Cytoskeletal Protein Dynamics & Intracellular Cargo Transport

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The Pro-apoptotic Protein Par-4 Regulates Myosin Light Chain Phosphorylation By Activating Myosin Phosphatase Activity

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Prostate apoptosis response-4 (Par-4) is a protein that has primarily been connected with apoptotic processes. Recently, we have shown that Par-4 also plays a role in smooth muscle contractility. Here, we have tested the hypothesis that Par-4 regulates the phosphorylation state of myosin regulatory light chain (LC20) by modulating the activity of myosin phosphatase. In the smooth muscle-derived cell line A7r5, Par-4 colocalizes with the targeting (MYPT1) and catalytic (PP1cδ) subunits of myosin phosphatase on actin filaments. Proximity ligation assays demonstrate a close proximity of Par-4 and MYPT1 in vivo. Moreover, endogenous MYPT1 and PP1cδ co-immunoprecipitate with endogenous Par-4 from A7r5 lysates. Direct binding of Par-4 and MYPT1 is shown by surface plasmon resonance, and the leucine zipper of MYPT1 is required for direct binding. The domain of Par-4 that mediates interaction with MYPT1 has been mapped to the leucine zipper motif in co-immunoprecipitation experiments and in proximity ligation assays. LC20 phosphorylation assays using the proximity ligation assay revealed that overexpression of Par-4 and a phosphorylation site mutant of Par-4 (T155A), but not a leucine-zipper defective mutant (L3A), leads to reduced phosphorylation levels of LC20, suggesting activation of myosin phosphatase by Par-4 in a leucine-zipper dependent manner. Moreover, the co-expression of either Par-4 mutant, but not wild type Par-4, with zipper-interacting protein kinase (ZIPK) interferes with ZIPK mediated LC20 phosphorylation. Our results demonstrate that Par-4 interacts with subunits of the myosin phosphatase in vivo, possibly as an accessory protein that supports the catalytic activity of myosin phosphatase by an as yet unknown mechanism. At the same time, Par-4 is required for efficient inhibition of myosin phosphatase by ZIPK. Support: HL31704, HL80003, HL86655, AR41637 from the NIH, AHA postdoctoral fellowship to SV.

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Microinjection of Smooth Muscle Myosin in Cultured Cells

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Our current knowledge on the ultrastructure of smooth muscle (SM) cells does not satisfactorily account for the particular length-tension behavior of SM, which seems to be able to adapt its contractile apparatus to generate great force over a wide range of lengths. A key question in the field remains: Are contractile filaments in SM cells a fixed array or dynamic structures? There is insufficient knowledge as to whether the myosin filaments can indeed reorganize themselves in vivo as has been shown through proposed models. We are proposing here to develop a microinjection technique to address this question.
Methods: Smooth muscle myosin was purified from chicken gizzard using serial precipitation. As a control, monomeric myosin and self-assembled filaments of the purified myosin were tested for functionality using an in vitro motility assay. The myosin was then labeled with a rhodamine-derivative fluorophore, tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA). The labeled myosin was then self-assembled in vitro into filaments and images were taken using a spinning-disk confocal microscope. The labeled filaments, as well as monomeric myosin were then respectively microinjected into cultured A5R7 vascular SM cells. The volume of the cells was estimated by measuring them using a calibrated microscopic ruler. The volume of myosin solution that was microinjected represented 2 to 5% of the estimated volume of the cells. The cells' viability was estimated by observing their morphology after the microinjection.
Results: The purified myosin was functional in the in vitro motility assay, both in a monomeric and filamentous form. The microinjection technique could be done with little modifications of the morphology of the injected cells in a reasonable number of cells.
Conclusion: The microinjection technique of purified myosin can be used for further studies on the ultrastructure and dynamics of myosin filaments in smooth muscle cells.

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Essential Features of a Non-processive Class V Myosin from Budding Yeast for *ASH1* mRNA Transport

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A feature of most class V myosins is their ability to move processively on actin. The budding yeast *Saccharomyces cerevisiae* has a non-processive class V myosin, Myo4p, which is a single-headed but high duty cycle motor. Its cellular role is to asymmetrically transport more than 20 different mRNAs, a widely used strategy to polarize a protein within the cell. The most studied mRNA is *ASH1*, which is moved by Myo4p to the bud tip to repress mating type switching in the daughter cell. Here we determine the features of Myo4p that are necessary for correct localization of *ASH1* mRNA to the daughter cell. This process requires the adapter protein She3p, and the mRNA binding protein She2p, which binds *ASH1* at specific localization elements called zip codes. Based on a series of chimeric constructs, we showed that the rod region of Myo4p, but not the globular tail, is essential for correct localization of *ASH1* mRNA. The rod thus contains the primary binding site for She3p, consistent with our earlier *in vitro* studies (Hodges et al., 2008). To test if mRNA localization is more efficient when two motors are coupled together, we compared transport by a constitutive dimer of Myo4p/She3p with a constitutive monomer. Correct *ASH1* mRNA localization was achieved equally well with both constructs. This may reflect the fact that many mRNAs and thus many motors are part of the translocation complex. Our results show that the most important feature for correct localization is the retention of coupling between all the members of the complex (Myo4p- She3p-She2p-*ASH1* mRNA), which is aided by She3p being a tightly bound subunit of Myo4p.

