which produces polarized actin bundles with 13 nm filament spacing. We applied a modified particle tracking program, which allowed us to analyze thousands of simultaneous myosin tracks and determine the run lengths and velocities typical of processive movement on the bundled networks. Myosin V moved processively on all types of in vitro actin structures. Myosin X moved well on polarized fascin cross-linked bundles, but movement was impaired or nonexistent on non-polarized alpha-actinin bundles. We hypothesize that forward runs of myosin X on alpha-actinin cross-linked bundles are inhibited because myosin X might makes ''sidesteps" to a neighboring filament, which stalls the run. The presence of an SAH domain in the lever arm of myosin X could increase the working stroke or flexibility of the lever arm allowing it to more easily sidestep across the larger alpha-actinin filament spacing.

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Cargo-mediated dimerization of Myosin VI

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Myosin VI is one of 18 known classes of the molecular motor superfamily called myosin (1,2). All myosins rapidly bind and hydrolyze ATP in the presence or absence of actin. Until recently it was thought that all myosins moved toward the barbed (+) end of the actin filament. Myosin VI is the exception to that rule and may be unique among the myosin family members in that it moves toward the pointed (-) end of the actin filament (3).

Our working model for myosin VI in a cell is that the full-length protein exists as a monomer if not bound to cargo. Binding of myosin VI monomers to cargo alters the conformation of the molecule, possibly exposing the high probability coiled-coil region (dimerization domain). Once dimerized, the myosin VI can move a vesicle processively toward the minus-end of an actin filament. GiPC and optineurin, two of the known myosin VI binding partners can dimerize, and thus potentially can initiate the dimerization of myosin VI when it binds. Both GiPC and optineurin has been expressed in insect Sf9 cells. Surface plasmon resonance (SPR) analysis showed that both GiPC and optineurin interact with full-length myosin VI within the nanomolar range. Both GiPC and optineurin when incubated with full-length myosin VI initiated its dimerization showed by ATPase assays, EM and TIRF microscopy.

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727-Pos Board B606

Characterization of drosophila myosin 7a mechanics

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Myosin 7a is an unconventional myosin which participates in the sensory cell functions of numerous organisms, including humans, zebra fish, and flies. In drosophila, myosin 7a (DmM7a) appears responsible for bristle morphology, including the antennae involved in auditory transduction. The composition of motifs within the molecule is as follows: a motor head, containing sub-domains broadly typical of the myosin super-family, which connects to 5 IQ's, followed by the tail region. Within the tail are a putative coiled-coil followed by two tandem MyTH4-FERM domains separated by an SH3 domain. Here, data obtained using the optical trap three bead assay - the practice of using photon force to manipulate micrometer-scale beads to observe single molecule events - are presented for DmM7a. A truncated DmM7a construct (DmM7aTD1), cropped after the tail SH3 domain, was observed to interact with an actin filament at low ionic strength (50 mM KCl). Under the same conditions no interactions were seen with the full length version (DmM7aFL), however, at high ionic strength (200 mM) DmM7aFL became active. These findings are in agreement with recent studies demonstrating that the tail performs an internal regulatory function which is electrostatic in nature. The actin detachment rates (Kdet), calculated from dwell times, were similar for DmM7aTD1 and DmM7aFL at 10 μM ATP, approximately 0.2 s^{-1}. The Kdet for DmM7aFL was dependent on ATP concentration, and was increased at 1 mM ATP. These data support previous studies showing M7a to be a high duty motor with slow ATPase activity. Attempts to dimerise DmM7a on actin were unsuccessful based on the absence of ''stepping'' events which are a hallmark of processivity. This supports the case for DmM7a having a role in tension maintenance.

