

likely to undergo exocytosis than those without such associations. When endocytosis was inhibited with Dynasore (inhibitor of dynamin 1 and 2), membrane *p/s* changes following fusion were long-lived (10s of seconds). This is consistent with endocytosis occurring at the site of exocytosis. These experiments are the first to directly visualize plasma membrane changes occurring with secretion. They reveal an association of the granule and plasma membrane for seconds before exocytosis and provide direct evidence for maintained curvature of the plasma membrane after exocytosis.

2937-Plat

Actin Cytoskeleton Controls Movement Of Intracellular Organelles In Epithelia

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Background: Intracellular organelles move along microtubules and filamentous actin (F-actin) using molecular motors. Previously we reported that movement of secretory granules was hindered by Ca^{2+} -dependent formation of a dense cytoplasmic F-actin meshwork in pancreatic duct epithelial cells (PDEC). **Aim:** Here we addressed whether other organelles such as mitochondria and lysosomes also are controlled by the same mechanism. **Methods:** Mitochondria and lysosomes were labeled with fluorescent MitoTracker Red and LysoTracker Red, respectively. Their movements were monitored every 1.2 s with high resolution fluorescence microscopy and measured by comparing subsequent images. Cells were stimulated with UTP to activate an endogenous P2Y_2 G-protein coupled receptor to increase intracellular Ca^{2+} . **Results:** Mitochondria and lysosomes moving actively at rest stopped rapidly ($\tau \sim 2.6$ s) after Ca^{2+} rise. This organelle 'freezing' was accompanied by the formation of F-actin in the whole cytoplasm as stained with phalloidin-Alexa 488. EGFP-actin binding domain 2 (ABD2) expressed in PDEC, indicated a rapid formation of cytoplasmic F-actin ($\tau \sim 7.8$ s). In addition, the freezing of the organelles was blocked by latrunculin B, an inhibitor of F-actin formation. **Conclusion:** Ca^{2+} -dependent formation of a fine F-actin meshwork reduces the movement of intracellular organelles physically in PDEC. The freezing mechanism controls exocytosis of secretory granules and may affect local ATP supply by controlling mitochondrial distribution in PDEC.

Supported by UW Royalty Research Fund and an NIH grant (GM083913).

Platform BG: Microtubular Motors

2938-Plat

Crystal Structure of Nucleotide-free Kinesin-1 Motor Domain Explains Coordinated Walking Mechanism

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Kinesin is a highly processive motor that moves along microtubules in a hand-over-hand manner. To understand structural basis for the walking mechanism, atomic-detailed structural information of kinesin at various nucleotide states are essential, however the nucleotide-free crystal structure has been unavailable. Here we report the first crystal structure of kinesin-1 motor domain without bound nucleotide solved at a 2.8Å resolution. The structure fits very well to the 9Å cryo-EM density map of kinesin-microtubule complex without nucleotide (Sindelar et al. 2007), demonstrating that the structure represents the ADP-released microtubule-bound kinesin head. The nucleotide-free structure showed marked differences from the previously solved ATP- and ADP-like crystal structures. First, compared to ADP-like structure, the switch II helix extends and this extension is stabilized through the interactions with both switch I/II loops and tubulin subunits. Furthermore, switch I uncoils and extends toward the nucleotide pocket. We suggest that microtubule-binding is sensed via the extension of switch II helix, which then promotes ADP release through switch I/II loops. Another striking feature is that $\alpha 6$ helix that directly connects to the neck linker moved such that the beginning of the neck linker collides with the switch II helix. The neck linker itself is mobile but this steric hindrance prohibits extension of the neck linker to the forward direction. Using crystal structures docked to the EM density map, we modeled dimeric kinesin on microtubule and found that kinesin cannot adapt two-head-bound state when both heads are nucleotide-free due to the constraint posed on the neck linker, and the tethered head can bind only to the forward tubulin-binding site after the trailing head becomes ATP-like structure. These findings provide structural basis for the coordinated processive movement of two kinesin motor domains.

