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Review Letter

Dynamics of intermediate filaments

Recent progress and unanswered questions*

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Intermediate filaments (IFs) have always been considered as the most static and 'skeletal' cellular elements. This view is now changing: new information reveals that IFs exchange subunits at steady-state, that IF networks can be assembled de novo, and that IF proteins are subject to elaborate chemical modification and de-modification during mitosis. I describe below some of the key observations which have made us realize that IFs are dynamic structures. I also discuss some of the remaining questions pertinent to the pathways of IF assembly under in vivo conditions.

Intermediate filament; Dynamics; In vivo assembly; Phosphorylation

1. INTRODUCTION

Intermediate filaments (IFs) are major components of the cytoskeleton and the nuclear lamina of almost all eukaryotic cells. Despite their ubiquity, these elements have not been implicated in any vital cellular function. However, that a system as ubiquitous and organized as this would play no major biological role is somewhat counterintuitive. It would seem more reasonable to assume that the inability to define the function (or functions) of IFs originates from the lack of precise, mechanistic information.

Realizing the importance of learning details, several laboratories have started investigating the interactions and the properties of IFs on a molecular level. The fruits of this effort are already visible; new observations allow us to re-define IFs as a dynamic and highly interactive cellular assembly. To highlight these developments, I will focus here on a few observations which, in my opinion, have substantiated the dynamic character of IFs. By the same token, I will also try to tackle some questions pertinent to the regulation of IF assembly under in vivo conditions.

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*This article is dedicated to Stavros and Adamantia Politis.

2. POLYMERIZATION IN VITRO VERSUS ASSEMBLY IN VIVO

The family of IF-forming proteins comprises more than forty sequence-related polypeptide chains [1,2] and includes cytoplasmic as well as nuclear envelope-associated proteins (lamins). IF proteins contain a central α-helical domain ('rod'), which conforms to a heptad repeat motif and shows secondary structure similarity to a variety of other proteins (myosin, tropomyosin, microtubule motors, leucine zipper proteins, etc.). The rod is flanked by the NH_2-terminal ('head') and the COOH-terminal ('tail') domains and can be divided into four helical subdomains, the so-called coils 1a, 1b, 2a and 2b.

Helix packing rules dictate the lateral association of the heptad repeat regions of two IF protein chains and the formation of a two-stranded superhelix, the so called 'coiled-coil' (for a comprehensive review see [3]). IF aggregation seems to follow the pathway: monomers → (coiled coil) dimers → tetramers (protofilaments) → octamers (protofibrils) → higher oligomers → filaments [4]. While the structure of the higher assembly intermediates is unknown, the organization of the dimers and the protofilaments is now understood in some detail. The consensus view is that each dimer consists of two parallel and in-register chains, whereas each protofilament contains two antiparallel, staggered dimers [5,6], for another opinion see [7]. From analysis of paracrystals it has been postulated that the protofilaments are laterally and longitudinally associated in a way that the coil 2b regions of two adjacent dimers overlap by 2–3 nm [6].

A variety of studies have established that IF assembly in vitro proceeds spontaneously when purified IF proteins are transferred from a denaturing or low ionic strength environment to buffered salt solutions. Polymerization involves a dynamic equilibrium; beyond a certain (critical) concentration, the levels of non-polymerized subunits (which coexist with the polymer) remain constant, while the extent of polymerization depends on the total protein concentration and is saturable [8–10]. The existence of a dynamic state during in vitro assembly is further supported by the fact that non-polymerizing peptides modelled after short IF protein sequences can cause the disassembly of *preformed* filaments [11–13]. Excluding unsaturable binding (which formally remains a possibility), this could mean that the peptides exchange with (and replace) intact subunits dissociating from the preassembled polymer.

There are some features which clearly distinguish IF assembly in the test tube from assembly in a cellular context. First, although IF proteins self-assemble in vitro, this may not always be the case under in vivo conditions. For example, whereas purified small neurofilament subunit (NF-L) readily polymerizes in vitro, the same protein fails to form filaments when expressed (by transfection) in certain 'IF-negative' cells (G.Y. Ching, P. Macioce and R.K.H. Liem, personal communication). The ability of NF-L to assemble can be restored if the medium-size neurofilament protein (NF-M) is co-expressed (D. Cleveland, personal communication). Second, cellular IFs are known to resist dilution and to be refractory to chemical treatments with various chaotropic agents and non-ionic detergents. This behavior, unexpected from an 'equilibrium polymer' held together by non-covalent interactions, is probably due to the stabilization of the ends and the core of the filaments in situ. Supporting this view is the fact that the cellular IFs expose very few free ends, are extensively anastomosed or cross-linked, and maintain 'end-on' and 'side-on' attachments to several membranous organelles (for a review see [14]). Third, whereas in vitro assembled IFs do not self-organize into a network, the IFs inside the living cell form elaborate architectural patterns. The factors responsible for the in vivo organization of IF networks probably occur in limiting numbers, because, under conditions of IF protein overexpression, masses of disorganized and abnormally distributed filaments are formed (for recent examples see [15–17]).

