brane excitability and stabilization of neuronal resting potential; charge balance during endosome acidification; fluid secretion, and regulation of cell volume. As a consequence dysfunctions in regulating membrane chloride permeability originate various diseases. For instance cystic fibrosis arises from a genetic alteration of a transmembrane conductance regulatory channel (CFTR).

At present processes regulating intracellular chloride ion concentrations are still widely unexplored mainly as a consequence of limiting methods to quantify chloride fluxes in living cells.

In the present work a novel genetically-encoded sensor with double ratiometric readout is presented. The sensor was obtained by fusion of a red fluorescent protein (DsRed-monomer), insensitive to chloride and pH, to a GFP variant containing a specific chloride-binding site (GFP-Chl) [1]. The latter mutant is obtained from EGFP by the single-point mutation T203Y. GFP-Chl binds the chloride ion following a fluorescence static quenching mechanism that allows to measure the intracellular pH in a chloride independent manner. Chloride concentration is subsequently determined from the Grynkiewicz equation using the appropriate pH-dependent chloride affinity.

The sensor was successfully tested in vivo with two different cell lines cultured in a pH range 5.5–8.5 with chloride concentration up to 200 mM. Applicability to high-throughput screening, range of validity and accuracy of time-lapse maps will be discussed.

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2241-Pos

WITHDRAWN

2242-Pos Automated Internal Solution Exchange On A Planar Patch Clamp Surface

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Nanion Technologies, Munich, Germany.

Board B357

Ion channels have become important targets in academic and pharmaceutical research alike. Understanding how ion channels function is critical in determining underlying disease mechanisms and drug interactions. Patch clamp electrophysiology has been used for decades as a tool for understanding ion channel function. Planar patch clamp platforms have also gained a place in recent years in ion channel research for those requiring higher data throughput. Nanion's planar patch clamp devices, the Port-a-Patch and Patchliner, provide higher throughput whilst maintaining high quality, typically obtaining Giga-seals with a 60–80% success rate.

In addition to providing higher throughput, the planar patch clamp chip also gives unprecedented access to the internal surface of the membrane. Nanion has exploited this feature by designing a device which can exchange the internal solution of the cell in an automated fashion. The device for internal perfusion described in this study is suitable for the rapid administration of up to 4 different solutions directly to the internal side of the membrane. This greatly expands the experimental possibilities open to the ion channel researcher. For example, studies of second messenger systems by exchanging the internal solution become possible. In addition, compounds can be applied internally to examine differences in potency or mechanism when applied to the internal vs. the external surface of the cell.

In this study data will be described for exchanging the internal solution when recording from cells expressing K+ currents. For example, the effect of blockers such as ions or small molecules on the Kv1.3 channel when applied internally will be shown. The data described here are just the beginning of the ever-expanding possibilities of planar patch clamp.

Myosin & Myosin-family Proteins - I

2243-Pos Testing Cargo-induced Dimerization of Myosin VI

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Board B358

The propensity of the unconventional Myosin VI (M6) to form processive dimers through coiled-coil interactions in its tail domain is thought to be initiated by adapter proteins that bind to the globular tail domains of two monomeric M6 molecules. In Drosophila, embryonic neuroblasts target cell fate determinants basally, rotate their spindles to align them apically, and divide asymmetrically. The spindle orientation, and the localization of cell fate determinants and their adaptor proteins such as Miranda are all specified by the same apical complex. As Miranda localization requires M6, our studies test whether the presence of Miranda in vitro could convert an otherwise nonprocessive, single-headed M6 to a processive, two-headed complex.

2244-Pos Probing the Reverse Directionality of Myosin VI

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Board B359

Myosin VI moves toward the (−) end of actin filaments, despite sharing extensive sequence and structural homology with (+) end directed myosins. A unique insert between the converter domain and the lever arm was proposed to provide the structural basis of directionality reversal (J. Menetrey, et al, Nature 2005). Single molecule analysis of truncated versions of myosin VI strongly supports this model (Z. Bryant, D. Altman, and J.A. Spudich, PNAS 2007). To further explore the structural determinants of directionality, we designed a series of myosin VI constructs with artificial lever arms fused at different locations beyond the converter domain. Computational modeling including molecular dynamics simulation were used to predict the behavior of chimeric designs and select optimal fusion locations. In vitro motility assays were used to characterize the directionality and velocity of the chimeras. Our results demonstrated that the calmodulin-bound distal portion of the unique insert is not an integral structural or mechanistic component of the reverse stroke. Furthermore, we showed that as few as 18 residues are required to change the directionality of myosin.

2245-Pos Strain-dependent Search And Capture Mechanism For Directional Motion Of Myosin-VI

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Board B360

Myosin-VI has a two-headed structure on endocytic vesicles in cells and processively moves along actin helical pitches in a hand-over-hand fashion. When the two heads span the actin helical pitch, the front and rear heads are exposed to backward and forward strains, respectively. It is well established that the movement is started by binding of ATP to the rear head in a strain-dependent manner to release it from actin. However, how the released head searches for the forward actin target and captures it has remained unsolved. The detached head with ATP or ADP and Pi complex bound undergoes Brownian motion back and forth along an actin filament, repeating rapid association and dissociation with it (weak binding). The detached head should be exposed to backward strain near the forward target region at a moment when the head attached to actin. Thus we examined the possibility that the backward strain might accelerate strong binding to the target actin. We applied forward and backward strain to a single-headed myosin-VI at weak binding state tethered to an optically-trapped bead by moving the bead back and forth at various speeds. The result showed that the transition from weak to strong binding state was greatly accelerated when the backward strain was applied at sufficiently large speed for the head to be exposed to it at a moment of bound. Thus, we propose a strain-dependent search and capture mechanism for directional motion of myosin-VI.

2246-Pos Mechanism Of Load-Induced Modulation Of ADP Affinity In Myosins V And VI

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Board B361

Individual dimeric molecules of unconventional myosins V and VI travel long distances toward opposite ends of actin filaments in cells, taking multiple ∼36-nm steps in a "hand-over-hand" fashion before dissociating. For efficient directional processive movement of a dimeric myosin via a "hand-over-hand" mechanism, the two heads must communicate with each other, resulting in the easier dissociation of ADP from the trailing head than from the leading head. We previously presented the direct experimental evidence that external loads applied to individual actomyosin V or VI bonds affect ADP affinity in an asymmetric manner, such that ADP binds weaker under loads acting in the direction of motility and stronger under loads opposing motility.