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Prefoldin 4 (PFD4): A putative new partner of myosin Va (MyoVa) in melanosome transport

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Recruitment of MyoVa and the proper transport of melanosomes during pigment dispersion requires the central region of melanophilin (Mlph, 90 kDa) to bind MyoVa and the N-terminal region bound to the melanosome membrane via Rab27a. The interaction among these proteins is the key to melanosome transport. Previously, we identified and mapped for the first time the interaction between PFD4 (~14kDa) and Mlph using the yeast 2-hybrid system (in vivo) and a biochemical assay (in vitro). PFD4 is a subunit of prefoldin (PFD, ~87kDa), a chaperone that delivers unfolded proteins to a chaperonin for correct folding. Our in-vivo results suggest that PFD4 interacts with Mlph at the same MyoVa binding site. Here we confirm that interaction using pull-down assays and fluorescence spectroscopy; PFD4 competes with MyoVa for the Mlph binding site and residues 400-590 (putative coiled coil) of Mlph are crucial for PFD4 binding. In-vitro fluorescence anisotropy reveals interaction of fluorescein-labeled full-length Mlph with MyoVa tail or PFD4, by an increase in anisotropy and polarization values. Neither mutated A453P full-length Mlph nor the 400-590 segment caused a significant change in anisotropy when incubated with MyoVa; thus these constructs do not bind Mlph. Full-length Mlph also did not bind muscle myosin II. The MyoVa binding domain for Mlph and fragments 150-400, 300-433, 400-590, and Mlph A453P seems to be intrinsically unstructured. When we pre-incubated Mlph with PFD4 or MyoVa the circular dichroism spectrum showed that binding Mlph 150-400 and 150-590 with PFD4 and MyoVa tail possibly causes an increase in α-helix content. Support: CNPq, FAPERJ, PRONEX, CAPES (Brazil); Wellcome Trust (UK)

729-Pos Board B608

The Mechanism of Filament Rotation in Gliding Assays with Non-Processive Myosin Motors

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We present a model study of gliding assays in which actin filaments are moved by non-processive myosin motors. We show that even if the power stroke of the motor protein has no lateral asymmetry, the filaments will move in a helical, rather than straight fashion. Notably, the handedness of this twirling motion is the opposite from that of the actin filaments. It stems from the fact that the gliding actin filament has "target zones" where its subunits are oriented towards the surface and are therefore more accessible for myosin heads. Because each myosin head has a higher binding probability before it reaches the center of the target zone than afterwards, this results in a left-handed helical motion of the actin filament. We present a stochastic simulation and an approximative analytical solution to study this effect. We show that the pitch of the helix depends on the filament velocity, which in turn depends on the ATP concentration. It reaches about 400nm for slow gliding and increases with higher speeds. These values are in good agreement with recent experiments.

730-Pos Board B609

Non-muscle Myosin IIb Is A Processive Actin-based Motor

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Proper tension maintainence in the cytoskeleton is essential for regulated cell polarity, cell motility and division. Non-muscle myosin IIB (NmIIB) generates tension in the actin cortex of non-muscle cells. Recent biochemical studies show that both heads of a NmIIB dimer can interact with a single actin filament and that this conformation demonstrates load dependent release of ADP. Using a three bead optical trapping assay we recorded NmIIB interactions with actin filaments to determine if a NmIIB dimer cycles along an actin filament in a processive manner. Our results show for the first time that NmIIB is the first myosin II to exhibit evidence of processive stepping behavior. Analysis of this data reveals a forward displacement of ~5 nm. Surprisingly, NmIIB can and does take frequent backward steps of ~5 nm. The short step size of NmIIB suggests that this motor twists actin. Actin twisting could facilitate the removal of actin crosslinking proteins from the cytoskeleton. Our data supports a model in which NmIIB takes processive forward steps to generate additional tension and also takes backwards steps to relieve tension in the actin cytoskeleton, suggesting that NmIIb is a general regulator of cytoskeleton tension.

