Unconventional Myosins

899-Pos Board B654
The Structure of Vertebrate Myosin-I Reveals New Insights into Mechanochemical Tuning of Myosins
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Myosins are actin based motors that are mechanically and kinetically tuned to function in a myriad of cellular processes. The myosin-I family member Myosin-IB (Myo1b) has very slow kinetics and a force sensitivity that is greater than any other characterized myosin, enabling it to function as a tension-sensing anchor. Because there have been no high-resolution structures of myosins that act as tension-sensitive anchors, we determined the 2.3-Å resolution structure of the motor domain and first IQ-motif of Myo1b with bound calmodulin in the absence of nucleotide. The structure reveals several striking features not yet seen in other myosins, including a unique positioning of the light chain binding domain (LCBD) and converter region. This unique conformation results in a cavity that sandwiches the N-terminal region (NTR) of the heavy-chain between the motor domain and LCBD. Single molecule and ensemble biochemical analyses show that the NTR plays an important role in stabilizing the post-power stroke conformation of Myo1b and in tuning the rate of the force-sensitive transitions that limit actin detachment. The sequence of the NTR is highly variable within the myosin-I family, so we propose that together with unique LCBD and converter conformations, the NTR plays a role in tuning the mechanochemical properties of the motor.

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Myosin-3B and its Light Chains
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Unconventional myosin-3B is a single-headed myosin containing an amino-terminal kinase domain. In vitro, we find full length and truncated versions of the molecule to bind the regulatory light chain (RLC) and calmodulin (CaM). The RLC binds to the first IQ-like motif and CaM to the second IQ motif within the neck region of the molecule. Uniquely, we find the RLC to be reversibly exchangeable with CaM in the presence of calcium. Calcium-free conditions favor RLC binding. Exchange of RLC versus CaM to the first IQ motif significantly increases the steady-state ATPase activity in the presence of calcium and modulates the interaction of the molecule with F-actin. This observation shows that the calcium-dependent light chain exchange triggers the activity of human myosin-3B as a molecular motor and suggests that myosin motor function is directly influenced by the set of light chains bound to the neck region.

901-Pos Board B656
Dynamics of the Lever-Arm Swing in Myosin V
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Myosins utilize a conserved structural mechanism to convert the energy from ATP hydrolysis to a large swing in the force generating lever-arm. However, there remains an ongoing controversy about the kinetics of lever-arm swing in relation to the steps in the ATPase cycle. To address this question we have developed a novel FRET system in myosin V (MV) that utilizes several donor-acceptor pairs to examine the dynamics of lever arm motion. MV containing a single IQ motif and an N-terminal (NT) tetracysteine site was labeled with the bisarsenical dye FlAsH (MV.NT.FlAsH). The first IQ motif of MV.NT.FlAsH was exchanged either with IAANS labeled CaM, a donor, or QSY-9 labeled CaM, a non-fluorescent acceptor. Steady-state and transient kinetic experiments reveal a decrease in FRET upon ATP binding (recovery stroke) in both donor-acceptor pairs. We utilized transient kinetic experiments to demonstrate that upon mixing the MV.ADP.Pi complex with actin there was a FRET increase that occurred in two phases, and the fast and slow phases correlated well with the release rates of Pi and ADP, respectively. We also labeled the upper-50kDa tetracysteine site with FlAsH (MV.U50.FlAsH) and exchanged the QSY labeled CaM on to the first IQ motif. We observed structural changes during ATP binding that were very similar to the MV.NT.FlAsH results. During actin-activated product release we observed two-phases, a rapid increase in FRET followed by a slower decrease in FRET, which correlated well with ADP release. We find that the force generating motion of the lever arm occurs in two steps which are closely coupled to the product release steps. Our results also indicate that the conformational changes in the lever arm associated with the power stroke may follow a unique pathway that is not simply the reversal of the recovery stroke.

902-Pos Board B657
Two Molecule of Two Headed Myosin 5C on a DNA Scaffold Steps Processively along Actin Filaments
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Myosin 5c is a low duty ratio motor and doesn't move processively along actin filaments as a single molecule. Here, we characterized the kinetic properties of double-headed myosin 5c and examined the possibility that multiple myosin 5c molecules could possess processive movement. Two myosin 5c molecules were conjugated to DNA scaffolds and their processivity was analyzed by single molecule techniques with TIRF and FIONA. ATPase activity assay and ADP dissociation kinetics demonstrated that the interaction between the two heads of myosin 5c increases its F-actin-binding affinity via the ''gated-gate'' mechanism. Single molecule study revealed that two myosin 5c molecules in a complex moved processively along actin filaments. Moreover, the space distance between the myosin 5c molecules is an important factor for processive movements. Our results provided a novel possibility for a non-processive motor to achieve processive movement that could be used to transport cargo in cells.