These findings have been expanded by performing the model analysis, based on the experimental data. This allowed us to separately determine the load-dependence of both dissociation and binding rates of ADP, which revealed the similar mechanism of the load-induced modulation of biochemical kinetics in both heads of myosins V and VI. Furthermore, we were able to estimate the value of the internal strain, which is exerted on the two heads of these motors during double-headed binding to actin, and found that it is smaller than previous estimates.

The asymmetric ADP affinities under directional load determined in this study provide strong support to models in which the intramolecular strain, generated during double-headed binding to actin, effectively contributes to high processivity of dimeric molecular by coordinating the catalytic cycles of the two heads.

2247-Pos Off-axis strain-dependent features of ADP dissociation kinetics in Myosin V

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Individual molecules of myosin V travel along an actin filament using two identical heads, alternately switching the leading and trailing positions with ATPase cycling. For such motion, which is explained by a hand-over-hand model, the ATPase cycles in both

heads should be coordinated, such that ADP dissociation is modulated to occur faster in the trailing head than in the leading head. Actually, we found that ADP dissociation is modulated by loads, namely, it was strongly inhibited under backward loads and slightly accelerated by forward loads, and we stressed that the internal stain should exist in native dimeric myosin V, ensuring this modulation during actual stepping.

In our previous experiments, loads applied to a single myosin V molecule to test these load-dependent features of ADP dissociation, probably mimicking the internal strain, were acting along the long-axis of the actin filament. However, in some previous studies, it was found that a single myosin V molecule walks on an actin filament along the left-handed spiral, and in the cell myosin V may walk on the branched actin meshwork, thus myosin V has to choose whether to stay on the straight mother filament or to switch to the daughter filament at the intersection. In these cases the internal strain during stepping is no longer acting along the actin filament. Therefore, we must consider the effect of the off-axis load on the ADP dissociation to understand the details of actual stepping mechanism. We are discussing this behavior, which is considered to be important for maintaining high proccesivity of myosin V in cells.

2248-Pos Mechanism of tail domain inhibition of myosin Va motor function

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Board B363

Myosin Va is a well-known processive motor involved in transport of organelles. A tail-inhibition model is generally accepted for the regulation of myosin Va: inhibited myosin Va is in a folded conformation such that the tail domain interacts with and inhibits myosin Va motor activity. Recent studies indicate that it is the C-terminal globular tail domain (GTD) that directly inhibits the motor activity of myosin Va. In the present study, we identified a conserved acidic residue in the motor domain (Asp136) and two conserved basic residues in the GTD (Lys1706 and Lys1779) as critical residues for this regulation. Alanine mutations of these conserved charged residues not only abolished the inhibition of motor activity by the GTD, but also prevented myosin Va from forming a folded conformation. We propose that Asp136 forms ionic interactions with Lys1706 and Lys1779. This assignment locates the GTD-binding site in a pocket of the motor domain, formed by the N-terminal domain, converter, and the calmodulin in the first IQ motif. We propose that binding of the GTD to the motor domain prevents the movement of the converter/lever arm during ATP hydrolysis cycle, thus inhibiting the chemical cycle of the motor domain.

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2249-Pos Spectroscopic And Molecular Dynamics Simulation Analysis Of Conformational Changes In The Nucleotide Pocket Of Myosin V

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We have used EPR spectroscopy and computational modeling of nucleotide-analog spin probes to investigate conformational changes at the nucleotide site of myosin V. We find that the mobility of a diphosphate analog bound at the active site of myosin V increases when the myosin V-diphosphate complex binds to actin, implying an opening of the active site when myosin V binds to actin. The probe mobilities observed are similar to those previously seen with fast and slow skeletal myosins. Molecular dynamics (MD) simulation has been used to understand better these results in terms of available x-ray structures. The x-ray structure of myosin V bound to ADP-BeFx shows a closed nucleotide site similar to that seen in other myosin x-ray structures. The myosin V-ADP structure shows an open nucleotide site due to a displacement of the switch 1 domain. A nucleotide analog spin probe was docked at the nucleotide site of the open and closed myosin V x-ray structures. MD simulation of the closed conformation gave a probe mobility comparable to that seen in EPR spectra of the myosin V-ADP complex. The open myosin V-ADP nucleotide pocket seen in the x-ray structure closed during MD simulation yielding a nucleotide pocket similar to the closed x-ray structure and comparable probe mobility. This implies flexibility in this region. The 50 kDa cleft remained closed. Thus there does not appear to be an obligatory coupling of the opening and closing of the nucleotide pocket and the 50 kDa cleft. Additional simulations are in progress.

2250-Pos Myosin V and Kinesin act as Tethers to Enhance Each Others' Processivity

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Organelle transport to the periphery of the cell involves coordinated transport between the processive motors kinesin and myosin V. Long range transport takes place on microtubule tracks, while final delivery involves shorter actin-based movements. The concept that motors only interact with their appropriate track required modification with the recent observation that myosin V undergoes a one-dimensional diffusional search on microtubules (Ali et al., 2007). Here we show, using single molecule techniques, that a functional consequence of the diffusive search by myosin V is to effectively enhance the processive run length of kinesin when both motors are

present on the same cargo. The complementary effect occurs on actin, with myosin V undergoing effectively longer processive runs when kinesin is present on the same cargo. The process that causes run length enhancement on both cytoskeletal tracks is electrostatic in nature. Myosin V mutants with increased or decreased charge in loop 2 (Hodges et al., 2007) were used to assess if this positively charged surface loop interacts with the negatively charged E-hook on the microtubule. The results showed a correlation between positive charge in loop 2 and run length enhancement, suggesting that this element contributes to the electrostatic interaction between myosin V and the microtubule. We propose that one motor acts as a tether for the other, preventing its diffusion away from the track and allowing multiple processive runs to occur before dissociation. The resulting run length enhancement likely contributes to the successful delivery of cargo in the cell.

