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Identifying the Role of Specific Motifs in the Lens Fiber Cell– Specific Intermediate Filament Phakosin

Joshua T. Pittenger, John F. Hess, and Paul G. FitzGerald

Department of Cell Biology and Human Anatomy, School of Medicine, University of California, Davis, California

Abstract

Purpose—Phakosin and filensin are lens fiber cell–specific intermediate filament (IF) proteins. Unlike every other cytoplasmic IF protein, they assemble into a beaded filament (BF) rather than an IF. Why the lens fiber cell requires two unique IF proteins and why and how they assemble into a structure other than an IF are unknown. In this report we test specific motifs/domains in phakosin to identify changes that have adapted phakosin to lens-specific structure and function.

Methods—Phakosin shows the highest level of sequence identity to K18, whose natural assembly partner is K8. We therefore exchanged conserved keratin motifs between phakosin and K18 to determine whether phakosin's divergent motifs could redirect the assembly of chimeric K18 and K8. Modified proteins were bacterially expressed and purified. Assembly competence was assessed by electron microscopy.

Results—Substitution of the phakosin helix initiation motif (HIM) into K18 does not alter assembly with K8, establishing that the radical divergence in phakosin HIM is not by itself the mechanism by which IF assembly is redirected to BF assembly. Unexpectedly, K18 bearing phakosin HIM resulted in normal IF assembly, despite the presence of an otherwise disease-causing R-C substitution, and two helix-disrupting glycines. This disproves the widely held belief that mutation of the R is catastrophic to IF assembly. Additional data are presented that suggest normal IF assembly is dependent on sequence-specific interactions between the IF head domain and the HIM.

Conclusions—In the lens fiber cell, two members of the IF family have evolved to produce BFs instead of IFs, a change that presumably adapts the IF to a fiber cell–specific function. The authors establish here that the most striking divergence seen in phakosin is not, as hypothesized, the cause of this altered assembly outcome. The authors further establish that the HIM of IFs is far more tolerant of mutations, such as those that cause some corneal dystrophies and Alexander disease, than previously hypothesized and that normal assembly involves sequence-specific interactions between the head domain and the HIM.

The family of intermediate filament (IF) proteins, with more than 60 members, is one of the largest in the human genome. Although every cell assembles an IF cytoskeleton, most cells use only one to three IF proteins to do so, with expression strictly regulated as a function of cell type and state of differentiation. IF proteins vary considerably in primary sequence but show strong conservation of predicted secondary structure, with well-delineated head, tail,

and central rod domains (for reviews, see Herrmann and Aebi¹, Parry and Steinert,² and Steinert and Parry³; see also Fig. 1). The size and primary sequence of the head and tail domains vary extensively in the IF family. However, the central rod domain, though showing significant variation in primary sequence, also shows very strong conservation of predicted secondary structure, consisting of α -helical, coiled-coil domains (coils) interrupted by short nonhelical domains (linkers). The size and number of these coil and linker regions are well conserved in the IF family.

Much experimental work has explored the role of specific IF domains in IF assembly. However, the results vary considerably, with outcome influence by several key experimental parameters: whether the mutation is in an IF protein that forms heterodimers (e.g., keratins) or homodimers (e.g., vimentin), whether filament assembly was assayed by in vitro assembly from purified proteins, and whether it was conducted by transfection into cells, where results are confounded by endogenous IF networks and variation in expression levels. Even within transfection studies, the choice of cell type can influence the outcome.^{4–6} Finally, there is a high degree of sequence difference among IF proteins, which may impose some limits on the degree to which conclusions may be generalized across the IF family. Nevertheless, it appears that the central rod domain is critical to filament assembly, whereas the tail domain is often unnecessary. The head domain, or part of it, is essential in some, though not all, studies and seems more important in in vitro studies than in transfection assays.^{1–3,7–11}

In contrast to the head and tail domains and much of the rod domain, where sequence variation can be substantial, two short motifs in IFs show a very high degree of sequence conservation across the IF family. These motifs are located at the beginning and end of the central rod domain and thus have been nicknamed the helix initiation motif (HIM) and the helix termination motif (HTM). Most human IF diseases result from point mutations in these motifs, particularly HIM.^{12–15}

