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COMMENTARY

To bead or not to bead? Lens-specific intermediate filaments revisited

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SUMMARY

For nearly three decades cytoplasmic intermediate filaments (IFs) have been described as 10 nm thick, unbranched ropes radiating from the cell nucleus and extending to the plasma membrane. This stereotype is now being challenged by the discovery and molecular characterization of the *beaded filaments* (BFs), a novel class of IFs composed of the lens-specific proteins filensin and phakinin. In contrast to 'mainstream' IFs, BFs have a distinctly nodular appearance and form a meshwork underneath the plasma membrane of the lens fiber cells. In vitro assembly studies, expression of filensin and phakinin in cultured cells, and analysis of the corresponding genes reveal that these proteins have evolved from two different subfamilies of IF proteins, thus yielding a unique structure. The new information provides a basis for understanding how the various forms of tissue-specific IF proteins might have developed adopting to the constraints of a specialized environment.

Key words: Cytoskeleton, Intermediate filament, Beaded filament, Eye lens, Filensin, Phakinin, In vitro assembly, Electron microscopy

THE BASIC FRAMEWORK

The intermediate-size, 10 nm filaments (IFs) are long fibrous polymers and constitute a major component of the cytoskeleton. Although they appear morphologically similar, the IFs of the various cell types are chemically heterogeneous and consist of different proteins. Recent counts raise the number of individual IF proteins to about sixty for any given animal species, and the list is still growing. All IF proteins share a common molecular blueprint and are comprised of three distinct structural domains: an N-terminal head, a central rod and a C-terminal tail. The two end domains possess variable length and amino acid sequence, whereas the central rod domain has a well-defined size (either 310 or 352 residues) and contains highly conserved sequence motifs (e.g. Geisler and Weber, 1983; Hanukoglu and Fuchs, 1982; Steinert et al., 1983; reviewed by Steinert and Roop, 1988). It can be separated into four subdomains (i.e. coils 1a, 1b, 2a and 2b), conforming to an α-helix and being interconnected by non-helical linkers (i.e. linkers L1, L1-2 and L2). Sequence-wise, the α-helical subdomains are made of heptad repeats, thereby affording the formation of two-stranded α-helical coiled-coils (e.g. Parry et al., 1985; Quinlan et al., 1986; Aebi et al., 1986, 1988; reviewed by Parry and Steinert, 1992).

A variety of in vitro studies have now established that 10-nm filaments assemble from parallel, unstaggered coiled-coil dimers which further oligomerize into anti-parallel, approximately half-staggered tetramers (protofilaments), octamers (protofibrils) and higher order intermediates (e.g. Steinert and Parry, 1993; Steinert et al., 1993a,b,c; Geisler et al., 1992; Geisler, 1993; Heins et al., 1993; Downing, 1995; reviewed by Fuchs and Weber, 1994; Heins and Aebi, 1994). How the partially overlapping coiled-coils interact in the context of the mature 10 nm filament is not exactly known. However, based on cross-linking experiments and structural analyses, it is now clear that at either end of the central rod domain there is a short segment that plays a pivotal role in the longitudinal growth of the polymer (reviewed by Heins and Aebi, 1994). It is, therefore, no accident that these two rod end segments represent the most conserved regions among the different IF proteins (e.g. Conway and Parry, 1988; Letai et al., 1992) and are the target of debilitating genetic diseases which result in a structurally compromised cytoskeleton (e.g. Bonifas et al., 1991; Coulombe et al., 1991; Lane et al., 1992; Rothnagel et al., 1992; Cheng et al., 1992; Chipev et al., 1992; for reviews see Fuchs and Weber, 1994; Fuchs, 1995, 1996).