2251-Pos Myosin V Passing over Arp2/3 Junctions: Branching Ratio Calculated from the Elastic Lever Arm Model

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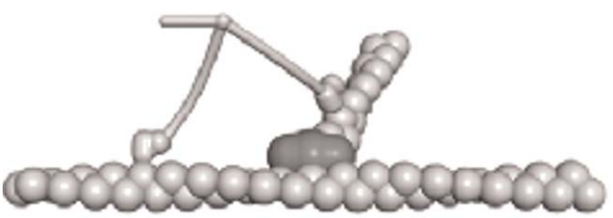
Board B366

Myosin-V is a two-headed processive motor protein that walks in a hand-over-hand fashion along actin filaments. Here we study the behaviour of a myosin-V molecule encountering an actin junction formed by the Arp2/3 complex. Our calculation is based on the elastic lever arm model for myosin-V [1], which describes the dimeric molecule as two identical heads, connected with elastic lever arms. To predict the branching probability at Arp2/3 junctions, we calculate the shapes and bending energies of all relevant configurations in which the trail head is bound to the actin filament before Arp2/3 and the lead head is bound either to the mother or to the daughter filament. We assume that the probability that the lead head binds to a certain actin subunit is proportional to the Boltzmann factor obtained from the elastic energy. Our model predicts a branching ratio of 27% for the daughter and 73% for the mother filament. This result is in good agreement with recent experimental data [2].



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2252-Pos Twirling Of Actin By Myosin Isoforms

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Several factors are expected to determine whether actin experiences a torque during active myosin-directed sliding: the direction of the force vector between actin and myosin, the distribution of myosin binding sites on actin, and cooperation between myosins translocating an individual actin filament. In order to learn more about these possible mechanisms we use a modified gliding filament assay to observe rotations of the actin filament about its longitudinal axis (twirling). The angular velocity and pitch of the filament is inferred from measuring the 3D orientation of single tetra-methylrhodamine fluorophores bound to actin using polarized total internal reflection microscopy. Here we compare twirling results for 4 different forms: tissue purified myosin II and V, and recombinant myosin V with 4 and 6 IQ motifs. Myosins II, V, and V-6IQ twirl actin filaments with a relatively long ($\sim$1 μm) left-handed pitch, opposite to the intrinsic right-handed $\sim$74 nm pitch of the actin filament. The shorter-length lever arm of myosin V- 4IQ resulted in similar magnitude left-handed pitch in most twirling filaments, but some filaments twirled right-handed. A more thorough study of twirling vs. actin filament length, ATP concentration and myosin loading was performed for myosin II in order to explore the mechanisms underlying twirling. The pitch of myosin II is relatively insensitive to filament length, myosin density, and ATP concentration. The observed twirling motions are most easily explained by an applied torque between actin and myosin which differs among isoforms.

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2253-Pos Myosin-V Makes Two Brownian 90° Rotations per 36 nm-Step

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Myosin-V processively walks on actin filaments in a hand-over-hand fashion. The identical head structure predicts a symmetric hand-over-hand mechanism where regular, unidirectional rotation occurs during a 36 nm step. We investigated this rotation by observing how fixed myosin-V rotates actin filaments. Actin filaments randomly rotated 90° both clockwise and counter clockwise during each step. Furthermore ATP dependent rotations were regularly followed by ATP independent ones. Kinetic analysis indicated that the two 90° rotations related to the coordinated unbinding and rebinding of the heads from actin. We propose a Brownian rotation hand-over-hand model in which myosin-V randomly rotates by thermally twisting its elastic neck domains during

the 36 nm step. The Brownian rotation may be advantageous for cargo transport through a crowded actin meshwork and to carrying cargos reliably via multiple myosin-V molecules in a cell.

2254-Pos Tracing the Ratchet Mechanism of Myosin-V

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Board B369

Molecular motors capable of converting chemical energy, e.g. from ATP, to mechanical work, are essential for the correct functioning of cells. Detailed knowledge about their mode of operation is therefore important. Myosin-V is a linear molecular motor that moves processively towards the plus end of actin filaments. In the cell, it colocalizes with other, stronger motors like kinesin and therefore experiences forces both below and above its own stall force.

Application of high external forces to the motor reveals a ratchet like behaviour of the two-headed myosin-V. High backward loads can induce rapid and processive backward steps along the actin filament, even in the absence of ATP. In contrast, forward forces cannot induce ATP-independent forward steps. To further investigate the origin of the ratchet mechanism, we use optical tweezers to apply force on a single headed construct of myosin-V. We find that the asymmetry between both directions of force application is already present in the interaction of one motor head with actin.

2255-Pos Structural basis for gated release of ADP from Myosin 5 on actin

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Two-headed myosin 5 strides processively along actin filaments carrying cargoes at the end of its tail. Processivity is enhanced by intramolecular gating between the two heads when both are attached to an actin filament. The trailing head strongly inhibits dissociation of ADP from the leading head, while the leading head may stimulate ADP release from the trailing head. Our previous electron microscopy showed that the lever of the leading head is in a pre-powerstroke position. This may inhibit ADP release. The lever of myosin 5 comprises 6IQ motifs that bind 6 calmodulin light chains. Truncation to 2IQ motifs in an HMM construct produces a molecule with very low processivity, whereas 4IQ and 8IQ constructs are similar to 6IQ. We have now found that unlike the other constructs, 2IQ HMM has weak gating of ADP release: a single fast rate constant describes release from both heads. We have used a rapid-mixing apparatus and electron microscopy to determine the structural basis for this behaviour. 2IQ HMM was mixed with 1.25 ATP/head, mixed with actin, then ejected onto an EM grid and quickly negative stained. We find in contrast to other constructs that have widely separated heads, that 2IQ HMM heads bind to adjacent actin subunits. The levers of both heads are in the post-powerstroke position, accounting for the lack of slow ADP release from the leading head. This actin-attached structure of 2IQ HMM closely resembles myosin 2 HMM which also has two light chains per head. Thus gating of product release from wild type myosin 5 derives from its long levers placing the leading head far away from the trailing head so that the trailing head mechanically tethers its lever.

Supported by AHA (to EF), Wellcome Trust and NIH.

2256-Pos Direct observation of mechanochemical coupling in single-headed myosin V

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Board B371

Myosin V is an actin-based processive molecular motor driven by the chemical energy of ATP hydrolysis. Recently it was reported that one step by single-headed myosin V is composed of two successive steps (a large step and a following small step). Although how the ATP hydrolysis is coupled to these force producing steps is postulated by separate experiments of structural, mechanical and biochemical studies, there is no direct evidence for the connection between them. Therefore the relationship between force generation and ATP hydrolysis remain unclear.