2939-Plat

Conformational Specificity in Allosteric Signaling: High-throughput Measurement of Modular Secondary Structural Changes within Human Eg5 Kinesin

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Communication within allosteric enzymes requires long-range interactions. The ATPase activity of human Eg5 kinesin (HsEg5) is allosterically inhibited upon noncovalent binding of monastrol or S-trityl-L-cysteine (STC) with the L5 loop and subsequent distal conformation changes. Our hypothesis is that the E116 and E118 carboxylates, flanking an isolated β -bridge in the L5 loop, are required for allosteric signaling during small chemical inhibition. Surprisingly, assessment by molecular and biochemical methods revealed that substitution of these carboxylates, irrespective of its nature, has a positional dependence on HsEg5 ATPase rates. Single-site substitution of E118 increased basal ATP hydrolysis rates of Eg5, whereas substitution of E116 resulted in lower ATPase rates. Thus, sequence variation at residues 116 and 118 of the L5 loop can drive upregulation and downregulation of ATP hydrolysis *in vitro*, respectively. We conducted high-throughput infrared measurements of the secondary structure composition of the upregulated and downregulated proteins in solution. Vibrational signatures from upregulated proteins were distinct from the net changes measured from downregulated proteins. Compared to control samples, in HsEg5 motors with E118 substitutions, amide I' components between 1642-1620 cm^{-1} decreased in normalized area, whereas proteins with alterations of E116 exhibited an increased contribution in the amide I' area for modes between 1684 and 1646 cm^{-1} . Moreover, the spectral changes exhibited by E116 variants were similar to those of wildtype HsEg5 during allosteric inhibition by monastrol or STC. We conclude that allosteric inhibition by amino acid substitution or by small molecules both result in convergent steady-state changes to the secondary structure of the HsEg5 motor domain in solution, and these rapid methods will provide insight into how long-range structural changes impact motor function.

2940-Plat

The Motility of Monomeric and Dimeric Variants of Eg5 studied in the Presence of the Kinesin-5-specific Inhibitor Monastrol

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The homo-tetrameric motor-protein Eg5 from *Xenopus laevis* drives relative sliding of anti-parallel microtubules, most likely by the processive action of its two sets of dimeric motor domains at each end. As recently shown by Kwok et al. (NCB 2006) and Kapitein et al. (JCB 2008), tetrameric motors move on a single microtubule in a fashion including diffusional and directional episodes, while motors moving between anti-parallel microtubules act in a highly directional and processive fashion. We have studied the processive behavior of a dimeric chimera (Eg5Kin) carrying the Eg5-motor and neck-linker and the Kinesin-1 neck and stalk. While Eg5Kin displays essentially the same motile properties as a truncated Eg5 (Eg5-513 his, Krysiak et al., JBC 2006, Valentine et al., NCB, 2006) its processivity is 40x increased to about 240 consecutive 8nm-steps on average, at a velocity of 95 nm/s. With increasing monastrol concentrations we find a dose-dependent and cooperative reduction in run length, but not in speed, indicating that two monastrol molecules are required to terminate a processive run. To further study the allosteric effect of monastrol on the motility of Eg5-motors, we generated monomeric and dimeric Eg5-constructs and compared their surface gliding-velocities in the presence of increasing concentrations monastrol.