The unique properties of cellular IFs do not necessarily contradict the conclusions reached by in vitro studies. They simply imply that the potential of IF proteins to polymerize on their own may not be realized as such in a living cell and that, instead, post-translational modifications, assembly-promoting and inhibiting factors, and blocking of the filament ends may regulate the extent of assembly and the organization of IF networks. Adopting this basic thesis, I will focus next on three pivotal questions: (i) the influence of phosphorylation on IF assembly; (ii) the potential modes of assembly in vivo; and (iii) the role of IF-associated proteins and organelles in network formation.

3. THE ROLE OF PHOSPHORYLATION

Several studies have shown that site-specific modification of IF proteins by various protein kinases renders the subunits incompetent to assemble in vitro. This has been shown for kinase A-, kinase C- and, more recently, for cdc2-mediated phosphorylation (e.g. [18–20]). Unfortunately, most of these studies have been performed at constant subunit concentrations and in a 'all-or-none' fashion. Thus, it remains unclear whether phosphorylation raises the critical assembly concentration, or whether it (more or less irreversibly) 'poisons' some critical site(s) in the subunit molecules. This distinction is of some mechanistic value; a phosphorylation-induced increase of the critical concentration could be overcome at higher subunit concentrations, whereas an inhibitory effect that can only be relieved by the action of phosphatases would mean a very tight enzymatic regulation of the assembly process.

To understand how phosphorylation could affect the ability of IF proteins to polymerize, one should examine more specifically the location of the modified sites in various IF proteins. For example, recent experiments indicate that the nuclear lamins are hyperphosphorylated during mitosis at sites flanking the coil 1a and coil 2b regions [21–24]. This may influence potential coil 2b–coil 2b, or coil 2b–coil 1a interactions and inhibit filament elongation. Consistently, phosphorylation of nuclear lamin B by purified cdc2 kinase (which modifies this protein at the same sites which are modified during mitosis) blocks polymer elongation in vitro [23]. It should be noted, however, that this may be a unique situation because the lamins appear to polymerize differently than cytoplasmic IFs; whereas the latter assemble by the simultaneous lateral and longitudinal growth of tetrameric protofilaments, the former assemble by head-to-tail elongation of dimers followed by a phase of lateral growth which, eventually, leads to thick paracrystals [25]. Phosphorylation of cytoplasmic IF proteins by the cdc2 kinase seems to involve sites located at the NH_2-terminal domain (e.g. [26]). Although it is clear that cdc2-modified subunits do not assemble in vitro, it is not obvious whether the kinase can act to the same extent and dissociate assembled IFs under in vivo conditions. Furthermore, it is presently unknown how the modification of the head domain could inhibit the polymerization process.

Some IF proteins have been shown to be hyperphosphorylated during mitosis (e.g. [27,28]); however, phosphorylation-dependent depolymerization under in vivo conditions has been directly demonstrated only in the case of the nuclear lamins [28]. The siuation is remarkably variable in other cell types. Although in sev-

eral cell lines cytoplasmic IFs are extensively remodelled during mitosis [18,29–35], there are other examples where mitotic IFs maintain their continuity without any indication of physical breakage ([29,36] and our unpublished observations). An interesting phenomenon is seen in the maturing *Xenopus laevis* oocyte, where keratin filaments are gradually converted to soluble aggregates of a relatively low sedimentation coefficient (*S*) value. It has been proposed (but not directly shown) that these aggregates could represent 'severed' IFs [37].