731-Pos Board B610

Lever Arm Length Determines The Azimuthal But Not The Axial Orientation Of Myosin V During Processive Motility

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In order to assess how lever arm length affects the three-dimensional motions of myosin V during processive motility, two constructs were studied using single molecule polarized total internal reflection fluorescence (polTIRF) microscopy. MyoV6IQ and MyoV4IQ contain 6 and 4 calmodulin (CaM) binding IQ motifs, and otherwise consist of the native myosin V excluding the tail domain. Bifunctional rhodamine labeled CaM replaced a native CaM, giving probe angles β_P relative to the actin axis and α_P, the azimuth around actin. As with other processive myosins, α_P and β_P exhibited tilting of the probe with each step. With MyoV6IQ, α_P often returned to its initial value after two steps, as expected for nearly straight walking. This behavior enabled us to determine the orientation of the lever arm, α_L and β_L, as well as θ_L and f_L, the probe angles relative to CaM. β_L was 100° and 40° in the leading and trailing heads, respectively. In MyoV4IQ, β_P was similar to 6IQ, but α_P seldom returned to its earlier value after two steps. This indicates considerable net azimuthal rotation, as expected for smaller step sizes. Thus, lever arm length determines the azimuthal angular path, whereas the axial orientation is likely determined by structural constraints in the motor domain. Modified gliding filament assays were performed using polTIRF to detect twirling of actin about its axis during motility. MyoV6IQ twirled almost exclusively left-handed with a pitch of 1.4 μm. MyoV4IQ twirled with both right- and left-handed pitches of 1.0 and 1.2 μm, respectively. Bidirectional twirling of MyoV4IQ contrasts with every isoform of myosin previously tested (II, native V, VI and X) all of which twirled with a single handedness. This work was supported by NIH grant AR05117.

732-Pos Board B611
Structural and Mechanistic Determinants of Myosin VI Processivity and Anchoring
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Myosin VI is a molecular motor that can function both as a transporter and anchor in cells. Its role is regulated through load, being capable of taking multiple consecutive 36 nm steps along an actin filament under zero load and switching to an anchor by stalling when placed under piconewton levels of load. The parameters necessary for such processivity and anchoring are not fully understood. We use high-speed gold nanoparticle tracking to study single molecules of myosin VI with millisecond resolution in the absence of load. Optical tweezers are used to observe the behavior of the molecules when perturbed by load. In order to probe the contribution of the myosin VI tail domain to processivity and anchoring, we have created and characterized a number of mutant tail domain constructs. Our results reveal the resiliency of myosin VI as a transporter and suggest that it has evolved its unusual tail domain for purposes other than efficient cargo transport in the absence of applied load. We present preliminary data investigating the role of the myosin VI tail domain in important cellular processes such as transport against load and load-induced anchoring.

733-Pos Board B612
Myosin VI Dimerizes And Walks Processively Along Actin
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Myosin VI is an unconventional motor protein that can move processively along the actin filament in an opposite direction towards the minus-end, contrary to all other known myosins. Despite its short lever arm, represented by a single IQ domain, myosin VI demonstrates large step sizes (30-36nm), typically characteristic of motor proteins with longer lever arms, viz. myosin V with 6 IQ domains. In cells, myosin VI is involved in diverse functions including Golgi transport, endocytosis and stereocilia maintenance. Though it is possible that myosin VI can function either as a dimer or a monomer in cells, based on our studies on the functional properties of the protein, it is likely that a dimeric protein can undergo intramolecular strain to become a more efficient actin anchor which makes it more competent as a transporter. Previous studies from our lab have shown that both full-length as well HMM fragments are capable of forming stable, processive dimers upon clustering, indicating that myosin VI monomers need to be in close proximity to initiate dimerization. Our recent studies show dimerization of full-length myosin VI can be triggered by cargo binding and the cargo-bound motors walk processively on actin filaments with the expected step size. Following the IQ motif, the lever arm extension of about hundred amino acid residues contains the sequence sufficient for dimerization. However, the accurate location of dimer formation remains controversial since the putative dimerization domain in myosin VI has non-native coiled-coil sequences. Our working hypothesis is that dimerization triggers the unfolding of a 3-helix bundle creating the 12nm extension required for proper myosin VI walking. Based on a series of truncations, we are in the process of testing this hypothesis and defining the nature and sequence of the dimerization domain.