JUDGING BY APPEARANCE: BEADS, NODES, AND PEARLS

A textbook 'fact' that we all teach and propagate is that the three major constitutents of the filamentous cytoskeleton (i.e. actin-containing microfilaments, intermediate filaments, and tubulin-containing microtubules) are smooth, helical polymers that can be readily distinguished in the electron microscope

(EM) by their characteristic diameter (hence the term '10 nm or intermediate filaments' which denotes fibers thinner than the microtubules (i.e. 20-25 nm), but thicker than the microfilaments (i.e. 8-10 nm)). Although 'sidearms' are sometimes seen to project at more or less regular intervals from the surface of these cytoskeletal filaments, except for native neurofilaments (e.g. Hisanaga and Hirokawa, 1988; Troncoso et al., 1990), such spikes are thought to represent structural anomalies arising from fluctuations in the ionic environment, or co-purifying factors extrinsic to the filament proper. Not to leave any doubt about that, all respectable IF experts finish their lectures by projecting electron micrographs of 'properly' assembled filaments exhibiting a 'perfectly uniform thickness' throughout their length. Although the esoteric discussant may occasionally mention to their class that IFs prepared for visualization in the EM by glycerol spraying/low-angle rotary metal shadowing reveal a distinct 'beading' with a 21-23 nm axial repeat (e.g. Milam and Erickson, 1982; Henderson et al., 1982; Aebi et al., 1983; Hisanaga and Hirokawa, 1988; Troncoso et al., 1989, 1990; Gotow et al., 1992; reviewed by Heins and Aebi, 1994), very few bother to recall a 'strange' type of cytoskeletal filament uniquely located to the fiber cells of the eye lens, the 'beaded-chain filament' (BF) (Maisel and Perry, 1972).

BFs did not see the light of publicity until very recently. The long delay in recognizing the structural significance and the biological relevance of these structures has its roots in two reservations. First, when it comes to assessing the unanticipated, conventional wisdom dictates that we should discard the idea of something really new and, instead, consider the trivial: for example, BFs could easily be actin filaments decorated by sticky ribosomes, or IFs contaminated by crystallin aggregates. If this line does not discourage the curious, then comes the powerful 'irrelevance principle': because the lens is a rather specialized system, it would be highly unlikely that a structure confined to this organ can tell us something substantial for the organization of the average eukaryotic cell.

Irony aside, the road to the characterization of the BFs has been troublesome, but quite didactic. Analyzing crude fractions of chicken lens, Maisel and Perry (1972) were the first to observe fibers consisting of a 5-6 nm thick core filament and irregularly spaced 'grapes' of globular particles 15-20 nm in diameter. Fortunately, these workers did not stop at the level of 'appearance' and continued their analysis by making an astute biochemical observation: they noticed that subcellular fractions enriched in BFs contained basically two proteins, CP95 and CP49 (Maisel and Perry, 1972; Ireland and Maisel, 1984, 1989; FitzGerald, 1988; FitzGerald and Gottlieb, 1989). As it now turns out, these two polypeptides are indeed the molecular building blocks of the BFs, and they are clearly distinct from other cytoskeletal proteins. Accepting that this cytoskeletal filament system is indeed new, the question then remains: are the BFs really intrinsically beaded, or do the beads represent extrinsic material (e.g. lens crystallins) decorating the intrinsically smooth BFs? What is the structural basis of this characteristic beading?

A circumstantial, yet very interesting observation pertinent to this question was made several years ago by Granger and Lazarides (1984). In an attempt to localize the IF-associated protein synemin in lens fiber cells, this group obtained striking electron micrographs of metal shadowed samples revealing side-by-side vimentin filaments and BFs attaching to mats of the plasma membrane. Whereas vimentin IFs exhibited a smooth surface morphology and were heavily decorated by anti-synemin antibodies, BFs were synemin-free and their surface was decorated by regularly spaced 'nodes' (Granger and Lazarides, 1984). These observations established for the first time that the native BFs of non-manipulated cells are *regularly* beaded. It took nearly ten more years until CP95 (also referred to as CP94 and CP115) and CP49 (also called CP45) were cloned and sequenced (Masaki and Watanabe, 1992; Gounari et al., 1993; Remington, 1993; Merdes et al., 1993; Hess et al., 1993, 1996; Sawada et al, 1995). These proteins have now been renamed 'filensin' (CP95) and 'phakinin' (CP49) when their amino acid sequence became known to avoid confusion and to keep up with the tradition of giving Greek and Latin names to newly characterized proteins (Gounari et al., 1993; Merdes et al., 1993). Both polypeptides were found to share primary and secondary structure homology to a variety of IF proteins and were able to co-polymerize into filaments in vitro. Surprisingly, when visualized in the EM by negative staining, these in vitro reconstituted filensin/phakinin filaments exhibited no resemblance to BFs and, instead, looked most similar to the classical smooth-surface 10 nm filaments which assemble from other IF proteins (Merdes et al., 1993).

