A Proline Residue in the α-Helical Rod Domain of Type I Keratin 16 Destabilizes Keratin Heterotetramers*

(Received for publication, July 30, 1997, and in revised form, September 29, 1997)

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The type I keratins (K14 and K16) are distinct in their assembly properties and their expression pattern despite a high degree of sequence identity. Understanding K16 function and regulation is of interest, given its strong induction in keratinocytes located at the wound edge after injury to stratified epithelia. We reported previously that, compared with K14, K16 forms unstable heterotetramers with either K5 or K6 as the type II keratin pairing partner (Paladini, R. D., Takahashi, K., Bravo, N. S., and Coulombe, P. A. (1996) J. Cell Biol. 132, 381–397). We show here that yet another related type I keratin, K17, forms stable heterotetramers with a variety of type II keratins, further accentuating the unique nature of K16. Analysis of chimeric K14-K16 proteins in a heterotetramer formation assay indicated that the instability determinant resides in a 220-amino acid segment within the α-helical rod domain of K16. Site-directed mutagenesis revealed that Pro 188, an amino acid residue located in subdomain 1B of the rod, accounts quantitatively for the instability of K16-containing heterotetramers under denaturing conditions. In vivo polymerization studies suggest that the absence of Pro 188 correlates with a reduction in assembly efficiency. In addition to their implications for the stable conformation of the keratin heterotetramers, these findings suggest that the tetramer-forming properties of K16 may influence its partitioning between the soluble and polymer pools, and hence contribute to its regulation in epithelial cells under resting and wound repair conditions.

The family of intermediate filament (IF) genes comprises >50 members and encodes proteins that assemble into filaments defined by their flexibility, an apparent lack of polarity, and a diameter of ∼10–12 nm. IFs are ubiquitous cellular components in higher multicellular organisms and have been subdivided into five major types based on nucleotide sequence homology and genomic structure (1). Type I and type II IF genes encode the acidic and basic-neutral keratins, respectively, which co-polymerize into extended IFs in the cytoplasm of epithelial cells. The expression of the >12 basic-neutral and >12 acidic keratins is co-regulated in a pairwise fashion in the various epithelia of the body, depending on the epithelial tissue type and program of terminal differentiation executed (2). The diversity and differentiation-specific expression of keratin genes is prevalent among higher vertebrates, although the underlying molecular basis remains obscure.

Keratins and other cytoplasmic IF proteins show a conserved tripartite domain structure consisting of a ∼310-residue-long, α-helical central domain flanked by non-helical head and tail domains of various length and primary sequence. The rod domain features extended heptad repeats (abcdefg) in which the first (a) and fourth (d) residues are hydrophobic. Short linker sequences interrupt these repeats at three conserved locations, defining subdomains 1A, 1B, 2A, and 2B (1, 3). Such heptad repeats foster coiled-coil interactions between monomeric α-helices, producing a thermodynamically stable dimer. Keratins are unique among IF proteins in that the coiled-coil is a heterodimer involving a type I and a type II protein (4–6). As for all other IF proteins, however, the two keratin chains in the dimer are aligned in parallel and in register (see, e.g., Refs. 5 and 7–10). Cytoplasmic IF dimers readily associate with an antiparallel orientation to form a tetramer, whose conformation is still debated (see, e.g., Refs. 8–11). The tetramer is the only assembly intermediate obtainable in high yield in vitro and may play a major role in the rapid elongation phase that ensues nucleation of 10-nm filament assembly (12, 13). In addition, the available evidence indicates that the tetramer is the predominant species in the cytoplasmic pool of soluble assembly subunits in various mammalian cell types in culture (see Refs. 14 and 15, and references therein; for a different situation in Xenopus oocytes, see Ref. 16). As such, it represents a logical candidate for being an exchangeable subunit in the context of dynamic IFs in vitro (14). While these issues require further attention, they nevertheless identify the tetramer as a determinant of the assembly, regulation, and dynamics of cytoplasmic IFs, at least in mammalian cells.

The molecular architecture of IFs remains to be defined. 10-nm filaments assembled from various IF protein types show consistent features when examined by electron microscopy. These filaments show an axial beading repeat of 21–22 nm when analyzed by glycerol spraying and rotary shadowing, and the occasional unraveling of the filament backbone reveals a subfibrillar structure with three to four protofibrils per filament cross-section (17). The current models for the structure of IFs assume that there are 16 dimers per filament cross-section (which may represent the “ideal” case), and propose a packing of dimer subunits that satisfies the many constraints imposed by the biochemical and ultrastructural data accumulated to date (1, 9–11). However, scanning transmission electron microscopy (STEM) analyses showed that the number of mono-

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Keratin expression is altered after tissue injury and under conditions that promote altered differentiation in stratified epithelia. For example, induction of the type II K6 and the type I K16 and K17 genes occurs in the suprabasal (post-mitotic) layers of the wounded stratified epithelium (20–23). We showed that the rapid induction and the subsequent accumulation of these keratins during the first 24 h after acute injury to human skin correlates with striking changes in the organization of keratin IFs and cytoarchitecture of keratinocytes at the wound edge (23). By comparing the assembly properties of K6-K16 and K5-K14, we demonstrated that K16, in particular, features unusual properties in that it formed unstable heterotetramers that polymerized into relatively short 10-nm filaments in vitro. Here we report on the biochemical basis underlying K16’s peculiar tetramer assembly properties, provide evidence for its relevance to 10-nm filament assembly and regulation, and present a model that integrates the regulation of K16 protein levels and its function in stratified epithelia under normal and wound repair conditions.

