847-Pos
Mechanistic Analysis of Kar3Cik1 for Mitotic Function
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Kar3Cik1 is a S. cerevisae Kinesin-14 motor protein that functions to shorten cytoplasmic microtubule (MT) during yeast mating for nuclear fusion, yet cross-links interpolar MTs (ipMTs) during anaphase. The Kar3 head contains both an ATP and MT binding site, yet the Cik1 head lacks an ATP catalytic site. Pre-steady-state and steady state experiments were conducted to define the mechanochemical pathway by which Kar3Cik1 stabilizes anti-parallel ipMTs for its mitotic function. To initiate the cycle, we used a high ADP strategy to promote MT-binding by the Cik1 head at 4.9 ∈ 1/4M-1s-1. The initial association is then followed by a 4-5 s-1 conformational change to induce Kar3 head binding to the MT with rapid ADP release from the active site at 109 s-1. MantATP binding to the nucleotide free MT•Kar3Cik1 is fast at 2.1 ∈ 1/4M-1s-1 with koff = 16.6 s-1. Pulse-chase methodology further reveals that MgATP binding to MT•Kar3Cik1 follows a two step process, formation of a collision complex followed by a 64 s-1 isomerization step. ATP hydrolysis occurs at 26 s-1 followed by motor detachment from microtubule at 11.5 s-1. The rate-limiting step for steady-state ATP turnover at 5 s-1 is hypothesized to be the conformational change leading to Kar3 head binding to MT. These initial results suggest a model in which Kar3-Cik1 interacts with the MT through an alternating cycle of Cik1 binding followed by Kar3 binding. Because Cik1 does not have a nucleotide binding site, we propose that head-head communication is mediated by a strain-dependent mechanism. Supported by NIH GM54141.

848-Pos
Probing the Regulatory Mechanisms of KCBP using EPR Spectroscopy
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KCBP, a plant Kinesin-14, is involved in cell division and trichome morphogenesis by structuring and organizing bundles of microtubules. Our published crystal structure of KCBP in complex with its regulator KIC suggests that bound KIC would inhibit KCBP function by physically blocking binding of the motor to microtubules. The neck mimic of KCBP, a linker found at the C-terminus of motor domain, homologous to the neck linker of Kinesin-1, appears immobilized upon KIC binding. The capture of this structural module would disrupt the nucleotide-controlled conformational cycling necessary for generating force and normal function.
Our new experiments demonstrate that tail and motor of KCBP interact directly. Binding of tail has an inhibitory regulatory effect on KCBP motor. Interestingly, binding of KIC to motor tears the motor-tail complex apart suggesting that tail and KIC may share the binding site on the motor, including the neck mimic.
To test the proposed regulatory mechanisms, we assessed the conformational freedom of the neck mimic in KCBP (a.a. 876-1261, no neck) in the presence and the absence of KIC or tail (a.a. 12-503) using EPR spectroscopy. Spectra of spin probes attached to single Cys (S1220C or S1215C) on the neck mimic showed a mobile and an immobilized component. There was a significantly higher content of the immobile component in the spectrum of the Cys1220 mutant. The addition of KIC resulted in a shift of the spectrum into more mobile region in the Cys1220 mutant but not in the Cys1215 mutant. When the tail was added to the labeled motor, the EPR spectra did not change in either mutant. The spectra clearly resolve 2 conformations for each probe and binding of KIC leads to a less structured conformation of a portion of the neck mimic under conditions studied.

849-Pos
ATPase Cycle of the Nonmotile Kinesin NOD Allows Microtubule End Tracking and Drives Chromosome Movement
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Segregation of nonexchange chromosomes during Drosophila melanogaster meiosis requires the proper function of NOD, a nonmotile kinesin-10. We have determined the X-ray crystal structure of the NOD catalytic domain in the ADP- and AMPPNP-bound states. These structures reveal an alternate conformation of the microtubule binding region as well as a nucleotide-sensitive relay of hydrogen bonds at the active site. Additionally, a cryo-electron microscopy reconstruction of the nucleotide-free microtubule-NOD complex shows an atypical binding orientation. Thermodynamic studies show that NOD binds tightly to microtubules in the nucleotide-free state, yet other nucleotide states, including AMPPNP, are weakened. Our pre-steady-state kinetic analysis demonstrates that NOD interaction with microtubules occurs slowly with weak activation of ADP product release. Upon rapid substrate binding, NOD detaches from the microtubule prior to the rate-limiting step of ATP hydrolysis, which is also atypical for a kinesin. We propose a model for NOD's microtubule plus-end tracking that drives chromosome movement.