To resolve this paradox, we have recently examined in detail the structure of filensin/phakinin co-polymers assembled in vitro from native or recombinant proteins (Goulielmos et al., 1996a,b). Interestingly, unstained freeze-dried filensin/phakinin co-polymers unveiled a distinctly beaded appearance by scanning transmission electron microscopy (STEM). The beading was regular (i.e. with a 19-21 nm axial repeat), but became less distinctive when the unfixed sample was diluted or subjected to uranyl salts. Thus, although unstained filensin/phakinin co-polymers looked regularly beaded by STEM, they lacked an obvious beading when visualized after negative staining. Filensin/phakinin filaments stripped of their beads (i.e. by diluting them) tended to unravel and had a lower mass-per-length (MPL) value than the original beaded filaments. This lower MPL value equaled the mass of four phakinin tetramers per filament cross-section (i.e. four phakinin protofilaments). On the other hand, the difference in MPL between beaded and stripped filaments corresponded to the mass of the bead-like particles commonly found in the background, and it was close to the mass of filensin/phakinin heterodimers or integer multiples thereof.

As illustrated schematically in Fig. 1, from these data it was reasonable to conclude that BFs comprise a 'core filament' composed of four homotypic phakinin protofilaments (or two homotypic octameric phakinin protofibrils) surrounded by a 'shell' composed of up to four heterotypic filensin/phakinin protofilaments, thus giving rise to regularly spaced (19-21 nm) 'beads' (12-15 nm in diameter) along the length of the core filament (see also Goulielmos et al., 1996a). Such a nonuniform architecture of phakinin homopolymers and filensin/phakinin heteropolymers could then explain another surprising finding, i.e. that filensin and phakinin co-assemble in a 1:3 molar stoichiometry, both in vivo and in vitro (Merdes et al., 1993; Carter et al., 1995). As yet, such a non-stoichiometric association has never been observed with other heterotypic IFs (e.g. keratin filaments or neurofilaments).

