Dynamics of the cytoskeleton in live cells

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Actin filaments, microtubules, and intermediate filaments, have all been found to be dynamic structures in living cells. Recent studies have shed important light on the assembly, disassembly, and mobility of these structures. In addition, a growing emphasis has been placed on the regulation of cytoskeletal activities by various signal transduction pathways.

Introduction

Previous observations of cellular motile activities, such as the shortening of spindle fibers during mitosis and the ruffling of actin-rich lamellipodia, suggest that cytoskeletal structures are highly dynamic in live cells. It is not until recent years, however, that direct studies of the reorganization of cytoskeleton have been carried out. It appears that most cytoskeletal components are engaged in active assembly, disassembly, and/or translocation throughout the cell cycle, under the regulation of a complex network of control mechanisms. This high degree of dynamics is likely to be crucial for the achievement of a wide spectrum of transient functions.

To a large degree, recent advances have relied on video-enhanced microscopy and microinjection techniques, which allow the direct analysis of a large variety of probes/structures inside living cells. The application of gene transfection techniques has further facilitated the analysis of the functions of specific proteins. While it is important to recognize the limitations of these techniques, such as potential artefacts caused by the sudden change in protein concentration or by the autoregulation/compensation mechanisms, these different approaches generally complement one another and have created a vast number of ways to probe, manipulate, and observe living cells.

Although significant progress was made during the past year in understanding the assembly and mobility of various cytoskeletal structures, major emphasis has also been placed on their regulatory mechanisms. This review focuses on some of the most important advances. For a comprehensive review of studies with microinjection techniques the reader is referred to a recent paper by Sanders and myself [1] and to other articles in this issue for reviews of mechanochemical enzymes and the dynamics of microtubules in mitotic cells.

Cortical activities of actin and myosin in interphase and mitotic cells

Cortical actin and myosin are known to play a crucial role in such activities as cell locomotion, cytokinesis, and membrane capping. Although these functions clearly involve cortical reorganization, our understanding of the dynamics of the cell cortex is still very limited. It has been proposed for many years, based on indirect evidence, that a directional flow of cortical cytoskeleton may underlie a wide range of activities [2].

Much of the cortical activity may be driven by the myosin molecule. In a time-lapse study of 3T3 cells microinjected with fluorescently labeled myosin II, McKenna *et al.* [3••] observed a *de novo* assembly of bead-like myosin minifilaments immediately behind the leading lamellipodia, and a backward flow of minifilaments on the anterior cortex. Subsequent studies by Giuliano and Taylor [4•] indicated that similar activities occurred in serum-deprived cells, where myosin filaments were organized predominantly along stress fiber-like structures. Thus, the behavior of myosin appears to parallel closely that of cortical actin, which is known to assemble continuously at the leading edge and move centripetally on the cortex at least under some conditions [2,5,6]. Such a simultaneous backward movement of actin and myosin is consistent with models involving either differential cortical tensions or a shearing interaction between the cortex and the inner cytoplasm.

It is clear that the current picture of cortical dynamics is far from complete. In order to maintain a steady state, the backward-moving filaments must disassemble and/or dissociate from the cortex somewhere in the posterior region and recycle back to the anterior region. Such activities remain to be demonstrated directly under improved optical conditions.

Equally important is the determination of the mechanisms for triggering the assembly–disassembly of the fila-

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ments and for determining the directionality of the cortical flow. The possible role of calcium ions in stimulating the advancement of the nerve growth cone, which closely resembles the leading lamella of fibroblasts, is still under active investigation [7]. In addition, there is a possibility that microtubules may play a role in modulating the assembly and mobility of cortical structures; treatment of KE37 lymphoblastic cells with nocodazole in order to disassemble microtubules was shown to induce an increase in the incorporation of myosin into the cortex and an intriguing back-and-forth movement of myosin-rich cortical constrictions [8••].