From all the data discussed above it seems quite clear that cytoplasmic IFs do not completely and universally depolymerize upon mitotic phosphorylation as the nuclear lamins do. Thus, the modification of the subunit proteins in the context of a filament may have fundamentally different consequences from the phosphorylation of dissociated or newly synthesized subunits. Some possibilities which may be worth testing are: (1) whether phosphorylation in mitosis affects the lateral packing of IFs (the mitotic IFs often appear thicker than usual); (2) whether phosphorylation changes the interactions of IFs with other cell structures; and (3) whether there are IF-associated proteins which redistribute upon modification of IFs during the M-phase.

4. PATHWAYS OF IN VIVO ASSEMBLY

4.1. *Co-translational and post-translational subunit incorporation*

Previous experiments [38,39] have demonstrated the existence of soluble IF subunits in a variety of cell types. These forms are non-filamentous (containing oligomers up to tetramers), but can polymerize if purified and appropriately concentrated [39]. Consistent with the idea that the soluble pool contains the precursors of the filaments, most of the soluble material is rapidly labeled by metabolic tracers and can be chased into the cytoskeletal fraction with half-times ranging from 7 to 20 min [38]. More recently pulse-chase studies indicate two, kinetically distinct, modes of IF subunit integration into the polymer: a rapid incorporation and a relatively slow incorporation [40]. Interestingly, [^{3}H]puromycin-labeled IF nascent chains appear to associate with some detergent-resistant structure. On this basis, it has been proposed that the fast-assembling fraction includes subunits incorporating into the cytoskeleton co-translationally and that the slow-assembling fraction (which varies depending on the cell type) comprises subunits incorporating post-translationally [40].

Co-translational incorporation of nascent chains into preexisting IFs has yet to be directly demonstrated; nevertheless, the postulated co-translational assembly pathway brings on the scene the possibility of 'preferred' IF assembly sites inside the living cell, because most of the vimentin-coding mRNA has been previously localized (by in situ hybridization) along the perinuclear region [41]. In principle, a co-translational assembly mechanism implies that the IF protein-synthesizing polysomes are anchored to perinuclear IFs, that nascent chains are directly inserted into preexisting filaments, and that this (steady-state) incorporation is more pronounced around the cell nucleus. If these postulates were correct, one would have to assume that mechanisms exist whereby IFs sort out and 'capture' IF protein-coding RNPs as they exit through the nuclear pores.

Whereas co-translational assembly could constitute a 'one way' process, post-translational assembly could be expected to involve a dynamic equilibrium, justifying the co-existence of polymerized and non-polymerized subunits inside the living cell. Should the size of the soluble pool become greater than the critical concentration (for example, under conditions of overexpression), two things may happen: either 'excessive assembly' of IFs, or 'buffering' of the excess subunits by an assembly-inhibiting factor, or a chaperonin. ATP-dependent in vitro binding of IF proteins to HSP-70 has indeed been reported [S.D. Georgatos, G. Blobel and W. Chirico (1989) J. Cell Biol. 109, 257a-abstract], but the physiological relevance of this interaction remains to be demonstrated. Recent experiments also indicate the existence of host-specific assembly-inhibiting factors in the cytoplasm of the *Xenopus laevis* oocyte (J.A. Dent, J.B. Bachant, R.B. Cary, A. Domingo and M. Klymkowsky, personal communication).

4.2. *Mechanism of subunit incorporation into preexisting filaments*

The forced expression of various IF proteins in cultured cells has revealed that newly made subunits can integrate into the sides of preexisting IFs [42–47]. Although these data have established the ability of newly synthesized IF proteins to incorporate into preassembled filaments, they have not clarified whether the mechanism of integration involves subunit exchange.

The idea of subunit exchange is, nevertheless, supported by recent studies employing fluorescence recovery after photobleaching [48]. In these experiments, IFs labeled with microinjected x-rhodamine-subunits have been focally bleached and examined after various periods of time. Because the bleached areas have been found to regain their fluorescence within 30–40 min (i.e. much longer than expected for the diffusion of soluble subunits into the bleached zone), it has been postulated that the material which fills the fluorescence gaps is provided by the dissociation of unbleached subunits from labeled filaments. Although these results are consistent with the fact that IFs are not uniform and appear to contain variable numbers of subunits per unit length [49,50], several issues need to be further explored. First, it is not entirely clear whether or not photobleaching results in breakage of vimentin filaments. This point could be directly addressed by serial sectioning and electron microscopy. Second, the relatively slow rate of fluores-

cence recovery does not necessarily mean that assembly is exclusively limited by subunit dissociation from preexisting filaments. For example, if the soluble IF subunits were transiently associated with some assembly-inhibiting factors (see above), the dissociation rate of *these* complexes would also affect the rate of subunit exchange between the soluble pool and the filaments. Finally, since the recovery rate would be a function of the relative association and dissociation rates of fluorescent versus unmodified subunits, it should be closely investigated how the labeling of vimentin affects its critical concentration.