903-Pos Board B658
A Role for Myosin VI in Retinal Pigment Epithelium Phagocytosis
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Retinal pigment epithelium (RPE) cell phagocytosis of shed photoreceptor rod outer segments (ROS) is vital for retinal function. We examined the role of myosin VI in this process by utilizing micron-diameter plastic beads as a proxy for ROS waste. When placed in the extracellular medium, the beads were phagocytosed by a cultured human RPE cell line (ARPE-19). At the same time, these cells were made to over-express myosin VI with perturbed motor function. We observed that the rates of trafficking of beads within the cell were significantly reduced in the presence of the perturbed myosin VI compared to control cells, indicating that myosin VI plays a role, either directly or indirectly, in RPE phagocytosis.

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Single Molecule Characterization of Human Myosin VIIA
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Human myosin VIIA is responsible for Usher Syndrome (USH) type 1B. Although human myosin VIIA is thought to be involved in transportation of USH1 proteins and melanosomes, it is obscure that human myosin VIIA is a suitable motor as a cargo transporter.
In this study, we analyzed the motor characteristics of tail truncated human myosin VIIA (HM7DTail/LZ) at single molecule level. We found that HM7DTail/LZ moves processively with large ~35 nm forward and small ~23 nm backward steps at physiological ATP concentration. The forward step of HM7DTail/LZ was slightly larger than that of Drosophila myosin VIIA [~30 nm, Yang *et al.* (2006) PNAS, 103, 5746-5751]. The average run-length of HM7DTail/LZ was ~0.7 μm on single actin filaments. This means that HM7DTail/LZ can move ~20 steps on single actin filaments on average. Dwell time distribution gave the average waiting time of ~3.4 s, yielding 0.3 s^{-1} for the mechanical turnover rate. This rate is consistent with the V_{max} value of actin activated ATPase activity (~0.3 s^{-1}) of tailless human myosin VIIA. On the other hands, the velocity of HM7DTail/LZ was extremely slow, 11 nm/sec. This value is ~7 times slower than that of Drosophila myosin VIIA [72 nm/s, Yang *et al.* (2006)]. Recent studies have suggested that mammalian myosin VIIA may function in tethering melanosomes on actin filaments. Thus, the slow velocity may imply that human myosin VIIA is more suitable for cargo-actin tethering motor than Drosophila myosin VIIA, and may link transportation processes such as USH protein transportation. We recently found that human myosin VIIA localizes at the filopodia tip in cells when it dimerizes [Sakai *et al.* (2011) PNAS,108, 7028-7033]. Currently, we

are examining the movement of HM7DTail/LZ on actin bundles in filopodia using de-membraned cell system. The result is underway.

905-Pos Board B660

Myosin-10 Produces its Power-Stroke in Two Phases and Moves Processively along a Single Actin Filament under Low-Load

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Myosin-10 is an actin-activated ATPase that participates in essential intracellular processes such as filopodia formation/extension, phagocytosis, cell migration and mitotic spindle maintenance. The myosin-10 duty-cycle ratio - *i.e.* the fraction of time the motor remains tightly bound to actin during its total ATPase cycle - from previous biochemical studies are inconclusive, thus whether this myosin displays intermediate or high duty ratio is still under debate. To study this motor protein's mechano-chemical properties we have used a recombinant, truncated form of myosin-10 consisting of the first 940 amino acids, followed by a GCN4 leucine zipper motif to force dimerization. Negative-stain electron microscopy reveals that the majority of molecules (~87%) are dimeric with a head-to-head contour distance of ~50 nm. *In vitro* motility assays show that myosin-10 moves actin filaments smoothly with a velocity of 150 - 400 nm s^{-1}. Steady-state and transient kinetic analysis of the ATPase cycle shows that the ADP release rate (~13 s^{-1}) is similar to the maximum ATPase activity (~12 - 14 s^{-1}) and, therefore, contributes to rate-limitation of the enzymatic cycle. Single molecule optical tweezers experiments show that under intermediate load (~0.5 pN) myosin-10 interacts intermittently with actin and produces a working stroke of ~17 nm, composed of an initial 15 nm and subsequent 2 nm movement. At low optical trap loads, we observed staircase-like processive movements of myosin-10 interacting with the actin filament, consisting of up to six, ~35 nm, steps per binding interaction. Here we describe the kinetics and mechanics of myosin-10, interrogating bulk and single molecule biophysical/biochemical properties to further our understanding of its ATP-driven, motor mechanism and how this relates to its cellular functions.