MATERIALS AND METHODS

Cloning of Human Keratin 17 cDNA—The full-length coding sequence for human K17 was obtained by reverse transcription-PCR using oligonucleotide primers designed from the published sequence (24) and total RNA isolated from cultured human epidermal keratinocytes as described (25). Several clones were subjected to dyeoxygen sequencing and a wild-type sequence was reconstructed, with the exception that the creation of a NcoI restriction site (CATGTC) codon resulted in a Thr (ACC) → Ala (GCC) substitution at the first amino acid. The final cDNA clone was subcloned into plasmid pET-3d (26) for bacterial expression.

Preparation of Recombinant and Native Human Keratins, and Immunological Analyses—We used an Escherichia coli expression system based on the T7 RNA polymerase gene (26) to generate recombinant human keratins as described (4). Plasmids pET-K5 and pET-K14 (4), pET-K6b and pET-K16 (26), PET-K8 (27), pET-K19a, b and pET-K17 were individually transformed into E. coli strain BL21 (DE3). cDNAs were inserted in the NcoI restriction site (CATGTC) codon of K16 and pSpI1 at positions 928 and 1220, respectively. Constructs with a Thr (ACC) → Ala (GCC) substitution at the first amino acid were sequenced prior to their transfer back into the parental pET constructs. Construction of the pET-K14 Arg925→Cys mutant has been described (30).

In Vitro Keratin Filament Assembly—Mono Q fractions containing heterotypic keratin complexes were used for in vitro polymerization assays as described (23). Polymerization was achieved by dialysis of 0.25 ml samples at 200 μM K6, 10 mM K16, K14, 8 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 8.1 (Q buffer). Each keratin was purified to near-homogeneity by chromatography in Q buffer on a Pharmacia FPLC Hi Trap column operated at 1.0 ml/min and 1.0 M KCl. Each keratin was transferred to nitrocellulose, and the blots incubated with primary antisera raised against and monospecific for K6 (29), K14 (22), K6b and K17, and K16 (30), and mouse monoclonal antibodies directed against K18 (15), K17, and K19 (Sigma).

Heterotypic Complex Formation and Analysis—Purified recombinant type I and type II keratins were mixed in a 5:45:55 molar ratio, incubated for 1 h at room temperature and fractionated by anion-exchange chromatography on a Mono Q column in Q buffer as described above. For wild-type keratins, we tested concentrations ranging from 200 to 1200 μg/ml and obtained identical results. All combinations involving mutant keratins were tested at 350 μg/ml. Collected fractions (0.5 ml) were analyzed by SDS-PAGE, and those containing type I-type II heterotypic complexes were pooled and dialyzed against 25 mM sodium phosphate and 10 mM β-ME, containing 6.5 mM or 8 mM urea at pH 7.4, to remove Tris ions (which interfere with cross-linking). Protein concentration was adjusted to 200 μg/ml (0.3 mM keratin). Chemical cross-linking was performed by adding bis(sulfosuccinimidyl) suberate (Pierce) to a final concentration of 5 mM for 1 h at room temperature. Under these conditions, individual keratins do not cross-link into larger-size products (23). Cross-linked products (6 μg of proteins) were resolved on 4–16% gradient SDS-PAGE and stained with Coomassie Blue. The apparent Mₐ of cross-linked species was calculated from a standard curve established with protein standards of known Mₐ values (Sigma). Densitometry was performed using the NIH Image 1.59 software to estimate the relative yield of each major product of the reaction. For the competition assays, ~250–300 μg of K14, K16, and either K5 or K6b were mixed in a molar ratio of ~1:1:1 to generate a final mixture having a 1.2 mg/ml protein concentration. Depending on the experiment, two keratins were mixed first, and the third one was added 45 min later. After a further 45-min incubation at room temperature, the mixture was analyzed by chromatography and SDS-PAGE as described above.

Chimerogenesis and Site-directed Mutagenesis—The recognition sequence for the Kpnl and SpI1 restriction enzymes are unique and conserved in location between the human K14 and K16 cDNAs. The Kpnl site occurs in a segment encoding linker LI, located between the 1A and 1B subdomains of the rod, while the SpI1 site occurs in the middle of subdomain 2B (25, 31). We took advantage of these conserved sites to generate six chimeric cDNAs, designated A–F, using standard procedures. All K14-K16 junctions were sequenced in the chimeric cDNAs generated. No addition, deletion, or substitution of amino acids was introduced in the coding sequence of these keratins. Site-directed mutagenesis was performed using a PCR method as described (32) to produce the point mutants K16 Pro188→Val, K16 Pro188→Asn, K14 Val196→Pro, and K17 Ile155→Pro. All PCR-generated fragments were sequenced prior to their transfer back into the parental pET constructs. Construction of the pET-K14 Arg925→Cys mutant has been described (30).

