including the kidney filter protein nephrin. In addition to discrete binding sites for actin and nephrin, CD2AP possesses three SH3 domains and a proline-rich region containing, in turn, binding sites for SH3 domains. CD2AP is implicated in dynamic actin remodeling and membrane trafficking that occurs during receptor endocytosis and cytokinesis. We have initiated structural studies of recombinant CD2AP protein using electron microscopy and single particle image analysis. Negative stain electron microscopy of revealed uniform particles with a size and morphology suggesting a tetrameric organization, subsequently verified with chemical crosslinking. Single particle image analysis was used to generate a three-dimensional map of the CD2AP tetramer at 21 Å resolution. The electron density map reveals an extended structure allowing the identification of specific subdomains. The tetramer is organized around a central core, including density assigned to the C-terminal coiled-coil domain, surrounded by four loosely attached arms radiating out from the center, which we have assigned to the N-terminal SH3 domains. We have further identified CD2AP as a substrate for cytoplasmic cathepsin L, a protease that is induced in early podocyte damage. Cleavage of CD2AP with cathepsin L results in a C-terminal core domain that is structurally competent but releases the CD2AP binding partner dendrin resulting in translocation of dendrin to the nucleus where it promotes apoptosis. Based on our analysis of the cathepsin L cleavage sites within CD2AP we conclude that cytosolic cathepsin L releases the N-terminal arms producing a structurally competent C-terminal core domain.

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Prestress-dependent Rheology of Semiflexible Polymers of the Cytoskeleton

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Rheological properties of living cells are essential for their physiological functions. Microrheological measurements have shown that cytoskeletal contractile stress (or prestress) and weak power-law viscoelasticity are governing principles of cell rheology, and that these two properties are closely associated in living cells for reasons that are largely unknown. In this study, we develop a stochastic model of a semiflexible polymer of the cytoskeleton that links the power-law rheology to the prestress. We describe a semiflexible polymer chain as a three-dimensional elastically-jointed chain composed of nonlinearly elastic bonds jointed by linearly elastic torsional springs. Assuming that the chain dynamics is thermally driven, we use a Monte-Carlo-based algorithm to obtain numerical simulations of the chain's creep behavior during uniaxial stretching. We obtain that the creep curves follow a power-law and that this behavior changes with prestress in a manner that is consistent with previously reported data from living cells and reconstituted crosslinked actin gels. We show that the power-law creep results from a finite-speed propagation of free energy from the chain's end points towards the center of the chain in response to externally applied stretching force. We also show that the power-law dependence on the prestress results from the chain's nonlinear, stiffening behavior that originates from both entropic and enthalpic contributions. Based on qualitative similarities between model simulations and experimental data from living cells and actin gels, it is conceivable that the mechanisms embodied in our model may also be key determinants of the overall viscoelastic properties of living cells and actin gels.

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Straining the Laws of Attraction: Mechanotransduction Studied Through Changes in Intracellular Binding Energy

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Mechanical force modulates myriad cellular functions including migration, alignment, proliferation, and gene transcription. Mechanotransduction, the transmission of mechanical forces and its translation into biochemical signals, may be mediated by force-induced protein conformation changes, and subsequently result in the modulation of protein signaling cascades. For the paxillin and focal adhesion kinase interaction, we demonstrate that mechano-induced changes in protein complex conformation, dissociation constant, and Gibbs free energy of binding can be quantified by lifetime-resolved fluorescence energy transfer microscopy and fluorescence correlation spectroscopy. A comparison with in vitro data shows that this interaction is allosteric in vivo, and spatially resolved imaging indicates that this binding constant is equal in both the cytosol and focal adhesion complexes. Further, inhibitor assays show that the mechano-sensitivity of this interaction must be mediated by soluble factors not based on protein tyrosine phosphorylation.