Crystal structure has not been established for the IF or for any IF protein, though crystal structure has been achieved for some IF protein fragments.^{16,17} Data generated using a variety of approaches, including these partial crystal structures, site-directed spin labeling in concert with electron paramagnetic resonance,^{18–20} cross-linking, and size estimation of IFs during in vitro assembly,^{1,7,21–28} have resulted in a tentative model for IF structure. The most fundamental subunit is an in-parallel, in-register, coiled-coil dimer. These dimers are arranged in at least two different anti-parallel, staggered-registry tetramers. However, longer range relationships have been more difficult to determine. Mass-to-volume measurements suggest that a given IF is approximately 16 dimers in cross-section.⁷

Purified IF proteins are not soluble in physiological conditions, so assembly is typically conducted by protein solubilization in 8 M urea, followed by dialysis to more physiological buffer. Successful assembly is manifested by long smooth filaments. However, manipulation of conditions can yield particles thought to be tetramers, which can then be triggered to form unit length filaments (ULFs). These are full-width (approximately 10 nm across) but very short. ULFs are considered an assembly intermediate that can rapidly join end-to-end to form full-length filaments, at least under these in vitro conditions, on dialysis from urea. IFs can also be visualized as parallel “protofilaments” in unraveled IFs.^{29,30} How these ULFs and protofilaments relate to each other is unclear for the moment.

IF proteins are surprisingly tolerant of mutations, and most mutations will permit assembly of full-length filaments. However, some mutations interrupt assembly and result in a variety of different sized intermediates, or aggregates. The exact structure of these intermediates remains unclear. It does seem certain, however, that assembly of IFs is a multistage process

and that this process can be interrupted at many different points depending on the impact of a specific mutation.

IF proteins can be grouped into classes or types on the basis of primary sequence identity, gene structure, and assembly and expression characteristics. IF proteins within a class share a level of sequence identity typically greater than 60; between classes, the level of sequence identity may drop below 20%. Types I and II keratins form the IFs of epithelia. These begin assembly with an obligate heterodimer consisting of a type I and a type II keratin. Generally, any type I can coassemble with any type II. In contrast, type III IF proteins are homodimers. However, any type III can coassemble with any other type III. Thus, within classes, minor sequence variations do not compromise the capacity to coassemble.

It is common for IF protein expression to switch during development. Developing muscle initially expresses vimentin, for example, but this is replaced by desmin. Similarly, the terminal differentiation of skin coincides with keratin expression changing from a K5/K14 pair to a K1/K10 pair.^{31,32} The developmental switching that occurs is almost always a switch within a particular class of IF proteins. The lens shows developmental switching as well. Lens epithelial cells and younger fiber cells express vimentin, a type III IF protein.^{33–38} As fiber cell elongation proceeds, however, vimentin expression is dialed down, and two fiber cell-specific IF proteins, phakosin and filensin, are turned on.^{33,39–45} These two IF proteins have proven to be the most divergent members of the IF family. Filensin has a primary sequence and gene structure that does not match any existing IF class, whereas phakosin shows a sequence identity and gene structure that clearly marks it as a type I keratin, though with a relatively low level of sequence identity to other type I keratins.^{46–48}

These two fiber cell-specific IF proteins are the only cytoplasmic IF proteins that have not been shown to assemble into 10-nm IFs. Instead they localize to a structure called the beaded filament (BF).^{39,40,42,45,49,50} The BF has been shown by gene knockout approaches to confer extended structural stability to lens fiber cells. In the absence of either protein, the lens develops light scatter that becomes progressively worse with age. This light scatter appears to derive from a loss of fiber cell shape and long-range order.^{51–53} What is unclear at this juncture is what changes in the BF proteins result in a redirection of assembly from IFs to BFs and why this change is important to lens biology. What function is served by the BF that could not be filled by any of the other 60 IF proteins?