In vivo Keratin Filament Assembly—Mono Q fractions containing heterotypic keratin complexes were used for in vitro polymerization assays as described (23). Polymerization was achieved by dialysis of 0.25 ml samples at 200 μg/ml against 2.5 mM Tris-HCl, 10 mM β-ME, pH 7.5, for 16 h at 4 °C. These conditions are different than those reported previously to be optimal for K5-K14 or K6b-K14 assembly (5 and 6). The purified keratins migrate with the expected mobility in 10% SDS-PAGE, using reagents purchased from Bio-Rad. SDS-PAGE. The purified keratins were stained with Coomassie Blue, and reacted with previously characterized monospecific antibodies. Native keratin filaments were prepared from cultured human keratinocytes using the high salt extraction method (28). The final keratin filament preparation was analyzed by SDS-PAGE, and those containing type I-type II heterotypic complexes were pooled and dialyzed against 25 mM sodium phosphate and 10 mM β-ME, containing 6.5 or 8 mM urea at pH 7.4, to remove Tris ions (which interfere with cross-linking). Protein concentration was adjusted to 200 μg/ml (0.3 mM keratin). Chemical cross-linking was performed by adding bis(sulfosuccinimidyl) suberate (Pierce) to a final concentration of 5 mM for 1 h at room temperature. Under these conditions, individual keratins do not cross-link into larger-size products (23). Cross-linked products (6 μg of proteins) were resolved on 4–16% gradient SDS-PAGE and stained with Coomassie Blue. The apparent Mₐ of cross-linked species was calculated from a standard curve established with protein standards of known Mₐ values (Sigma). Densitometry was performed using the NIH Image 1.59 software to estimate the relative yield of each major product of the reaction. For the competition assays, ~250–300 μg of K14, K16, and either K5 or K6b were mixed in a molar ratio of ~1:1:1 to generate a final mixture having a 1.2 mg/ml protein concentration. Depending on the experiment, two keratins were mixed first, and the third one was added 45 min later. After a further 45-min incubation at room temperature, the mixture was analyzed by chromatography and SDS-PAGE as described above.

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In vitro Keratin Filament Assembly—Mono Q fractions containing heterotypic keratin complexes were used for in vitro polymerization assays as described (23). Polymerization was achieved by dialysis of 0.25 ml samples at 200 μg/ml against 2.5 mM Tris-HCl, 10 mM β-ME, pH 7.5, for 16 h at 4 °C. These conditions are different than those reported previously to be optimal for K5-K14 or K6b-K14 assembly (5 and 6). The purified keratins migrate with the expected mobility and react with previously characterized monospecific antibodies. Native human keratin filaments were isolated from cultured human fore skin keratinocytes using the high salt extraction method (28). The final pellet was solubilized in Q buffer and subjected to Mono Q chromatography (0.5 ml/min flow rate). Protein concentration was determined using reagents purchased from Bio-Rad.

Immunological Analyses—For immunoblot analyses, known quantities of recombinant or native human keratins were electrophoresed, transferred to nitrocellulose, and the blots incubated with primary antisera diluted in blocking buffer (Tris-buffered saline with 0.5% Tween 20 and 5% dry milk). Bound primary antibodies were revealed by alkaline phosphatase-conjugated secondary antibodies as recommended by the manufacturer (Bio-Rad). The primary antisera included rabbit polyclonal antisera raised against and monospecific for K5 (29), K14 (22), K6b and K17, and K16 (30), and mouse monoclonal antibodies directed against K8 and K18 (15), K17, and K19 (Sigma).
processed for indirect double immunofluorescence. We used the mouse monoclonal antibody L2A1 to detect the endogenous K8-K18 network (15), and either the anti-K14 or anti-K16 antisera (see above) to detect the transfected human keratin. As controls in all labeling protocols, mock-transfected cells were processed in parallel with the relevant antisera.

**RESULTS**

**Keratin 16 Stands Out in Its Inability to Form Stable Heterotetramers**—Acidic type I and basic-neutral type II keratins (35) display different charge properties when in the form of monomers or small oligomers (see, e.g., Ref. 4). In a Mono Q anion-exchange chromatography assay performed at pH 8.1 in the presence of 6.5 M urea, type II monomers elute at ~80 mM guanidine-HCl, designated as “peak 1,” while type I monomers elute at ~130–140 mM guanidine-HCl, designated as “peak 2.” When mixed prior to chromatography, type I and type II keratins form heterotypic complexes that elute at a higher salt concentration, ~175 mM guanidine-HCl, in a peak (“peak 3”) that can be resolved from the two monomer types (4, 23). Such a complete shift in elution of the two keratins and their recovery in a 1:1 molar ratio indicate highly efficient heterotypic complex formation. Chemical cross-linking and gel filtration analyses established that the heterotypic complexes that form under these conditions are heterotetramers (4, 23). We used this assay to show that, compared with K14, K16 forms relatively unstable heterotetramers with either K5 or K6b as type II keratin pairing partner. Accordingly, type I-type II keratin combinations involving K16 show a different elution profile on Mono Q, characterized by substantial amounts of type I and type II keratins in peak 2 fractions. When subjected to chemical cross-linking, isolated fractions containing K6b-K16 or K5-K16 (peak 2/3) give rise to mostly heterodimers and few heterotetramers (23). These results showed that: (a) there is a good concordance between the yields of dimers and tetramers by chemical cross-linking and the elution profile of type I-type II keratin complexes in the Mono Q chromatography assay; (b) the charge properties of keratin heterodimers are the same as type I monomers, while heterotetramers are more acidic; and (c) K16 forms poorly stable heterotetramers when compared with the highly related K14.

