INVESTIGATIONS INTO STRUCTURAL AND BIOCHEMICAL DETERMINANTS OF
KINESIN PROCESSIVITY

A Dissertation in
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by
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ABSTRACT

Kinesins are biological motors that utilize chemical energy from ATP hydrolysis to carry out intracellular transport activities. The kinesin superfamily of proteins is comprised of 14 subfamilies that are involved in diverse cellular tasks ranging from cargo transport in neurons to mitotic spindle organization. Understanding motor function has potential applications in elucidating mechanisms of cell division, slowing the progression of cancer, and treating infertility and neuronal diseases. Though extensive studies of the structure, biochemistry and force producing properties of diverse kinesins have contributed greatly to knowledge of the mechanism of motor function, the precise transitions underlying the kinetic cycle and the molecular basis of motor processivity are not completely understood. The overall goal of this thesis is to investigate the structural and biophysical determinants of kinesin function in order to relate the motor properties of diverse kinesins to their cellular functions.

The kinesin neck-linker, a 14-18 amino acid flexible domain that connects the catalytic core to the coiled-coil rod, is the key mechanical element that underlies coordinated stepping of dimeric motors. Consistent with their diverse cellular tasks, the processivity of kinesins – the ability to take multiple steps per interaction with a microtubule – varies from ~10 to ~1000. To test the degree to which the processivity of N-terminal kinesins is determined by their neck-linker domains, kinesins-1, 2, 3, 5 and 7 were engineered with different neck-linker lengths. Using total internal reflection fluorescence microscopy, single kinesin molecules walking along microtubules were visualized and their run lengths were characterized. With native neck-linkers, which range from 14 to 18 amino acids, run lengths of kinesins-1, 2, 3, 5 & 7 ranged from below 200 nm to 2 μm. Surprisingly, when their neck-linkers were shortened to 14 amino acids, kinesin-3 and kinesin-5 run lengths matched that of kinesin-1 as did kinesin-2, after changing a kinked proline to an alanine and kinesin-7, after the end of α-6 was changed to match the kinesin-1 sequence.
This convergence of processivity is observed even though the speeds of these motors varied over a 25-fold range. From stochastic simulations of the hydrolysis cycle, these effects can best be explained by either a slower strain-dependent detachment of the rear head (disrupting rear head gating) or a reduced strain-dependent inhibition of ATP binding to the leading head (disrupting front head gating). These results suggest that diverse N-terminal kinesins are inherently processive to the same degree, and their wild-type behavior results from differences in the length and sequence of their neck-linker domains.

Many intracellular transport activities including long range cargo transport and bidirectional neuronal transport involve multiple motors working in a coordinated manner. Though single motor properties have been studied in great detail, much less is known about the mechanism of collective motor behavior. By linking single molecule biophysics and in-vitro characterization of multi-motor behavior to cell biology, mechanistic details of cellular functions can be uncovered. Using the kinesins generated for the neck linker investigations, fast and slow motors in varying ratios were adsorbed onto flow cells and the speed at which they moved microtubules was measured. When mixed with kinesin-3 and kinesin-5 motors, kinesin-1 and kinesin-7 speeds dominate, and influence the mechanical properties of kinesins-3 & 5.

This study has established that inter-head tension controls kinesin processivity by altering either front-head gating or rear-head gating, while motor speeds are controlled by a different mechanism.
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Chapter 1

Introduction to kinesin structure, function and mechanochemistry

Kinesins are ATPases that interact with microtubule tracks to carry out active intracellular transport including movement of organelles, proteins, vesicles and chromosomes (Vale and Fletterick, 1997). In addition to helping us understand the biological roles of these protein machines, biophysical characterization of kinesin promises to enhance our knowledge of the interplay of biochemistry and mechanics at the molecular level. This thesis contributes to the realization of this promise for the N-terminal proteins of the kinesin superfamily.