We have begun to explore how these two lens-specific IF proteins have been adapted to lens biology by trying to identify the function of specific motifs within these lens proteins, with a particular emphasis on identification of changes in the lens proteins that redirect assembly from IFs to BFs. Phakosin is most closely related to K18, whose natural assembly partner is K8.⁵⁴ We have queried the role of specific phakosin motifs by exchanging homologous K18 and phakosin motifs, then assessing the impact of that motif on the capacity of the K18/phakosin chimera to assemble with K8. At a minimum, this clarifies whether specific divergences in phakosin preclude normal IF assembly or are capable of redirecting normal IF assembly.

We have targeted changes in the HIM as the most obvious candidate for altering assembly and function for several reasons: (1) the HIM is the most highly conserved motif in IFs; (2) among the dozen or so type I keratins, the HIM is absolutely conserved, with a sequence of LNDR; (3) the HIM is intolerant of mutations, particularly an R-C mutation at the fourth residue, which accounts for a disproportionately high number of human diseases,^{12,14,55–59} including some forms of corneal dystrophy^{60–63}; and (4) phakosin diverges from the type I HIM at three of four residues, from LNDR to LGGC.^{54,64} Phakosin's HIM thus includes the R-C mutation that is disease causing in any other IF protein but also includes two α -helix–

disrupting glycines, changes that should alter the otherwise highly conserved α -helical central rod domain format. The extreme degree of divergence in phakosin's HIM would, a priori, lead naturally to the hypothesis that it is key to the lens fiber cell-specific adaptations that result in redirecting IF assembly into BF assembly.

Materials and Methods

Construction of Plasmid Vectors for the Production of Mutant and Chimeric Proteins

PT7 plasmids and arabinose-inducible BL21 cells were used for the expression of wild-type, mutant, and chimeric human phakosin and K8 and K18 proteins. DNA sequence changes were created using a kit (QuikChange; Stratagene, La Jolla, CA; <http://www.stratagene.com>) on K18 and phakosin clones in pT7 vectors and verified by DNA sequencing. Specifically, the phakosin rod N-terminal mutations were accomplished using the kit (QuikChange; Stratagene) on phakosin clones in pT7 mutating amino acid positions 112–114 from LGGC to LNDR (phakosin-LNDR). Amino acids 404–413 representing the rod C-terminal sequence were changed from SYHALLDREE to TYRRLLEDGE (phakosin-TYRRLLEDGE). The clone containing both rod N- and C-terminal mutations was constructed by swapping the *Nde-EcoRI* fragment of phakosin-LNDR with the same fragment in a phakosin-TYRRLLEDGE construct with subsequent ligation. The K18 and K8 clones with LGGC substitution were produced using the kit (Quikchange; Stratagene) on appropriate amino acid positions.

K18 head and phakosin rod and tail clones (K18-H/phakosin-RT) were created by introducing a *Nco* cut site at base pair position 240 of K18, and subsequently inserting *Nde-Nco* fragments of wild-type K8 into an *Nde-Nco* cut wild-type phakosin construct. Phakosin head and K18 rod/tail clones (phakosin-H/K18-RT) were constructed by creating an *Nco* site at base pair position 232 in K18, and subsequently swapping *Nde-Nco* fragments of wild-type phakosin and K18. This clone was then used to create the phakosin-H/K18-RT-LGGC construct by using the kit (Quikchange; Stratagene) to mutate the LNDR amino acids to LGGC. K18 head, phakosin rod, and K18 tail clones (K18-H/phakosin-R/K18-T) were constructed by adding the final 39 amino acid residues of the tail domain of K18 to the K18-H/phakosin-RT clone produced previously.

Protein Expression and Purification

Inclusion bodies were purified from bacteria by a short lysozyme/DNAse treatment followed by high- and low-salt washes.^{65,66} Once purified, inclusion bodies were dissolved in 8 M urea and chromatographed over a gel filtration column (SuperDex; GE Healthcare Life Sciences, Piscataway, NJ) using an FPLC system (Amersham Biosciences, <http://www.amersham.com/>). Fractions were run and analyzed by 10% SDS-PAGE. Peak protein fractions were pooled into a primary stock and stored at -80°C .