We examined the behavior of additional human keratins in these assays to better assess the significance of these findings. K17, a type I keratin, is co-regulated with K16 in many epithelia and is remarkably similar to both K14 and K16 at the predicted amino acid sequence level (Ref. 24; for our K16 sequence, see Ref. 25). We found that K17 forms stable heterotetramers with very high efficiency when paired with K6b, since it is part of a 1:1 complex that elutes in peak 3 fractions on Mono Q (Fig. 1), which upon chemical cross-linking yields a single major product of size ~240 kDa (Fig. 2). Similar results were obtained with K5 as the type II pairing partner (data not shown). Thus, K17 behaves in a manner indistinguishable from K14 in these assays. These data contrast with those involving mixtures of K6b-K16 or K5-K16, which elute mostly in peak 2 fractions (see K6b-K16 in Fig. 1) and produce a major product of ~130 kDa, i.e. a heterodimer, upon cross-linking (Fig. 2). We repeated these analyses with K19, a short-tailed type I keratin expressed in a variety of simple and stratified epithelia, but which is less related to K14 and K16 in its primary sequence (36, 37). We found that K19 shows an intermediate behavior, in that the heterotetramers it forms with either K5 or K6b are more stable than those containing K16 but less stable than those involving K14 or K17. Finally, we examined the behavior of these type I keratins when paired with human K8, the major type II keratin expressed in simple epithelia (35). We found that the K8-K14 and K8-K17 combination form relatively stable tetramers, while the K8-K16 combination clearly does not (Fig. 2). Altogether, these studies provide clear evidence that the recombinant type I K16 stands out in its inability to form urea-stable heterotetramers, independent of the type II keratin partner involved.

To determine whether the behavior of recombinant K16 reflects that of the corresponding native protein, we extracted keratin filaments from cultured human primary keratinocytes, denatured them in 6.5 M urea Q buffer, and performed Mono Q chromatography. Fractions were collected and analyzed by SDS-PAGE and immunoblotting using antisera to K16, K14, and K17. The bulk of the K14 and K17 immunoreactivity elutes in peak 3 fractions, corresponding to the elution position for recombinant keratin tetramers, while the pool of K16 protein is nearly equally distributed between the peak 2 (monomers/dimers) and peak 3 fractions (Fig. 3). We did not analyze this material using chemical cross-linking due to complications arising from the formation of mixed tetramers in vivo. Of note, the major type II keratins available for subunit formation in cultured primary keratinocytes are K5 and K6, present in approximately equimolar amounts (data not shown). These data establish that the behavior of recombinant human K14, K16 and K17 reflect that of the corresponding native proteins in these assays.

**Solving the Biochemical Basis of K16's Tetramer Instability by Mutagenesis**—Keratins 14 and 16 are very homologous at the primary sequence level, with identity scores of 90% for the non-helical amino-terminal head domain, 88% for the central α-helical rod domain, and ~45% for the non-helical carboxyl-terminal tail domain. We exploited the occurrence of conserved restriction sites within the nucleotide sequences coding for linker L1 (between subdomains 1A and 1B) and the middle of subdomain 2B to construct a series of K14-K16 chimeric cDNAs

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4 J. Fradette and P. A. Coulombe, unpublished data.
FIG. 2. Chemical cross-linking of type I-type II keratin heterotypic complexes. Type I-type II heterotypic complexes were isolated by Mono Q anion-exchange chromatography and dialyzed against 25 mM sodium phosphate buffer containing 6.5 M urea at pH 7.4. Protein concentration was adjusted to 200 μg/ml; biotin(sulfosuccinimidyl) suberate was added to a final concentration of 5 mM, and the samples were incubated for 1 h at room temperature. Cross-linked products were resolved on a 4–16% gradient SDS-PAGE and stained with Coomassie Blue. The migration of molecular size standards and that of the type I-type II tetramer (T, ~240 kDa), type I-type II dimer (D, ~135 kDa), and type I and type II monomers (M) are indicated for both gels. The K6b-K14 and K6b-K17 combinations form stable heterotetramers, while K6b-K16 forms mostly heterodimers. Even when paired with an unnatural partner, the type II keratins K8, K14, and K17 are clearly superior to K16 in their ability to form urea-stable heterotetramers.

FIG. 3. Stability of native keratin heterotypic complexes containing K14, K16, and K17. An IF extract was prepared from cultured primary human keratinocytes, solubilized, and subjected to anion-exchange chromatography in Q buffer containing 6.5 M urea (see Fig. 1). Aliquots from fractions encompassing peak 1 (fractions 17–19), peak 2 (fractions 22–25), and peak 3 (fractions 27–30) were resolved by SDS-PAGE in triplicate and transferred onto nitrocellulose. In each case, mixtures containing known amounts of purified recombinant keratins are retrieved in peak 1 fractions. In contrast, only half of the native K14 and K17 pools are retrieved in peak 3 fractions. These results are nearly identical to those obtained using the recombinant versions of these type I keratins (see Fig. 1).