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Salt Dependence of Neurofilament Gel Phase Behavior - A Synchrotron X-ray Scattering Study

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Neurofilaments (NFs) are cytoskeletal proteins expressed in neuronal cells, and are believed to play a role in the determination and maintenance of the neuronal cell shape and mechanical integrity. NFs self-assemble as flexible cylinders from 3 protein subunits: NF-Low (NF-L), NF-Medium (NF-M), and NF-High (NF-H). The three subunits are structurally conserved with the exception of their ''tail'' domains, composed of amino acid strands of increasing length and charge respectively. Screening of tail charges is achieved by varying the salinity of the in vitro buffer. At high concentrations, the filaments interact amongst themselves through their unstructured tails that branch out from the filament core and form a viscous gel. Polarized microscopy was used to map out phase diagrams of the resultant neurofilament hydrogels, and thus characterize salt dependent phase behavior. Reassembled separate networks of NF-L + NF-M and NF-L + NF-H show phase transitions from isotropic to nematic gel phases at distinct salt concentrations, thus reflecting the differences in the governing sidearm interactions [1].
We will describe synchrotron x-ray scattering experiments that have allowed us to quantitatively study the microscopic structure of the NF gels: shifts in average interfilament spacing demonstrate the phase boundary between the isotropic and anisotropic NF-liquid crystal gel phases and how they are shifted as a result of varying the in vitro buffer salt concentrations. Funding provided by DOE DE-FG-02-06ER46314, NIH GM-59288, NSF DMR-0503347.
[1] J.B. Jones, C.R. Safinya, Biophys. J. 95, 823 (2008).

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Multi-Parameter Analysis of Spindle and Cell Cycle Dynamics in Asymmetric Cell Division

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Budding yeast is a unicellular organism which undergoes an asymmetric cell division. Successful completion of this process requires the assembly and translocation of the mitotic spindle from the mother cavity interior to the plane of cell division prior to segregation of the chromosomes. We use confocal fluorescence microscopy and automated image analysis algorithms to quantify the movement of the mitotic spindle, throughout the cell life cycle, at high spatial and temporal resolution. By selecting fluorescent proteins which specifically label the spindle poles and cell periphery, the spindle dynamics can be characterised within a coordinate system relevant to the cell division. Spindle length and orientation as well as the mother and bud cavity volumes have been extensively quantified in wild type cells. Our observations reveal fluctuations in spindle length and angle in the short pre-anaphase spindles that are distinct from longer anaphase spindles. Furthermore they indicate an apparent correlation between cell morphology and spindle dynamics. To elucidate the contributions of individual intercellular force generators to the fluctuations observed during spindle assembly and positioning, we apply the above methods to cells bearing deletions of the kinesins cin8, kip1 and kip3. It is expected that this work will ultimately enable a connection between observable single cell dynamics and biochemical signal pathways measurable by bulk assays.

Microtubular Motors I

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Synthesis Of Novel Fluorescent Atp Analogue And Interaction With Nucleotide Dependent Motor Proteins

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Previously, several kind of fluorescent ATP analogues have been synthesized for the application to the kinetic study of ATPase. However, some of the ATP analogues exist as mixture of isomers and showed small fluorescence changes during ATP hydrolysis.
For instance, 2′(3′)-O-NBD-ATP and 2′(3′)-O-Mant-ATP have isomer of 2′ and 3′ in their ribose moiety and each isomer performs differently as substrate for the ATPases. In the present study, we have tried to synthesize new fluorescence ATP analogues that have no isomer and show significant fluorescence change during ATP hydrolysis. The fluorescent ATP analogue 6-(N- (7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino) ethyl triphosphate (NBDTP) and N-methylanthraniloyl amino ethyl triphosphate (MANTTP) have been designed and synthesized, which are similar to non-nucleotide ATP analogue 2-[(4-azido-2-nitrophenyl) amino] ethyl triphosphate (NANTP). It is known that NANTP

are good substrate for skeletal myosin and induce actin gliding in vitro motility assay. The synthesis of the two ATP analogues have been confirmed by FAB-MS. Excitation and emission maximums in the fluorescence spectrum of the ATP analogues were 474nm and 533nm for NBDTP, and 374nm and 430nm for MANTTP, respectively. NBDTP showed microtubules dependent ATP hydrolysis for conventional kinesin at the almost same level to that of regular ATP. The fluorescence changes of the ATP analogues during ATP hydrolysis have been also studied.

