The Cytoskeleton

Cellular architecture and choreography

ALICE B. FULTON

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Editors' Foreword

The student of biological science in his final years as an undergraduate and his first years as a graduate is expected to gain some familiarity with current research at the frontiers of his discipline. New research work is published in a perplexing diversity of publications and is inevitably concerned with the minutiae of the subject. The sheer number of research journals and papers also causes confusion and difficulties of assimilation. Review articles usually presuppose a background knowledge of the field and are inevitably rather restricted in scope. There is thus a need for short but authoritative introductions to those areas of modern biological research which are either not dealt with in standard introductory textbooks or are not dealt with in sufficient detail to enable the student to go on from them to read scholarly reviews with profit. This series of books is designed to satisfy this need. The authors have been asked to produce a brief outline of their subject assuming that their readers will have read and remembered much of a standard introductory textbook of biology. This outline then sets out to provide by building on this basis, the conceptual framework within which modern research work is progressing and aims to give the reader an indication of the problems, both conceptual and practical, which must be overcome if progress is to be maintained. We hope that students will go on to read the more detailed reviews and articles to which reference is made with a greater insight and understanding of how they fit into the overall scheme of modern research effort and may thus be helped to choose where to make their own contribution to this effort. These books are guidebooks, not textbooks. Modern research pays scant regard for the academic divisions into which biological teaching and introductory textbooks must, to a certain extent, be divided. We have thus concentrated in this series on providing guides to those areas which fall between, or which involve, several different academic disciplines. It is here that the gap between the textbook and the research paper is widest and where the need for guidance is greatest. In so doing we hope to have extended or supplemented but not supplanted main texts, and to have given students assistance in seeing how modern biological research is progressing, while at the same time providing a foundation for self help in the achievement of successful examination results.

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1 Introduction

In eucaryotic cells, Brownian motion is a sign of death. The living cell orients its organelles and subcellular particles, so that everything not at rest undergoes directed and metabolically driven motion. Moreover, the patterns of motion and cell shape are specific; every cell type can be characterized by a particular configuration. This ceaseless and pervasive organization is mediated through the cytoskeleton; the cytoskeleton can be functionally defined as the structures responsible for this spatial organization.

What is the cytoskeleton? For some, the cytoskeleton is solely the network of intermediate filaments, or of microfilaments, or of microtubules. For others, it is a combination of two or three of these systems of filaments and their associated proteins. It is unlikely, however, that any one of these filament systems has an exclusive claim to being the cytoskeleton, since these three filament systems interact extensively.

The preceding definitions of the cytoskeleton are optative. An operational approach to the structural basis for cellular shape and location is to define the cytoskeletal framework operationally as the structures that remain after a non-ionic detergent extraction. The cytoskeletal framework includes the aforementioned filament systems when the extraction is performed appropriately. However, the structure obtained is complex and preserves many relationships to the nuclear matrix and cell membrane not included in the narrower definitions above. A yet more inclusive term for the structures responsible for spatial organization is the cytoplasmic matrix, visible when intact cells are fixed. It probably includes proteins whose associations with the cytoskeletal framework are briefer than the cytoskeletal filaments in the stricter sense. The cytoplasmic matrix includes many small heterogeneous elements termed microtrabeculae, that interconnect elements of the cytoskeleton, the cytoskeletal framework and subcellular organelles.

These definitions shade into each other; any attempt to be overstrict leads to difficulties such as those by which peripheral membrane proteins would be excluded from membranes proper. What should be clear from this discussion is the hierarchical nature of the cytoskeleton. The mechanisms whereby the cell is spatially organized lie in the chemical properties of the cytoskeletal proteins. These proteins are integrated in a tissue-specific and cell-specific manner into complex, dynamic networks and structures that interact with each other. These structures,

in their turn, are responsible for the co-ordinated spatial behavior of the cell. In the end, a complete explanation of the spatial behavior of the cell must be found in the molecules responsible for it. Likewise, our understanding of the cytoskeleton will not be complete until we can account for the full and complex choreography of the cell.

