paracrystals of the rod domain of GFAP (Stewart et al. 1989). Here a specific contact between the motif sequences of neighboring antiparallelly oriented tetramers is deduced. While our results could be compatible with such views, they emphasize an obligatory role of the unchanged motif sequence for normal IF formation.

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