1.1 Cellular Functions of the Kinesin Superfamily

The 14 families under the kinesin superfamily (Lawrence et al., 2004) are comprised of motor proteins with N-terminal motor domains (Kinesins 1-12), C-terminal motor domains (Kinesin-14) and internal motor domains (Kinesin-13). These various kinesins use chemical energy from ATP binding, hydrolysis and release to move along microtubules and carry out diverse and essential cellular tasks. Kinesin-1 (Conventional kinesin, KHC) motors are involved in fast transport including axonal transport (Brady et al., 1990) and nuclear movement (Holzinger and Lutz-Meindl, 2002). Kinesin-2 (KIF3) motors, in tandem with dyenins, are critical for maintaining bidirectional transport along flagella in chlamydomonas (Kozmsinski et al., 1995). KIF3B knock-out mice exhibited loss of nodal cilia and loss of left-right asymmetry and randomization of heart loops and tail turning (Nonaka et al., 1998). Kinesin-3 (Unc/KIF1) motors are involved in the transport of synaptic vesicles in neurons (Hall and Hedgecock, 1991). Kinesin-5 (Eg5) motors are mitotic kinesins involved in bipolar spindle formation (Enos and
Morris, 1990), while Kinesin-7 (CENPE) are involved in chromosome alignment during mitosis (Schaar et al., 1997; Wood et al., 1997). Kinesin-8 and Kinesin-13 are known to be depolymerizing kinesins responsible for regulating microtubule dynamics (Desai et al., 1999; Gupta et al., 2006; Varga et al., 2006).

A hallmark of many of these kinesins is their processivity, the ability to walk on microtubule tracks (Block et al., 1990; Howard et al., 1989), taking multiple 8 nm steps (Svoboda et al., 1993) - corresponding to the size of each tubulin subunit they step along. Kinesins are capable of working against external loads (Svoboda and Block, 1994; Svoboda et al., 1993; Valentine et al., 2006; Yardimci et al., 2008). Their ability to walk long distances and to carry loads makes kinesins efficient vehicles for transporting organelles, chromosomes and sliding of anti-parallel microtubules in the mitotic spindle.

1.2 Kinesin Structure

The overall structure of kinesin-1, as seen in figure 1, is composed of heavy chains containing the dimeric motor and light chains which contain cargo binding sites (Bloom et al., 1988; Cole et al., 1993). The head domains contain a nucleotide binding site, a microtubule binding site (Mandelkow and Hoenger, 1999) and a flexible 14 amino acid chain called the neck-linker (Sack et al., 1997). The two heads are dimerized through an alpha-helical stalk region (Sack et al., 1997), which also acts as a tether to separate the cargo-binding tail domain from the microtubule-binding head domain. The flexible neck-linkers connect the two catalytic core domains to the stalk. Crystal structures of the various other kinesin family members have been solved more recently and show a high degree of structural similarity with kinesin-1 (Garcia-Saez et al., 2004; Turner et al., 2001).
1.3 Kinesin mechanochemistry

Kinesin-1, so named as it was the first discovered member, has been the focus of intensive study and most of our understanding of how these motor proteins function is from structural, biophysical and biochemical characterization of kinesin-1. Recent investigations into different kinesin family members have broadened our knowledge of kinesin mechanochemistry.

The very first single molecule assays of kinesin established that single kinesin-1 molecules are processive motors capable of transporting microtubules and cargo (Block et al., 1990; Howard et al., 1989). Bead experiments showed that single kinesin-1 motors can pull external loads, and motor speeds slowed down linearly with applied force (Meyhöfer and Howard, 1995; Schnitzer et al., 2000; Svoboda and Block, 1994), suggesting that there are at least two load-dependent transitions in the stepping cycle of kinesin. Stall forces for different kinesins
have been recorded in optical trapping experiments with kinesin-1 and kinesin-7 both capable of resisting forces up to ~6 pN (Schnitzer et al., 2000; Svoboda and Block, 1994; Yardimci et al., 2008). Dimeric constructs of kinesin-5 are processive and able to pull loads of up to 5 pN, though they detach from microtubules before they stall (Valentine et al., 2006).