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2 Protein chemistry

The proteins that compose the filaments of the three major cytoskeletal networks have been isolated and their behavior *in vitro* is beginning to be well known. Actin, tubulin and the intermediate filament proteins can all be disassociated and reassembled *in vitro* to permit analysis of their chemical properties. Several common features have emerged which reflect the functional constraints and interactions of these systems. All three proteins form filaments, an economical structural member. These filaments are polar; their heads and tails are chemically distinct. In addition, these filaments are helical. Helical structures possess multiple equivalent binding sites; thus, they can form a multiplicity of structures generated by probabilistic interactions rather than the deterministic pathways followed during phage assembly [1]. These proteins all exist in multiple forms. Thus, there are several tissue-specific isoforms of actin and tubulin. Nonetheless, they are evolutionarily highly conserved [2]. For the intermediate filament proteins, tissue-specific variation is more conspicuous and evolutionary conservation less so [3, 4]. Nonetheless, antibodies exist that recognize intermediate filaments from *Drosophila* and mammals. Calcium plays a role in the regulation and function of all three filament systems and enters into the behavior of the cytoskeleton in so many highly specific and regulatory ways, that calcium might almost be regarded as the cell's transformer of soluble signals into solid cytoskeletal configurations. All three of these filament-forming proteins can associate with other proteins, either in the filament form or otherwise, and it is these specific associations that will be discussed at length in this chapter. Phosphorylation plays many roles in these interactions, either through the phosphorylation of associated proteins or of the filament-forming protein itself. Finally, the cytoskeletal filament proteins, are conspicuous by their acidity. The amino-terminus of actin, the carboxy-terminus of tubulin and the intermediate filament proteins in general are highly acidic and the role that this acidity plays is still being uncovered.

2.1 Actin and actin-binding proteins

2.1.1 *Actin polymerization*

Actin monomer (G-actin, 42 kD) is a globular protein with binding sites for divalent cations and nucleotides. Under physiological conditions

these binding sites are occupied by magnesium and ATP. Actin polymerization is a multistep process; the influence of actin-binding proteins can only be understood if the full complexity of the process is kept in mind [5]. Polymerization is represented diagrammatically in Fig. 2.1, and results in filaments (F-actin).

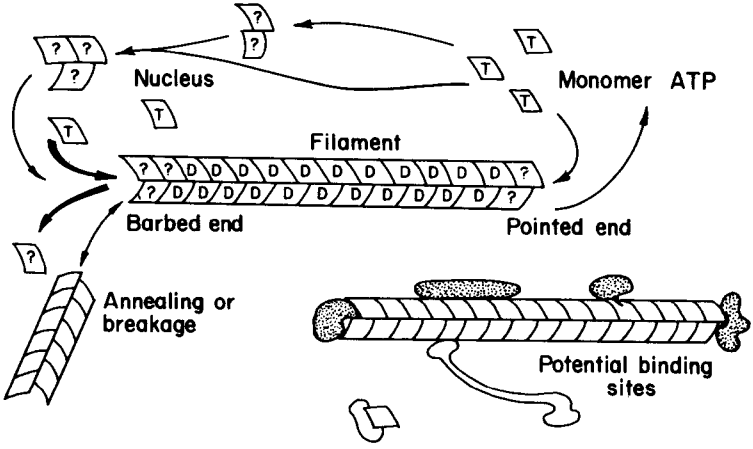

Fig. 2.1

Actin monomers can associate to form both dimers and trimers, but only the trimer is sufficiently stable to nucleate further polymerization. Once a nucleus is formed (probably trimeric), elongation can proceed rapidly. Elongation from a nucleus can proceed from both ends of the filament, but under most conditions the rates of elongation will not be identical. The polarity of the filament can be revealed by decoration with heavy meromyosin, which produces an arrow-headed pattern on the filament. The barbed end of the filament after myosin decoration is also the fast assembly end and the end of the filament with the lower critical concentration in the presence of ATP. The pointed end is the slower growing end and has a higher critical concentration. Polymerization is associated with ATP hydrolysis, but does not require it since polymerization goes equally well in the presence of non-hydrolyzable analogs [5]. Finally, as filaments elongate, thermal agitation may lead to breakage, and filaments may anneal with other filaments.