850-Pos
Analysis of the Role of Unique Loop L5 in Rice Kinesin K16 Motor Domain
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L5 is one of the unique loops locates in the vicinity of ATP binding site of kinesin. We have previously demonstrated that the point mutation at the L5 dramatically alters ATPase activity and interaction with microtubules. Therefore, the loop may be functional key region. The novel rice plant specific kinesin K16 has several unique enzymatic characteristics comparing with conventional kinesin. The most interesting property is that the ADP-free K16 motor domain is very stable, contrast to conventional kinesin that is very labile in ADP-free state. Recently, we have determined crystal structure of the novel rice kinesin K16 motor domain. The crystal structure revealed that the length of the loop L5 was much shorter than that of conventional kinesin. In the present study, we have tried to clarify how the shorter L5 relate to the function of K16. The K16 mutants that have elongated different length L5 were prepared. Microtubule dependent ATPase activity of K16 mutant that has same size of L5 to that of Eg5 was almost abolished. And the monastrol that is known as Eg5 specific inhibitor did not bind to the mutant. On the other hand the K16 mutant that has same size of L5 to that of conventional kinesin showed normal range of ATPase activity. We have also prepared the K16 mutant Q101C that has a single reactive cysteine reside in the L5. Photochromic molecule of azobenzene derivative PAM was incorporated into the cysteine residue to induce conformational change of L5 by ultraviolet and visible light irradiation. However, the kinesin modified by PAM did not alter the ATPase activity by light irradiations.

851-Pos
Interaction of the Eg5 Loop 5 with the Nucleotide Binding Site
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Loop 5 (L5) is a conserved loop that projects from the α2-helix adjacent to the P-loop at the nucleotide site of all kinesin super-family motors. In kinesin-1 and kinesin-3 motors, L5 is 6-8 amino acids in length. Kinesin-5 motors such as Eg5 have longer L5 loops, ~17 a.a. in length. X-ray structures show that L5 is the binding site for small molecules that inhibit microtubule-stimulated ADP release by Eg5. However, crystallography has failed to identify the function of L5 because all Eg5 structures, both with and without bound inhibitors, show similar conformations for L5. It is bent away from the nucleotide site with an unusual loop W127 residue interacting with hydrophobic surface patches and the inhibitor. The proximity of the Eg5 L5 to the nucleotide site suggests it could interact with a bound nucleotide, modulating function. Larson (this meeting) presents EPR spectroscopy data supporting this conclusion. We have used molecular modeling and molecular dynamics (MD) simulations to investigate the potential interaction of L5 and the nucleotide. The L5 domain of the Eg5•ADP x-ray structure was manually deformed via phi-psi backbone rotations. L5 was sufficiently lengthy that W127 could be located in proximity to the adenine ring of ADP. The modified structure was solvated in a box of explicit waters and energy minimized. After 1000 ps of MD simulation, a stable structure was obtained. The structure shows L5 interacting with the adenine ring of ADP via W127 in a pocket formed by the hydrophobic portions of the side chains of E129, D130, and by P27. The structure shows significant impingement on the ribose hydroxyls, consistent with the experimental results of Larson. Thus the simulations provide support for the hypothesis that L5 modulates Eg5 function via interaction with the nucleotide-binding site.