2941-Plat

Cross-Species Analysis of Kinesin-14s: Human HSET Functions in Fission Yeast to Regulate Spindle Bipolarity

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The discovery of Kinesin-like proteins in 1990, five years after conventional Kinesin, has blossomed into the identification of hundreds of Klps that are classified into fourteen families and one orphan family. Not all Klp families are conserved between eukaryotes, however the Kinesin-14 Klps are ubiquitous and key regulators of bipolar spindle assembly and microtubule dynamics and organization. Kinesin-14 Klps associate with microtubules through ATP-dependent binding of their motor domains, but an additional ATP-independent microtubule binding site in the stalk domain is proposed to allow bundling of microtubules. Kinesin-14 members represent some of the most extensively studied Klps, however limited analysis has been done to examine conserved

domains or mechanisms. Kinesin-14s share a domain structure in the stalk that consists of an N-terminal region followed by a region of coiled coil. They can vary drastically in size and share minimal sequence similarities. Features of a second microtubule-binding domain are unknown as well as regulatory elements. Most recently this lab has determined that Kinesin-14/ γ -tubulin interactions at the γ -TuSC MTOC can regulate bipolarity. Other genetic and biochemical analysis indicates that Kinesin-14 interactions with other MTOC proteins also occur. We are using deletion analysis, site-directed mutagenesis and chimeric Kinesin-14 proteins with human HSET and *Drosophila* Ned to relate structural and sequence domains in Kinesin-14s to their functional importance. Twenty-five Kinesin-14 constructs have been generated and are helping to define the minimal Kinesin-14 and to separate domains for bipolar spindle assembly versus later mitotic roles. Length requirements of the coiled-coil domain are also being examined as a critical component in models that emphasize microtubule cross-linking by Kinesin-14s. This work provides insights into Kinesin-14 functional needs and unique tools for assessing Kinesin-14 interactions with tubulin and non-tubulin proteins in conserved molecular mechanisms.

2942-Plat

Reconstitution Of Microtubule-driven Movement and Force Production by the Ndc80 Kinetochore Complex

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The kinetochore-microtubule junction is a central but poorly understood part of the molecular machinery that organizes and separates chromosomes during mitosis. Kinetochores maintain load-bearing attachments between chromosomes and microtubule tips, even as the tips assemble and disassemble under their grip. This end-on coupling is conserved from yeast to humans, suggesting it may be mediated by a common molecular mechanism but the existence of a conserved coupler remains uncertain. Using techniques for manipulating and tracking individual molecules, we discovered that end-on coupling can be reconstituted with the Ndc80 complex, a widely conserved component of the outer kinetochore. Beads decorated with pure Ndc80 complex bind individual microtubules through the N-terminal globular domains of Ndc80p and Nuf2p. Like kinetochores, these Ndc80-based couplers are carried by disassembling tips and they remain tip-bound even under tensile loads up to 2.5 pN. Microtubule-driven movement and force production persist at low surface densities where the number of Ndc80 complexes interacting with the filament is close to the number found at kinetochores *in vivo*. At the single molecule level, fluorescent-tagged Ndc80 complexes diffuse randomly along microtubules except at disassembling tips where their motion becomes biased in the direction of filament shortening. Our results indicate that Ndc80-based coupling likely depends upon a 'fluctuating ensemble' mechanism similar to that proposed by Hill on purely theoretical grounds more than two decades ago*. We also demonstrate that both the yeast and human Ndc80 complexes support microtubule-driven bead movement and force production. This conservation of function, together with the established importance of the Ndc80 complex for kinetochore-microtubule coupling across eukaryotes, strongly suggests that a fluctuating ensemble of Ndc80 complexes represents a core attachment module within the kinetochore.

* Hill, T. L. (1985) Proc Natl Acad Sci USA 82:4404-4408.