To elucidate the mechanism of the energy conversion processe in myosin V, we measured the mechanical event (force generation) and chemical event (ATP hydrolysis) of single-headed myosin V simultaneously. Steps of myosin V were measured using optical trap while ATP hydrolysis of myosin V were visualized using fluorescent ATP analogues with TIRF microscopy. The nucleotide fluorescence disappeared from 0 to 200 msec following the force generation, which is considered to the large step. This disappearance is probably due to the release of hydrolyzed ADP. Next, myosin V detached from the actin filament within 20 msec of the nucleotide fluorescence appearing. These results suggest that ADP release is not coupled with the first large step (although it is unclear whether ADP release is responsible for the second small step) and the detachment of myosin V occurs immediately after ATP binding to myosin V.

2257-Pos Stiffness of Neck Region in Myosin-V HMM

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Myosin-V moves processively along actin tracks. Each step is powered by converting ATP hydrolysis energy to mechanical work by amplifying a small conformational change in the motor domain to a large scale rotation of neck region. The long neck of myosin-V (6 IQ motifs) allows it to span the half-pitch of actin helical repeat (36nm) when binding. In the presence of calcium, calmodulins on IQ motifs dissociate and myosin-V loses the ability to move processively. To understand the structure of neck region, we measure the stiffness of IQ motifs of myosin V HMM. To do so, we have created myosin V HMM-like mutants with different neck length, some with shorter (2IQ and 4IQ) and another with a longer (8IQ) neck, which are tagged at the C-terminus with a FLAG epitope. We also have created a His-tagged construct containing only the coiled-coil portion. Single-headed HMM-like mutants were prepared by coexpress in the HMM-like heavy chain constructs with His-tagged coil-coil constructs. We selectively purified single-headed myosin-V HMM IQ mutants by using FLAG tag affinity columns and Ni-NTA resin for a 6x histidine tag. Single headed myosin-V-HMM were bound to the surface of a cover slip via their coiled-coil tail region and to fluorescently labeled actin filaments ($\sim$2 μm) via their head domain. The rotation of the actin filaments due to Brownian motion were observed and analyzed at 30fps; the angle with respect to the horizontal was taken for the IQ mutants in the presence and absence of calcium. The range of angles of actin filaments increased as the neck length increased of myosin-V-HMM IQ mutants. In the presence of calcium (1 $\sim$10 μM), the fluctuations of the actin filament angles also increased.

2258-Pos Relationship between Myosin Va ATPase activity and motility

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Board B373

Myosin V is a member of the myosin family, a group of molecular motors that transport organelle in cells. Myosin V is composed of a motor domain, which hydrolyzes ATP at the N terminal, a neck domain and a global tail domain, which binds to the vesicle at the C terminal. Furthermore, myosin V is a dimer as predicted by second amino acid structures and confirmed by electron microscope images of an isolated recombinant molecule. Some researchers have reported that both the long neck domain and the dimer structure are required for processive movement, but this only remains a theory. In fact, single myosin V molecules can also move over an actin filament using hydrolysis energy of ATP like natural dimers.

In order to investigate the mechanism of the chemical-mechanical conversion, we prepared myosin Va-HMM (WT) and myosin Va-HMM mutants at the ATP binding site (P-Loop, Switch I, Switch II) using a baculovirus expression system and observed the behavior of recombinants at the single molecule level by imaging and at the multimolecule level by ATPase and in vitro motility assays.

2259-Pos Simultaneous observation of individual mechanochemical events of myosin V during processive 36 nm steps

Mitsuhiro Sugawa, So Nishikawa, Atsuko Hikikoshi Iwane, Toshio Yanagida

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Board B374

Myosin V is a dimeric molecular motor that moves processively on an actin filament. A coordination of mechanochemical cycles between front and rear heads has been widely believed to be important for this processive movement. However, the coordinated ATPase activity during the processive movement of the two headed myosin V has not been observed directly. Here we attached a quantum dot to the tail domain of myosin V and observed the step movements of the tail (36 nm). Simultaneously, the ATPase turnover events by the head were visualized using Cy3-labeled ATP. We found that the lifetime of Cy3-nucleotide bound to the head was much longer than that expected from the biochemical kinetic study of single-headed myosin V ATPase activity. The timing between the mechanical and Cy3-nucleotide release events indicates that ADP release from the front head is inhibited during processive movement on an actin filament. Therefore, our results favor a model in which the ADP release of the front head was prevented by the backward intramolecular strain, and hence ADP release followed by ATP binding predominantly occur at the rear head. Thus, myosin V achieves the processive movement without dissociating from an actin filament.

2260-Pos Visualization of the mechanochemical coupling in myosin V using deac-aminoATP

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Myosin-V is a two-headed motor, which moves processively along actin with ADP-release as the rate limiting step. The kinetic cycles of the two heads are gated by the internal strain each places on the other. Thus ADP release in the trailing head is accelerated by strain from the leading head and that of the leading head is slowed by the strain exerted by the trailing head. This pathway ensures that ADP is typically released from the trailing head, while the leading head still has bound nucleotide. To understand how myosin-V coordinates ATP binding and ADP release in ATPase cycle, we wish to visualize the ATP events and stepping of the myosin-V molecule simultaneously. A major difficulty in doing so has been that most fluorescently ATP derivatives have a strong nonspecific binding to the cover-slip, making it difficult to distinguish which fluorophores are actually bound to myosin. A new ATP analog, termed deac-ami-

noATP, has been developed which shows a 15–25-fold increase in fluorescent intensity when bound to the active site of myosin-V. To test at single molecule level whether the fluorescence of deac-aminoATP also increased greatly when bound to myosin on a cover-slip surface, we have observed the intensities of single spots of deac-aminoATP in flow chambers in the presence and the absence of myosin-V, which were immobilized on the surface at very low concentrations. In the absence of myosin-V, deac-aminoATP bound nonspecifically to the surface and was visualized as spots. The camera gain was decreased until no spots were observed. When deac-aminoATP was added to surface-bound myosin-V individual spots could again be seen at this same camera gain. Using this method, we should be able to observe ATP binding and ADP releasing from myosin-V as it steps processively along actin.

