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Extra View

Toward the Structure of Dynamic Membrane-Anchored Actin Networks

An Approach Using Cryo-Electron Tomography

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ABSTRACT

In the cortex of a motile cell, membrane-anchored actin filaments assemble into structures of varying shape and function. Filopodia are distinguished by a core of bundled actin filaments within finger-like extensions of the membrane. In a recent paper by Medalia et al[1] cryo-electron tomography has been used to reconstruct, from filopodia of *Dictyostelium* cells, the 3-dimensional organization of actin filaments in connection with the plasma membrane. A special arrangement of short filaments converging toward the filopod's tip has been called a "terminal cone". In this region force is applied for protrusion of the membrane. Here we discuss actin organization in the filopodia of *Dictyostelium* in the light of current views on forces that are generated by polymerizing actin filaments, and on the resistance of membranes against deformation that counteracts these forces.

VISUALIZING ACTIN NETWORKS IN THEIR NATIVE CONNECTION TO MEMBRANES

The network of actin filaments beneath the membrane is the major substrate for the generation of force that drives motility and shape changes in a eukaryotic cell. In highly motile cells such as those of *Dictyostelium*, organization of actin networks does change on the sub-second scale.[2] There are two major difficulties in preserving the structure of such networks for electron microscopic investigation: the networks are membrane-anchored and the filaments are highly dynamic because regulatory proteins will rapidly bind and dissociate. Moreover, actin filaments are not easily seen in thin sections. A convenient technique to visualize these filaments involves removal of the cell membrane by detergent and extraction of soluble proteins, accompanied by stabilization of the cytoskeleton with glutar- or formaldehyde.[3] Alternatively, cells firmly attached to a substrate surface can be unroofed by sonication whereby the cytoskeleton on the bottom surface of the cells is stabilized by fixatives.[4] These techniques have provided us with basic information on the organization of actin networks and their specialization at the leading edge of a cell or at the membrane of intracellular organelles. Nevertheless, there is a demand for procedures that prevent delicate actin networks from collapsing when their anchorage to the membrane is disrupted. Details of the structure may be altered and short filaments may get lost during the extraction of proteins due to the slowness of chemical fixation. In order to preserve actin structures in context with the membranes to which they are anchored, we have adapted techniques of cryo-electron tomography to eukaryotic cells. Initially, these techniques have been established for the 3-dimensional reconstruction of purified macromolecular complexes, and of membrane structures in prokaryotes.[5-7]

Cryo-electron tomography combines vitrification of the specimen with tomography, to reconstruct 3-dimensional images of protein complexes inside intact cells. Advantages of cryo-electron tomography are the instantaneous arrest of cellular structures in their actual state of activity, the preservation of the cytoskeleton in its native context with the membrane, and the possibility of omitting any chemical treatment for fixation or metal decoration of cellular structures. Because of these benefits, cryo-electron tomography of intact cells can complement other approaches even if it may not replace them. The thickness of an accessible portion of a eukaryotic cell is currently limited to 600 nm. This implies that, without injuring the cells, excess fluid has to be removed by blotting until a film of <1 μm thickness is left. This procedure makes it difficult to record the activities of a cell by light microscopy up to preparing the specimen for electron microscopy, as it can be done by live-cell video microscopy followed by an extraction technique.[3]

ACTIN FILAMENTS ARE DISCONTINUOUS IN THE FAST GROWING FILOPODIA OF *DICTYOSTELIUM*

Filopodia are actin-rich cell-surface protrusions of about 100–200 nm in diameter and several micrometers in length, which are optimally suited to cryo-electron tomographic examination of actin-membrane linkages. Actin filaments in the comprehensively studied filopodia of melanocytes and other mammalian cells are proposed to be nucleated once at the beginning of filopod formation and to grow continuously as the filopod elongates.[3] Our data suggest that in *Dictyostelium* cells the arrangement and length of actin filaments varies along the filopodia. The following discussion will be based on the data obtained by electron-tomography in *Dictyostelium*. It should be kept in mind, however, that different types of filopodia might have different structures and different modes of elongation.