2943-Plat

"Cortical" Dynein Can Capture And Pull On Dynamic Microtubule Ends In Vitro

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The interplay between dynamic microtubules (MTs) and cytoplasmic dynein plays an important role in many different cellular processes. Dynein at the cortex positions the mitotic spindle through interaction with astral MTs. Within the spindle MTs interact with dynein at the kinetochore, contributing to poleward movement of the chromosomes. Despite the apparent importance of MT-dynein interactions in these and other cellular processes, very little is known about how dynein mechanically interacts with dynamic MT ends. We have set-up different *in vitro* assays that mimic the MT-cortex/kinetochore interaction. Dynamic MTs grow against a micro fabricated barrier, coated with purified dynein. An optical tweezers based technique, previously

developed in our lab, allows us to monitor MT dynamics and force generation while MTs grow and shrink against the dynein-coated barrier. We show that dynein, artificially anchored to a barrier, dramatically alters MT dynamics (dynein in solution does not alter MT dynamics). It captures MT ends, inhibits growth and induces catastrophes. Subsequent shrinking of the MT is slow compared to the free walking velocity of the motor and the free shrinking velocity of the MT. In addition, the MT-dynein connection at the barrier can generate pulling forces up to 5 ± 1 pN. Our results show that the MT-dynein connection, if it is anchored to a barrier, forms a simple force-generating unit that does not require other factors to behave in a way that is similar to what is observed in living cells.

2944-Plat

Structure and Evolution of Tubulin C-terminal Tails

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Both α - and β -tubulin have a highly charged, acidic, unstructured, C-terminal "tail" peptide (CTT) that extends from the outer surface of the microtubule (MT). These peptides, ~10 residues for α and ~20 for β , do not appear in crystal structures or in cryo-EM reconstructions but are critical to the interaction of other proteins with tubulin in dimer or MT form. The CTT are the locus of iso-type-defining sequence differences as well as the site of most posttranslational modifications (PTM) of tubulin. Molecular modeling shows that the CTT are flexible and mobile compared to the body of the protein, explaining why they don't diffract. MT are covered with a sparse negatively charged brush composed of these peptides: their length is comparable to their grafting distance. CTT mediate interactions with many proteins, including the dimer with the mitochondrial outer membrane protein VDAC. Modeling shows that the tails fit into the channel, but not with branch-producing PTMs (polyglutamylation or polyglycylation). Thus PTMs could alter (prevent) tubulin from acting as a regulator of VDAC, and hence mitochondrial, function. Evolutionarily the tails are highly conserved, but not in exact sequence. Rather the residue length and charge of each CTT are closely maintained. This is found throughout the eukaryotic world, except in organisms that lack mitochondria, in which case the tail can be much shorter and/or less charged. Bacterial tubulin ($\alpha\beta$ -tubulin, not FtsZ), arriving from an unknown eukaryote by horizontal gene transfer, shows great divergence in tail length (from total loss to much increased length) and charge (including some with many positive charges). We conclude that the interactions of the tail peptides with other proteins, including VDAC on mitochondria, provide a major selective constraint on this critical part of the tubulin molecule.

2945-Plat

Modeling of Motor Mediated Microtubule Bending

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Microtubules are often viewed as mechanically rigid compressive struts that help maintain cell shape and aid in the transport of cellular cargo by serving as tracks for the molecular motors. However, individual microtubules in living cells are often highly bent, and even though the different force mechanisms that contribute to microtubule deformations are known, it is not clear how all these mechanisms act together in a given cell type. Recent experiments on LLC-PK1 epithelial cells strongly suggest that F-actin dynamics and polymerization of microtubules play a minor role, and that the dominant mechanism is anterograde transport via molecular motors. Surprisingly, quantitative analysis of these deformations using curvature distributions exhibit striking similarities to *in vitro* gliding assays. Motivated by these experiments, both *in vivo* and *in vitro*, we have modeled the deformation of microtubules under the influence of molecular motor forces by coarse-grained simulations. In the simulations, microtubules are modeled as semi-flexible polymers with rigid bond constraints embedded in a solvent. Molecular motors exert forces on the microtubules, and walk along microtubule tracks according to their known force-velocity relations, bind and unbind stochastically. Simulation results support our experimental findings and further elucidate on the interplay between molecular motors and passive cross-linkers. Our results suggest that molecular motors are not necessarily just cargo carriers, but can play a dynamic role in the deformation and positioning the microtubule array.