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ULTRASTRUCTURE OF PROTRUSIVE ACTIN FILAMENT ARRAYS

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Abstract

The actin cytoskeleton is the major force-generating machinery in the cell, which can produce pushing, pulling, and resistance forces. To accomplish these diverse functions, actin filaments, with help of numerous accessory proteins, form higher order ensembles, networks and bundles, adapted to specific tasks. Moreover, dynamic properties of the actin cytoskeleton allow a cell to constantly build, renew, and redesign actin structures according to its changing needs. High resolution architecture of actin filament arrays provides key information for understanding mechanisms of force generation. To generate pushing force, cells use coordinated polymerization of multiple actin filaments organized into branched (dendritic) networks or parallel bundles. This review summarizes our current knowledge of the structural organization of these two actin filament arrays.

Introduction

Coordinated polymerization of multiple interconnected actin filaments is key strategy for generating pushing force by the actin cytoskeleton. Acting as a cohort, actin filaments can achieve necessary stiffness to produce large forces. These forces are typically applied to the plasma membrane or membrane-enclosed organelles in order to change their shape or induce motility. Mechanistic understanding of any complex machinery greatly benefits from structural information. Determining the actin cytoskeleton structure, however, is challenging, because actin filaments are too tightly packed in the cell to be adequately resolved by light microscopy. Although new techniques such as subdiffraction fluorescence microscopy and atomic-force microscopy may overcome this barrier in future, most information about actin cytoskeleton structure has been obtained using different types of electron microscopy (EM).

Traditional thin section EM of plastic-embedded samples has been instrumental for characterizing relatively stable structures, such as actin filament bundles. However, dynamic actin filaments are not adequately preserved by this technique and their structure was revealed using whole-mount cell preparations contrasted by negative [1] or positive [2] staining or by metal shadowing [3,4]. More recently, cryo EM that analyzes ice-embedded samples in order to minimize preparation artifacts, in combination with electron tomography, began to contribute to structural studies of the actin cytoskeleton [5]. However,

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an extremely low contrast of cryo EM samples and their sensitivity to irradiation limits utility of the method and requires extensive image processing, which frequently produces unnaturally looking images of actin filaments with ambiguous gaps and fusions. Despite technical limitations of individual EM approaches, together they can provide an informative picture of the actin cytoskeletal architecture.

Dendritic Networks

A molecular mechanism of pushing force generation by dendritic networks is described by the dendritic nucleation model [6]. According to this model (Figure 1A), branched actin filaments are produced by the Arp2/3 complex, which upon activation by nucleation-promoting factors nucleates a new actin filament off the side of a pre-existing (“mother”) filament at 70° angle and structurally maintains the resulting branch junction. The specific architecture and dynamic properties of the dendritic network are controlled by additional proteins that promote (Ena/VASP proteins and formins) or block (capping proteins) elongation of actin filaments, regulate branch stability (cortactin, coronin, GMF), and cross-link (α -actinin, filamin) or disassemble (ADF/cofilin) the actin network. The dendritic nucleation mechanism is best studied in lamellipodia, flat protrusions at the leading edge of migrating cells, but the Arp2/3 complex and its partners also participate in many other cellular activities, such as various types of endocytosis, motility and biogenesis of intracellular organelles, and formation of diverse cell-cell junctions, suggesting that branched networks are present in these other locations (Figure 2).

Lamellipodia

Branched actin filaments were discovered in lamellipodia of migrating cells by metal shadowing EM and their significance for pushing force generation was suggested [7]. Subsequent studies with purified proteins, cytoplasmic extracts, and live cells revealed key molecules and events involved in actin-based protrusion. However, despite vast amount of diverse data supporting the dendritic nucleation model, the structural evidence for the existence of branched actin filaments in lamellipodia provided by metal shadowing EM [7,8] was repeatedly challenged by Victor Small and colleagues based on their inability to detect branches by negative staining EM [9,10]. Only recently this issue has been finally settled, when we detected branched actin filaments in their published electron tomograms of negatively stained lamellipodia [11**]. Therefore, the branched architecture of the lamellipodial actin network is currently confirmed by two independent EM techniques, although it unfortunately took more than ten years to come to a consensus on this point.