The arrangement of actin filaments in the filopod's tip region is of special interest. In this region actin is going to assemble,[8] while filament-bundling proteins diffuse into the filopod from the base.[9] In the filopodia of *Dictyostelium* cells, filaments are short in the tip region, and arranged in a cone pointing to the hemispherical tip of the filopod (Fig. 1). With one end these filaments are connected to the middle region of the hemisphere and with their opposite end they are connected to lateral sites of the membrane. We refer to this arrangement as the "terminal cone" of actin filaments in a filopod.

There are no straight filaments found to connect the tip region with the shaft of the filopodia in *Dictyostelium* cells. On the contrary, the transition zone between the terminal cone and the shaft is characterized by transversal filamentous elements. Further down along the shaft, actin filaments are mostly bundled and arranged in axial direction. Nevertheless, there is no general continuity of filaments within this region. This is immediately obvious for filaments that attach with either their distal or proximal end to the lateral membrane of the shaft.

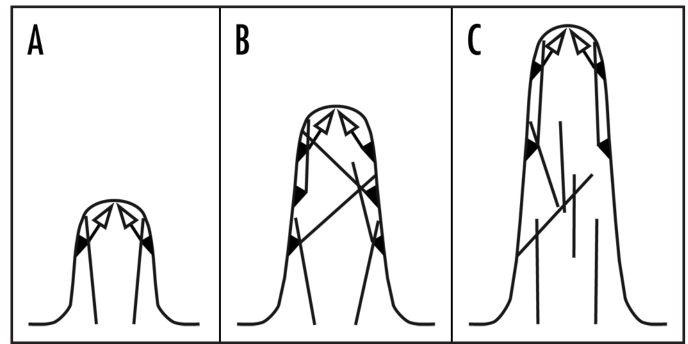

Figure 1. Model of actin organization in growing filopodia. This diagram illustrates the arrangement of actin filaments as revealed by cryo-electron tomography, in particular the conical arrangement of short filaments at the tip of filopodia in *Dictyostelium* cells. As an essential aspect of the model, de-novo nucleation of actin polymerization along with the growth of a filopod is proposed (A). This assumption is based on the finding that actin filaments in the tip region contact the membrane with both their ends. We assume that the proximal (lateral) membrane contact represents the nucleation site (filled triangles) and the distal (central) contact the elongation site (open arrowheads). At the borderline between tip region and shaft, filaments that stopped growing are interspersed with newly nucleated ones (B). Further down along the filopodia, most filaments separate from the membrane, but some remain in contact with the membrane at their proximal or distal ends (C). For reasons of clarity, the extensive bundling and membrane association along the length of actin filaments has not been included into this diagram, and also the possibility that actin filaments are getting severed has not been taken in account.

RATE OF FILOPOD GROWTH AND PUTATIVE FILAMENT REARRANGEMENTS

To relate actin network structure to filopod protrusion and retraction in live cells, we have complemented cryo-electron tomography with live-cell recording of filopod dynamics. Filopodia in the fast moving *Dictyostelium* cells extend with velocities of about 1 μm x sec^{-1} or more, this means at rates at least 60-fold higher than the actin assembly rate in neuronal filopodia.[8] Taking the increment in the filament length of 2.7 nm per actin monomer added, and assuming an average angle of 20° for the orientation of actin filaments relative to the axis of a filopod, the filopod will be elongated by 2.5 μm upon the addition of 1000 subunits to each working filament (neglecting elastic deformation in the actin network). A filopod protrusion rate of 1 μm x sec^{-1} will then correspond to the incorporation of 4 x 10^2 subunits per second into an actin filament. The rate in fast growing filopodia is thus close to the average polymerization rate of actin filaments in the cortical network of *Dictyostelium* cells, which corresponds to 11 x 10^2 subunits per second and is thought to be a formin mediated process.[2] The localization of DdDia2 to the filopod tip and its involvement in the formation of filopodia[10] strongly suggests a role of this formin in actin polymerization at the terminal cone of actin filaments.

Since the filaments in the tip region end up with both their proximal and distal ends at the membrane they need to be rearranged in order to be converted into the array of mostly parallel and bundled filaments that fill the shaft of the filopod. Rearrangements of actin filaments have been comprehensively analyzed during bristle formation in Drosophila.[11] In the bristles, modules of bundled actin filaments are formed at the growing tip and are processed into long bundles by filament elongation, possibly sliding and, most importantly, by the grafting of modules in a head-to-tail configuration. This process is followed by smoothing of the overlaps. Filopodia in *Dictyostelium* are much shorter than the bristles and their rate of extension is much higher. The filopod length of a few micrometers is the length of one module in the bristles, and the filopod extension rates are 1 μm per second or more as compared to 25 μm per hour for the bristles. Because of these differences, the way of rearrangement in the filopodia most likely differs from that in the bristles.

