transition state of the force dependent step) to be 12 nm by a fit of the force dependence of actin attachment times in the presence of 50 micromolar ATP. In this study, we examine the force dependence of a myo1b truncation mutant that contains only the IQ motif closest to the motor domain. Although the unloaded biochemical kinetics of this mutant are nearly identical to the 5 IQ construct, we found the actin detachment rate to be substantially less force sensitive. These experiments suggest that the length of the regulatory domain modulates force sensitivity.

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Single-molecule Measurements Of Myo1c-PIP2 Detachment Forces Using Optical Tweezers

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Optical tweezers has become one of the most efficient techniques to accurately measure small forces (pN) and displacements (nm) upon interactions of individual biological molecules, particularly molecular motors. In the current study we extend the application of optical tweezers to measure protein-lipid detachment forces at the single-molecule level. Myo1c is a single headed, force-generating motor that links cell membranes to the underlying actin cytoskeleton. Actin binding occurs via the motor domain, while the tail domain interacts with phosphatidylinositol 4,5-bisphosphate (PIP2). To determine the forces required to detach the myo1c tail domain from PIP2, we used spherical supported bilayers composed of 1 μm diameter silica beads coated with 2% PIP2 and 98% dioleoyl-phosphatidylcholine (PC). The efficiency of coating was verified by examining the distribution of fluorescently labeled lipids, and the specificity of binding was confirmed by sedimentation assays. The myo1c tail domain binds to the spherical supported bilayers containing a PC/PIP2 mixture, but not PC alone. Additionally, myo1c dissociates from the PC/PIP2 beads in the presence of inositol 1,4,5-trisphosphate, which effectively competes with PIP2 for binding. For the laser trap measurements, 2 μm diameter silica pedestals were immobilized under a layer of nitrocellulose on a coverslip and were coated with either anti-his-antibody or neutravidin to anchor tetra-His-tagged or biotinylated constructs of myosin-I in a specifically oriented manner. The bilayer coated beads trapped by a laser beam were brought into contact with spherical pedestals decorated with the myo1c tail domain. Upon repeated contact and retraction cycles, binding events and subsequent disruption forces were measured. The most probable rupture force of the PIP2/myosin-I tail interactions are ~ 7.1 pN at a loading rate of 360 pN/s.

717-Pos Board B596

High Speed Imaging For Myosin VI

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Myosin-family molecular motors have been a subject of extensive research recently. While there is a general consensus on how do these motor molecules perform their function, myosin VI presents a serious challenge to a conventional view. It has been reported that myosin VI moves processively in a hand-over-hand manner toward the minus end of actin filament, opposite to other classes of myosin. The head takes large steps (50 - 70 nm) relative to its short lever arms (7.2 nm), and frequently moves backward with half the size of a forward step. Using a video rate FIONA technique, we observed the above features under the several ATP concentrations (10 uM ATP, ~67 ± 19 nm, N=206; 100 uM ATP, ~73 ± 13 nm, N=362; 1000 uM ATP, ~73 ± 19 nm, N=105). The back-steps frequently occur (~8%), and its size is half of the forward step (42 ± 14 nm, N=57). The mystifying observation of a large step size and its large distribution has been explained in terms of the proximal tail domain to unwind which allows the myosin VI molecule to stretch out. However, this hypothesis does not support the exerted mechanical force, which was measured in myosin VI to be 2 pN without slowing.

We believe that the major action takes place during the rapid stepping transition (<30 msec) between a quick release of a rear head from the actin molecule and a strong rebinding event. To address all the above questions, we are currently working on the direct observation of the one head dynamics using a dark-field imaging microscopy with much-improved microsecond time resolution.