852-Pos
Multivariate Data Analyses for Classifying Allosteric Inhibition in Human Eg5 Kinesin
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Like other motor proteins, the human Eg5 kinesin couples ATP hydrolysis to large conformational changes distal from the nucleotide site, thereby driving movement along microtubules. The Eg5 motor domain uniquely possesses an allosteric L5 loop, responsible for sensitivity to small-molecule inhibitors. Prior studies support a common kinetic mode of inhibition by monastrol, S-trityl-L-cysteine, and ispinesib. However, missing is the role of individual residues

within the L5 loop in allosteric communication and why there are differing efficacies in drug inhibition. Here we demonstrate an integrated approach to build, test, and refine a model of how the L5 loop alters the conformation of the motor domain in solution. Over 30 perturbations of the L5 loop, either by sequence variation or drug binding, were analyzed using kinetic data, vibrational spectroscopy, and multivariate analysis. Principal component analysis organized mutant kinesins into two populations of Eg5 conformers, distinguished by changes mainly in 3_{10} helices and unordered regions. The presence of inhibitors also resulted in coincident, steady-state structural changes in this kinesin. We surmise that the above conformational changes are localized to the L5 loop. Unexpectedly, conformational changes were not restricted to the drug-binding pocket alone: we have directly measured long-distance changes to the beta-sheet core of the kinesin protein, a requirement for allostery that is quantifiable in this analysis. Such tools can ultimately permit prediction of pleiotropic changes in structure and consequently protein function and drug efficacy.

853-Pos

Structure-Function Studies of Loop L5 in the Mitotic Kinesin Eg5

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All processive kinesins contain an unusual structural motif consisting of an alpha helix (α2) interrupted in the middle by a stem and loop motif known as L5. The role of L5 in the overall enzymatic function of kinesin motors remains unknown. However, its importance is highlighted by the finding that a variety of small molecule inhibitors of the mitotic kinesin Eg5, which contains the longest L5 in the kinesin superfamily, bind to this region with high affinity. These inhibitors induce a folding of L5, and trap the motor in an ADP bound, weak microtubule binding conformation. In order to gain greater insight into the function of L5, we have characterized three site-directed mutants in the loop (position 121), stem (position 131) or in the portion of α2 amino-terminal to this stem and loop motif (position 113). These three mutations have profound effects on the kinetics of structural transitions that occur with nucleotide and microtubule binding, and our results indicate that this conserved structural motif plays an important role in tuning the kinetics of kinesin motors.

854-Pos

A Conserved Element in Kinesin-5 Motors Couples ADP Release to a Forward Step

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Kinesin superfamily motor proteins contain a structurally conserved loop near the ATP binding site, termed L5. The function of L5 is unknown, although several drug inhibitors of the mitotic kinesin Eg5 bind to L5. Here, we performed electron paramagnetic resonance spectroscopy on Eg5 with spin labels site-specifically attached to ADP, to L5, and to the neck linker element that docks along the enzymatic head to drive forward motility on microtubules. Our results indicate that L5 undergoes a conformational change that enables Eg5 to bind to microtubules in a pre-powerstroke state, with its neck linker undocked. Deletion or inhibition of L5 blocks this pre-powerstroke state and abolishes the fast, coordinated stepping of the Eg5 dimer. This L5-dependent motile mechanism should prevent single Eg5 dimers from moving on two separate microtubules and forming aberrant microtubule cross-links in the mitotic spindle.

855-Pos

Single Molecule Analysis of the Mitotic Kinesin Eg5

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In vertebrates, proper formation of the mitotic spindle requires the activity of Eg5, a motor protein of the kinesin-5 family. Loss of Eg5 function leads to monopolar spindles and mitotic arrest. Eg5's homotetrameric configuration, in which two pairs of motor domains are connected by a central stalk, allows it to crosslink and slide microtubules. Previous single molecule fluorescence studies of Eg5 have shown that in physiological ionic strength its motion along single microtubules is predominantly diffusive and ATP-independent, but becomes predominantly directional upon crosslinking two microtubules. The structural and mechanistic basis of this allosteric regulation of Eg5's motor activity is poorly understood. One possibility is that Eg5's non-motor domains, including the central stalk and C-terminal tail, transmit signals of microtubule binding state across the Eg5 tetramer and regulate motor activity. To probe the roles of Eg5's non-motor domains and to establish structure-function relationships for these domains, we have generated a series of GFP-labeled Eg5 deletion constructs, including a dimeric version and tetrameric versions containing deletions of the motor domain and of the C-terminal tail domain. Each of the constructs, including one that lacks the kinesin motor domain, binds microtubules with varying affinities. In a multiple motor microtubule gliding assay, deletion of the tail domain results in a 50% increase in gliding velocity, from 15 nm/s for full-length Eg5, to 23 nm/s for tail-less Eg5. This indicates that the tail domain is capable of attenuating Eg5 motility. To understand the contributions of the Eg5's domains to its directional and diffusive movement, we are using single molecule fluorescence microscopy to characterize the motility along microtubules for each construct. These studies suggest a model wherein the non-motor domains of Eg5 modulate its motor activity and contribute to its ability to associate with microtubules.