The specific geometry of a dendritic network in lamellipodia, such as density, length and orientation of filaments and frequency and distribution of branches, can vary depending on cell type and conditions, although these correlations have not been specifically addressed. Network geometry can be controlled, endogenously or experimentally, by absolute and relative rates of actin filament nucleation, elongation, and capping. For example, downregulation of capping protein [12–14] or upregulation of elongation factors, formin mDia2 [15] or VASP [16], increase actin filament lengths in branched networks. Conversely, interference with mDia2 [15] or Ena/VASP activities [16] produces very short branched actin filaments in lamellipodia. Dendritic network architecture also correlates with the protrusive behavior. It appears that when actin filament elongation dominates over branching, lamellipodia protrude faster, but they are less robust and frequently switch to ruffling or transform into filopodia [12,16,17]. Endogenous regulation of actin nucleation, elongation, and capping can be used by the cell to adjust the network architecture to its specific needs. For example, nascent lamellipodia formed shortly after a release from serum starvation [8] or at the edge of an intracellular wound [18], appear to contain a greater density of branches and shorter individual filaments compared with established lamellipodia

undergoing steady state protrusion. The density of branches can also vary locally within the same lamellipodium (Figure 2). For example, when the lamellipodial dendritic network begins to reorganize into filopodial bundles, the density of branches locally decreases, while the filament length increases [19]. Accordingly, in neuronal growth cones where filopodia are abundant, branched network is seen only in small areas between filopodia [20]. Conversely, fish or frog epidermal keratocytes that lack filopodia display relatively uniform distribution of branches along the leading edge [7,8,18].

The Arp2/3 complex not only nucleates actin filaments at the plasma membrane, but also acts as an end-to-side cross-linker. Therefore, the Arp2/3 complex and actin branches are present throughout the lamellipodium with a peak at the leading edge and a gradual decline toward the rear [8]. The slope of the decline may be controlled by relative rates of actin nucleation, debranching, and depolymerization and likely varies among systems. Thus, Vinzenz et al. found that actin branches were evenly distributed throughout the lamellipodium, or only slightly enriched at the front [18]. Although authors interpreted this profile as a lack of dependence between force generation and branching, this interpretation seems weak, because it remains unclear whether and how the front-to-rear distribution of branches affects protrusive behavior of lamellipodia. Also, since Vinzenz et al. selected for EM analysis thinner regions of lamellipodia that are more suitable for tomographic tracking, their data may not represent the entire range of lamellipodial architectures. Technical problems, such as a loss of very short filaments at the lamellipodial edge during drying of negatively stained samples or blotting of cryo samples, are also not excluded.

Actin filament orientation determines the direction of pushing force and therefore is another important parameter of dendritic networks. Although repeating nucleation of actin branches at 70° angle should eventually reverse the force direction, most actin filaments in lamellipodia remain oriented with their growing “barbed” ends roughly forward. This phenomenon can be explained by several non-exclusive mechanisms, such as targeting of Arp2/3 complex activators to the plasma membrane, preferential capping of backward-facing barbed ends [21,22], biased binding of the Arp2/3 complex to the convex surface of mother filaments deformed by membrane resistance [23*], and selective elimination of mechanically “unfit” filaments [24]. More subtle differences in the actin filament orientation in lamellipodia may correlate with the speed of protrusion. Thus, actin filaments in lamellipodia of fast moving keratocytes displayed a bimodal distribution of orientation with preferred angles of $\pm 35^\circ$ relative to the direction of movement [21], whereas slower moving keratocytes had most filaments oriented roughly orthogonal to the leading edge [25]. In contrast, pausing lamellipodia of macrophages display many tangentially oriented filaments [26]. However, tangential filaments were also dominant in protruding lamellipodia of migrating dendritic cells [27]. It remains unclear whether these filaments contribute to the pushing force or play a different role.

Membrane Organelles

The Arp2/3 complex and other components of the dendritic nucleation machinery function in trafficking and/or morphogenesis of membrane organelles [28]. However, structure of the organelle-associated actin cytoskeleton is mostly unknown except for actin patches during clathrin-mediated endocytosis and for bacterial comet tails, which are thought to represent an exaggerated version of endosomal trafficking machinery hijacked by pathogens.