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Photo-activation Of Atpase Activity Of Caged-kinesin

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Photo-responsive caged compounds have high potency in the application concerning functional biological molecules as photo-switching device. Kinesin is a motor protein that moves along microtubule by the energy generated from ATP hydrolysis. The structure of conventional kinesin has been well studied and the key regions related to the function were clarfied. In the present study, the photo-regulation of the catalytic activity of mouse brain and C. elegans kinesins were investigated by treating with a caging reagent, 4,5-dimethoxy-2-nitrobenzyl bromide (DMNBB). The mouse brain kinesin mutants that have a single reactive cysteine at 96C were prepared and modified by DMNBB in the presence and absence of ATP. For the kinesin modified in the absence of ADP, the ATPase activity was increased by 300% within 10 minutes. In the presence of ADP, the change of the ATPase activity was slower than that in the absence of ATP. Upon UV irradiation, the ATPase activity of the kinesin modified by DMNBB recovered to the level before modification. Wild type of C. elegans unc-116 kinesin motor domain derived from C. elegans, which has a single reactive cysteine residue. Modification with DMNBB and photo-irradiation on the wild type of C.elegans kinesin unc-116 showed also significant reduction and restoration of activity. We have identified the amino acid residue of kinesin unc-116, which affects activity by the modification with DMNBB as Cys16.

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Interaction of Processive Motor Proteins with ATP analogue Having *Syn* Conformation with respect to the Adenine-ribose bond

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It is known that ATP analogues such as 8-Br-ATP with bulky substitution at the eight position of the adenine ring predominantly assume the *syn* conformation with respect to the adenine-ribose bond. Previously we have demonstrated that 8-Br-ATP induces intrinsic trp fluorescence enhancement of smooth muscle myosin that reflect the formation of the M**·ADP·Pi state. Moreover, the phosphorylated smooth muscle myosin supported actin translocation using 8-Br-ATP. Contrary, for skeletal muscle myosin, 8-Br-ATP induced neither trp fluorescence enhancement nor actin translocation. Kinesin is also ATP driven motor protein that has strikingly similar structure of motor domain to myosin. In the present study, interaction of kinesin with 8-Br-ATP has been examined. Interestingly, conventional kinesin supported microtubules translocation using 8-Br-ATP. This suggests conventional kinesin adopts the 8-Br-ATP in the normal conformation. However, the sliding velocity was approximately one-fifth of regular ATP. Moreover single molecular measurment using optical tweezers revealed that for kinesin, 8-Br-ATP induced nearly similar force generation with that of ATP. Myosin V is also processive motor protein like kinesin. Interaction of unconventional myosin V with 8-Br-ATP was also analyzed. Myosin V supported actin translocation using 8-Br-ATP. Currently, we are examining single molecular measurment of myosin V in the presence of 8-Br-ATP.

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Photo-regulation of Kinesin ATPase Activity using Photochromic Molecule

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Azobenzene is a photochromic molecule that undergoes rapid and reversible isomerization between the cis- and trans-forms in response to ultraviolet (UV) and visible (VIS) light irradiation, respectively. Previously, we have introduced the sulfhydryl-reactive azobenzene derivative 4-phenylazophenyl maleimide (PAM) into the functional region of kinesin to reversibly regulate the ATPase activity of kinesin by photoirradiation. The five kinesin motor domain mutants, A247C, L249C, A252C, G272C and S275C, which contained a single reactive cysteine residue in loops L11 and L12 were prepared. The PAM-modified S275C and L249C mutants exhibited reversible alterations in ATPase activity accompanied by cis-trans isomerization upon UV and VIS light irradiation. In the present study, we prepared the six new mutants (A21C, G26C, S66C, R16C, R25C, M96C) that have single cysteine at near the ATP binding site in order to regulate kinesin activity more effectively than previous mutants. PAM was stoichiometrically incorporated into the cysteine residues in A21C, resulting in reduction of ATPase activity. However, PAM-modified A21C mutant did not exhibit reversible alterations in ATPase activity on UV-VIS light irradiation. PAM-modified G26C mutant also did no show the change in the ATPase activity upon UV-VIS light irradiation. On the other hand, PAM-modified S66C mutant exhibit reversible alterations in ATPase activity on UV-VIS light irradiation in a preliminary experiment.