It has been proposed that cortical flow may play an important role during cytokinesis [2] which involves an extensive reorganization of actin and myosin in the cortex (see [9•] for a recent study of this process in living cells). Other mechanisms, however, including *de novo* assembly of filaments or local rearrangement of pre-existing structures, have also been proposed. This question was recently addressed by microinjecting fluorescent phalloidin into mitotic cells [10•,11••]. Pre-existing, phalloidin-labeled actin filaments were shown to deplete at the polar cortex and dramatically concentrate in the equatorial region during cytokinesis, indicating a recruitment of pre-assembled filaments into the cleavage furrow. In addition, directional translocation of actin during cytokinesis was indicated by the movement of small aggregates of labeled filaments, first from the cytoplasm into the cortex and then toward the cleavage furrow. Although the incorporation of actin subunits into the equatorial cortex may occur at the same time, these observations suggest that cortical flow is primarily responsible for the reorganization of actin during cytokinesis. Most notable is the apparent similarity between the cortical activities in interphase cells and in dividing cells. Thus, it is possible that cytokinesis may be coupled to the establishment of opposite anterior–posterior polarities in the two daughter cells, and that a common mechanism may underlie the regulation of cortical activities in interphase and dividing cells.

Dynamics of microtubules in neurites

Cytoskeletal dynamics in axons (or neurites) are manifested as various classes of axonal transport, as indicated by pulse labeling with radioisotopes. Whereas fast axonal transport is involved in moving organelles and is believed to be driven by microtubule-based motors [12], little is known about the mechanism of slow transport, which appears to involve the anterograde movement of pre-assembled actin filaments and microtubules [12].

Attempts have been made recently to observe directly slow axonal transport in live, cultured neurons. Lim *et al.* [13•] microinjected PC12 cells with a fluorescently labeled tubulin and photobleached small spots along the neurites using laser microbeams. Surprisingly, the bleached spots failed to show any directional movement over a period of up to 4 h This study was subsequently

extended by Okabe and Hirokawa [14••] and by Lim *et al.* [15••] to dorsal root ganglia cells that underwent active neurite extension. Again, no movement of the bleached spots was observed despite active neurite elongation. In all of these studies, a small fraction (10–20%) of fluorescence recovered rapidly, reflecting either the diffusion of tubulin dimers or a rapid movement of a subpopulation of microtubules. The subsequent recovery occurred over a period of 30 minutes to several hours without a detectable decrease in the width of the bleached spot. This pattern of recovery is inconsistent with a sliding of microtubules along the length of the neurite, but could be explained by the exchange of tubulin along the entire bleached segment with unbleached molecules that moved in during the period of rapid recovery.

The lack of movement of microtubules during neurite growth clearly confirms previous suggestions that new microtubules assemble near the growth cone [16]. This, however, directly contradicts the idea of slow axonal transport, which would predict a movement of the bleached spot toward the growth cone at a rate of $> 10 \mu m\, h^{-1}$. Although there is no definitive answer to this paradox, several explanations should be considered. Firstly, the transport may involve a limited fraction of microtubules and thus may be very difficult to detect with photobleaching techniques. Secondly, the transport may not occur or may occur more slowly under culture conditions, possibly because of the lack of certain crucial factors. Thirdly, the transport may be inhibited by photodamages that are difficult to rule out despite careful controls. It would be worthwhile to approach this question using independent methods such as fluorescence photoactivation techniques or direct observations of microinjected, taxol-stabilized fluorescent microtubules [17].

Dynamics of intermediate filaments

Intermediate filaments are commonly perceived as stable structures because of their low solubility under physiological ionic conditions. A certain degree of dynamics in live cells, however, can be predicted from the extensive reorganization of intermediate filaments during mitosis [18], from the exchange of protein subunits among neurofilaments *in vitro* [19], and from the finite half-life of the intermediate filament proteins *in vivo*. Moreover, expression of a mutated keratin in PtK cells has been shown to disrupt the organization of pre-existing keratin structures [20], and direct evidence for the dynamics of vimentin filaments has also been obtained based on the incorporation of biotinylated vimentin into endogenous vimentin filaments within 4 h of microinjection into BHK-21 cells [21].

Two recent studies have extended these investigations using elegant molecular biological approaches to study the incorporation of vimentin molecules. Ngai *et al.* [22••] transfected mouse 3T3 cells with plasmids containing an inducible chicken vimentin gene. After induction, the chicken vimentin was detected along the endogenous vi-

mentin filaments throughout the cell, with no preferential incorporation into the filaments in the perinuclear region. Similar results were obtained by Sarria *et al.* [23••] with the expression of mouse vimentin in HeLa cells. Interestingly, newly synthesized vimentin molecules appeared to concentrate in small clusters interspersed by extended segments of endogenous vimentin, suggesting that the incorporation sites were not randomly distributed along the length of filaments. Because of difficulty in resolving the ends within a crosslinked network, however, it was difficult to determine whether or not the incorporation occurred more preferentially at the ends of existing filaments.