Contrary to the notion that the BFs represent a specific

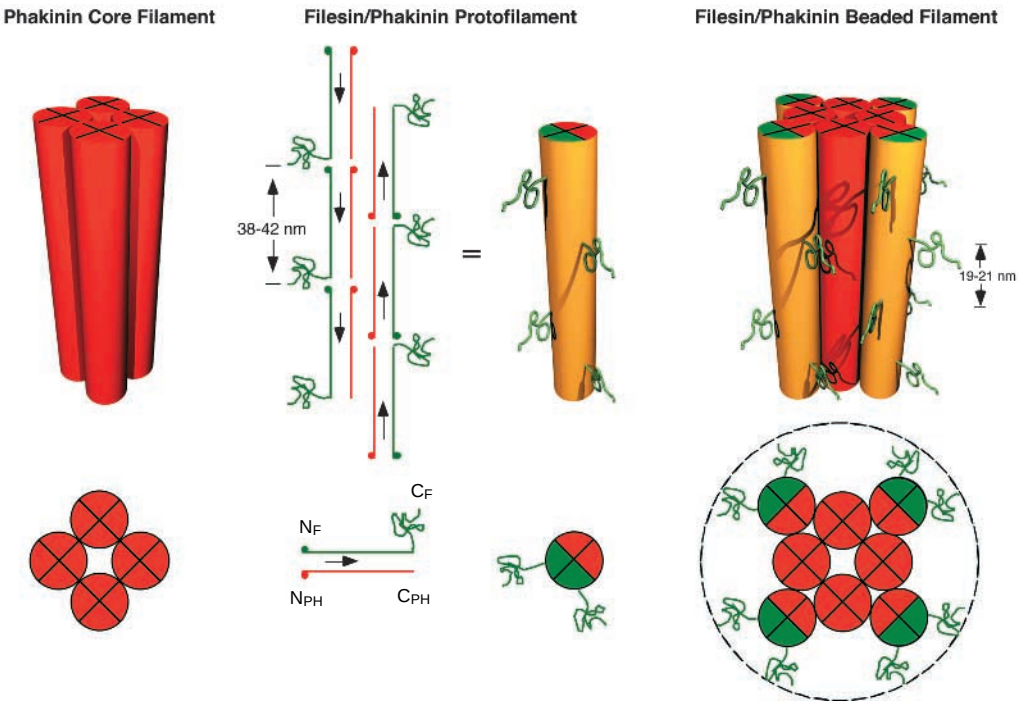

Fig. 1. Tentative model representing the molecular architecture of a lens-specific filensin/phakinin beaded filament. The filament is depicted as a multi-stranded assembly being built of a phakinin 'core filament' (i.e. consisting of four homotypic phakinin protofilaments shown in red), surrounded by a filensin/phakinin 'shell' (i.e. composed of four heterotypic filensin/phakinin protofilaments shown in yellow). The C-terminal tail domain of filensin is depicted as a 'folded wire' (shown in green) projecting from the filament core with an axial periodicity of 19-21 nm. Also shown is the schematic packing of filensin (in green) and phakinin (in red) polypeptide chains into filensin/phakinin heterodimers and heterotypic filensin/phakinin protofilaments. C_F and N_F represent the C-terminal tail and the N-terminal head domains of filensin, respectively, whereas N_{PH} represents the N-terminal head domain of tailless phakinin. At the bottom, cross-sections (i.e. axial projections) of a phakinin core filament (left), a filensin/phakinin heterotypic protofilament (middle), and a filensin/phakinin beaded filament (right) are displayed. This figure has been adapted from Fig. 7 of Goulielmos et al. (1996a).

device that can develop only in the specialized environment of the lens fiber cells, filensin and phakinin were found to polymerize de novo when co-expressed in IF-containing, or IF-deficient non-lenticular cells (Goulielmos et al., 1996a). Interestingly, the two proteins would co-distribute with vimentin filaments when expressed in fibroblastic cells, but segregate from the keratin filaments when co-expressed in epithelial cells. In an epithelial background, de novo assembled filensin and phakinin filaments started to grow from distinct sites associated with the plasma membrane and nuclear envelope, and they gradually formed thick laminae around these membranous organelles. This submembranous distribution appeared to mimic the deployment of BFs in the lens fiber cells (e.g. Ramaekers et al., 1982; Merdes et al., 1991, 1993).

FILENSIN AND PHAKININ DO NOT REPRESENT A KERATIN PAIR

As mentioned above, filensin and phakinin, the building blocks of BFs are structurally related to several different IF proteins. For instance, bovine filensin exhibits regional homology to the neurofilament triplet protein NF-L (43% identity in the coil 1a) and nestin (25% identity in coil 2b) (Gounari et al., 1993), while phakinin's rod is most similar to the rod of keratin 18 (31% sequence identity) (Merdes et al., 1993). However, there have also been several differences. More specifically, bovine and chicken filensin have a 'truncated' rod domain which lacks 29 residues in the area of coil 2a (Gounari et al., 1993; Remington, 1993). In contrast, mouse filensin possesses five additional residues in this area, but still differs from the canonical rod domain of other IF proteins (Gounari et al., 1997). Moreover, bovine filensin harbors six and a half tandem repeats in its C-terminal tail domain which match analogous motifs in the neurofilament triplet proteins NF-M and NF-H. Interestingly, these tandem repeats are missing in chicken and mouse filensin. Bovine and human phakinin are equipped with a normal-size rod. However, salient features among the highly conserved rod end segments of IF proteins are different in these two lens-specific IF homologs, and furthermore, they are completely tailless, i.e. they lack a C-terminal tail domain (Merdes et al., 1993; Hess et al., 1993, 1996; Sawada et al., 1995).

Based on the epithelial origin of lens fiber cells, the heterotypic polymer constitution of BFs, and the sequence homology of phakinin with type I keratins, some authors have suggested that filensin and phakinin represent a lens-specific keratin pair (e.g. Quinlan et al., 1996). However, the sequence peculiarities and assembly behavior of filensin and phakinin discussed above point to a different scenario. For example, it has been shown that isolated filensin and phakinin are unable to form regular-looking 10 nm filaments when combined at a 1:1 molar ratio and do not co-assemble with type I or type II keratins (Merdes et al., 1993). For comparison, it should be emphasized that the obligatory co-assembly of type I and type II keratins in a 1:1 molar ratio is a diagnostic feature of this subfamily of IF poteins. Another property which clearly distinguishes phakinin from keratins is its ability to self-assemble into long, non-IF-like fibers without the need of a partner (Goulielmos et al., 1996a).