Dialysis and Electron Microscopy

For dialysis, protein fractions were pooled in an approximately 1:1 molar ratio, with control reactions of K8/K18 (positive control) and K8/phakosin (negative control) run in parallel. Filament assembly was conducted by stepwise dialysis, at room temperature, for 2 hours each as follows: 8 M urea, 20 mM Tris, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol (DTT); 4 M urea, 20 mM Tris, pH 8.0, 2 mM EDTA, 2 mM DTT; 10 mM Tris, pH 8.0, 2 mM DTT; 10 mM Tris, pH 7.0, 1 mM MgCl_2 , 2 mM DTT; and overnight in 10 mM Tris, pH 7.0, 1 mM MgCl_2 , 50mM NaCl, 2 mM DTT. Filament formation was assessed by electron microscopy of negatively stained 10- μL samples removed from the dialysis reaction and stained with 1% uranyl acetate on polyvinyl formal (Form-var)-coated carbon grids with the

electron microscope (CM120; Phillips, Eindhoven, The Netherlands) operated at 80 kV acceleration voltage.

Results

Schematic diagrams of wild-type and mutant constructs created for this study, and a summary of their assembly behavior, are shown in Figure 1.

Protein Purification

Examples of the bacterially expressed, purified K8, K18, and phakosin used for assembly studies are shown in Figure 2. Subsequent constructs were all purified to a comparable level of purity for assembly studies.

IF Assembly

To verify that the starting constructs, purification procedures, and assembly conditions were permissive to assembly, we assessed the ability of wild-type K8 and K18 to assemble under the conditions used. Figure 3a shows that wild-type K8 and K18 formed elongated, uniformly sized IFs, the generally accepted standard for successful assembly.⁶⁷ We then mixed phakosin and K8 to verify that phakosin, though a type I keratin, was unable to assemble with K8.⁶⁸ Figure 3b shows an absence of intact normal IFs, indicating that assembly is not capable of going to completion. We note, however, the presence of aggregates that may represent assembly intermediates incapable of completing the assembly process (examples indicated by arrows, Fig. 3b). The presence of uniformly sized particles has been interpreted as assembly intermediates that fail to complete the assembly process.⁴

Helix Initiation and Termination Motifs

Except in phakosin, the HIM of type I keratins is absolutely conserved as LNDR. Phakosin shows a dramatic change in the HIM from LNDR to LGGC. These changes include not only the disease-causing R-C mutation but two glycines in an area predicted to be α -helical.⁵⁴ To test whether the extreme changes in phakosin HIM were responsible for the inability of phakosin to assemble with K8, we created phakosin constructs that carried the wild-type K18 HIM (phakosin-LNDR). This was then tested for the ability to assemble with K8, with results shown in Figure 4a. Though short rodlets were seen, suggesting that the initial phases of assembly may have occurred, no intact filaments were observed, indicating that the “correction” of phakosin HIM to the consensus LNDR was insufficient to restore assembly with a type II keratin.

At the opposite end of the rod domain is the second most conserved motif among IFs, the HTM. Though this is not nearly as well conserved as the HIM, phakosin shows considerable divergence in this motif as well. To test whether the altered HTM of phakosin accounted for the inability to coassemble with K8, we replaced phakosin's divergent HTM with that of K18 and tested for assembly with K8 (Fig. 4b). These data again show evidence of early assembly products but a failure to achieve complete assembly.

We then created constructs in which both the HIM and the HTM were replaced. These also showed the same result: evidence of initial assembly but a failure to complete assembly (Fig. 4c).

The previous experiments showed that replacing the wild-type phakosin HIM and HTM with IF consensus sequences did not confer assembly competence on phakosin with a type II cytokeratin. This was a negative result, however, that did not show whether these sequences were prohibitive to keratin assembly or whether the failure to assemble was attributed to

other sites within phakosin. To test whether the divergent phakosin HIM and HTM blocked assembly, we conducted the “converse” experiment: rather than placing the K18 LNDR motif into phakosin, we placed the phakosin LGGC motif into K18. We hypothesized that this mutant would be unable to form filaments with K8 because in every reported case, an R-C mutation in this motif negatively affected assembly. Further, the IF HIM is predicted to be part of an α -helical domain, and the LGGC contains two glycines, which would be predicted to preclude α -helix formation.