2261-Pos Mechanical Model Of Myosin-V: Simulation Of The One-head-bound State

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Board B376

The double-headed, unconventional myosin, myosin-V, transports vesicles through cells by walking toward the plus end of actin filaments in a hand-over-hand fashion. Recent single molecule experiments with high spatial and temporal resolution have investigated the dynamics of the detached head during the one-head-bound state in order to discern the physical mechanism by which the lagging head becomes the new leading head. We address mechanistic questions about myosin-V transport using a coarse-grained physical model in which the neck domains are treated as semi-flexible filaments and the lever arm rotation of the leading head is realized through state-dependent changes in the equilibrium angle between the neck and head domains. In particular, we present Langevin dynamics simulations of the tethered diffusion of the motor during the one-head-bound state, in order to answer specific functional questions, which will help with the interpretation of single-molecule experimental data:

1. Which is the rate-limiting part of the rebinding process: ATP-hydrolysis, or tethered diffusion of the detached head to an available binding site?
2. How does an external load force affect the distribution of strain throughout the molecule, and the relative probability for binding to a site in the plus end direction or the minus end direction?

2262-Pos Non-processive Molecular Motors On A Leash: A Novel Single-molecule, Microsecond-resolution Force Clamp

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Many motility functions fundamental for cell life are performed by molecular motors that are not processive (such as, for example, skeletal muscle myosin), or by processive motors that are ultimately constituted by non-processive subunits. Single molecule techniques allow a direct observation of the different mechanical and biochemical states displayed by a single non-processive motor and, in particular, force-clamp methodologies allow the study of how the transitions between these different conformations are affected by load. However, due to the intermittent interaction of a non-processive motor with its track, current force-clamp techniques apply a force few milliseconds after attachment of the motor to its track, during which the motor protein goes through its working stroke. Moreover, the typical time resolution at which the protein mechanical states are sampled ($\sim$0.1 ms) and the minimum duration of the detected events ($\sim$5 ms) might hide some important feature within the motor cycle. Here, we developed a novel single-molecule assay for the study of the interaction between a single non-processive myosin motor and an actin filament, under constant force. A constant positive or negative force is continuously applied to the actin filament, so that the delay between myosin binding and force application is abolished. Force in the range between 0 and $\sim\pm$12 pN are successfully applied. Data are acquired at sample intervals of 5 microseconds and events as short as 100 microseconds can be clearly detected due to the high signal-to-noise ratio of the method. The method allows following the actomyosin interaction under constant force from the very beginning of the cycle and with unprecedented time resolution, and might be applied to a wide range of non-processive molecular motors, single heads of processive motors, or ensemble of motor proteins.

2263-Pos Human Myosin-Vc is a Low Duty Ratio, Non-Processive Molecular Motor

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Myosin-Vc (MVc) is one of the three genes of myosin-V found in vertebrates and is widely found in secretory and glandular tissues, with a possible involvement in transferrin trafficking. Here we show the kinetic characterization of human MVc. Transient and steady-state kinetic studies of MVc were performed using a recombinant, truncated, single-headed construct whose single IQ motif was bound with calmodulin. Actin activated steady-state ATPase measure-

ments revealed a V_{max} of 1.8±0.3 s^{-1} and a K_{ATPase} of 43±11 μM. Unlike previously studied vertebrate myosin-Vs, the rate-limiting step in the acto-MVc ATPase pathway is the release of inorganic phosphate (P_i) (∼1.5 s^{-1}), rather than the ADP release step (∼12.0–16.0 s^{-1}). Nevertheless, the ADP affinity of acto-MVc (K_d = 0.25±0.02 μM) is higher than acto-myosin-Va. Using the measured kinetic rates, the calculated duty ratio of MVc was ∼10%, much less than that reported for mammalian myosins-Va and Vb. Kinetic simulations revealed that the high ADP affinity of acto-MVc may lead to elevations of the duty ratio of MVc to as high as 50% under possible physiological ADP concentrations, and may serve as a regulatory mechanism that may be sensitive to moderate changes in [ADP]. Furthermore, the motile behavior of a recombinant, double-headed heavy meromyosin, containing six IQ motifs and the coiled-coil region was examined using the *in vitro* actin gliding and single molecule total internal reflection fluorescence (TIRF) microscopy assays. The actin gliding assay revealed that the double-headed construct of MVc translocated TRITC-phalloidin-labeled actin filaments at a velocity of ∼24 nm/s, while the single molecule TIRF assay revealed no processive motion of MVc. We conclude that MVc is a low-duty ratio, non-processive myosin-V isoform, which perhaps uses a different mechanism to undergo cytoplasmic transport of cargo compared to other isoforms.

2264-Pos The Myo4p/She3p Complex is a Single-headed Class V Myosin from Budding Yeast

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Vertebrate myosin V's are dimeric processive motors that walk in a hand-over-hand fashion on actin to deliver cargo over long distances. In contrast, the two class V myosins in budding yeast, Myo2p and Myo4p, have been reported to be non-processive (Reck-Peterson *et al.*, 2001, *J. Cell Biol.*). However, a chimera with the motor domain of Myo4p on the backbone of mouse myosin Va was processive, demonstrating that the Myo4p motor domain has a high-duty ratio (Krementsova *et al.*, 2006, *J. Biol. Chem.*). To understand the basis for the lack of processivity, we next examined the properties of a chimera containing the Myo4p rod. Here we show that She3p, a known binding partner of Myo4p, binds to the coiled-coil region of Myo4p and forms a homogeneous single-headed myosin/She3p complex, based on sedimentation equilibrium and velocity data. We propose that She3p forms a hetero-coiled coil with Myo4p and is a subunit of the motor. She3p does not affect the maximal actin-activated ATPase in solution, nor the velocity of movement in an ensemble *in vitro* motility assay. At the single molecule level, the monomeric myosin/She3p complex showed no processivity. We added a leucine zipper to the Myo4p rod in order to simulate possible dimerization *in vivo* by a downstream binding partner such as She2p. Some processive runs were observed, but they were short and few in number. Robust continuous movement was observed when multiple monomeric myosin/She3p motors were bound to a quantum dot "cargo". We conclude that continuous transport of mRNA by Myo4p/She3p in yeast is probably accomplished by several high-duty cycle monomers, bound to multiple zipcodes on the mRNA cargo.