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Towards Custom-topology Tracks For Probing Myosin Motor Dynamics

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An important part of cellular transportation infrastructure is formed by myosin motors that use actin filaments as their tracks. Many questions concerning the mechano-chemistry of those motors and the degree of evolutionary adaption to the track topology remain elusive because we cannot tailor the geometric properties of F-actin.
Here, we employ three-dimensional DNA origami to construct hybrid DNA-actin filaments with precisely controlled geometric properties. We engineered building blocks exhibiting custom-shaped cavities that host F-actin tetramers such that myosin motors may still access their usual binding site on actin. We describe triggering the formation of filaments from those building blocks that grow to lengths above 10 microns and that have periodically aligned cavities for hosting actin segments. Binding-site orientation can be rotated by 90 degrees with respect to the filament axis to probe myosin's ability to take sidesteps. We propose the following strategy for hosting actin fragments: F-actin is labeled with thiol-modified oligos, followed by capture of this oligo by the DNA track building blocks, followed by filament bending into the custom shaped cavity on each building block, followed by filament breaking fueled by DNA hybridization.

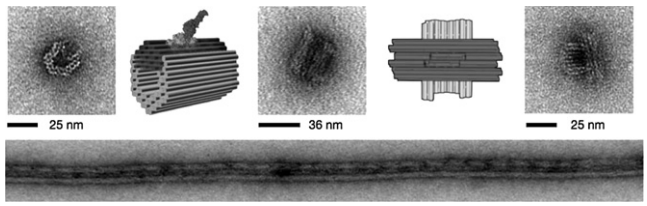

Figure 1. Upper panels: TEM micrographs and schematics of actin-hosting DNA track building blocks. Bottom: TEM micrograph of a synthetic filament with periodically spaced actin hosting cavities.

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An Open Model of Actin Dendritic Nucleation

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The availability of quantitative experimental data on the kinetics of actin assembly has enabled the construction of many mathematical models focused on explaining specific behaviors of this complex system. However these ad hoc models are generally not reusable or accessible by the large community of actin biologists. In this work, we present a comprehensive model that integrates and unifies much of the in vitro data on the components of the dendritic nucleation mechanism for actin dynamics. Over 300 simulations have been run based on compartmental and 3D spatial versions of this model. We have examined the behavior of the overall system as a function of the concentrations of actin, capping protein, thymosin-ß4, profilin and ADF/cofilin. We are able to provide an explanation for the sharp boundary between actin assembly and

disassembly in the lamelipodia of migrating cells. The model also provides insights on the critical interplay of capping protein and ADF/cofilin in the regulation of F-actin assembly. Because this model and the simulation results are ''open source'', in the sense that they are publicly available and editable through the *Virtual Cell* database (http://vcell.org), they can be accessed, analyzed, modified and extended. (Supported by NIH grants P41 RR013186, U54 RR022232 and U54 GM64346)

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Real-time Observation Of Actin Polymerization Regulated By The Gelsolin-family Of Proteins
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Cell motility is governed by the concerted assembly and disassembly of actin filaments.[1] Actin filament length is regulated by a variety of actin-modifying proteins such as gelsolin, vilin and adseverin. To understand the mechanism of cell movement in health and disease, a detailed understanding of the self-assembly of actin monomers into filaments and its regulation by the actin-modifying proteins is required. Conventional bulk measurements yield ensemble-averaged data which do not allow an understanding of the process at the single molecule level. Total internal reflection fluorescence (TIRF) microscopy coupled with fluorescence spectroscopy measurements provide a means to follow the actin dynamics with single molecule sensitivity. In the present work, *in vitro* real-time TIRF assays of actin polymerization in the presence of full length and truncated actin-regulating proteins such as gelsolin, vilin and adseverin will be presented. Use of calcium ions as a switch to activate gelsolin[2,3] is further corroborated in the time-lapse movies.
Footnotes
[1] T.D. Pollard, Annu. Rev. Biophys. Biomol. Struct. 2007. 36:451-477.
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Computational Modeling of Antigen Processing and Presentation by Dendritic Cells
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The stimulation of T lymphocytes (CD4+ or CD8+) by dendritic cells (DCs) is a key event in the initiation and establishment of immune responses against pathogens. Understanding the intracellular mechanisms that govern how DCs acquire, process, and present antigens would lead to more rational vaccine design. Although individual intracellular events have been elucidated, a quantitative view of how the various networks of antigen trafficking affect T cell stimulation is lacking. In this work, we developed a stochastic model to examine the critical steps involved in antigen delivery for T cell stimulation, including antigen internalization, trafficking in endosomal/lysosomal environments, access to various antigen presentation pathways, and stimulation of either CD4+ or CD8+ T cells. Kinetic parameters for various processes were either obtained from previous reports if available, or derived from our own experimental data. In particular, we aim to identify rate-limiting steps of antigen trafficking and processing that regulate T cell stimulation. Furthermore, we examine how characteristics of the vaccine, such as size and attachment of targeting ligands, affect whether delivered antigen stimulates CD4+ or CD8+ T cell responses. The development of the computation model of antigen delivery will lead to greater insight into the intracellular processes involved in the type of T cell response elicited and to more rational design of effective vaccines.