(Fig. 4). Purified chimeric proteins were tested for their ability to form tetramers with K6b using the chromatography and cross-linking assays. The results obtained are summarized in Fig. 4, and an example of the cross-linking data for chimeras E (K14-K16-K14) and F (K16-K14-K16) is shown in Fig. 5. These studies clearly show that the ~220-amino acid segment located within the central rod domain, between linker L1 and the middle of subdomain 2B, accounts nearly quantitatively for the instability of K16-containing tetramers.

An alignment of the predicted amino acid sequence of this 220-amino acid segment for K14, K16, K17, and K19 reveals that only a limited number of amino acid residues are unique to K16, and among these, many represent conservative substitutions. A notable exception is a proline residue located at position 188 of K16 (see Fig. 6). This proline substitution stands out because it occurs at a "d" position of the heptad repeat in the α-helical subdomain 1B, where nearly all other type I keratins feature a hydrophobic residue. Using site-directed mutagenesis, we engineered amino acid substitutions at Pro188 in K16 and the corresponding positions in K14 (Val186) and K17 (Ile155), and tested whether they had any effect on the formation of tetramers with K6b. The Mono Q elution profiles and the chemical cross-linking data obtained under 6.5 M urea buffer conditions were in complete agreement, and only the latter is reported in Fig. 5. Substituting Pro188 for valine in K16 led to formation of stable heterotetramers with K6b. Conversely, introducing a proline residue at the corresponding positions in K14 (Val186 → Pro) and K17 (Ile155 → Pro) significantly destabilized heterotetramers involving K6b (Fig. 5). These data directly implicate Pro188 in K16 as the major determinant of its inability to form urea-stable tetramers with K6b. This behavior...
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Fig. 5. Chemical cross-linking of heterotypic complexes containing mutant type I keratins. Type I-type II heterotypic complexes were isolated by Mono Q anion-exchange chromatography and dialyzed against 25 mM sodium phosphate buffer containing 6.5 M urea at pH 7.4. Protein concentration was then adjusted to 200 μg/ml. Bis[sulfosuccinimidyl] suberate was added to a final concentration of 5 mM, and the samples incubated for 1 h at room temperature. Cross-linked products were resolved on a 4–16% gradient SDS-PAGE and stained with Coomassie Blue. The migration of molecular size standards is indicated at left, while that of the type I-type II tetramer (T, ~240 kDa), type I-type II dimer (D, ~153 kDa), and type I and type II monomers (M) is indicated at right. Left, the K6b-chimera C forms stable heterotetramers, while the K6b-chimera E does not (see Fig. 4). Right, the K5-K14 Arg125→Cys, K6b-K16 Pro188→Asn, and K6b-K16 Pro188→Val combinations form stable heterotetramers. In contrast, the K6b-K17 Ile155→Pro and especially the K6b-K14 Val186→Pro combinations give rise to mostly dimers under the same buffer conditions. Altogether, these data demonstrate that Pro188 is responsible for the instability of K16-containing heterotetramers.

Fig. 6. Alignment of predicted amino acid sequences for several type I keratins. The sequences shown correspond to the predicted amino acid sequence for the L1 linker and coil 1B domain in the human K14 (31), human K16 (25), human K17 (24), and human K19 (55). An asterisk in the bottom row indicates identity for that particular codon among the keratin sequences surveyed. The position of Pro188 residue in K16 sequence and of the amino acid encoded at the corresponding position in the other keratin sequences shown is depicted by an arrow. It appears to be due to the presence of a proline residue rather than the loss of a bulky hydrophobic residue, since replacing Pro188 in K16 with Asn yields stable heterotetramers with K6b in 6.5 M urea buffer (Fig. 5). Repeating these cross-linking assays under more stringent denaturing conditions (8 M urea) showed that K6b-K16 Pro188→Val, K6b-K14, and K6b-K17 tetramers are destabilized to a comparable extent (data not shown).

Quantitation of the yields of cross-linked products reveals the existence of two distinct groups of type I keratins with respect to stable heterotetramer formation (Table I). The first group includes K14, K17, chimera F, and K16 Pro188→Val, which all form urea-stable tetramers with high efficiency when paired with K6b. The second group includes K16, chimera E, and K14 Val186→Pro, which form heterodimers in good yield but unstable tetramers. The behavior of the K16 Pro188→Asn and K17 Ile155→Pro mutants is somewhat intermediate under these conditions. Taken together, our results demonstrate that it is specifically the presence of a proline residue at position 188 in K16 that destabilizes tetramers containing this type I keratin. Our mutagenesis results further suggest that dimer formation is not affected as a result of the presence of Pro188 in K16 and at corresponding positions in K14 and K17 (Table I).