2265-Pos Single Molecule Mechanical Studies On Non-muscle Myosin IIa

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Non-muscle myosin II is involved in cytokinesis, maintenance of tension and contractility of non-muscle and smooth muscle cells and assembles into short filaments of about 10 molecules in each filament half. Compared to non-muscle myosin IIb (NMIIb), the unloaded kinetics of single-headed fragments of non-muscle myosin IIa (NMIIa) are faster and show a lower duty cycle ratio suited to achieve higher contractile speeds when interacting with actin filaments. Still, solution kinetics studies have shown NMIIa to be able to attach to an actin filament simultaneously with both heads, leading to a 4-fold increase in the ADP release rate in the trailing head and a 5 fold decrease in the leading head, consistent with the idea of intramolecular strain regulating the kinetics of nucleotide release in the two head bound state. Similar observations have been made previously for processive motors of the kinesin and myosin families. In order to characterise the mechanical properties of single NMIIa heads and to investigate whether a single dimeric molecule can move processively along actin we used an optical tweezers based force transducer and measured displacements and kinetics at different loads.

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2266-Pos EPR Studies At The Nucleotide Binding Site Of Wild Type And W239+ Dictyostelium Discoideum (Dd) Myosin Motor Domain Constructs

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We have used nitroxide spin labeled ATP analogs to monitor conformational changes at the nucleotide binding site of wild-type Dd myosin (WT) and a construct with a single tryptophan at position 239 in the switch 1 loop (W239+). EPR spectroscopy and tryptophan fluorescence have independently been used to investigate changes at the nucleotide site. The data now allow correlations between the conclusions of the two approaches. In the diphosphate state, the nucleotide pocket of the WT myosin is closed at all temperatures. Upon binding to actin, the nucleotide pocket showed two conformations, one similar to the closed conformation, and one more open, indicated by greater probe mobility. These results are

similar to skeletal myosin. For the W239+ mutant in the absence of actin, the pocket was partially opened at 25°C and closed at 2°C. Upon binding to actin it showed two conformations similar to the WT protein. In the triphosphate state, using ADP-AlFx and ADP-BeFx, the nucleotide pocket was always closed on myosin for both W239+ and WT. Our results with the W239+ mutant show a close correlation between the data obtained with EPR and fluorescence techniques, indicating that the conformational changes in switch 1 loop and nucleotide pocket are structurally coupled. The EPR spectra show that mutations of the W239+ construct have altered the energetics of the open to closed conformation of the nucleotide binding site, making the open conformation ~4–5 kJ/Mole more favorable in the myosin-ADP complex alone. However the energetics of W239+ and WT are similar to each other, and to skeletal myosin, in the complex with actin.

2267-Pos Mechanochemical Coupling In Actomyosin Motility With Fluctuation Analysis

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The central problem of molecular motor studies is to elucidate its mechanochemical coupling. For that purpose, we studied the actomyosin motility of myosin V and myosin II by means of the fluctuation analysis. In the case of myosin V, the dependence of the mean velocity on ATP concentration and the relationship between the mean and the variance of the velocity are both consistent with the Michaelis-Menten mechanism, which supports the tight mechanochemical coupling. On the other hand, in the case of myosin II, the dependence of the mean velocity on ATP concentration is consistent with Michaelis-Menten mechanism, while the relationship between the mean and the variance of the velocity is inconsistent with it. Introducing the molecular friction to Michaelis-Menten mechanism and employing the larger step size than that of expected from the proposed tight coupling model, we successfully explained the myosin II data. This supports the loose coupling view, and the realizing step size of myosin II functioning in ensemble is as large as 200nm by one ATP hydrolysis through cooperativity among myosin motors at zero load.

2268-Pos Mathematical Model Of Myosin Motor Focused On Interaction Length Of Single Myosin (stepsize) And Multiple Myosin System (sliding Distance)

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Interaction length, which is defined as interaction (sliding) distance of 1 myosin (head) per 1 ATP, has been one of most important experimental valus to construct mathematical models of myosin bio-motor. Recently, single molecule measurement techniques have been successfully applied to study the dynamic properties of myosins. By using optical trapping and/or scanning probe nanometry, interaction length (step size) of a single melecule of muscle myosin (myosin II) has been directly measured to be 5–15 nm when it is isolated. On the other hand, it has been shown that interaction length (sliding distance) is estimated to be 60–200 nm in multiple myosin system, such as surface assay, myosin filament assay and muscle. How does the stepsize become larger in multiple myosin system? Do myosins cooperate to convert ATP energy to sliding movment in multiple myosin system?

To approach these interesting questions, in this study, we have constructed mathematical model based on stochastic movement of myosin, which has been observed in single molecule measurements. We have constructed chemo-mechanical potential function which can produce stochastic movment of myosin observed in single molecule measurements. Then, we have simulated movment of multiple myosins connected with springs. With modifying potential function and connecting paramenters, we have calculated interaction length of multiple myosin system. Our results suggest that, in multiple myosin system, energy consumption per unit step would be lower, which could explain larger interaction length mentioned above, and that the larger interaction length could be supported by modifying chemo-mechanical pathways of myosins each other, which means increase of efficiency of conversion of ATP energy to sliding movement.

2269-Pos Step-size Fluctuations strongly affect Dynamics of Motor Proteins

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Recent single-molecule experiments indicate that motor proteins experience large spatial fluctuations during their motion along molecular tracks. We investigate these phenomena using discrete-state stochastic models that provide explicit expressions for dynamic properties of motor proteins. This approach has been used successfully to describe the motion of single processive enzymes. It is found that at zero force symmetric fluctuations do not change the mean velocities. However, fluctuations significantly affect dispersions. Our analysis also suggests that spatial fluctuations might change the stall forces of single motor proteins. Our theoretical approach is applied for understanding recent experimental observations on Myosin-V.

2270-Pos Structural Change and Nucleotide Dissociation of Myosin Motor Domain: Simulation Study using Dual Go Model

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We investigated the structural relaxation of myosin motor domain from the pre-power stroke state to the near-rigor state using molecular dynamics simulation of a coarse-grained protein model. To describe the spontaneous structural change, we introduce a variant of the Go-like model called "dual Go-model" that has two reference structures. The nucleotide molecule is also expressed by a coarse-grained model and its spontaneous dissociation process from myosin is observed.