906-Pos Board B661

Myosin X is Recruited to Focal Adhesion and Induces Filopodia Initiation

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Filopodia is the structure protruding from the edge of the cells that plays an important role in diverse cell motility. Myosin-X is involved in promoting filopodia formation and localizes at the tips of filopodia. However, the mechanism of myosin-X-induced filopodia formation remains largely unknown. Here we studied the mechanism of myosin-X-induced filopodia formation by directly monitoring the dynamics of myosin-X, actin and actin regulating proteins such as Arp2/3, vinculin and VASP during filopodia initiation and elongation. We found a specific local nucleation of actin and Arp2/3 at the cell's leading edge, where integrin was accumulated during filopodia initiation. Myosin X was then recruited to these sites by the lateral movement and gradual clustering along the actin nucleation sites, and initiated filopodia formation. During filopodia extension, we found the translocation of Arp2/3 and other actin regulating proteins along filopodia. Arp2/3 localized not only at the base of filopodia, but also at the middle of long filopodia, from where myosin X initiated the phased extension of filopodia, with the change of extension directions. Elimination of integrin-b by siRNA significantly attenuated myosin X induced filopodia formation and multiple phased elongations. Integrin-β is localized at the position where filopodia direction changes. Based upon present findings, we propose the following mechanism. Myosin X accumulates at focal adhesions at the cell's leading edge where integrin is localized. Myosin X promotes actin convergence to produce the base of filopodia. Myosin X transports VASP to filopodial tips to facilitate elongation of filopodia. At the tip where myosin X is accumulated, integrin is also accumulated and forms focal adhesion, from where myosin X promotes second phase elongation to further extend filopodia.

907-Pos Board B662

Dynamics of Myosin XI: The Family Speed Demon

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Myosin XI is the fastest known processive motor, and is implicated in organelle transport and propulsion of cytoplasmic streaming in plant cells. We studied the motility and dynamics of a recombinant myosin XI motor domain hybridized with the myosin Va lever arm with 6 calmodulin-bearing IQ motifs and the dimerization motif. Single molecule fluorescence tracking (FIONA) and polarization (polTIRF) measurements support the hand-over-hand stepping model for myosin XI. Its step size (~34.5 nm) is slightly shorter than the helical pitch of actin filaments, suggesting an overall left-handed helical walking path. The path was confirmed by polTIRF azimuthal angles as well as bead motility on suspended actin filaments. The hybrid myosin XI showed a maximal velocity of at least 4 μm/s, which is ~7-fold faster than its structurally similar cousin, myosin V, but they have similar run lengths of ~1 μm. FIONA measurements showed faster myosin XI stepping rates on actin filaments as compared to bundled actin. The gliding filament assay gave 5-10-fold slower velocities than single molecule processive runs. PolTIRF experiments identified several classes of molecules, with different leading and trailing probe angles. These classes may represent placement of the bifunctional rhodmaine probe on different calmodulins. Compared with myosin V, myosin XI showed more variable localization between steps and more off-axis motion, which may relate to myosin XI's role in cytoplasmic streaming on bundled actin beams. Supported by NIH grant GM086352.

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Purification and Characterization of Myosin-15, the Molecular Motor Mutated in DFNB3 Human Deafness

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Stereocilia are mechanosensitive organelles projecting from the surface of inner ear hair cells that detect nanometer deflections induced by sound, gravity or head movement. Unconventional myosin-15 (encoded by *Myo15*) is hypothesized to regulate stereocilia development by delivering cargoes such as whirlin and Eps8 to their tips. Whether myosin-15 actually functions as a transporter remains untested and little is known regarding its activity, structure and regulation within the highly specialized stereocilia compartment.

We have characterized the biochemical kinetics of a subfragment-1 (S1) like truncation of mouse myosin-15, comprising the catalytic ATPase/actin binding domain plus two IQ light chain binding sites. Expression of the recombinant S1 fragment in Sf9 insect cells required co-expression of multiple molecular chaperones in order to recover significant quantities of active protein. Unlike most unconventional myosin classes, the IQ regions of myosin-15 did not bind calmodulin with high affinity; instead preferentially associating with essential (MYL6) and regulatory (MYL12A) light chains that typically partner with myosin-2 isoforms. Single molecule TEM confirmed that the purified S1 was correctly folded and monomeric. The S1 fragment was mechanically active and moved actin filaments at ~170 $nm \cdot s^{-1}$ in a gliding motility assay. A powerstroke displacement of 7.8 nm was measured by optical trapping. Transient kinetics revealed that ATP binding to myosin-15 was slow and weak ($K_1k_2 = 0.17\ \mu M^{-1}s^{-1}$, $1/K1 = 1898\ \mu M$), and that ADP release (~$12s^{-1}$) was the rate-limiting step; indicating the predominant steady-state intermediates for myosin-15 would either be with ADP, or no nucleotide bound. Both states bind strongly to actin, suggesting that myosin-15 could be capable of longer-range processive motility if oligomerized *in vivo*. These data enable future kinetic and structural studies to investigate how deafness-associated mutations targeted within the ATPase ultimately disrupt stereocilia function.

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Human Myosin-18B - A Versatile Actin Binding Protein

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Class-18 myosins challenge our established view about myosins acting as molecular motors. No member of this class appears to have a significant ATPase activity, which is a prerequisite for motor activity. Humans express two myosin-18 isoforms, myosin-18A and myosin-18B. Whereas recent studies on myosin-18A shed some light on its cellular and biochemical mode of action, the molecular function of myosin-18B remains poorly understood. Class-18 myosins contain protein interaction domains outside their generic motor domain. In the case of myosin-18B this includes a large,