To further highlight the unique tetramer-forming properties of K16, we tested the K14 Arg125→Cys point mutant (33) in our assay system. K14 Arg125→Cys is a naturally occurring mutation that affects several human type I keratins (including K14, K10, K9, and K17) and accounts for a significant fraction of the reported incidences of keratin mutation-based epidermal blistering diseases (38, 39). As reported and consistent with its dominant negative behavior, K14 Arg125→Cys is indistinguishable from wild-type K14 in its ability to form stable tetramers with its natural type II pairing partner K5 (33). We extended these findings here, demonstrating that this is also the case with K6b (Fig. 5 and Table I). K14 Arg125→Cys thus provides an example of a dominantly acting mutation that severely impacts on keratin IF assembly at a step beyond tetramer formation. In contrast, the consequences associated with the presence of Pro188 in K16 are likely to be manifested before or at the level of tetramer formation.

Comparing the Affinities of K14 and K16 for Type II Keratins by Competition Assays—We further exploited the Mono Q chromatography assay to test type I-type II pairing in a context where K16 would have to compete with K14 for binding to a type II keratin partner. Such a situation mimics the in vivo environment, since K16 is expressed in post-mitotic keratinocytes that already feature substantial amounts of K14 (see Ref. 30). K5-K14 and K5-K16 heterotypic complexes were first generated and then mixed with 1× equivalent (on a molar basis) of the other type I keratin. After further incubation, the mixture was subjected to the Mono Q chromatography (see “Materials and Methods”). Collected fractions were analyzed by SDS-PAGE and densitometry. In both cases, K5 eluted in peak 3 fractions (Fig. 7), indicating efficient heterotetramer formation. Densitometry analysis revealed a ratio of ~3:1 in favor of K14 in peak 3 fractions, and conversely an inverted ratio in favor of K16 in the monomer pool that elutes in peak 2 fractions (in the absence of K5), in both mixtures (Fig. 7). Of note, nearly identical results were obtained when using K6b as the type II keratin (data not shown), and when K14 and K16 were mixed first followed by the addition of the type II keratin. From these competition assays we conclude that: (a) heterotetramer formation is reversible under these buffer conditions; (b) heterodimer formation is itself reversible, given the 3:1 ratio between K14 and K16.
and K16 in the heterotetramer peak; (c) an equilibrium is reached within an incubation period of 45 min, since the K14-K16 ratios observed in the peak 2 and 3 fractions are independent of the order of component addition; and (d) K14 shows a >3-fold greater affinity for K5 or K6b compared with K16.

The Presence of Pro<sup>188</sup> Correlates with Reduced Efficiency of Filament Formation in Vitro—We reported previously that, when prompted to undergo full assembly under optimal buffer conditions, K6b-K16 tetramers polymerize with a lower efficiency than K6b-K14 ones (85–90% versus 100%; Ref. 23). As this difference may be related to the unstable character of K16-containing heterotetramers observed under stringent urea buffer conditions, we investigated the behavior of the K14 Val<sup>186</sup> → Pro and K16 Pro<sup>188</sup> → Val mutants in a filament polymerization assay. Under conditions modified for this purpose (see “Materials and Methods”) and at a protein concentration of 200 µg/ml, we found that relative to K6b-K14 (normalized to 100%), 20% of the K6b-K16, 71% of the K6b-K14 Val<sup>186</sup> → Pro, and 91% of the K6b-K16 Pro<sup>188</sup> → Val samples could be pelleted (Fig. 8). These findings suggest that Pro<sup>188</sup> in K16 plays a role in determining the efficiency with which it assembles with its natural partner K6b, and that this property can be conferred upon K14 when introducing a Pro residue at the corresponding position.

Pro<sup>188</sup> Is Not Involved in K16’s Ability to Reorganize Keratin Filaments in Transfected PtK2 Cells—We reported previously that when expressed in transiently transfected PtK2 epithelial cells, K16 causes a reorganization of keratin IFs to a juxtanuclear location in ~50% of cells, while K14 causes a distinct type of alteration in only 10–15% of transfected cells (23). A similar reorganization of keratin IFs occurs when the intact human K16 gene is overexpressed in skin keratinocytes of transgenic mice (30). To assess whether Pro<sup>188</sup> plays a role in this phenomenon, we transfected the K16 Pro<sup>188</sup> → Val mutant in cultured PtK2 cells and examined IFs by immunofluorescence microscopy. We observed that ~55% of PtK2 cells expressing the K16 Pro<sup>188</sup> → Val mutant showed a reorganization of keratin IFs (Fig. 9). We did not assess the behavior of the K14 Val<sup>186</sup> → Pro or K17 Ile<sup>155</sup> → Pro in this assay system. Consistent with our transfection results, however, Letai et al. (40) reported that transient expression of K14 Asn<sup>183</sup> → Pro or K14 Arg<sup>232</sup> → Pro in the same PtK2 cell line or in SCC13 human skin keratinocytes did not alter the organization of endogenous keratin filaments. These findings suggest that in PtK2 cells, Pro<sup>188</sup> does not directly contribute to the ability of wild-type K16 to affect the organization of the keratin IF network.