We began by verifying that K18 bearing the R-C substitution (i.e., LNDR to LNDC) negatively affected *in vitro* assembly. The results are shown in Figure 5a, and it is clear that normal assembly is precluded, as predicted. This is in agreement with a large body of literature showing the R-C mutation to be deleterious to IF assembly and function.

Surprisingly, when the entire phakosin HIM (LGGC) was placed into K18, normal filament assembly occurred. This is shown in Figure 5b, where long smooth filaments are evident. Thus, the R-C substitution, considered catastrophic to filament integrity in a wide range of IF proteins of types I, II, and III, including Meesman corneal dystrophy, does not preclude filament assembly and can be “rescued” by the inclusion of the two glycine residues within the HIM motif.

Phakosin HIM in K8

Unlike the rest of the IF family, type II keratins do not contain the LNDR sequence motif. Instead they show a highly conserved LNNK motif. We sought to determine whether phakosin HIM (LGGC) was permissive to assembly when included in a type II keratin, as it proved to be when included in the type I K18. To this end, we constructed a K8 mutant that contained phakosin LGGC. Surprisingly, this also proved capable of normal assembly with K18 (Fig. 5c). Thus, phakosin's LGGC proved to be an acceptable substitute for type I LNDR and type II LNNK. When the phakosin LGGC was placed into *both* K8 and K18, however, assembly of this keratin pair failed to occur (not shown).

To determine whether the LGGC HIM sequence could act “in trans” and suppress the dominant negative effect of the R-C mutation, we mixed K8 LGGC with the disease-causing K18 LNDC. This combination was unable to form native-looking IFs (not shown).

Functional Equivalence of Phakosin and K18 Head, Rod, and Tail Domains

Correction of the phakosin HIM and HTM sequences failed to permit filament formation between phakosin and K8, raising the possibility that the incompatibility may reside in other phakosin domains. Viewed under the electron microscope, assembly between phakosin and K8 appeared to proceed partially, which we interpret as a failure of subunits to complete the assembly process. In other words, electron microscopy provides evidence that the early stage of assembly, involving rod-rod interactions, proceeded at least somewhat normally. Thus, we examined the head and tail domains for their ability to permit complete assembly of phakosin with K8. These domains had previously been shown to impact the assembly of keratin filaments. Phakosin lacks a true tail domain, so the small sequence after the HTM is referred to here as the “tail.” First we created a K18 head + phakosin rod + phakosin tail. This failed to coassemble with K8, suggesting that the incompatibility resided within the rod or tail domain or that it required some interaction between head and rod/tail (Fig. 6a).

We then tested the phakosin rod domain directly by replacing the K18 rod with the phakosin rod. This K18 head + phakosin rod + K18 tail chimera also failed to assemble with K8, evidence that the phakosin rod domain might be the source of incompatibility (Fig. 6b). When the phakosin rod was present, however, a uniform population of “rodlets” formed, suggesting again that the initial stages of assembly might have occurred but could not

progress to intact filaments. These mutants do show that phakosin rod domain blocks normal assembly and that inclusion of the K18 head and tail domains does not alleviate that block. This suggests that fundamental differences in the structure of the phakosin rod domain distinguish it from the rest of the type I keratins. To directly test this, we again conducted the complementary experiment, placing the phakosin head on the K18 rod/tail to determine whether the phakosin head domain was compatible with types I and II assembly. Previous studies of keratin proteins had shown that swapping head domains of keratin partners led to irregular filament formation but that some assembly did take place.⁶⁹ The phakosin head + K18/rod K18 tail failed to form normal filaments with K8 (Fig. 6a), though, again, some short filamentous products could be found, suggesting that initial phases of assembly might have occurred. Thus, the phakosin head by itself precludes normal filament assembly.

However, if we extended the definition of “head” domain to include the HIM and substituted the phakosin head HIM in place of the K18 head HIM, normal filament assembly occurred with K8. Thus, while the phakosin head fused to the K18 rod/tail blocked assembly, this blockage was rescued by the inclusion of the phakosin HIM (LGGC; Fig. 7b). The inclusion of the LGGC motif allowed the K18 and phakosin heads to be interchangeable with respect to filament formation, clear evidence of an interaction between the head and the HIM.