Thus, there are conceptually several distinct processes in actin polymerization: nucleation, addition at either end, dissociation from either end, filament breakage and annealing. Unfortunately, in a solution of actin induced to polymerize, most of these processes will be occurring simultaneously. In addition, each time filaments break they generate

new free ends that behave like nuclei. Thus, detailed analysis of the separate processes requires intervention in some form to discriminate between the different aspects of polymerization.

The method of measuring polymerization also constrains which aspect of polymerization will be visible. For example, viscosity is sensitive to filament length, but not to subunit exchange. Fluorescence quenching measurements are insensitive to filament length, and thus good measures of fractional incorporation into filaments, but they are sensitive to the presence or absence of subunit exchange [5]. Direct measurements of filament length in the electron microscope, unless limited to short filaments, encounter the difficulty of filament breakage. Thus, the complete characterization of the kinetic and equilibrium properties of actin polymerization of actin-binding proteins usually requires several different methods of measurement.

2.1.2 *Actin-binding proteins*

From the discussion above, it is clear there are five principal sites at which actin-binding proteins may have their effect. They may bind to the monomer, the pointed or slow assembly end of the filament, the barbed or fast assembly end of the filament, the side of the filament, or between two filaments to serve as crosslinkers (Fig. 2.1). In addition to these five modes of interaction, such proteins may be sensitive or insensitive to calcium. With so many varieties of interaction it is hardly surprising that a multiplicity of actin-binding proteins have been described, some with several modes of interaction.

Monomer-binding proteins inhibit nucleation by reducing the interaction of monomers. They may or may not lower the rate of elongation, depending upon whether or not the monomer associated with the actin-binding protein can associate with filaments. Two calcium-sensitive monomer-binding proteins are profilin and fragmin [6]. Both require calcium to bind to the actin monomer. The profilin–monomer complex can however polymerize onto existing filaments. Fragmin–actin complexes will not. Thus, profilin principally suppresses nucleation whereas fragmin both suppresses nucleation and inhibits the rate of elongation. Of the three calcium-insensitive monomer-binding proteins, DNase I [5] and vitamin D-binding protein [5] are both extracellular. The significance of their ability to bind actin is not known. There is an actin-depolymerizing protein from brain, however, which depolymerizes filaments by binding monomer and thus lowering the available concentration of actin [5].

The barbed or fast assembly end of actin filaments can be blocked either by capping proteins or the drug cytochalasin B or D. By blocking the fast assembly end, capping proteins promote nucleation but inhibit elongation and annealing, and their overall effect is often to shorten

filaments, both by creating more nuclei that compete for free monomer and by preventing annealing. At least four proteins are known to have these effects in the presence of calcium. Gelsolin [5], a 90 kD protein from platelets [5], villin [5], and fragmin [5] can all eliminate the lag during nucleation for pure monomer and also can shorten existing filaments in the presence of calcium. Calcium-insensitive capping proteins also exist. Acanthameba 31 kD and 28 kD proteins and a platelet 65 kD protein [6] have similar effects, whether or not calcium is present.