Some intracellular pathogens, such as *Listeria monocytogenes* and *Shigella flexneri*, assemble Arp2/3 complex-dependent actin-rich comet tails to propel themselves throughout the cytoplasm [29]. Although the structure of prototypical endosome-associated dendritic networks is not yet available, architecture of bacterial comet tails has been determined in several conditions. Comet tails formed in vitro in cytoplasmic extracts contained branched

actin filaments throughout the tail [30]. Similarly, in the bulk cytoplasm of infected cells, comet tails of *Listeria* or *Shigella* contained non-oriented short actin filaments [31,32], likely corresponding to the dendritic network. However, when moving *Listeria* induced filopodium-like protrusions from the plasma membrane, their comet tails contained many long axial actin filaments [33], suggesting that the dendritic network undergoes dramatic remodeling within the membrane-confined space (Figure 2A). Since the filopodial tip marker, myosin X [34], is recruited to *Listeria* or *Shigella* specifically when they induce surface protrusions [35*], this remodeling may be governed by the convergent elongation mechanism functioning during formation of leading edge filopodia [19], or it may follow an alternative pathway, like that acting during initiation of cell-cell adhesions in endothelial cells [36*].

During clathrin-mediated endocytosis in yeast and mammals, small Arp2/3 complex-dependent actin patches assemble at endocytic sites and facilitate endocytic vesicle formation. As revealed by metal shadowing EM [37*], endocytic actin patches in mammalian cells consist of very short and densely branched actin filaments, suggesting relatively high rates of nucleation and capping. Importantly, actin barbed ends have been found to face the neck of the clathrin-coated pit, and thus presumably push at this site (Figure 2A). This finding resolved an uncertainty regarding a mode of force application during clathrin-mediated endocytosis [38], at least for mammalian cells. In yeast, however, the situation remains controversial, because existing EM approaches cannot resolve individual actin filaments. Thin section EM of actin patches combined with either immunogold labelling [39*] or correlative fluorescence imaging [40*] revealed that actin assembly was either subsequent to or coincident with plasma membrane invagination, which suggests different geometries of actin networks. The former result is consistent with force applied to the tip of the invagination [39*], whereas in the latter case, the actin network can press against the plasma membrane and undergo retrograde flow simultaneously pulling the attached endocytic bud into the cytoplasm [40*]. Future studies should resolve this ambiguity.

Since the Arp2/3 complex is activated by a nucleation-promoting factor in conjunction with a pre-existing actin filament, the source of the first mother filament at the onset of the dendritic nucleation cycle remained an open question. EM analyses of endocytic actin patches in mammalian cells [37*] or nascent lamellipodia at the edge of a healing intracellular wound [18] suggested that a random actin filament present in the vicinity of the Arp2/3 complex activation site can serve as a mother filament. In yeast, where cytoplasmic actin filaments are scarce, it was proposed that actin fragments severed away from a disassembling actin patch by cofilin can serve as mother filaments to initiate a new patch [41].

Cell-Cell Junctions

The Arp2/3 complex functions in various cell-cell junctions, such as adherens junctions in epithelial and endothelial cells [42], in neuronal [43,44] and immune [45] synapses, and perfusion junctions in myoblasts [46]. Among them, dendritic actin networks have been ultrastructurally characterized in excitatory neuronal synapses [47*,48*] and at initial stages of adherens junction formation in endothelial cells [36*].

Excitatory synapses in neurons are formed between presynaptic terminals on axons and postsynaptic dendritic spines, actin-rich protrusions on dendrites frequently having mushroom-like shape. As revealed by metal shadowing EM of cultured hippocampal neurons, bulbous heads of dendritic spines, which are involved in synapse formation, are filled with a dense three-dimensional branched actin network immunopositive for typical components of dendritic nucleation machinery, such as the Arp2/3 complex and capping