856-Pos

Tetrameric Chimera DK4mer is a Tool to Study Mechanisms Of Kinesin-5 Regulation. A Tetrameric Chimera of a Kinesin 1 and a Kinesin 5 is a Fast Microtubule Sliding Motor

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The homo-tetrameric motor protein Eg5 from *X. laevis* drives relative sliding of anti-parallel microtubules by the processive action of its two opposing sets of dimeric motor. As shown by Kwok *et al.* (2006, Nat. Chem. Biol. 2:480) and Kapitein *et al.* (2008, J. Cell Biol. 182:421), tetrameric motors move slowly (~20nm/s), but processively on a single microtubule alternating between diffusional and directional episodes, while motors moving between two microtubules move in a highly directional and processive fashion.

In order to obtain a tetrameric model system with more clearly defined properties and motile phases, we have constructed a tetrameric chimera by replacing Eg5-motor domain and neck-linker by the homologous regions of D. melanogaster Kinesin 1 (DK4mer).

In surface-gliding assays, Dk4mer showed fast motility (553 ± 31nm/s), irrespective of a C-terminal his- or GFP-his-tag. Comparison to DmKHC shows a similar $k_{0.5,\ ATP}$ of ~0.06mM, suggesting that the GFP-tagged version is suitable for single-molecule fluorescence studies. Single GFP-tagged DK4mer motors moved processively along the MT at speeds comparable to those seen in surface-gliding assays (499 ± 3nm/s). We observe clearly distinguished directional and diffusional episodes and an overall run length of ~9µm on average. We further performed relative sliding assays using DK4mer and observe the expected trimodal distribution of velocities at v=0, v=v_1 and v=2v_1, v_1 being 500nm/s, clearly showing that DK4mer is capable of sliding microtubules apart simultaneously using both pairs of motor domains.

The DK4mer is thus an excellent model system to study regulatory aspects of Kinesin-5 due to its high speed, its long processivity and its clear separation of diffusive and directional motility and its fast and efficient relative sliding of microtubules.

(*authors contributed equally)

857-Pos

Analysis of Conformational Change of Conventional Kinesin Chimeric Protein Fused with GFP using Small Angle X-Ray Solution Scattering

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Recently, we have successfully dissolved the crystal structure of ADP bound K16 motor domain. The overall structure of the K16MD is similar to that of conventional kinesin motor domains, as expected from the high similarity of amino acid sequence. However, neck-linker region of K16 showed an ordered conformation in a position like that of Eg5. Previously, we have designed the K16 motor domain chimera protein fused with GFP at the neck-linker in order to monitor the conformational change of the neck-linker during ATP hydrolysis by small angle X-ray solution scattering. We determined the Radius gyration (Rg) values of K16-GFP in the presence or absence of nucleotides by X-ray solution scattering. The Rg of nucleotide-free K16-GFP was about 42 Å. In the presence of ADP and ATP, the Rg values were 38 Å and 39Å, respectively. In this study, conventional kinesin fused with GFP (KIF5A-GFP) was prepared and analyzed by small-angle X-ray scattering in order to compare its neck-linker conformation with K16-GFP. The Rg value of ADP and AMPPNP states are 34.5Å while that of nucleotide-free is 35.4Å. For KIF5A-GFP, the Rg difference between nucleotide-free state and nucleotide-docked state is three times smaller than K16. These results suggest that the conformational change of K16 neck-linker is more significant than KIF5A. Moreover, Eg5 fused with GFP (Eg5-GFP) was also successfully expressed in E.coli., which has different orientation of neck-linker in crystal structure. The conformation of the kinesin in the solution was also analyzed.