The pointed or slow assembly end of the filament is another potential site for protein interaction. A protein binding here could nucleate assembly and prevent annealing. It would affect the elongation rate of actin as a function of actin concentration; between the critical concentrations of the slow assembly end and the fast assembly end, a protein binding to the slow end would increase elongation by preventing loss of monomers from the pointed end. However, above the higher critical concentration, the net effect of a protein binding to the slow end would be to lower the overall elongation rate by blocking one of the two sites of addition. The net effect of these three properties (increased nucleation, decreased annealing and elongation) is to increase the number and shorten the length of filaments. These effects are similar to those of proteins that bind at the barbed end. Therefore, distinguishing between these two classes requires either determining a protein's effect in competition with proteins known to bind to the fast end, or using a seeded assembly assay [6, 5] to determine which end the protein affects. So far, only one protein is known that definitively binds to the pointed or slow assembly end of actin filaments, namely acumentin, an abundant protein from macrophages [7]. Another possible candidate is brevin [6], a serum protein which rapidly decreases the viscosity of F-actin solutions by shortening the filaments without increasing the free monomer concentration. Both brevin and acumentin are insensitive to the calcium concentration.

A fourth potential binding site is along the side of the actin filament without further interactions with other filaments. Proteins binding at the side of the filament might then either stabilize the filament or destabilize it. Tropomyosin binds in a calcium-insensitive way to stabilize F-actin filaments [5], while severin and villin bind to F-actin filaments and cut them in the presence of calcium [5, 8].

Probably the most dramatic of the actin-binding proteins are those which can crosslink actin filaments and form gels. By virtue of their binding to the F-actin form, such proteins generally nucleate as well as gel actins. At least four crosslinking proteins will cause F-actin to gel in the absence of calcium. Platelet α actinin [5], villin [6], fimbrin [9], and macrophage actinogelin [6] will all crosslink an F-actin solution into a rigid gel that will not permit the passage of a metal ball. The addition of calcium will dissolve this gel. All four of these proteins are monomeric. In the case of villin, the protein can be dissected into

separate domains, a core which is calcium sensitive and can bind to and cap actin filaments and a head region that is required for crosslinking filaments in the absence of calcium. There are also numerous calcium-insensitive crosslinking proteins. Two of these, filamin and actin binding protein [6], originally found in macrophages, share the properties of being a homodimer of long, flexible proteins. Alpha-actinin from muscle is also a calcium-insensitive crosslinking protein [5]. Vinculin and a high molecular weight protein from BHK cells are also capable of crosslinking without additional proteins. However, fascin from sea urchins can only generate actin needles by itselt. It requires the co-operation of a 220 kD protein to generate gels [6].

The spectrin family of proteins is one of the most interesting of the crosslinking proteins that are not directly affected by calcium. Spectrin is an $(\alpha\beta)_2$ tetramer first found in the membrane cytoskeleton of red blood cells. The $\alpha\beta$ dimers are associated tail-to-tail, leaving the heads of the molecule free to interact with actin oligomers. In addition, the α subunit of each dimer can interact with calmodulin, a calcium-binding protein involved in many calcium regulated activities. It is not known as yet what effect calmodulin binding has on the activity of spectrin. Spectrin-like molecules have now been found in a wide variety of cell types [10, 11, 12], so that it is more appropriate to speak of the spectrin family. The α subunit of red blood cell spectrin is 240 kD, and a 240 kD immunologically cross-reactive protein has been found in most cell types examined. However, the β subunit of red blood cell spectrin is 220 kD. Cells that display a 240 kD protein that reacts with α spectrin antibodies may also display, for example, a 260 kD subunit (found in the terminal web) or a 235 kD subunit (found in nerve cells and elsewhere). These cross-reacting species were originally described as separate families, called TW260/240 and fodrin. However, spectrin appears to be a tissue-specific family, as has been seen for so many cytoskeletal proteins. That all of these proteins contain a calmodulin-binding domain is a recent discovery and the consequences of that activity are yet to be understood.