The exchange of subunits along the filament backbone implies that IFs exhibit some 'breathing' under steady-state conditions. Relevant to this point may be the recent finding that the lateral packing of type III IFs could be regulated by an interaction between two sites (termed β and ε) located in the tail domain and the rod domain of the subunit proteins, respectively [51]. It will be interesting to examine whether phosphorylation or point mutations in these two regions could modify the ability of IF proteins to incorporate into preexisting filaments.

4.3. *De novo assembly*

Several transfection and microinjection studies have shown that new IF networks can be initiated from many, random, cytoplasmic sites [52], or from sites adjacent to preexisting IFs ([47]; see also below). Similar data have been obtained by expression of IF proteins in 'IF-negative' cells (e.g. [47]), although the evergrowing number of IF proteins makes it possible that such cells may, in fact, contain some yet unrecognized IF protein. The 'stochastic' mode of de novo assembly contrasts previous hypotheses postulating the existence of IF-organizing centers or vectorial assembly of IFs. Such ideas have been supported by the fact that a distinct polarity (from the nucleus to the cell surface) is detected when, following obliteration of the endogenous system, new IFs are allowed to assemble in living cells [42] and by the observation that IFs can be grown from the surfaces of isolated nuclei [53] or nuclear envelopes [54].

These differences may be due to a variety of reasons. The physical ends of preexisting IFs and the potential IF-organizing sites may be blocked under certain conditions. Thus, IF proteins produced by transfection, depending on the degree of expression, may be forced to choose the next thermodynamically permitted option, i.e. to spontaneously polymerize in the cytoplasm (very much like in a test tube). Such a behavior may only reflect a redundancy in the assembly pathways and not necessarily the main mechanism of filament growth during development and differentiation. This is also suggested by earlier and recent experiments done in three different systems. First, it is known that the keratin IFs of mammalian oocytes, which occur as patchy aggregates and cortical networks, reorganize after fertilization and appear to concentrate first around the embryonic nuclei and the plasma membrane before they fully develop into the cytoplasm [55]. Second, it has been observed that chick embryonic erythrocytes possess relatively few vimentin IFs which are associated with the nuclear surface but do not, in general, reach the cellular periphery. In contrast, more developed forms of red blood cells contain numerous filaments, extending from the nucleus to the plasma membrane [56]. Third, recent observations in CNS neurons induced to express the IF protein peripherin by appropriate environmental cues indicate a distinctly vectorial assembly from the cell body to the axonal and dendritic processes (K. Djabali, S.D. Georgatos and C. Dotti, in preparation). Although circumstantial, these paradigms suggest that de novo assembly under natural conditions may not occur via filament initiation from random cytoplasmic sites. It is conceivable that the formation of an IF network requires unmasking or rearrangement of IF-organizing centers, coordinated with changes in IF protein synthesis. Such events may not easily be attainable in transfection studies with cultured cells.

4.4. *Growth by branching?*

In certain cells which possess more than one IF systems (e.g. keratin and vimentin IFs), the two networks are seen to be non-overlapping. This has been generally interpreted as evidence against an interaction between certain species of heterotypic subunits. However, previous studies have suggested that the vimentin and the keratin IFs of some cells are interrelated [57], whereas other observations have shown that substoichiometric keratin–vimentin and keratin–desmin complexes can be produced in vitro [58]. These data are more consistent with the idea that, although homotypic interactions are stronger than heterotypic binding, a low degree of vimentin–keratin complexation could occur under a certain set of conditions. Further support for this is provided by the recent demonstration that assembly of desmin IFs in keratin-containing cells sometimes starts near endogenous keratin IFs [59] and that expression of truncated keratins in certain cell types affects both the keratin *and* the vimentin networks [60]. On this basis, one may propose that, when desmin (or vimentin) subunits are expressed in keratin-containing cells, some limited co-assembly may occur. Further desmin or vimentin assembly could then involve homotypic (desmin–desmin and vimentin–vimentin) rather than heterotypic (desmin–keratin and vimentin–keratin) binding, resulting in branches of homogeneous desmin or vimentin IFs developing from the sides of preexisting cytokeratin filaments.