With the exception of monomeric KIF1A which is shown to be processive in vitro (Okada and Hirokawa, 1999), processivity in kinesins is a feature of two-headed motors and requires coordination between the two heads (Hancock and Howard, 1998; Hancock and Howard, 1999). The two motor domains hydrolyze ATP alternately as they walk along microtubules (Hackney, 1994) and have different affinities for microtubules depending on the nucleotide environment. In the absence of nucleotides, only one of the motor heads binds to microtubules and this state is a high affinity state (Asenjo et al., 2003; Hackney, 1994; Kawaguchi and Ishiwata, 2001). In the presence of ADP, the heads bind weakly to microtubules and in the presence of ATP, binding is tight (Asenjo et al., 2003; Uemura et al., 2002). Processive stepping of kinesin-1 is tightly coupled to ATP hydrolysis, with the motor hydrolyzing one ATP per step (Coy et al., 1999b; Hua et al., 1997; Schnitzer and Block, 1997).

ATP binding to kinesin is force-dependent (Svoboda and Block, 1994; Visscher et al., 1999), indicating that this step is associated with a conformational change. The kinesin neck-linker undergoes a transition from a disordered to an ordered state upon ATP binding (Rice et al., 1999). This structural change is the key conformational change that the motor undergoes as it steps along a microtubule. According to the neck-linker docking model when a kinesin dimer encounters a microtubule, one of the heads releases its nucleotide and binds tightly to the filament. When ATP binds to this microtubule bound head, the neck-linker docks to the front head and this change is thought to displace the rear head towards the next binding site. The
tethered head then releases its ADP to bind to the microtubule and the other head hydrolyzes its ATP, which causes the neck-linker to undock and the motor is ready for its next step (figure 1.2). When residues in neck-linker of kinesin were mutated, motor speeds decreased 100-fold even though the ATPase rate did not change significantly (Case et al., 2000). These results illustrate the importance of the neck-linker in kinesin motility.

Figure 1.2: Structure of the docked kinesin neck-linker.
A) Rat kinesin-1 crystal structure showing docked neck-linker (Sack et al., 1997). B) Kinesin-1 docked to microtubule, neck-linker in purple (Sindelar and Downing).

Figure 1.3: Neck-linker docking model of kinesin stepping (Rice et al., 1999)
1.4 Processive stepping cycle of kinesin

To help delineate the mechanism by which kinesin motors walk processively along microtubules, models have been developed that incorporate chemical and mechanical transitions of the two head domains. To maintain processivity, the two heads must synchronize with each other such that they do not simultaneously detach from the microtubule. The overall consensus in the field is that the motors walk in a hand over hand model, where the heads take alternate steps and constantly exchange their forward and rear positions (Asbury et al., 2003; Hackney, 1994; Yildiz et al., 2004). Since the affinities of motor heads for microtubules differ with the nucleotide environment, a phase difference in their catalytic activity must be maintained to enable hand-over-hand movement. Internal mechanical tension is thought to control this ‘gating’ mechanism, and the two models that describe the mechanism are rear head gating and front head gating.

Detachment from microtubules in the ADP/ADP-Pi state of a single headed kinesin construct is ~10 fold lower than for a dimeric motor indicating that the detachment of one head is accelerated by the other head (Hancock and Howard, 1999). Using kinesin mutants that stall on microtubules after hydrolyzing one ATP, it was shown that the rear head detachment has to occur for ATP to bind to the front head (Klumpp et al., 2004). These results support a gated rear head model, where inter-head tension accelerates detachment of the rear head which is then followed by ATP binding, hydrolysis and forward stepping, continuing on with the processive cycle.