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Temperature Dependent Energy Transfer Measurements Reveal Flexibility in the Upper 50 kDa Domain of Myosin V

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Our previous work has demonstrated that labeling myosin V in the upper 50 kDa domain with the biarsenical dye FlAsH can serve as an acceptor for fluorescence resonance energy transfer studies with mant labeled nucleotides and IAEDANS actin. These FRET studies suggest that myosin V can adopt a conformation in which the nucleotide binding pocket and the actin binding cleft are in a closed conformation. Our studies suggest the upper 50 kDa domain may be highly flexible in certain nucleotide-states which allows tight binding to nucleotide and actin. Molecular geometric simulations demonstrate the upper 50 kDa domain is most flexible in the myosin V.ADP state, consistent with this state having a high affinity for ADP and actin. Currently, we examined the temperature dependence of the FRET signal between mantADP and MV FlAsH. We found that at low temperature (4-15°C) a high FRET state dominates (closed pocket) while at high temperature (30-37°C) a low FRET state dominates (open pocket). This transition is reversible suggesting a temperature-dependent conformational change. We also found that FlAsH labeled G440A MV, a non-hydrolyzable mutant, has a similar temperature-dependent transition in the presence of mantATP. In contrast, the transition does not occur in the presence of mantADP.BeFx or with the non-hydrolyzable E44A MV mutant in the presence of mantATP. Our results suggest coordination of the gamma-phosphate of ATP rigidifies the upper 50 kDa domain which results in a weak actin affinity state (open actin binding cleft and closed nucleotide binding pocket). However, upon phosphate release the upper 50kDa domain becomes more flexible which allows myosin to adopt a conformation in which it has a high affinity for both nucleotide and actin (closed nucleotide binding pocket and actin binding cleft).

719-Pos Board B598

Kinetics Of Myo1c Association To And Dissociation From Phosphoinositide-containing Vesicles

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Myo1c is a single-headed unconventional myosin that associates with negatively-charged lipids through electrostatic interactions. A putative pleckstrin homology (PH) domain has been identified in the myo1c tail that binds $PI(4,5)P_2$ with high affinity. However, the kinetics of association and dissociation, as well as the influence of membrane phospholipid composition and Mg^{2+} on the kinetics, remain unknown. Stopped-flow measurements were made using the increase in light scattering that occurred upon myo1c-tail binding to 100 nm diameter large unilamellar vesicles (LUVs).

We found that the association of myo1c-tail with phosphatidylcholine (PC) LUVs containing 2% $PI(4,5)P_2$ followed a 2-exponential time-course. The rate of the predominant, fast phase depended linearly upon the total lipid concentration. The apparent second order rate constant in the absence of Mg^{2+} was approximately diffusion-limited, indicating that no conformational change occurs upon binding. The molar ratio of anionic lipid was increased by adding phosphatidylserine (PS) or additional $PI(4,5)P_2$ to LUVs or by situating $PI(4,5)P_2$ in a more physiologically relevant lipid background (phosphatidylethanolamine, PC, PS, phosphatidylinositol, and sphingomyelin). None of these conditions increased the apparent association rate constant much more than two-fold. Dissociation of myo1c-tail was measured by chasing with excess inositol 1,4,5-trisphosphate ($InsP_3$). The presence of additional anionic phospholipid reduced the observed dissociation rate constant by orders of magnitude ($3.2\ s^{-1}$ vs. $0.03\ s^{-1}$). This suggests that once myo1c-tail interacts via its putative PH domain with $PI(4,5)P_2$, additional electrostatic interactions between positively-charged regions of the tail and negatively-charged lipids help to stabilize binding. The presence of Mg^{2+}, known to interact with polyvalent anions, did not alter these trends, though it did increase the dissociation rate for all lipid compositions. Finally, we measured the dissociation rate of myo1c-tail from $InsP_3$ and found that it approximates the dissociation rate from $PI(4,5)P_2$.