We found that the structural relaxation to the near-rigor conformation cannot be completed unless the nucleotide is dissociated. Moreover, the relaxation and the dissociation occurred cooperatively when the nucleotide was tightly bound to the myosin head. The result suggested that the primary role of the nucleotide is to suppress the structural relaxation (Takagi and Kikuchi, Biophys. J.(2007)).

The coupling of deformation and the dissociation seems to be relevant to the strain sensing mechanism. To clarify this, we performed simulations in which a constant force is applied to the converter domain. It was found that the nucleotide dissociation and the structural relaxation process are accelerated by the force in the direction of the cross-bridge movement, and decelerated by the force in the opposite direction. This effect of the strain on the structural relaxation and the dissociation process is consistent with experiments.

2271-Pos Mechanism of Switch-2 Mediated Mg^{2+}-Sensing by Unconventional Myosins

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Switch-2 loop is a highly conserved structural element that functions as a nucleotide sensor. Through hydrogen bond formation and Mg^{2+}-coordination, switch-2 is assumed to tightly control ATP-hydrolysis and product release. A tyrosine residue is found in the switch-2 consensus sequence DIYGFE at a position were fast skeletal muscle myosin-2 and a selection of myosins from other classes have an alanine, serine or phenylalanine. Recently, we have shown that physiological changes in the concentration of free Mg^{2+}-ions can modulate the kinetic and motor properties of class-1 and class-5 myosins that have a Tyr-residue at this position, but not those of class-1 and class-2 myosins with a substitution at this position. Therefore, we wanted to find out whether this particular tyrosine is a critical determinant for Mg^{2+}-dependent changes of myosin motor activity. In order to test this hypothesis, we generated *Dictyostelium* myosin-1B and myosin-1E motor domain constructs, in which the corresponding residues F387 (myosin-1B) and Y388 (myosin-1E) were replaced by tyrosine and phenylalanine, respectively. In the case of myosin-1B mutation F387Y led to Mg^{2+}-sensitive ADP-release kinetics, increased ADP-affinity in the actin-bound state, and Mg^{2+}-regulated motile activity. Exactly the opposite effects were observed for the myosin-1E mutant Y388F. Our results indicate the importance of this tyr-residue in mediating interactions between switch-2, Mg^{2+}, and nucleotide. They indicate that all myosins that have a tyr-residue at this position share the ability to sense physiological changes in the concentration of free Mg^{2+}-ions and to switch between rapid motility at low Mg^{2+}-concentrations and bearing tension at high concentrations of free Mg^{2+}-ions. Based on the results of our mutational analysis, structural data, and molecular dynamics simulations, a mechanistic model is proposed that explains how switch-2 can act as a Mg^{2+}-sensor.

2272-Pos *Chara Corallina* Myosin Displays Load-dependent Kinetics

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Chara myosin displays the fastest motor activity amongst the functionally characterized members of the myosin superfamily. To investigate the molecular mechanism of this fast movement, biochemical measurements and gliding assays were performed using a motor domain constructs with two α-actinin repeats serving as an artificial lever arm instead of the native neck region. This construct was efficiently produced in *Dictyostelium* cells and once purified to homogeneity displayed high ATPase activity with a Vmax of 500 Pi/s/head. In the *in vitro* gliding assay, a velocity of 16 μm/s was measured with this construct at 30°C and standard assay conditions. To avoid interference with the active sliding generated by other molecules when working in ensembles, each myosin molecule is expected to dissociate from an actin filament as soon as it sustains a negative load. Here, we characterized the effects of external loads on the kinetics displayed by this construct using laser trap nanometry. In the presence of 5 μM ATP, movement of the myosin was not processive but showed on-off interactions. Dwell times for the bound state were classified into bins according to load and averaged for each bin. The average dwell time was 146 ms at 0.13 pN but shortened to 66 ms when myosin sustained small negative load of −0.03 pN. The plot of the detachment rate, which corresponds to the rate of ATP binding, versus the load gives the interaction length of 6.5 nm. A value that is much larger than measured with myosin V. Our results indicate that even small changes in load affect the kinetics of Chara myosin and that this load dependence is important

for the mechanism of fast myosin-based motility displayed by this motor.

2273-Pos Effect of Calcium on Myosin 1c

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Class 1 myosins are thought to be involved in a wide range of cellular mechanisms where long persisting forces are needed, for instance in bracing and tensioning the cytoskeleton. They are non-filamentous motors containing between one and six calmodulin family light chains. Unlike myosins 2 and 5, they are not dimerized into a two headed molecule. Myosin 1c is implicated in hearing and may be under calcium control through reversible dissociation of calmodulin. We have used negative stain electron microscopy and single particle image averaging to study native full length myosin 1c and myosin 1c expressed with one, two, three and four IQ motifs. In the absence of calcium, image averages were detailed and consistent with crystal structure projections in both the motor domain and light chain regions, to a resolution of ~2 nm. The light chains nearest the motor were clearest and the calmodulin lobes on opposite sides of the heavy chain lever arm α-helix were resolved. The angle between the lever arm and the long axis of the motor was 30–40°. In the presence of calcium, image averages were less detailed but the motor domain shape was often still discernible. The lever arm was much shorter and sometimes appeared to have folded back on itself, suggesting that some or all of the light chains were dissociated by calcium.

2274-Pos Calcium Sensitivity Of The Cross-bridge Cycle Of Myo1c, The Adaptation Motor In The Inner Ear

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Considerable evidence points to the class I myosin, Myo1c, as a mediator of adaptation of mechanoelectrical transduction in the stereocilia of the inner ear. Adaptation, which is strongly affected by Ca2+, permits hair cells under prolonged stimuli to remain sensitive to new stimuli. Using a Myo1c fragment consisting of the motor domain and one IQ domain with associated calmodulin, that has biochemical and kinetic properties similar to the native molecule, we have performed a thorough analysis of the biochemical cross-bridge cycle. We determined that although the steady-state ATPase activity shows little calcium sensitivity, individual molecular events are calcium sensitive. Of significance is a 7-fold inhibition of the ATP hydrolysis step and a 10-fold acceleration of ADP release in calcium. These changes result in a calcium-induced shift in Myo1c from a high to a low-duty ratio motor and a change in the coupling of ADP and actin binding to the motor. These data support a model for slipping adaptation in which Myo1c reduces tip-link tension and allows the transduction channels to close following an excitatory stimulus involving a calcium transient.