**DISCUSSION**

A Proposed Mechanism for the Unstable Character of K16-containing Tetramers, and Its Significance for the Structure of the Tetramer—All IF proteins dimerize spontaneously via long range coiled-coil interactions with their central rod domain parallel and in exact axial register (see, e.g., Refs. 1 and 11). The primary feature of this interaction is the knob-in-hole packing of hydrophobic side chains projecting inward from amino acids alternately spaced 3 and 4 residues apart in each α-helix (41). IFs are unique in that...
they, along with a few other structural proteins, form a coiled-coil interface that is several hundred residues long. This notion, along with the unconstrained ability of these proteins to assemble into filaments, has made the task of obtaining structural data at a high resolution particularly difficult. It is very likely, however, that the general principles revealed by the characterization of short coiled-coil interfaces (41) apply to IF proteins.

The precise architecture of the IF tetramer is still being debated. Recent work involving the analysis of tetramer cross-links obtained by subjecting large-size desmin, vimentin, and keratin oligomers to partial cross-linking yielded evidence for multiple alignments of antiparallel dimers (8–10). These alignments are illustrated in Fig. 10, and feature the two dimers: (i) in near-perfect register (NC-CN type); (ii) with overlap of subdomain 1B (N-N type), (iii) with overlap of subdomain 2B (C-C type); and (iv) with a short (1–2 nm) overlap involving the extremities of the rod domain (N-C type). These tetramer conformations, which involve lateral interactions between distinct segments of the coiled-coil dimer, have all been integrated into a model of 10 nm filament structure devised by Aebi and colleagues (11). This particular model also incorporates the key structural features of IFs as revealed by the application of a variety of electron microscopy techniques (see Refs. 1, 9, and 10). A critical issue pending, however, is whether each of these tetramer configurations represents a true intermediate productive for 10-nm filament assembly. That is, when applied in the context of larger oligomers, partial cross-linking allows strictly for a nearest-neighbor analysis, and is not designed to examine the size and structure of the assembly competent subunit. In fact, Geisler et al. (8) found evidence for a single tetramer conformation, the N-N type, when applying cross-linking methods to tetramer-rich preparations of desmin. The N-N type tetramer conformation is supported by a number of early biochemical studies on various types of IF proteins (see Ref. 8, and references therein). On the other hand, the concept of polymorphic tetramer structure is appealing since it would provide a basis for the mass-per-unit-length heterogeneity that characterizes polymerized IFs, whether native or reconstituted in vitro. In addition to its implications for the assembly and structure of the IF polymer, the elucidation of the conformation of the tetramer would lead to a better understanding of several issues pertaining to the IF soluble pool.

Assuming that the cross-linking agent and conditions used in our study do not bias against any particular tetramer conformation (see Ref. 9), and that the denaturing conditions applied select for thermodynamically stable, assembly-productive tetramer conformations, our efforts show that Pro188 causes the
relative instability of K16-containing tetramers, and that this property is conferred upon K14 by introducing a proline at the corresponding position. These results strongly suggest that the N-N and/or NC-CN tetramer configurations predominate for the K5-K14 and K6-K16 combinations under urea buffer conditions since both involve the sub-α-helical domain (1B) that bears Pro$^{188}$ in K16 (Fig. 10). Indeed, given its position within the rod, this proline substitution is not expected to impact upon the stability of tetramers having the C-C or N-C configurations (Fig. 10). As the introduction of a Ile$^{155}$ → Pro substitution in K17 did not completely destabilize tetramers formed with K6b (Fig. 6, Table I), we cannot rule out the possibility that they could adopt the C-C conformation, although this would again have to represent a minor species in urea buffer. Consistent with the interpretation of our findings, Hatzfeld and Burba (42) showed that the proximal head domain of K8 and K18 plays a role in tetramer formation and stability. We conclude, therefore, that the N-N and/or the NC-CN conformations (see Fig. 10) are the most thermodynamically stable and thus the most prevalent for tetramers assembled from type I and type II keratins tested in our study. This conclusion is consistent with a number of previous studies showing that subdomain 1B is a critical determinant of tetramer formation (e.g. Refs. 34, 43, 44, and references therein).

Inspection of the central rod domain sequence in type I and type II keratins reveals a complete absence of proline residues from subdomains 1A, 1B, 2A, and 2B, which feature the signature heptad repeats (45). This is expected, since the rigid proline ring structure cannot be accommodated in the context of a classical right-handed α-helix with 3.6 residues per turn. In the E. coli GreA protein, for instance, the presence of a proline residue in a “d” (outside) position within an intramolecular coiled-coil motif has a major impact on the local helical backbone: helix α1 of GreA kinks “inward” at Pro$^{27}$, locally disrupting the hydrophobic interface between two interacting helices (46). Transposition of this information to K16, where Pro$^{188}$ occurs in a “d” (inside) position of the coiled-coil interface, suggests that the helix might be locally kinked “outward” with respect to its main axis. Such a kink would be expected to impact significantly upon the surface features the coiled-coiled dimer near residue Pro$^{188}$ in the participating K16 chain. This said, our data suggest that the formation and stability of heterodimers is not significantly different for K16 and the K14 Val$^{186}$ → Pro and K17 Ile$^{155}$ → Pro mutants, possibly because of a compensatory effect of the long range coiled-coil interface (see Fig. 10). The structural consequences of Pro$^{188}$ are clearly manifested at the next stage of assembly, i.e. tetramer formation, because it involves interactions between the lateral surfaces of the participating antiparallel dimers.