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Photo-control Of Atpase Activity Of The Kinesin Motor Domain Intermolecularly Cross-linked By Bifunctional Photochromic Compound

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Kinesin is an ATP driven dimeric motor protein carries cellular cargoes along microtubules. Azobenzene-dimaleimide (ABDM) is a bifunctional SH reactive photochromic compounds. We have previously demonstrated that ABDM was incorporated into the functional site of skeletal muscle myosin head (S1), and the global conformational change of S1 was induced by cis-trans isomerization of the cross-linked ABDM upon UV/VIS light irradiation, which may mimic the conformational change accompanied by energy transduction. We have also cross-linked kinesin using ABDM in order to photo-control the ATPase activity of kinesin. We have prepared the mutants of kinesin motor domain T242C, A244C, A247C, L249C, A252C, G272C, and S275C, which have a single cysteine residue in L11 or L12. Only A252C cross-linked by ABDM showed significant alteration of ATPase activity upon UV and VIS light irradiation. In the present study, we prepared novel mutants of kinesin motor domain Q21C, L25C, R26C, in L1, binding site, S66C, Q104C in ATP binding site, T196C in L9, K273C, K274C, Y276C in L12 to regulate ATPase activity efficiently. The mutants were cross-linked intermolecularly by ABDM at high efficiency 70–90%. These mutants cross-linked by ABDM showed alteration of ATPase activity between UV and VIS light irradiation. Especially, the kinesin mutant A252C was regulated most effectively. Additionally, We also prepared the mutant of dimmer kinesin A252C or T353C in order to photo-regulate motility of kinesin with ABDM.

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Processive Motility of Heterodimeric Kinesin That Has Defect in the Neck Linker Docking

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Kinesin-1 is a motor protein that moves processively along microtubules in a hand-over-hand manner. The neck linker, a short stretch that connects two motor heads, has been shown to undergo ATP-dependent conformational changes, although its role on the processive motility is still controversial. To address this question, we employed a kinesin mutant at switch I (R203K) that can normally bind ATP but is unable to hydrolyse ATP (Klumpp et al. JBC 2003). First we observed the neck linker structural state of monomeric R203K bound to the microtubule using single molecule FRET. We found that the neck linker remained undocked conformation even in the presence of saturating ATP, suggesting that ATP-binding is not sufficient to stabilize neck linker docked state. Next we constructed heterodimeric kinesin that is composed of a wild-type head and a mutant R203K head. As recently been shown by Thoresen and Gelles (Biochemistry 2008), this heterodimer showed slow processive movement along microtubules. Then we observed the conformational changes of this heterodimer using a single molecule FRET sensor as previously developed to distinguish one-head-bound and two-head-bound states (Mori et al. Nature 2007), and found that the heterodimer showed hand-over-hand movement. Unexpectedly, they spent most of the time in the two-head-bound state where wild-type head is in the front and the mutant head is in the rear, indicating that the displacement of rear wild-type head to the forward binding site is not the rate-limiting. These results suggest that the neck linker docking in the microtubule-bound head is not essential for the tethered head to translate and bind to the forward tubulin-binding site and rather is required for promoting ATP-hydrolysis and subsequent detachment of the trailing head from microtubule.

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Configuration Of The Kinesin1 Motor Domains In The ATP-waiting State As Revealed By Fluorescence Polarization Microscopy

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The molecular mechanism of coordination between kinesin1 heads during processive walking remains unclear, partly due to the lack of structural information on critical intermediates of the kinesin1 mechano-chemical cycle. To address this issue here we used ensemble and single molecule fluorescence