The first evidence of a strain-gated ATP binding to the front head was shown by pre-steady state kinetic measurements of ATP binding to nucleotide free motors in the presence of microtubules (Rosenfeld et al., 2003). More recent evidence for front head gating comes from experiments that investigated kinesin stepping in the presence of ATP analogs (Guydosh and
Block, 2006). In the event of analog binding, processive stepping of kinesin was paused and normal stepping could only occur when the motor underwent a back step in order to exchange its front and rear head positions and release the analog allowing ATP to bind for normal processive stepping to continue. In this model, internal strain between the two heads prevents premature ATP binding to the front head. This reduces the possibility of both heads being in a low affinity state, which would lead to dissociation from the microtubule. When the rear head detaches from the microtubule, that relieves the tension, allowing ATP to bind to the front head and continue on with its processive cycle.

The Hancock model of chemomechanical cycle shown in figure 2 starts with both motor heads bound to the microtubule (state 1) (Muthukrishnan et al., 2009). For the motor to continue walking ATP has to bind to the front head and the rear head has to detach and move forward. There is still debate in the field on the sequence of transitions that lead to stepping. In the gated rear head pathway, internal strain on the rear head speeds its detachment to (state 2) in the cycle, ATP binds subsequently in state 4 followed by a conformational change throwing the rear head forward, while in the other pathway ATP binding to the front head (state 3) leads to detachment of rear head and causes reorientation of the rear head towards the next binding site (state 4). ATP hydrolysis occurs (state 5) and the tethered head can release its ADP and bind to the next tubulin subunit (state 1) continuing on with its processive walking or it can detach from the microtubule terminating the cycle.
What limits processivity of motor proteins?

Despite the large body of knowledge of the kinesin hydrolysis cycle, there are still many unanswered questions. Where precisely in the hydrolysis cycle is the forward motion of kinesin produced? Do sub steps occur during kinesin’s 8nm stepping and on what scales do they occur? What is the rate limiting step in the motor hydrolysis cycle and is it the same for all the different kinesin family members? All of these questions ultimately bear on the question: what limits
kinesin processivity? Different kinesin family members are processive to different degrees, with run lengths ranging from ~10-1000 steps per interaction with a microtubule (Valentine et al., 2006; Varga et al., 2006) consistent with the diverse cellular tasks they carry out. These differences in processivity have been attributed to differences in the biochemical rates of their kinetic cycle (Rosenfeld et al., 2009; Rosenfeld et al., 2005). Electrostatic interactions between the motor and microtubules have also been shown to influence kinesin processivity (Okada and Hirokawa, 2000; Thorn et al., 2000). The precise mechanism by which kinesin processivity is controlled is still not understood completely.

1.6 Neck-linker - a mechanical element for transmission of force

Internal tension between the two heads, mediated through their neck-linkers, is thought to be the mechanical signal that gates the catalytic activity of the two heads and enables kinesin to step processively (Block, 2007; Hancock and Howard, 1999; Rosenfeld et al., 2003). Because the neck-linker is thought to be an important element that drives kinesin processivity, uncovering the precise role of the neck-linker in motor stepping is an important step for understanding the mechanism of kinesin processivity. When 6 additional amino acids were added to the kinesin-1 neck-linker, its biochemical processivity decreased by a factor of three (Hackney et al., 2003) and this finding was supported by data from Muthukrishnan et al, showing a factor of four decrease in processivity on insertion of 3 amino acids to the kinesin-1 neck-linker (Muthukrishnan et al., 2009). In contrast to these findings, Yildiz et al published data that showed no significant change in processivity even on addition of 13 amino acids (Yildiz et al., 2008). Because the neck-linker is thought to transmit inter-head tension, any changes to the length of the neck-linker is expected to influence motor stepping. Hence, the results from Yildiz et al are particularly intriguing and warrant a more thorough investigation into the role of the neck-linker, in kinesin processivity.
1.7 Diversity in the structure and function of kinesins