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Single Molecule Investigation of the Acto-Myosin-10 Complex Using Optical Tweezers

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Recent cell biological studies of myosin-10 have revealed that myosin-10 is essential to cellular processes such as filopodia extensions and phagocytosis. Steady-state and transient kinetic biochemical studies of the ATPase cycle of a single-headed, subfragment-1 like (S1), construct of myosin-10 show that it has an intermediate duty-cycle ratio. It remains tightly bound to actin for about 16% of its total ATPase cycle time but spends around 90% associated with actin in both weak and strongly bound states. Furthermore, the acto-myosin-10-S1 complex has two ADP bound states and a surprisingly low affinity for actin, comparable to that of the rigor complex between actin and skeletal muscle myosin II. To study the mechano-chemical coupling of myosin-10, we used the

actin filament sliding assay and a dual-beam, optical tweezers apparatus to perform single molecule mechanical studies. For these studies we used a two-headed, heavy meromyosin-like (HMM) construct of myosin-10 that contained a leucine-zipper at the C-terminal end to force dimerization. The actin filament gliding assay showed that myosin-10-HMM moves filaments at a velocity of ~125 nm s^{-1}. This is similar to the speed of intact, GFP-tagged, myosin-10 moving within filopodia of live mammalian cells (~140 nm s^{-1}) measured by TIRF microscopy. Optical trapping results showed that the average power-stroke size was ~10 nm, with the rate of ATP binding of ~1.3 μM^{-1} s^{-1}. In most of the raw data traces, we observed displacements of unitary size, however at low ATP concentrations (2 μM) we also observed a number of interactions that exhibited multiple, staircase-like movements consisting of up to 3 steps per binding interaction. This behaviour is characteristic of a *processive* molecular motor. We will discuss these measurements in the context of mechano-chemical coupling and the functional significance in the living cell.

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Bayesian Estimation for Hidden Information of a Single Molecular Motor

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In single-molecule experiments of molecular motors, it is a widely adopted strategy to visualize the motion of a molecule by attaching a large probe particle. In such a situation, only the motion of the probe can be monitored while the motion of the molecule is hidden. Therefore, in order to study a stepwise motion of the motor in detail, one has to estimate the system parameters and the hidden trajectory of the motor molecule at the same time on the basis of the observed trajectory of the probe. In this presentation, we will present a novel approach for this problem, which is based on the statistical mechanics and empirical Bayesian estimation. The method consists of several steps. First, we temporarily fix the parameters of the system. Then, we can utilize the Bayes theorem to estimate the trajectory of the motor from the trajectory of the probe. On the basis of the estimated trajectory, the marginal likelihood can be calculated using the WKB method. Finally, by maximizing the marginal likelihood, we can estimate the system parameters along with the trajectory of the motor. Although the above mentioned tasks appear straightforward, it is necessary to adopt appropriate approximation schemes in order to reduce the computational cost as well as artifacts resulting from the finite time resolution of the measurements. It is found that our method gives a reasonable solution to this problem. We will discuss the effectiveness of the method presented here by using a simple model that consists of two Brownian particles (a molecule and a probe) connected by a spring.

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ADP Affinity of Myosin VI is Regulated by Off-Axis Load

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Individual dimeric molecules of unconventional myosin VI move processively along an actin filament, taking multiple steps in a hand-over-hand fashion. Recently we showed experimentally that the efficient processive movement is achieved via asymmetric rates of ADP binding under the forward and the backward loads, that is, to the trailing and the leading heads, respectively, such that ADP dissociates more readily from the trailing head, though the difference in the ADP affinity between the heads is less pronounced than in case of myosin V-6IQ construct. Several lines of evidence, obtained by other groups, suggest that during the processive stepping both the azimuthal angle of myosin's VI lever arm and the path of the motor along an actin filament are very variable, which implies the existence of an off-axis load during the double-headed binding of myosin VI to actin. To reveal the effect of an off-axis load on the efficiency of the processive stepping, we directly measured the ADP affinity of individual single-headed myosin VI molecules bound to actin, under loads applied at certain angles to the long axis of actin filament, towards both barbed and pointed ends. The obtained results indicate that the off-axis load significantly affects ADP affinity of myosin VI motor domain and is an important factor regulating the efficient processive movement of the dimeric molecules of myosin VI.