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Regulation and single-molecule mechanics of microtubule-based motors in living Chlamydomonas
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Whether motors of different directionality are functionally coordinated in cells or operate in a semi-random ''tug of war'' is unclear. We tested the hypothesis that the microtubule-based motion of the transmembrane protein FMG-1 in the flagella of Chlamydomonas is functionally coordinated for unidirectional transport. A laser trap was used to position microspheres on the plasma membrane of paralyzed Chlamydomonas flagella. The anterograde and retrograde movements of the microsphere were measured with nanometer resolution as microtubule-based motors moved FMG-1. Based on stall forces, we find that an average of 10 motors act to move the microsphere in either direction, with mean step sizes of 4 and 8 nm. Reverse steps were uncommon, and quiescent periods separated every transport event, suggesting the exclusive activation of motors of one direction. Temperature-sensitive mutants of kinesin-2 showed exclusively retrograde steps after jumps to the non-permissive temperature. These data suggest that molecular motors in living cells can be reciprocally coordinated to engage in large numbers and for transport in a single direction, even when motors of mixed directionality are present. The predominance of 4 nm steps suggests sub-steps or other novel behavior of these motors in the cytoplasm. This novel technique should prove beneficial for studying the mechanics, regulation and bidirectional coordination of molecular motors in vivo.

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Multiple-Motor-Based Transport
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Intra-cellular transport via processive molecular motors plays an important role in maintaining cell structure and function. In many cases, cargoes move distances longer than expected for single motors; there is significant evidence that this increased travel is in part due to multiple motors working together to move cargoes. However, while we understand much about the function of single motors both experimentally and theoretically, our understanding of how multiple motors work together to move cargoes is less developed. We start with a Monte-Carlo model of single motor to theoretically investigate how multiple motors work together. We have investigated the effect of non-linear force-velocity curves and stochastic load sharing on multiple motor transport using stochastic model. We are particularly interested in cargo transport by a few molecular motors which is motivated by in-vivo results that only a few motors are engaged to transport cargo. Predictions for average travel distances and mean velocities obtained from stochastic model are significantly different from those predicted using steady-state model. Our theoretical study of multiple motor transport using stochastic model also shows that single-motor force-velocity curve plays an important role in determining the ensemble function when only a few motors are engaged.

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Transport Of Micrometer-Sized Vesicles By Kinesin In Vitro
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Cytoskeletal motor proteins (e.g., kinesin) are responsible for directed transport in cells. Motor proteins can also be used in artificial bionanotechnological systems to provide a controlled cargo transport. We explore this possibility by using giant unilamellar vesicles (GUVs) as a micrometer-sized cargo model and establish an in vitro system to transport this cargo by kinesin (rK430) molecules along surface-attached microtubules (MTs). Kinesin was linked to GUVs (diameter 1–4 μm) via biotin-streptavidin interaction. MTs and moving GUVs were visualized using fluorescence wide-field imaging microscopy. We observe directed transport of GUVs along MTs with traveling distances of up to 100 μm and velocities of ~0.7 μm/s being in a good agreement with the velocity of kinesin motion along MTs (~0.8 μm/s). The long walking distances, as well as the visualization of the GFP-labeled kinesin molecules by total internal reflection fluorescence imaging, suggest that a large number (>10) of kinesin molecules is involved in the transport of a single GUV. Apart from its biotechnological importance, this system might additionally be useful to gain further understanding of vesicle transport processes in cells.

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Intermittent Search Strategies for Delivering mRNA to Synaptic Targets
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We model the motor–driven transport of an mRNA containing granule along a dendrite in terms of a random intermittent search for a synaptic target. The granule is injected at one end of a one–dimensional track with an absorbing boundary at the other end. The particle switches between a stationary phase and a mobile phase that is biased in the anterograde direction. A single hidden target is located at a fixed but unknown location on the track. We calculate the hitting probability and conditional mean first passage time for finding the target, and determine conditions for an optimal search strategy.

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Microtubule elasticity: Connecting all-atom simulations with continuum mechanics
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The mechanical properties of microtubules have been extensively studied using a wide range of biophysical techniques. These experiments have sought to understand how the mechanics of these cylindrical polymers is related to their