Though the diverse kinesin family members exhibit a high degree of structural similarity, there are striking differences in their structure that bear directly on their specific cellular tasks. When the motor domains of 5 different N-terminal kinesins were aligned, it was observed that the length of their neck-linkers, though conserved within a specific family, varied from 14–18 amino acids between the different kinesin family members (Hariharan and Hancock, 2009). Although parts of the neck-linker are conserved across kinesin families, differences do exist and these may contribute to diversity in motor mechanical and biochemical properties. The neck-linker has been shown to regulate motor ATPase and differences in motor speeds can be attributed to differences in their neck-linker sequences as evidenced by neck-linker mutant studies (Case et al., 2000; Kalchishkova and Bohm, 2008; Nitta et al., 2008). When the crystal structures of kinesin-1 and kinesin-14 were compared it was observed that even though the catalytic cores of the two motors were very similar, there were differences in their surface loops and it was proposed that these features may dictate the different behaviors of the two motors (Sablin et al., 1996). Kinesin-3 motors are known to tether to microtubules through interactions with a lysine rich motif on their loop 12 (Okada and Hirokawa, 2000) and this feature may confer a high degree of processivity required for their long axonal transport functions. The loop 2 in kinesin-13 motors and kinesin-8 motors is longer than in other family members and has been shown to be important for the depolymerization activity of these motors (Moores et al., 2002; Ogawa et al., 2004; Peters et al., 2010). Kinesin-5 motors have a longer loop 5 and binding of monastrol, a kinesin-5 specific ligand, to loop 5, inhibits motor activity (Cochran et al., 2005). These differences in motor structure underscore the importance of specific structural features and the need for a better understanding of their function.
1.8 Collective motor behavior

Intracellular trafficking by assemblies of multiple motor have been reported for transport of endosomes, melanophores and lipid droplets (Levi et al., 2006; Shubeita et al., 2008; Soppina et al., 2009). In many of these cases including in mitochondrial transport and transport of lipid droplets, bidirectional movement along microtubules is a common mode of transport (Welte, 2004). The motor ensemble which includes both plus-end directed and minus-end directed motors receives cellular cues to transport cargo along microtubules in either anterograde or retrograde direction. Ensembles of the faster Osm-3 and the slower kinesin-2 motors work cooperatively to transport IFT particles to build sensory neurons in C. elegans (Snow et al., 2004). The slow motor exerts drag on the fast motor and the fast motor speeds up the slow motor in a concerted manner, thereby controlling the IFT pathway for building and maintaining cilia (Pan et al., 2006). This system serves as a model for understanding coordination between different motors as they transport cargo in cells. In both unidirectional and bidirectional multi-motor transport, coordination between the component motors is a key factor for efficient and precise cargo trafficking. Two important advantages to having multiple motors over a single motor pulling cargo are that longer run lengths and higher force capacities can be realized. Simulations of multi-motor systems have in fact shown that run lengths increase exponentially with increasing numbers of motors and these motors can pull larger forces (Klumpp and Lipowsky, 2005). Understanding collective motor behavior and the mechanics of motor coupling in these protein networks is a major research area and has been receiving much attention of late (Constantinou and Diehl, 2010; Howard, 2009; Kunwar et al., 2008; Leduc et al., 2010). Protein and DNA based scaffolds have recently been developed for organization of motor assemblies, thus allowing precise quantitative and mechanistic analysis of multi-motor systems (Diehl et al., 2006; Schweller et al., 2008). With existing structural, biophysical and biochemical data from study of
single motor properties, predictions of motor assembly behavior can be tested. Investigations of how motor proteins work in groups opens very exciting avenues of research into behavior of protein complexes and towards understanding many cellular processes.

1.9 Significance of studying kinesins

Forces generated by interaction of molecular motors with cytoskeletal filaments drive the formation and maintenance of the mitotic spindle leading to cell division (Scholey et al., 2003). Disrupting the process of cell division in cancer cells by targeting mitotic motors, has recently received a lot of attention as possible treatment strategies. Kinesins-1 and 3 are neuronal transport motors and understanding their role in propagation of neurodegenerative diseases may hold the key to finding cures for various disorders like Alzheimer’s and Parkinson’s. In eukaryotic cells, assembly and maintenance of cilia is required for normal function of kidneys and photoreceptor cells among others. Intra-flagellar transport driven by kinesin-2 and dynein motors helps maintain cilia and defects in intra-flagellar transport are linked to many pathologies (Pazour and Rosenbaum, 2002).