A model based on repetitive nucleation of actin filaments in the filopodia of *Dictyostelium* cells is outlined in Figure 1. The capacity of de-novo nucleation during the growth of a filopod is provided by the presence of DdDia2 specifically at the filopod tip. This formin has nucleating activity and is required in *Dictyostelium* cells for the formation and maintenance of filopodia.[10] De novo nucleation of actin filaments is indicated by the repeated branching of *Dictyostelium* filopodia whereby each branch retains the thickness of the common stem.[12] The model of Figure 1 is also consistent with previous findings indicating that actin polymerization is confined to the tip of a filopod,[8] as indicated in the diagram by arrow-headed filaments. According to this "sequential nucleation model", the filaments detach in the course of their growth with their distal and/or proximal ends from the membrane. Subsequently, the actin filaments are extensively bundled, and lateral connections of filaments to the membrane are formed along the filopod shaft. The number of filament ends may further increase by the severing of filaments along a filopod, in particular during the retraction phase. A role for severing

is indicated by the delayed filopod retraction at neuronal growth cones that is caused by knock out of the severing protein gelsolin in mice.[13]

Because of the discontinuity of actin filaments along the shaft of filopodia in *Dictyostelium*, the rigidity of the actin core and its capability of applying force on the membrane to lengthen the filopod critically depends on lateral struts of proteins that bundle or otherwise crosslink actin filaments. This network allows rapid reorganization and thereby bending of the filopodia, a flexibility important for the role of filopodia in catching bacteria and in subjecting them to phagocytosis on the surface of the cell body.

HOW IS FORCE GENERATED BY THE GROWING ACTIN FILAMENTS?

The protrusion of filopodia requires at their tip an optimal number of actin filaments with plus-ends that are open for subunit addition. Too few plus-ends cannot generate enough force to overcome the membrane resistance, whereas too many plus-ends deplete the pool of actin subunits and therefore grow slowly.[14] About 10 filaments in a terminal cone is the number found in the fast growing filopodia of *Dictyostelium* (Table 1 in the supplement of ref. 1).

If the free length of an actin filament is too high, the filament will buckle even under a small load.[15,16] Our data indicate that buckling is not a limiting factor in filopodial force generation. At the terminal cone, the filaments are short and their angles of incidence are small: even the longest filaments of about 250 nm in length are not evidently buckled. It may be relevant that the force required for buckling is raised by about one order of magnitude if the filament end contacting the membrane is tethered rather than free to slide.[17] The centering of actin filaments to the midpoint of the hemisphere suggests that the filament ends are indeed fixed within a small area at the tip of the filopod.

Two classes of theories address how actin subunits are incorporated at the growing tip of an actin filament in close apposition to the membrane of the cell. One class assumes the presence of a polymerization machine,[17] the other is based on Brownian ratchets and does not require a special apical complex.[15,16] ATP-driven molecular machines would raise the polymerization force beyond the thermodynamic limit that is given by the cellular concentration of ATP-actin and the inherent cleavage of ATP along the actin filament, by an additional amount of energy captured from ATP-hydrolysis.[17]

Brownian ratchet models for actin-driven membrane protrusion depend on the creation of free space between the plus-end of the filament and the membrane, where actin monomers can be added to the free end of the filament. This space can be created by thermal fluctuations of the membrane and by bending fluctuations of the filament, which depend on its bending stiffness, its free length, and on its angle of incidence to the membrane. Estimates of the forces produced and the velocities of protrusion achieved largely depend on the specific version of the ratchet model on which calculations are based; for instance whether filopod protrusion is thought to be effected by a co-axial bundle of strongly cross-linked actin filaments,[18] and whether the membrane on top of the actin filaments is assumed to be rigid or flexible enough to be deformed by single filaments.[19]