5. ROLE OF VARIOUS CELLULAR STRUCTURES IN IF NETWORK FORMATION

From an architectural viewpoint, it would seem rea-

sonable to assume that the formation of radial IF networks inside eukaryotic cells involves two fundamental processes: (a) establishment of contacts to the nuclear surface and the cell membrane; and (b) filament cross-linking and anastomosis. On this basis, one may postulate that specific factors exist which can mediate the anchorage of IFs to different organelles and the lateral or 'end-to-end' joining of individual filaments. The nature of the interactions between IFs and other cellular structures and the molecular features of IF-associated proteins have not yet been determined in detail. Thus, we can only discuss here some controversial (but yet interesting) ideas based on earlier and more recent studies.

It is generally agreed that cytoplasmic IFs are tenaciously associated with the cell nucleus and co-fractionate with nuclear envelope proteins [61,62]. An elegant demonstration of the fact that the IF-nucleus interaction involves more than a mere 'sticking' has been provided by in situ studies on skeletal muscle fibers. In these cells, desmin IFs emanating from the Z disks contact the nuclei at distinct focal sites where the surface of the nuclear envelope seems to be 'wrinkled' or 'elevated' [63]. The coupling of the nuclei to the myofibrils appears to be dynamic because the nuclear contour changes during the contraction–relaxation cycle of the muscle fibers. Direct connections between the nuclear envelope and desmin filaments have also been documented in situ in smooth muscle cells [64].

Electron microscopy of quickly-frozen, deeply-etched preparations demonstrates that the IFs of certain cells approach the nuclear surface in a tangential fashion [14]. Thin (~5 nm) fibrils have been seen to connect the core of IFs with the nuclear pores [14,65,66]. The nature of these fibrils remains elusive; they may correspond either to the pore-associated cytoplasmic fibers thought to be involved in nuclear transport, or to unraveled protofilaments originating from the IFs themselves. Unfortunately, the *Xenopus laevis* oocyte, which has been the principal model for analyzing pore complex ultrastructure, would not be the system of choice to investigate potential IF–nuclear pore associations because the IFs in this cell type are distributed predominantly around the cell cortex.

Earlier work by Penman and associates [67,68] has provided hints that cytoplasmic IFs may directly link to the nuclear matrix and to the nuclear lamina. The same has been claimed in subsequent morphological studies (e.g. [69]). However, this view has not been universally accepted because of the potential artifacts associated with the salt extraction and critical point drying methods used. It should, nevertheless, be pointed out that several of the initial findings of such ultrastructural studies (as, for example, the idea that the nucleoplasm is structured and contains distinct filamentous elements) have now been established by independent methods. A direct interaction of cytoplasmic IFs with the

