Cytoskeletal Protein Dynamics & Intracellular Cargo Transport

665-Pos Board B544

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.

666-Pos Board B545

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.

667-Pos Board B546

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.

668-Pos Board B547

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.

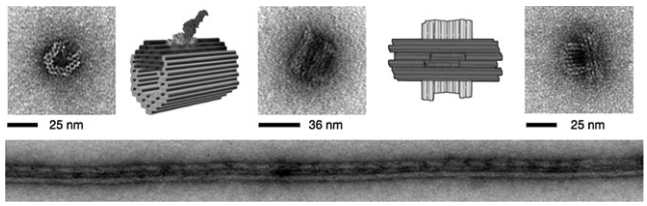

Figure 1. Upper panels: TEM micrographs and schematics of actin-hosting DNA track building blocks. Bottom: TEM micrograph of a synthetic filament with periodically spaced actin hosting cavities.

669-Pos Board B548

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