Structurally speaking, if one had to relate BFs to one of the known classes of IFs, the best choice would have been the neurofilaments. Neurofilaments are heterotypic fibrous polymers

composed of the three neurofilament triplet proteins NF-L, NF-M and NF-H (for a review see Liem, 1993). Similar to NF-M and NF-H (Delacourte et al., 1980; Geisler and Weber, 1981; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1988; Troncoso et al., 1990), bovine filensin appears to reside at the periphery of the heterotypic BFs (Goulielmos et al., 1996a) and possesses a large tail domain which, in the case of the bovine homolog, is punctuated by distinct tandem repeats (see above). Moreover, self-assembly of filensin in vitro yields short, kinked fibrils (Merdes et al., 1991; Goulielmos et al., 1996a) looking somewhat similar to those formed by purified NF-M or NF-H (Aebi et al., 1988; Troncoso et al., 1989). Similar to purified NF-L (Geisler and Weber, 1981; Liem and Hutchison, 1982; Heins et al., 1993), phakinin self-assembles into long filaments (Goulielmos et al., 1996a) which, however, are noticeably thicker than the 10 nm-like NF-L filaments. In fact, the tailless phakinin filaments are reminiscent of those produced by tailless NF-L (Gill et al., 1990; Heins et al., 1993). Very much like the neurofilament triplet proteins (Ching and Liem, 1993; Lee et al., 1993), filensin and phakinin form obligate heteropolymers in vivo (Goulielmos et al., 1996a,b).

THE MYSTERY LIES HIDDEN IN THE GENES

Although the arguments against the keratin similarity of filensin and phakinin outnumber those for it, the skeptic would like to wait until genomic information becomes available to decide which side is right and which wrong. That has happened very recently as the mouse filensin (Gounari et al., 1997) and the human phakinin (Hess et al., 1996) genes were cloned. Interestingly, as illustrated in Fig. 2, the human phakinin intron/exon junctions correspond precisely to those of type I keratins. However, when it comes to comparing amino acid sequences within the keratin family, phakinin seems to be a cousin, even in the area of coil 1a which includes the highly conserved LNDR motif. Overall, the sequence identity of human phakinin with type I keratins is about 36%, well below that among the various members of the type I keratin subfamily (50-90%). The intron/exon junctions of mouse filensin coincide almost entirely with those of type III IF proteins (vimentin, desmin, peripherin), while the gene lacks two characteristic introns diagnostic for keratins (see Fig. 2; Gounari et al., 1997). In addition, other than a N-terminal di-arginine motif encountered in the head domain and a low-level homology in the rod, the primary structure differences between filensin and classical IF proteins outweigh their similarities. Curiously, the seventh (out of eight) exon of mouse filensin aligns exactly with the region bridging the rod domain and the nuclear localization signal of nuclear lamins, while its length and boundaries correspond to an analogous exon of the non-neuronal *Helix aspersa* (an invertebrate) IF gene (see Fig. 2; Gounari et al., 1997; Moir et al., 1995; Dodemont et al., 1990).

WHERE DO WE GO FROM HERE?

Slowly but definitely, the long and winding road of characterizing filensin and phakinin is now approaching its end. It is clear from the genomic data that the two lens-specific IF proteins evolved from two different subfamilies of IF proteins, namely, type III (filensin) and type I (phakinin), diverging continuously and thereby acquiring new, rather radical structural features. In that respect, it should be remembered that bovine and mouse filensin already differ in their rod and C-terminal tail domains (see above), suggesting an important role in cell physiology. The lenses of the various species differ markedly in their architecture and ability to accommodate. For example, the mouse lens is perfectly spherical and minimally accommodating, while the lens of a bird or a human is biconvex and highly accommodating. Obviously, the accommodation reaction is directly related to the ability of the elongated fiber cells to deform upon contraction and relaxation of the ciliary muscle. If we take this into consideration, it may be easier to