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The Tail Binds To The Head-Neck Domain To Form A Folded-Back Conformation That Inhibits The Actin-Activated ATPase Activity Of Drosophila Myosin VIIA

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Myosin VIIA is an unconventional myosin, responsible for human Usher Syndrome type 1B, which causes hearing and visual loss. We have analyzed the molecular mechanism of regulation of myosin VIIA, which is not yet understood. While it was originally thought that myosin VIIA is a dimeric myosin, our electron microscopic (EM) observations revealed that full-length Drosophila myosin VIIA (DM7A) is monomeric. Interestingly, the tail domain markedly inhibits the actin-activated ATPase activity of tail-less DM7A in the absence of Ca2+ but not below pCa 6. By examining various deletion constructs, we found that deletion of the distal IQ domain, the C-terminal domain of the tail, or the N-terminal domain of the tail abolishes the tail-induced inhibition of ATPase activity.
EM study of full-length DM7A suggests that the tail domain folds back on to the head, such that the tail and the head/neck domain contact each other. Single particle analysis of full-length DM7A and of tail-less molecules to which exogenous tail has been added reveals that the tail contacts the head/neck domain at two sites, the motor core domain and the neck domain. These observations, together with the functional results, suggest that DM7A is inactivated by forming an inhibited conformation, in which the tail interacts with the motor and lever-arm domains. This is the first report to identify the regulatory mechanism of a single-headed unconventional myosin.

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The Medial-tail Domain of Myosin-VI as a Dimerization Region

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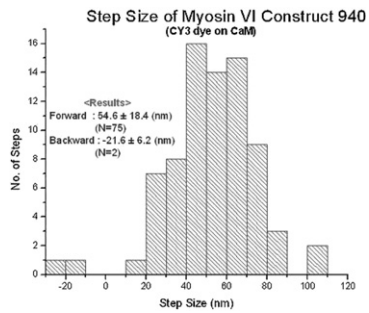
Myosin-VI has been one of the least-understood unconventional molecular motors due to its peculiar characteristics. For instance, each myosin-VI monomer has a short lever arm (~10nm) which contains only two Calmodulins while taking large steps (~36nm center-of-mass movements). Recently, the Spudich group proposed, based on bacterial expressed fragments, that the medial-tail domain of myosin-VI is a single alpha helix (~10nm), which can account for its large step sizes if dimerization occurs after the medial-tail domain. They also suggested that dimerization is formed via the cargo-binding domain. However, these results are contrary to our previous paper that showed that a myosin-VI construct without the cargo-binding domain can dimerize and walk processively. To solve this paradox, we prepared a myosin-VI construct truncated in the middle of the medial-tail domain (940-construct). A FIONA assay showed that this construct does dimerize either via antibody induction or actin-saturation method, and it walks processively with the same step size as the full-length myosin-VI construct. These results suggest that the medial-tail domain of myosin-VI is involved in dimerization and imply that the proximal-tail domain must be the major contributor to the unexpectedly large step size.



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Processivity of Myosin V and X on two-dimensional (2D) paracrystalline actin array

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Myosin movement in vivo takes place on a wide variety of F-actin structures, including single filaments and 2D/3D bundled networks. Using in vitro single molecule motility techniqes, we have investigated the processivity and stepping characteristics of myosin V HMM and myosin X HMM with a leucine zipper on single actin filaments and 2D actin bundles. To answer how myosin V and myosin X step on actin bundles, we observed single molecule motility of fluorescently labeled myosin V and X using total internal reflection fluorescent microscopy, and analyzed the step-size, run length, speed, and direction of the movements on actin-bundles. Actin was polymerized and cross-linked on a charged lipid monolayer in Teflon wells to create regular 2D actin arrays. Two cross-linking proteins were used: alpha-actinin, which produces non-polarized bundles with 40 nm filament spacing, and fascin