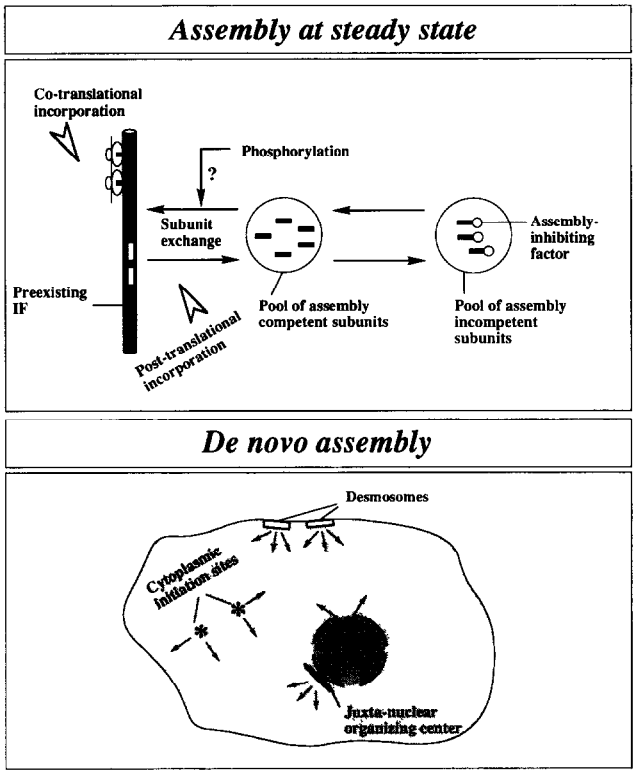

Fig. 1. Postulated mechanisms of in vivo assembly of IFs. Assembly at steady-state (upper panel) may involve the post-translational incorporation of newly synthesized subunits into preexisting filaments via exchange reactions along the filament walls. Specific 'buffering' factors may regulate the size of the assembly-competent subunits and influence the rate and the extent of post-translational exchange. In addition to this, co-translational incorporation may involve capturing of IF protein-synthesizing RNPs and direct insertion of nascent chains into the polymer. Assembly de novo (lower panel) may involve initiation of new IFs from random cytoplasmic sites, from the desmosomes or the plasma membrane, from juxta-nuclear organizing centers, or from the nuclear lamina. At the present time, the body of in vivo evidence favors assembly from random cytoplasmic sites (for details and alternative views see text).

nuclear lamins (or lamin-like proteins residing in other organelles) has also been suggested by in vitro binding studies and work with anti-idiotypic antibodies [70–75], but more and more precise information is needed to understand how cytoplasmic fibers would get access to the nuclear interior.

Pertinent to the last point is the unexpected finding that tail-truncated cytoplasmic IF proteins are transported and accumulate into the nucleus (e.g. [76,77]). Although the mechanisms involved in this process are still unclear, such results can be interpreted to mean that cellular factors exist which could mediate the transport of cytoplasmic IF subunits into the nucleus. That nuclear import of wild-type IF proteins does not occur under normal circumstances can be explained by postulating a strong binding of cytoplasmic IF proteins, via their tailpieces, to some cytoplasmic or plasma membrane structure. However, an alternative idea may be

that transport of wild-type subunits into the nucleus does take place, but that this is not easily detected because the putative intranuclear receptors for cytoplasmic IFs are spread 'thin' along the inner nuclear membrane and are rapidly saturated by a small number of imported IF protein molecules. According to this scenario, the transport of the tail-less IF subunits into the nucleus may be detectable precisely because the mutated proteins fail to associate with their nuclear envelope receptors and, therefore, accumulate in the nucleoplasm.

'End-on' contacts between IFs and the plasma membrane have been very well resolved in the fiber cells of the ocular lens [78], whereas lateral associations have been observed in avian erythrocytes [56]. Membrane-skeletal proteins such as ankyrin [79], spectrin [80], and filensin [81] have been proposed to be among the factors involved in these interactions. However, by and large, the most obvious contacts between IFs and the plasma membrane are those at the level of the desmosomes. Biochemical studies show that a peripheral protein of the desmosomal plaque, which shares antigenic determinants with the nuclear lamin B, may act as a connecting element and couple IFs to the cell surface [72]. Alternatively, other major plaque proteins may mediate this association (desmoplakins will be the best candidates but, so far, there is no evidence for a direct interaction between these proteins and the IFs).

Finally, a variety of proteins which are co-distributed with IFs are thought to provide cross-linking functions and stabilize the IF networks in vivo (for a catalogue see [1]). Most of these molecules appear to be expressed in a more or less tissue-specific fashion. The only widespread IF-associated protein is plectin, a high molecular weight polypeptide which has been found to interact with IFs in a phosphorylation-dependent fashion (reviewed in [82]). Plectin may provide an important element involved in regulation of IF 'meshing' and the global mechano-elastic properties of the IF networks.

6. CONCLUDING REMARKS

From the points discussed above it is apparent that IFs are modulated in many different ways. Regulation of assembly by the relative rates of subunit synthesis and accumulation in the soluble pool could be a determining factor at steady-state. Yet, the rapid remodelling of the filament networks during mitosis may involve phosphorylation-driven reactions which shift the assembly equilibrium and affect the critical concentration. A new promising direction in IF research may be the identification of assembly-inhibiting and assembly-promoting factors. It is clear from work done in other systems that assembly of multicomponent systems in vivo usually involves the action of chaperonins.

All said, one realizes that a consensus model which describes sufficiently well all modes of IF assembly in vivo has not yet been reached. Important distinctions, as for example the relative contributions of co-translational and post-translational assembly, remain to be settled in future studies. The same holds for subunit incorporation into preexisting filaments and de novo assembly, two processes which may involve fundamentally different molecular mechanisms. Connections of IFs to the membranous organelles are probably very important, as indicated by the fact that the 'primordial IFs' (lamins) are in contact to and assemble onto the inner nuclear membrane. However, many topological problems have to be elucidated before we titrate into a consensus mechanism which explains how filaments link to membranes.

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