protein [48*]. Electron tomography of thin-sectioned brain samples [47*] also showed a dense fibrillar meshwork in dendritic spines. Although tracing individual filaments was rather difficult, this technique revealed that actin meshwork density was maximal immediately adjacent to the postsynaptic density, the signalling platform of dendritic spines, and that sites of actin-membrane interactions contained electron-dense material, possibly, corresponding to barbed end-associated elongation factors, such as VASP [49]. Unexpectedly, presynaptic sites in cultured hippocampal neurons were also found to contain a branched actin network, albeit less elaborate than in dendritic spines [48*]. The two networks, presynaptic and postsynaptic, are in close proximity at the junction site marked by the presence of N-cadherin, suggesting that these networks press against each other to promote junctional interaction of plasma membranes (Figure 2B). It is likely that Arp2/3 complex-dependent networks play a similar role in other cell-cell junctions.

Adherens junctions are essential for compartmentalization of tissues by epithelial and endothelial sheets. Cell-cell interaction is mediated by cadherins and other adhesion molecules, which can form continuous lines or discrete puncta, and stabilized by the actin cytoskeleton [42]. Ultrastructurally, adherens junctions appear associated mostly with actin bundles, whereas light microscopy approaches revealed contribution of dynamic Arp2/3 complex-dependent actin structures [42]. Metal shadowing EM was recently used to define actin organization during adherens junction initiation in endothelial cells [36*]. It showed that the first contact between cells is made by lamellipodia containing typical dendritic actin networks (Figure 2A). Similar to neuronal synapses [48*], networks of two adjacent cells can press against each other to bring membranes in close proximity and thus facilitate engagement of adhesion molecules. The lamellipodial interaction is followed by cell edge retraction, which leaves behind filopodia-like bridges connected by punctate adherens junctions, which then transform into contractile stress fibers [36*]. This reorganization, probably, strengthens adherens junctions through tension-dependent mechanisms [50,51*]. Although filopodial bridges and conventional filopodia at the leading edge of migrating cells are both formed by reorganization of dendritic networks, the mechanisms of reorganization are quite different. Instead of forming from tip to base, like leading edge filopodia [19], filopodial bridges propagate in the opposite direction and transiently retain mini-lamellipodia at their tips during this process [36*] (Figure 2A).

Parallel Bundles

Formation of tight bundles of uniformly oriented and synchronously polymerizing actin filaments is another major strategy for generating pushing force by the actin cytoskeleton (Figure 1B). This mechanism is best understood for leading edge filopodia of migrating cells [52,53]. Similar bundles are also present in other cylindrical cell surface extensions, such as brush border microvilli in intestinal epithelium and hair cell stereocilia in the inner ear. Unlike filopodia, microvilli and stereocilia maintain a constant length and exhibit very slow (microvilli) [54] or negligible (stereocilia) dynamics [55]. Notably, not all cylindrical membrane protrusions contain parallel actin bundles. For example, narrow elongated necks of dendritic spines (Figure 2), as well as dendritic filopodia, the precursors of dendritic spines, contain branched actin networks and differently oriented actin filaments [48*].

Leading edge filopodia are needle-like membrane protrusions functioning in cell motility and navigation (Figure 2A). Actin filaments within the filopodial bundle undergo treadmilling, whereby subunits are added to barbed ends at the plasma membrane, move away from the tip as a part of the filament lattice, and are released at the rear. Uninterrupted and fast elongation of individual actin filaments in the bundle is assisted by barbed-end associated proteins of formin and Ena/VASP families. Elongation-promoting activities of these otherwise unrelated protein families include protection of barbed ends from capping,

recruitment of profilin-actin complexes to increase local concentration of polymerizable subunits, and processive attachment of elongating barbed ends to the membrane to increase efficiency of pushing [56]. Unconventional myosin X also promotes filopodium elongation, probably, by delivering actin monomers or pushing the membrane away from barbed ends for easier monomer addition [34,35*]. All these proteins are enriched at filopodial tips and likely reside in the structurally recognizable filopodial tip complex [19]. As a cohesive multimolecular scaffold, the tip complex may also synchronize polymerization of filopodial filaments.