Myosin is the only actin-associated protein that can generate mechanical force. This ATP derived mechanical work is the basis for muscle contraction and is believed to generate the tension exerted by fibroblasts and other cells in contact with the extracellular matrix. The interaction of myosin and actin is complex, so much so that it has been discussed in another book of this series. Myosin performs work by a cyclical interaction with actin. Myosin–ADP can bind actin filaments. A change in conformation occurs that is accompanied by ADP release. If ATP is available in solution to replace the ADP released from the myosin, it causes the release of the actin filament; ATP hydrolysis permits the initiation of a second cycle. Calcium regulates this interaction at any of several sites. In some muscle cells, calcium interacts with troponin to control the binding of tropomyosin to actin. These cells

are considered to be regulated at the thin filament. In other muscles, calcium acts on the myosin molecule, either directly or by activating enzymes that phosphorylate the light chains of the myosin molecule. In some non-muscle cells, calcium regulates contraction at the level of assembly of the myosin filaments.

The relationship between these different classes of actin-binding proteins gains some focus when considered in terms proposed by Florey's gel theory. Gel theory predicts that, when polymers have a greater than even probability of connecting to other polymers in the system, a three-dimensionally connected network will result. This predicts an abrupt transition from solution to gel, called the gel point, which has some mathematical similarities to other transitions, such as melting or vaporization. Beyond the gel point, further crosslinking leads to differences in rigidity only. Thus, crosslinking proteins can transform a viscous solution of F-actin into a solid gel. Filament-breaking proteins or proteins which increase the filament number can dissolve this gel by decreasing the average length of polymer without increasing the number of crosslinks. When the necessary density of crosslinks falls below that required for the gel point, the actin gel will dissolve. Myosin can interact with a gel to contract it. Gel theory is helpful in connecting the properties of the different classes of actin-binding proteins, and it has proven valuable for developing assays for different functions. It is probably important to bear in mind, however, that gel theory discusses isotropic structures and does not concern itself with precise topological connections. As will be clear later, these topological properties are extremely important properties of the cytoskeleton that cannot yet be predicted by gel theory.

Interpreting protein chemistry in a meaningful way requires detailed knowledge of the conditions that obtain within the cell. This includes the precise stoichiometry of all the relevant proteins and determinations of other controlling conditions such as pH, pCa nucleotide concentrations, and probably other variables such as phospholipid composition of adjoining membranes. When proteins can be effective at stoichiometries of 1:500 in promoting phenomena that exhibit cooperative and abrupt transitions, quantitative prediction will clearly be a challenging task.

2.2 Tubulin and microtubule polymerization

Microtubules, like microfilaments, are linear polymers, formed from tubulin subunits. However, the tubulin subunit is an $\alpha\beta$ dimer; both α- and β-tubulin are approximately 55 kD and can bind GTP or GDP. In the dimer, however, only nucleotide bound to β tubulin can exchange with GTP in solution. Like actin, tubulin has a highly conserved protein sequence. The α and β peptides diverged early in eucaryotic evolution; subsequent change has been less extensive [13].

Tubulin polymerization shares many characteristics with actin

polymerization. The subunits must associate to form a nucleus from which there is biased bi-directional growth with associated hydrolysis of the bound nucleotide triphosphate [13]. As a consequence, the two ends of the microtubule have different critical concentrations and permit potentially the treadmilling of subunits through the microtubule when the free subunit concentration lies between the critical concentrations of the two ends [14]. Again, as for actin filaments, breakage and annealing of the microtubules can change the number density of microtubule ends without changing the number of subunits found in the polymer.

Microtubule polymerization is affected by the concentration of divalent cations and temperature; i.e., it is inhibited by calcium, EDTA, and cold. Hydrolysis of GTP is not required for polymerization since non-hydrolyzable analogs support polymerization at normal rates. The microtubules so formed are now stable to calcium.