734-Pos Board B613
Cryo-Electron Microscopy of Myosin 5 on Actin
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Single molecules of myosin 5 move processively along actin filaments by a hand-over-hand mechanism. In an earlier study we found by negative staining that at micromolar, rate limiting ATP concentrations both heads of the HMM fragment of myosin 5 were attached to actin, usually 13 actin subunits apart and the leading head had its converter subdomain in a pre-powerstroke position with variable leading lever conformation. To determine whether any of these results were artefacts of our experimental method, we have now gathered data by cryo-EM of unstained samples flash frozen at saturating (0.2 mM) ATP concentrations and low calcium concentrations. We used full length mouse myosin 5a (melanocyte isoform), expressed in sf9 cells. From our recent work, we expect many molecules to be detached from actin and folded into a triangular shape, and some molecules to be unfolded and actively moving along actin. We observe both forms of molecule. Unfolded molecules attached to actin by both heads were analyzed by single particle methods. The heads are mostly spaced 13 actin subunits apart with small proportions at 11 and 15 subunit spacings. Trailing heads, expected to contain ADP under these conditions in contrast to no nucleotide in our earlier study, have the conformation expected for post-powerstroke heads, and straight levers. Levers of leading heads emerge from the motor domain at a pre-powerstroke position and are somewhat curved. The initial segment of the tail is sometimes visible. It is usually angled in the trailing direction, as noted in negative stain, suggesting that the head-tail junction of active molecules is not a freely mobile joint. These results confirm and refine our earlier conclusions. Supported by the Wellcome Trust (076057).

Ion Motive ATPases

735-Pos Board B614
Kinetic Analysis Of ATP Synthesis Catalyzed By E. coli FoF1 ATP Synthase Reconstituted Into Egg Yolk Liposomes: Evidence For Bi-site Activation
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Escherichia coli F_OF_1 ATP synthase was reconstituted into liposomes made of asolectin, soybean PC or egg yolk PC. The reconstitution system with egg yolk proteoliposomes gave the highest ATP synthase activity and ATP yield, and was used for analysis of the reaction characteristics. Under optimal conditions (ΔpH 3.4 at 37°C, $\Delta\Psi = 109$ mV, 10 μM valinomycin), the steady state rate of ATP synthesis reached 400 s^{-1}. The dependency for Pi was hyperbolic over a range from 0.01 -5 mM. In contrast, variation of ADP concentration over a broad range (20 nM-2000 μM) revealed two apparent K_m, one much less than 1 μM and the second at 11 μM. The apparent K_m values for both substrates were independent of the membrane potential, $\Delta\Psi$. We propose that filling of two catalytic sites is sufficient and necessary for steady state ATP synthesis. Also, thiophosphate was found to be an uncompetitive inhibitor of ATP synthesis with respect to ADP, which implies an ordered substrate binding with ADP binding preceding phosphate binding. The data are in agreement with a reversible ATP synthesis-hydrolysis catalytic step with the ratio of the forward and reverse rate constants close to unity (Baylis Scanlon et al. *J. Biol. Chem.* 283, 26228-26240, 2008). In contrast to ATP hydrolysis where binding of Mg·ATP to the third catalytic site drives rotational catalysis, our results show that steady state ATP synthesis only requires binding of substrates to the second site.

736-Pos Board B615
Ca^{2+} Binding to Site I of the Cardiac Ca^{2+} Pump (SERCA2a) is Sufficient to Dissociate Phospholamban (PLB)
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Our model of mutually exclusive binding of PLB and Ca^{2+} to SERCA2a suggests that the Ca^{2+}-bound form of SERCA2a (E1) cannot interact with PLB. However, it is unclear whether Ca^{2+} binding to site I, site II, or both sites of SERCA2a is sufficient to dissociate PLB. To investigate this, we made several SERCA2a mutants: mutants lacking Ca^{2+} binding site I (E770Q or T798A), Ca^{2+} binding site II (E309Q or N795A), or both sites (D799N, or E309Q, E770Q double mutant). When individually expressed in insect cell microsomes, all these mutants failed to transport Ca^{2+}, but were readily phosphorylated by Pi to form E2~P (measured in Ca^{2+}-free buffer favoring formation of E2, the low Ca^{2+} affinity conformation). Ca^{2+} inhibition of E2~P formation