Discussion

The differentiated lens fiber cell expresses two fiber cell-specific IF proteins, phakosin and filensin. In addition to being the most divergent members of this large IF family, they are also the only known examples of IF proteins that do not assemble into IFs. Instead, they are assembled into a structure with a thinner filament decorated with periodic beads, referred to as the beaded filament.^{39,40,42,45,49,70,71} Knockout (gene) studies have established that removal of either phakosin or filensin eliminates BF from the lens. The knockouts further establish that the BF is not required for normal lens development or for the differentiation of the fiber cell through the stage at which it loses organelles. However, though gross lens development appears normal, mice lacking either BF protein develop light scattering that worsens with age.^{51–53} A similar progression of lens failure is described in humans bearing point mutations in the phakosin.^{72,73} Thus, though BFs are not required to achieve clarity, they are required to sustain it.

How the BF serves this function is not yet known, though evidence has been presented to suggest that BFs may serve to stabilize the differentiated cell phenotype, a function that generally fits with the known function of IFs: provision of mechanical stability.⁵³ What is puzzling is why two unique proteins must be dedicated to this task in the fiber cell and why and how they achieve a different assembly outcome (BF versus IF).

We have approached this question by swapping motifs between phakosin and K18, both type I keratins, and determining what these motifs contribute to the process of IF assembly. We have placed particular emphasis on the HIM. Consistent with the high degree of sequence conservation in the HIM, the literature shows that mutations in these sequences are not well tolerated.⁷⁴ An R-C mutation in type I HIM accounts for approximately 40% of known disease-causing mutations in keratins,^{12,31,60,61,71,75,76} resulting in skin and corneal diseases. Mouse models have shown that the R-C mutation in the mouse K18 disrupts filament assembly in insect and mammalian cells *in vitro* and that transgenic mice expressing this protein exhibit chronic hepatitis.^{77–81} This same mutation also causes disease in the type III protein GFAP.⁸² Thus, across all IF classes, the R-C mutation is considered catastrophic to the function of IF proteins.

The determination that phakosin was a highly diverged type I cytokeratin but that it contained the R-C substitution within the HIM was, therefore, unexpected.^{46,64} Further, the phakosin HIM also has two glycine residues. Coils analysis of K18 predicts that the α -helical rod domain begins 10 residues *before* the HIM. If the phakosin LGGC is substituted into K18, the coiled-coil structure is predicted to begin one residue *after* the HIM, a shift of 15 residues. Thus, by all accounts, the change from LNDR to LGGC in K18 should have a significant impact on normal assembly. At the opposite end of the rod domain, phakosin also diverged from the consensus sequence of the HTM, though the sequence substitutions are more conservative. All these changes led to the hypothesis, shown to be true, that phakosin would not assemble with keratins.⁶⁸

Given these predictions and the sensitivity of the HIM to mutation, the most obvious question was whether the dramatic change in phakosin HIM alone accounted for the failure to coassemble with K8. We report here that “correction” of the wild-type phakosin HIM or HTM motifs to consensus type I sequences, either alone or in tandem, did not enable phakosin to assemble with K8. In contrast, though introduction of the disease-causing R-C mutation into K18 (LNDR to LNDC) negatively affected assembly, further mutation of the K18 HIM to phakosin's LGGC suppressed the negative impact of the R-C substitution alone. This is particularly surprising because the two glycines are not conservative substitutions and are predicted to eliminate the α -helical structure at this critical site.

Collectively, the data presented here demonstrate that the highly divergent phakosin HIM and HTM motifs are not the source of assembly failure between phakosin and K8 and do not account for the redirection of IF assembly into fiber cell-specific BF_s, that the R-C mutation, heretofore considered catastrophic to IF assembly, is not incompatible with IF assembly and can be “rescued” by inclusion of two glycines, and that the α -helical nature of the HIM is likely not to be critical to IF assembly.

In addition, an unexpected finding was that replacement of the conserved type II HIM (LNNK) with phakosin's LGGC was similarly permissive to K18/K8 assembly. Though the LNNK region is found only in type II cytokeratins and has not been identified as a major cause of IF diseases, it is nonetheless highly conserved in type II cytokeratins. Further, coils analysis predicts the HIM of type II cytokeratins is also α -helical and therefore should also be susceptible to the presence of the glycine residues.