In order to determine the role of pre-existing filaments in the organization of newly synthesized vimentin, Sarria *et al.* [23••] have also transfected vimentin into a cell line (SW-13/cl.2 vim⁻) that does not contain detectable endogenous intermediate filaments. Newly synthesized vimentin molecules were initially localized in discrete, short segments throughout the cell, which subsequently extended into a network similar to that found in HeLa cells. As in HeLa cells, no preferential assembly in the perinuclear region was detected. Although it is possible that the SW-13/cl.2 vim⁻ cells may lack organizing centers for the intermediate filaments, the random distribution of the nascent segments argues against the involvement of a perinuclear organizing center for the initial nucleation of intermediate filaments. The results are in contrast to those of Vikstrom *et al.* [21], who observed an initial incorporation of microinjected, biotinylated vimentin into vimentin filaments in the perinuclear region. One important aspect of the microinjected vimentin, however, was its initial distribution as aggregates in the perinuclear region, possibly resulting in a preferential release of solubilized vimentin molecules in this region.

While intermediate filaments may be more dynamic than previously anticipated, many questions remain to be answered. On the one hand, the incorporation occurs over many hours and may simply reflect the normal turnover of intermediate filament proteins. On the other hand, it is still unclear whether protein subunits exchange among different filaments and whether any movement of intermediate filaments can take place. To date, all experiments have focused on the association reaction under a limited supply of soluble/synthesized subunits. Thus, it would be informative if the rate of dissociation, which probably represents the rate-limiting factor for the dynamics, could also be investigated using such techniques as fluorescence photobleaching, or photoactivation.

Regulation of cytoskeletal dynamics

Current biochemical studies have painted an extremely complicated picture for the regulation of the cytoskeleton, ranging from the levels of protein expression, self-assembly, and crosslinking, to force generation. Most of the known cellular regulatory mechanisms, including divalent cations, phosphoinositides, GTP-binding proteins, and

post-translational modifications, have been implicated in the controlling mechanism. This is perhaps not surprising given the involvement of cytoskeleton in a vast array of transient functions.

One unresolved question concerns the polymerization of actin, which appears to be tightly regulated both spatially and temporally in the cell. A study by Sanders and Wang [24•] indicated that the cell probably contains an excess of factors for inhibiting the polymerization of actin subunits, but not for capping the ends of existing filaments. Although profilin has been a favorite candidate for this inhibitory function, recent biochemical analyses have raised valid questions about both its abundance in the cell [25,26], and possible inactivation by lipid molecules [27]. On the other hand, strong indications, based on the electroporation of GTP-γ-S into neutrophils [28•,29,30], have emerged suggesting the involvement of GTP-binding proteins in the regulation of actin assembly.

It is therefore important to identify the GTP-binding proteins that are involved and to determine the mechanism of regulation. A simple test for identifying the involvement of membrane GTP-binding proteins, by examining the sensitivity of GTP-γ-S-induced polymerization to pertussis toxin, has so far yielded negative results [28•]. In the meantime, there are indications that other classes of GTP-binding proteins may be involved. Paterson *et al.* [31•] reported that the microinjection of a constitutively active mutant form of rho (a small GTP-binding protein related to *ras* [32]) can induce a dramatic contraction of non-confluent 3T3 cells and formation of stress fibers in confluent cultures, although it was unclear whether these effects were coupled to a stimulation of actin polymerization. A different class of GTP-binding protein, the elongation factor (EF)-1a, has also been implicated in the regulation of actin cytoskeleton [33,34••]. The protein has been shown to bind both G- and F-actins and undergo a cAMP-stimulated association with filopodia in *Dictyostelium*. Although the exact role of EF-1a in regulating actin structures awaits further clarification, these results raise exciting possibilities directly linking the dynamics of cytoskeleton to the synthesis of proteins.