2275-Pos Myosin-I Can Act as a Force Sensor

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Myosin-Is are the single-headed members of the myosin superfamily that associate directly with cell membranes and play roles in powering membrane dynamics and retraction. Myo1b, a widely expressed vertebrate myosin-I isoform, is a low duty ratio motor as determined by biochemical measurements, i.e., it spends < 10 % of its ATPase cycle strongly-bound to actin. We examined the force and displacement generated by single myo1b molecules, and we determined the actin-attachment kinetics under varying loads using an optical trap combined with a feedback force clamp. We confirm that myo1b has a two-step powerstroke, and we are able to correlate the lifetimes of these steps with the rates of ADP release and ATP binding. We discovered the kinetics of the actin-myo1b attachment to be extremely strain dependent, with resistive loads increasing the lifetime of the actin-bound state up to 100-fold. The increased attachment lifetime increases the effective duty ratio of the myosin from 0.08 to > 0.80. This is the largest change in attachment lifetime seen for any myosin and is the first direct evidence that myosin-I can act as a force sensor.

2276-Pos Predicted coiled coil of *Dictyostelium* myosin M forms a stable single α-helix that can contribute to the myosin 5 lever

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Myosins use a lever region to amplify conformational changes in the motor domain. Myosin 5's long lever (6IQ motifs that bind 6 calmodulins) allows 36nm processive strides along actin. Trunca-

tion to 2IQs reduces stride length proportionately and almost abolishes processive movement. We previously showed that the predicted coiled-coil domain of myosin 10 forms a stable single α-helix (SAH) that could contribute to its lever. Sequence comparisons suggested that predicted coiled coil in myosins 6, 7a and *Dictyostelium discoideum* MyoM might also be SAH domains. To test these proposals, we have created a chimera from GFP-myosin 5 2IQ HMM by inserting 112 residues of predicted coiled coil from MyoM after the IQs (2IQ-SAH-HMM). If these residues formed a SAH domain, the lever length would be comparable to the wild type (6IQ) HMM. In single molecule total internal reflection fluorescence assays, 2IQ-SAH-HMM was processive, moving on actin at similar velocity to 6IQ HMM. Step length measurements in this assay showed 2IQ-HMM takes short steps consistent with its truncated lever whereas 2IQ-SAH-HMM takes long steps similar to 6IQ-HMM but with a broader distribution. These data show the MyoM residues can increase step length but allow more choice of target actins. Electron microscopy of metal shadowed 2IQ-SAH-HMM shows a thin SAH domain connecting each 2IQ head to the tail. SAH domain length is consistent with an α-helix of 112 residues, and there is no evidence that it dimerises to form an extension of the myosin 5 coiled-coil tail. We conclude that the predicted coiled coil of MyoM is a SAH domain, and that this domain can contribute to myosin stepping along actin.

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2277-Pos Electron Microscopy of Myosin 18 Molecules

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Myosin 18A is a recently identified non-muscle myosin implicated in haematopoiesis. Thus far most of its properties have been inferred from sequence predictions and have not been tested experimentally. The heavy chain C-terminal to the single IQ motif comprises ~700 AA and is mostly predicted to be coiled-coil α-helix. On this basis the molecule was expected to be a dimer with two heads. However, helix propensity plots are less strong than for other myosin tails, such as classes 2 and 5. The α isoform of myosin 18A also contains an N-terminal 300 AA KE-PDZ sequence. We have studied the shape and flexibility of myosin 18A by negative stain electron microscopy. Both α and β isoforms of HMM (truncated at residue 1363) were dimeric. Image averages of expressed α isoform heads showed extra density that may be the KE-PDZ domain near where the N-terminus is expected. The α isoform heads also bundled actin in the presence or absence of ATP, but this was not observed for the β isoform. The tail of the full length molecule was 96 +/− 8 nm long, which is approximately what is predicted for the coiled-coil. The full length molecule also formed bipolar filaments at roughly physiological ionic strength. The results are consistent with the suggestion that the tail region of myosin 18 has similarities to class 2 myosins.

Myosin & Myosin-family Proteins - II

2278-Pos Fluorescent Probes on the N-terminal Domain of the Regulatory Light Chain of Smooth Muscle Myosin: Kinetics of Nucleotide Binding

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Previous studies[1] showed that nucleotide binding to unphosphorylated smooth muscle heavy meromyosin (UP-HMM) with acrylodan-labeled A23C on the regulatory light chain (RLC), increased the polarity and decreased the solvent exposure of the fluorophore, but not in S1 or in phosphorylated HMM (TP-HMM). This suggests that the acrylodan signal is specific to constructs that are regulated by phosphorylation. Here we further investigate the relationship between nucleotide binding kinetics as measured by tryptophan fluorescence *versus* that measured by acrylodan. We show that the second order rate constant for ATP binding reported by tryptophan (K_1k_{+2}) is the same in S1, UP-HMM and P-HMM. It is similar to the fast phase of a biphasic response of acrylodan to [ATP]. Interestingly, the total amplitude change is greater for UP-HMM (−8%) with an acrylodan on both heads than for UP-HMM with acrylodan on only one head (−2%). This suggests a mechanism of ATP-induced self-quenching of the fluorophores, requiring proximity. We propose that A23 in UP-HMM and P-HMM are in similar environments, but only in UP-HMM can ATP cause the two fluorophores to self-quench. In relation to ADP binding, we report a biphasic response of tryptophan fluorescence, only in HMM not in S1. The second order rate constant for ADP binding as measured by tryptophan was similar for S1, UP-HMM and P-HMM. Acrylodan fluorescence changes in response to ADP are still under investigation. Fluorescence anisotropy experiments are underway to determine whether nucleotide can induce changes in rotational mobility of acrylodan at A23C and other positions within the N-terminal domain.

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2279-Pos The Mechanism of Blebbistatin Inhibition on Actin-Myosin Mechanics

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Blebbistatin is a small molecule inhibitor of both muscle force and unloaded shortening. Its primary kinetic effect is to trap myosin in a products complex that has a low affinity for actin. However, the link between blebbistatin's mechanical and kinetic effects remains