Letai et al. (40) reported that single proline substitutions at position 183 and 232 in human K14 did not alter its ability to form urea-stable heterotypic complexes with K5. This is of interest, since codons 183 (Asn) and 186 (Val) correspond to the “a” and “d” positions, respectively, of the third heptad repeat in subdomain 1B of K14. A direct comparison of the results obtained for K14 Asn$^{183}$ → Pro (40) with ours involving K14 Val$^{186}$ → Pro may not be appropriate since these authors did not ascertain the size of the complexes formed in their study. Still, the apparent difference between the tetramer-forming properties of K14 Asn$^{183}$ → Pro and K14 Val$^{186}$ → Pro is perplexing. If real, such a difference implies that the presence of a proline residue is more consequential when introduced at position “d” compared with position “a” of the third heptad repeat in subdomain 1B. In support of this, Letai et al. (40) showed that the net effect of introducing prolines in the rod domain of K14 is context-dependent (40). In that regard, position “a” of this heptad is invariably occupied by an asparagine in type I keratin sequences, while position “d” is occupied by a hydrophobic amino acid (Val, Ile) in all cases but K16 (Pro), K9 (Thr), and K20 (Cys) (45). Our finding that K16 Pro$^{188}$ → Val forms slightly more stable tetramers compared with K16 Pro$^{188}$ → Asn (Table I) is consistent with the need for a hydrophobic side chain at the “d” position to confer maximal stability to tetramer(s).

Implication of Our Findings for the Regulation of K16 in Vivo—The precise mechanisms that regulate assembly and disassembly of intermediate filaments and those that control exchange between the polymer and the soluble pools remain to be defined, although phosphorylation and interactions with non-IF proteins are clearly involved (14, 47–50). Still, the available evidence suggests that the assembly subunit incorporating into the growing IF polymer is larger than a dimer (see Introduction), such that it can be assumed that tetramer formation represents an obligatory step preceding the incorporation of an assembly subunit into the polymer pool in vivo. Four lines of evidence support this assumption.

First, it has been nearly impossible to isolate dimers in any substantial yield while working with cytoplasmic IF proteins in vitro (cf. Introduction). Second, studies conducted in mammalian cells in culture have shown that, at least for keratin and vimentin IFs, the tetramer is the prevalent assembly subunit in the small soluble pool (see Refs. 15 and 16, and references therein). Third, keratin mutants that disrupt IF function to the point of causing severe blistering disease are nevertheless capable of forming stable heterotetramers (e.g. this study), a property that may underlie their ability to act in a dominant-negative fashion in vivo. Fourth, in vitro assembly studies reported here suggest a correlation between tetramer stability and the efficiency of filament polymerization (Fig. 8).

Therefore, one hypothesis is that the tetramer-forming properties of K16 may cause it to incorporate less readily into the polymer pool as compared with other type I keratins capable of forming stable tetramers. The instability of K16 tetramers could be exploited to set a threshold of protein expression which must be overcome in order to allow for efficient filament incorporation and reorganization. Such a situation may occur in the physiological context of wound repair where K16 appears to be more abundantly expressed (23). Consistent with this concept, we previously showed the ability of K16 to promote cytoarchitectural changes in keratinocytes of transgenic mouse skin to be dose dependent (30). This does not exclude the possibility that post-translational modifications (K16 is a phosphoprotein; see Ref. 51) and interacting proteins may also be directly involved in regulating the partitioning between the soluble and polymer pools. Alternatively, it may be that Pro$^{188}$ does not influence the kinetics of incorporation or exchange of K16-containing subunits, but alters the form in which these interact with the polymer (e.g. as a dimer instead of a tetramer or a larger species). More direct evidence for the role of Pro$^{188}$ in K16 assembly and regulation will require an examination of the rate of its incorporation and turnover in vitro as well as in vivo. We believe that an understanding of these issues is key to a better understanding of the regulation of K16 in stratified epithelia under normal as well as injury conditions (23).

Our results show that despite its significant effects on tetramer subunit stability and efficiency of filament formation in vitro, Pro$^{188}$ is not single-handedly responsible for the ability of wild-type K16 to promote a reorganization of endogenous keratin IFs in transfected PtK2 epithelial cells. The results of ongoing transgenic mouse studies in the laboratory clearly point to the carboxyl-terminal tail portion of K16 as playing a
substantial role in that phenomenon. The data accumulated so far thus indicate that the interesting properties of K16 are the result of at least two biochemical determinants, that is, Pro188 and the carboxyl-terminal 100 amino acids. It should prove interesting to investigate further how these and other yet to be identified determinants contribute to define the regulation and function of K16 in vivo.

Acknowledgments—We are very grateful to Chia Che Chang for efforts toward the cloning of the human K17 cDNA, to Julie Fradette for sharing unpublished results on keratin 19, to Dr. M. Bishr Omary for the gift of monoclonal antibody L11A, and to Dr. Cynthia Wolberger for advice.

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