Regulation of structural assembly may also be mediated through various accessory proteins. The functional effects of these proteins, however, are often difficult to assess on the basis of biochemical characterization alone. An elegant example in defining the function of actin-binding proteins *in vivo* involved the transfection of fibroblasts with plasmids containing the gene for villin [35••], one of the major actin-binding proteins found in the microvilli of intestinal brush border [36]. Expression of intact villin induced a dramatic formation of microvilli on the dorsal surface of the transfected cells, while no effect was detected with the expression of the crosslinking incompetent core domain, indicating that the bundling activity of villin is crucial for microvilli morphogenesis. The important question remains: how did the microvilli form? As villin does not appear to be directly responsible for membrane binding in intestinal microvilli [36], it is likely that other proteins may be involved, and villin may induce the formation of microvilli by initiating a cas-

cade of binding events or by modifying existing cortical structures. Thus, it will be of great interest to determine the protein composition of the induced microvilli and the pathway of their formation. Villin also carries a number of interesting calcium-activated activities such nucleating actin polymerization and severing existing filaments [36], which may be evaluated in future studies by triggering the release of calcium ions in transfected cells.

A similar study was performed to examine the effects of tau, a microtubule-binding protein normally found in neuronal cells, on the organization of microtubules [37•]. The expression of tau in L-cells was coupled to the formation of striking bundles of microtubules that were not associated with the microtubule-organizing center, suggesting that tau may be able to mediate the formation of microtubule bundles and possibly also nucleate the assembly of microtubules. The expression of tau, however, also affected the amount of total tubulin and possibly the expression of other microtubule-binding proteins, raising the possibility that the formation of microtubule bundles may be caused by changes in a number of proteins.

Effects of protein phosphorylation–dephosphorylation on cytoskeletal dynamics

Although various kinases have been implicated in the regulation of cytoskeleton, on the basis of biochemical studies, little is known about their roles in living cells. Recently, Shoemaker et al. [38•] investigated the functions of muscle light chain kinase (MLCK) by microinjecting antisense oligodeoxynucleotides against the MLCK gene sequence. The suppression of MLCK synthesis appeared to induce a cell rounding similar to that induced by the microinjection of MLCK antibodies [39], although detailed studies of the organization of actin and myosin were not performed. These investigators have also obtained plasmids that code for various mutant forms of MLCK, and it should be quite informative to study their effects on cell structure, locomotion, and division upon transfection into cultured cells.

Recently, the cell-cycle-dependent kinase p34^{cdc2}, has attracted a lot of attention [40]. Because all the three cytoskeletal systems undergo pronounced changes during mitosis, it is possible that some cytoskeletal components may be targeted by this enzyme [41,42]. Lamb et al. [43•] recently showed that the microinjection of active p34^{cdc2} into interphase REF-52 cells could induce a dramatic retraction of cells and shortening of stress fibers. Microtubules appeared partially disrupted, without the formation of any mitotic spindle-like structures. Although these effects were reminiscent of changes during the early stages of mitosis, it is possible that similar events may be induced through unrelated pathways such as the release of free calcium ions as observed with the microinjection of peptides of the PSTAIR domain of p34^{cdc2} [44]. Of particular interest will be the fate of vimentin and myosin filaments in such injected cells, because p34^{cdc2} has been found to mediate the phosphorylation of the myosin light

chain and vimentin, and induce the disassembly of these filaments in vitro [42,45].

An equally important question concerns the possible role of dephosphorylation in regulating cytoskeletal structures. To date, little is known about the enzymes involved or about their controlling mechanisms. A recent study by Fernandez et al. [46•] demonstrated the ability of type 1 protein phosphatase to induce the dephosphorylation of the myosin regulatory light chain in vitro and in vivo. After microinjection into fibroblasts, this phosphatase induced a transient disappearance of stress fibers and an apparent net disassembly of actin filaments, as judged by the decreased staining with fluorescent phalloidin. Although such effects may be mediated through the dephosphorylation of the myosin light chain, it will be important to determine whether type I phosphatase has other cytoskeletal substrates, thus possibly regulating the cytoskeleton through multiple pathways.

Conclusion

Despite the advancement in recent years, our understanding of cytoskeletal dynamics is still very limited. On the morphological level, continual efforts are required to determine where and when protein molecules assemble and disassemble, and how structures move within the cell. On the molecular level, the functions of many proteins in vivo have to be clarified, and pathways of signal transduction that affect cytoskeletal structures must be defined. Although the complexity of the problem is enormous, there is little doubt that significant progress will be made with the recent refinement and combination of various powerful approaches. Clearly, living cells will provide a fertile ground for the ultimate integration of biochemistry, molecular biology, and morphology.

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Indicates a potential role of type 1 phosphatase in regulating myosin molecules and actin cytoskeleton, by microinjecting type 1 and type 2a phosphatases into living cells and observing both protein phosphorylation and stress-fiber morphology.

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