Microtubule polymerization, however, has a larger number of potential pathways than actin polymerization. *In vitro* several polymorphs have been observed, depending upon the conditions of polymerization [13]. These polymorphs led to efforts to find factors that promote polymerization, often under rather unphysiological conditions. In principle, tubulin-binding proteins could be classified as we have classified actin-binding proteins, that is, as binding to the subunit, the fast assembly or the slow assembly end, or the side of the filament. However, for historical reasons, the majority of microtubule-associated proteins have been studied either in terms of co-polymerization with microtubules, or of their ability to stimulate assembly. From the discussion above for microfilaments, it is clear that 'assembly' is the sum of nucleation, elongation, breakage and annealing, and that each of these steps can be affected by proteins that bind to tubulin. In addition, since the nucleus required for microtubule elongation is larger than that required for microfilaments, nucleation is especially sensitive to tubulin concentration. Any factor which stabilizes nuclei will be most conspicuous in its enhancement of nucleation, whether or not that is its function or site of association *in vivo*. With these caveats, the microtubule-associated proteins will be discussed individually below.

2.2.1 *Tubulin associated proteins*

Two major groups of microtubule-associated proteins were originally identified either by their co-purification with tubulin during cycles of assembly and disassembly or by their association with tubulin during purification by other means. A persistent association with tubulin is not an adequate criterion for specific association, since tubulin is a highly charged protein and can behave as an ion exchange resin. However, these two major groups have also been found on microtubules in cells fixed without extraction.

One group of microtubule-associated proteins (MAPs) is the group of high molecular weight (HMW) MAPs, between 290 and 350 kD in

weight, which are especially common in brain microtubules [15]. A second group of microtubule associated proteins, called tau, are between 55 and 70 kD [16, 17]. Although both HMW and tau were primarily described as promoting polymerization, the appearance of microtubules decorated with MAPs from either group and the location of these proteins in the cell make it clear that both HMW and tau associate with the side of microtubules. Their enhancement of polymerization appears to be by promoting nucleation, presumably by a mechanism similar to the side-binding proteins that associate with actin. In addition, microtubules decorated with HMWs display side arms that give the microtubules a furry appearance. These side arms are capable of associating with secretory granules; this association is reversed by ATP [18].

A different method of identifying microtubule-associated proteins, which exploits associations with microtubules *in vivo*, has identified a number of MAP proteins in various cells [19]. These include a 69 kD protein that is homologous to the tau proteins, and an 80 kD MAP protein with peptide homologies to the 69 kD protein. Both the 69 kD and 80 kD proteins are phosphorylated to various extents; the more highly phosphorylated forms are more extensively associated with microtubules [19]. This is one of the few cases in which the phosphorylation of a cytoskeletal protein has a measurable effect on its affinity for the cytoskeleton.

Nucleoside diphosphate kinase has been found associated with microtubules and can associate at a constant specific activity through three cycles of polymerization [13]. It is not identical to either of the HMW proteins or to tau. It is capable of phosphorylating both GDP and ADP. However, its contribution to microtubule function is not yet known.

Tubulin L-tyrosine ligase is an enzyme found in two isoforms. Tyrosine ligase catalyzes the addition of a tyrosine post-translationally to the C-terminal glutamate of the α tubulin protein. Variations in the distribution of both the enzyme and of the tyrosinylated tubulin have been noted, but the functional significance is still unknown [13].

Cyclic AMP-dependent protein kinase has been found to co-purify with microtubules purified by several different techniques. This kinase is associated with MAP 2 and will phosphorylate both MAP 2 and tau in the presence of cyclic AMP and ATP [20]. In light of the increased association of phosphorylated tau proteins with microtubules, it seems likely that such phosphorylation might increase the stabilizing effect of these proteins on microtubules or increase the extent of association of these proteins to microtubules.

The high molecular weight MAPs, MAP 1 (approximately 350 kD) and MAP 2 (approximately 270 kD), are both heterogeneous on SDS PAGE gels. MAP 2 has been separated into two species, 2A and 2B. MAP 1 consists of at least three species, MAP 1A, 1B, and 1C. MAP 1 possesses smaller polypeptides, called light chains 1 and 2, approximately 30 and 28 kD, which appear to be present in a 1:1 stoichiometry.