Bundling of actin filaments increases their collective stiffness and allows them to overcome membrane resistance and avoid buckling during protrusion. The major actin filament cross-linker in leading edge filopodia is fascin [57,58], a bivalent monomeric protein, whose actin-binding sites have been only recently characterized [59*,60*,61]. Although fascin forms stiff and stable bundles in filopodia, it undergoes fast turnover within bundles [58,62], which may result from its ability to switch between active and inactive conformations [61]. Fascin-mediated bundling changes the twist of actin filaments [63], which may affect binding of other proteins to actin filaments, such as cofilin [64,65] and myosin X [66,67].

In contrast to overall consensus regarding the actin filament organization and dynamics in established filopodia, the origin of filopodial filaments remains a matter of debate [53]. Two types of actin filament nucleators, the Arp2/3 complex and formins, participate in filopodial protrusion. Whereas the role of the Arp2/3 complex in this process is best explained by its nucleating activity, it remains unclear whether formins promote filopodia by nucleating new filaments or by elongating the pre-existing ones. Since cells that are completely or largely devoid of the Arp2/3 complex still make filopodia [68*,69*], formins or other actin nucleators may indeed generate filopodial actin filaments [15,70]. High resolution structural analysis correlated with de novo filopodia formation is required to understand the mechanism of filopodia initiation in these systems.

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Figure 1. Molecular architecture of protrusive actin filament arrays

(A) Dendritic networks in lamellipodia. (1) The Arp2/3 complex is coactivated at the plasma membrane by binding both a nucleation promoting factor (NPF) and a mother filament; (2,3) elongation of the newly nucleated branch, as well as of the mother filament, is transiently assisted by Ena/VASP (2) and/or formin (3) family proteins, which protect barbed ends from capping, recruit actin-profilin complexes, and mediate processive attachment of barbed ends to the plasma membrane. (4) During plasma membrane advance or retrograde flow, the Arp2/3 complex-containing actin filament branch becomes a part of the dendritic network and can be stabilized by cortactin. (5) After a period of elongation, some barbed ends lose elongation factors and become capped by capping protein; this process balances continuous nucleation of new filaments and is a part of filament length control in the dendritic network. Disassembly of the network is mediated by severing activity of ADF/cofilin and dissociation of branches (not depicted).

(B) Parallel bundles in filopodia. Long actin filaments in the bundle are oriented with their barbed ends to the plasma membrane at the tip of the filopodium. Their elongation is assisted by formins and Ena/VASP proteins, as well as myosin X. Filaments are bundled by fascin and laterally attached to the plasma membrane by ERM proteins.

A Various cell types

B Neurons

Figure 2. Functions of protrusive actin filament arrays

(A) Many cell types, such as fibroblasts, epithelial, endothelial, and immune cells employ dendritic networks and parallel bundle for different purposes. (1) Dendritic networks and parallel bundles at the leading edge drive membrane protrusion. (2) Dendritic networks induced by bacterial pathogens propel these bacteria throughout the bulk cytoplasm of a host cell; this motility is thought to mimic a normal membrane trafficking process. (3) During clathrin-mediated endocytosis, dendritic networks associate with clathrin-coated structures to promote their invagination, constriction and departure. (4) When bacterial comet tails become enclosed within a filopodium-like protrusion, the dendritic actin network appears to reorganize into a parallel bundle, although the continuing presence of a branched network at the actin-bacterium interface is a speculation at present. (5, lower part) Lamellipodial dendritic networks in contacting cells press against each other to bring their membranes into close proximity and facilitate cell-cell adhesion during adherens junction formation. (6, upper part) Retraction of an attached lamellipodium transforms its dendritic network into a parallel bundle in a base-to-tip direction, so that a mini-lamellipodium transiently remains at

the tip of the bridge; the bundle subsequently recruits myosin II to exert tension on the forming adhesion and thus enhance its strength.

(B) Neurons use protrusive actin arrays for similar purposes, but within neuron-specific structures. (7) Dendritic networks are present asymmetrically on both sides of an excitatory synapse, in the dendritic spine (left) and axonal presynaptic terminal (right), where they likely facilitate membrane juxtaposition; despite its narrow elongated shape, the spine neck also contains branched network. (8) Parallel bundles in filopodia are dominant protrusive structures in neuronal growth cones mediating elongation of axons and dendrites; however, dendritic networks are also present between growth cone filopodia and participate in filopodia initiation.