MEMBRANE PROTRUSION IN THE TERMINAL CONE

In order to push the filopod membrane ahead, the short filaments at the filopod's tip have to act against the resistance of the membrane. Theoretically, force that resists deformation of the membrane has three components: bending elasticity of the membrane, in-plane membrane tension, and adhesion between the lipid bilayer and the underlying cortical cytoskeleton. For practical purposes, membrane tension and cytoskeleton adhesion are combined in an "effective membrane tension" term, T.[20]

To closely mimic the process of filopod extension, optically trapped beads have been used to pull thin membrane extensions, the tethers, out of the plasma membrane. The static tether force, F_t, needed to keep a membrane tether at a constant length, is approximated by $F_t = \pi B/R_t + 2\pi R_t T$, where B is the bending modulus and T the effective membrane tension.[21] Although thin tethers have not been pulled out of the membrane of *Dictyostelium* cells, deflection of the membrane in response to shear forces has been measured in order to determine B and T.[22] Using the values of B = 2 pN x µm and T = 3 pN x μm^{-1} obtained by this method, and assuming their uniform distribution over the membrane, F_t = 64 pN is calculated for filopodia with a radius of 100 nm. This is taken as a lower limit since much higher values for F_t have been obtained by deforming *Dictyostelium* cells using micropipette aspiration.[23] Moreover, for the protrusion of filopodia with velocities of 1 µm x sec^{-1} or higher—as found in *Dictyostelium*—forces higher than the static tether force are needed.

WHO HELPS ACTIN FILAMENTS TO PUSH OUT A FILOPOD?

Direct measurement in vitro of the force produced by the formin-assisted polymerization of single actin filaments resulted in an estimate of ≥1.3 pN in a solution of 0.7 µM ATP-actin,[24] a concentration that is not far from equilibrium.[25] Taking this conservative measure of force generation one would end up, for the filopodia of *Dictyostelium*, with an average of only 12 pN for the cumulated forces produced by the actin filaments in a terminal cone (see Table 1 in the supplement of ref. 1). Therefore, it appears to be a gap between the forces required to overcome resistance of the membrane and the forces produced by actin polymerization under these conditions. This apparent gap may be filled under the non-equilibrium conditions of a living cell by enhancing the force produced by actin polymerization, and also by mechanisms that lower the resistance of the membrane at the site of filopod formation. In accord with this notion, theoretical considerations indicate that lengths of filopodia >2 µm and growth rates >0.1 µm x sec^{-1} cannot be explained by a simple model based on the diffusion of actin monomers and the existence of a linear bundle of cross-linked filaments within a filopod.[18]

The involvement of a molecular device in the elongation of filopodial actin filaments is suggested by the presence of DdDia2 at the interface between actin filaments and the curved membrane of the filopod tip.[10] DdDia2 belongs to the family of formins that act at the plus-end of actin filaments as processive polymerization machines.[26] Moreover, a plus-end directed motor protein, myosin VII, is localized to the filopod tips in *Dictyostelium* and plays a role in the extension of filopodia.[27]

Alternatively, or in addition, membrane protrusion could be facilitated either by disconnection of the membrane from the cytoskeleton or by local changes in lipid or protein composition in a way that favors bending.[28] Evidence for the latter mechanism is

provided by the requirement for filopodia induction of proteins that deform membranes into tubules.[29] The proteins IRSp53 and MIM (missing-in-metastasis) share a domain (IMD) at their N-terminus, which binds to PI(4,5)P_2-rich membranes from the inside and thereby converts them into tubular structures.

OUTLOOK ON FURNISHING THEORIES WITH EXPERIMENTAL DATA

The data obtained by cryo-electron tomography are a step toward a comprehensive model of actin-based membrane protrusion in motile cells. Our 3D-reconstructions invoke a comparison with simulations for the stochastic growth of actin filaments taking into account polymerization and depolymerization, capping, membrane resistance, and branching by the Arp2/3 complex.[30] The simulated three-dimensional networks have been analyzed using measures of filament orientation, network density and velocity of growth. Although the Arp2/3 complex does not seem to play a role in filopod elongation, there are similarities between the modeled structures and the electron tomograms. For instance, under conditions of plus-end uncapping near the obstacle, some filaments become much longer than the average in the simulated networks, in accord with the broad range of filament lengths found in the reconstructed filopodia of *Dictyostelium*. Comparison of such analysis in silico with quantitative data extracted from electron tomograms will provide a detailed view on the mechanics of membrane-anchored actin networks in their relation to membrane protrusion.

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