Although all the aforementioned mutations are located in the rod domain, these studies also tested the assembly competence of the highly divergent head and tail domains of phakosin and K18. K18 bearing the phakosin head domain was not capable of assembly with K8, but this block was “rescued” if the definition of the phakosin head was expanded 11 residues further into the rod domain to include the HIM. Thus, there appeared to be a “compatibility” requirement between the head and HIM domains, suggesting interaction between these domains. It is unclear, however, whether this was within a protein or between adjacent proteins.

The existing assumption that phakosin and K8 do not form dimers and therefore lack the appropriate recognition motifs is based on the failure of phakosin and K8 to form filaments. This end point assessment clearly does not distinguish between different stages of assembly. Although these studies show that substitution of conserved HIM/HTM keratin motifs do not facilitate phakosin and K18 filament assembly, they do show the formation of a population of particles relatively uniform in size, as opposed to variable aggregates that sometime result from mutation/assembly studies. The presence of uniformly sized particles is thus often interpreted as assembly that has been initiated but is unable to go to completion. An example of this was provided by Coulombe et al.,⁴ who showed that removal of the entire helix 1A

segment resulted in length-truncated but full-width subunits that failed to complete assembly. Determination of the structure of these phakosin/K8 assembly intermediates is under investigation.

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Figure 1. Mutant phakosin and K18 proteins. *Left:* different protein domains contained in wild-type and mutant phakosin and K18 proteins that were created. *Right:* name assigned to each protein and filament assembly competency with K8.

Figure 2. SDS-PAGE of bacterially expressed, chromatographically purified K18, phakosin, and K8. Relative molecular weights in kilodaltons, derived from standards, are indicated.

Figure 3. Comparison of types I and II keratin assembly with phakosin and type II assembly. **(a)** Type I keratin K18 dialyzed with natural type II keratin partner K8 forms normal 10-nm intermediate filaments. **(b)** Lens-specific intermediate filament protein phakosin dialyzed with K8 fails to form intermediate filaments.

Figure 4.

Correction of phakosin helix initiation and termination motifs. **(a)** Phakosin with wild-type K18 helix initiation motif LNDK (phakosin-LNDK) dialyzed with K8. **(b)** Phakosin with wild-type K18 helix termination motif of TYRRLLEDGE (phakosin-TYRR) dialyzed with K8. **(c)** Phakosin with wild-type K18 helix initiation and termination motifs (phakosin-LNDK-TYRR) dialyzed with K8. All three corrected phakosin mutants failed to assemble with K8 to form intermediate filaments.

Figure 5.

Replacement of wild-type cytokeratin HIM with phakosin sequence. **(a)** Type I keratin K18 with LNDC mutation in the helix initiation motif (K18-LNDC), which eliminates assembly competency with k8. **(b)** Type I keratin K18 with LGGC helix initiation sequence (K18-LGGC) and normal filament assembly with K8. **(c)** Type II keratin K8 with LGGC helix initiation sequence replacing wild-type LNNK motif (K8-LGGC) and normal filament assembly with K18.

Figure 6.

Phakosin rod domain is assembly incompetent with type II keratins. **(a)** K18 head domain fused to phakosin rod domain (K18-H/phakosin-RT) and dialyzed with K8. *Arrow*: some form of higher order structure but incomplete filament assembly. **(b)** K18 head and tail domain fused to phakosin rod domain (K18-H/phakosin-R/K18-T) and dialyzed with K8. Both proteins containing the phakosin rod domain failed to assemble into intermediate filaments with K8, though some rodlets were detected.

Figure 7.

Phakosin head domain assembly competent with K8. **(a)** Phakosin head domain fused to wild-type K18 rod and tail domain (phakosin-H/K18-RT) and dialyzed with K8 fails to form intermediate filaments. **(b)** Phakosin head domain fused to K18 rod and tail domain containing a helix initiation mutation of LNDR to LGGC (phakosin-H/K18-RT-LGGC) dialyzed with K8 forms normal intermediate filaments.