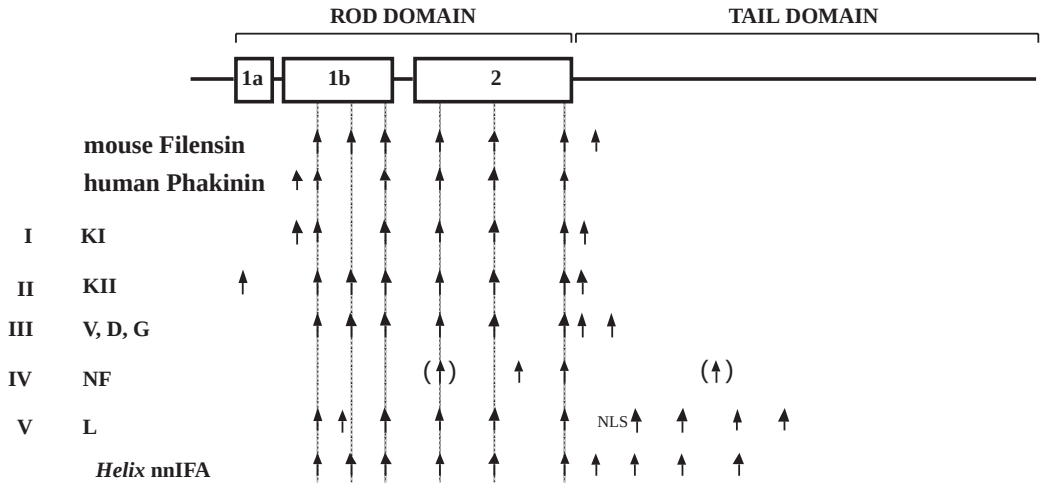

Fig. 2. The filensin and phakinin intron positions are compared to the different types of IF genes. Intron positions (arrows) are shown with respect to the typical IF protein structure. Boxes represent the α-helical coiled-coil segments of the central rod domain. Abbreviations are as follows: I KI, type I neutral-basic keratins; II KII, type II acidic keratins; III V, D, G, type III vimentin, desmin and glial fibrillary acidic protein; IV NF, type IV neurofilament triplet proteins (the two arrows in parentheses mark intron positions which are only present in one of the three NF genes analyzed); V L, type V nuclear lamins (NLS marks the nuclear localization signal); and *Helix* nnIFA, *Helix aspersa* non-neuronal IF protein A. The intron-exon organisation of filensin and phakinin was taken from Gounari et al. (1997) and Hess et al. (1996), respectively. Data for the types I to IV IF genes were taken from Steinert and Roop (1988) and references therein. Data for the *H. aspersa* IF gene are from Dodemont et al. (1990), and for the nuclear lamin gene from Döring and Stick (1990).

understand why the lens-specific IFs are made of such polymorphic subunits. However, that the lens-specific IFs are a finely tailored structure that meets the needs of a highly specialized environment does not necessarily subtract from their usefulness as a model system. Hence, we should view the sequence peculiarities and the assembly idiosynchracies of filensin and phakinin as a unique opportunity to explore structure-function relationships in naturally varying subunits without having to resort to mutagenesis. That the different forms of filensin afford filament assembly despite deletions and insertions in the sacred area of the central rod domain, that the tailless phakinin self-assembles into thick filaments in the absence of filensin and into 10-nm filaments in its presence, and that the two proteins form heterotypic filaments with a 3:1 stoichiometry of phakinin to filensin (see Fig. 1; Merdes et al., 1993; Goulielmos et al., 1996a), are all indications for alternative assembly pathways of IF proteins that we have not thought of before

The nodular architecture of the BFs is a provocation for the structural biologist and an invitation to the cell biologist to look harder for ligand binding sites and thus define the dynamic properties and in vivo partners of the BFs. The exquisite tissue-specificity of the BFs is a challenge to the molecular biologist which should be met by careful analysis of the *cis*-acting elements of the filensin and phakinin genes by looking for factors that regulate the expression of these two polypeptides during development and differentiation. Last, but not least, the fact that human BFs spend about 75 years in the cytoplasm of an anucleate cell that is unable to renew its components, might perhaps tempt those concerned with aging or protein stability.

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