Amyloid precursor proteins which lead to formation of amyloid plaques in Alzheimer’s patients are transported by kinesin-1 molecules (Kamal et al., 2000). In vertebrate cells, site specific knock-out of kinesin-2 resulted in apoptosis mediated by opsin mislocalization (Marszalek et al., 1999) and kinesin-2 motors are known to play a role in the development and transport of sperm (Kierszenbaum, 2002; Miller et al., 1999). Kinesin-5 inhibitors have been the focus of clinical trials as anti-mitotic drugs for cancer treatment (DeBonis et al., 2004; Mayer et al., 1999). A recently identified small molecule inhibitor of kinesin-7 has been shown to arrest mitosis and shows promise as an anti-tumor drug (Wood et al., 2010). Understanding the function
of these remarkable protein machines will enhance our knowledge of the diseases associated with them and help chalk potential treatments and develop therapeutic reagents. As a mechanoenzyme, kinesin serves as a model system for understanding chemomechanical interactions at the molecular level.

1.10 Thesis motivation and summary

The overall goal of this thesis is to understand the molecular basis of processive motility of the diverse kinesins and to define the structural, biophysical and biochemical determinates of motor function. Motors belonging to five different kinesin families were assayed in single molecule and multi-motor experiments using total internal reflection microscopy (TIRF), fluorescence microscopy, Forster resonance energy transfer (FRET) and stochastic simulations of the motor chemo-mechanical cycle.

Kinesin-2 motors contain two different catalytic domains dimerized by a stalk region and are thus unique in the superfamily of motors. Understanding their structure and function will shed light on the working of other kinesins. In single molecule studies, KIF3A/B a mouse ortholog, moved at 60% of kinesin-1 speed and was 4 fold less processive than kinesin-1 (Muthukrishnan et al., 2009). The neck-linker of kinesin-2 motors contains 17 amino acids and is 3 amino acids longer than kinesin-1. To understand how the neck-linker length influences processivity, run lengths of kinesin-1 and kinesin-2 motors with varying neck-linker lengths were compared with each other and data was interpreted in the context of our chemomechanical cycle using stochastic simulations.
Processivity of the different kinesin families varies over a wide range and has been attributed to differences in their biochemical rate constants, dimerization strength of their stalk region and to presence of charged residues on their stalks. Our data on kinesin-2 processivity prompted us to re-evaluate the current understanding of the molecular basis of kinesin processivity. To test if the neck-linker length determines processivity in other kinesins single molecule run lengths of 5 different N-terminal kinesins were characterized in chapter 3. Stochastic simulations of motor stepping were carried out to understand the chemomechanical cycle and to place constraints for identifying the rate limiting transition.

In order to probe the hydrolysis cycle in greater detail, experiments were designed to measure FRET between kinesin heads and microtubules in chapter 4. Novel strategies for selective labeling of motor heads were explored and in the process, mutant kinesins whose activity was regulated by inhibitors were engineered. In chapter 5, the pitch of the research was changed to interrogate behavior of an ensemble of motors to relate to their cellular activity using motors generated in chapter 3. Motors with varying speeds were mixed in different ratios and their influence on each other was assayed to predict how mechanical coordination within a network of motors regulates many intracellular processes. In chapter 6 results from the thesis modules are interpreted in terms of the current model of kinesin processivity to delineate motor function and to set future research goals.
1.11 References


Chapter 2

Neck-linker Length Determines the Degree of Processivity in Kinesin-1 and Kinesin-2 motors

2.1 Summary

Defining the mechanical and biochemical determinates of kinesin processivity is important for understanding how diverse kinesins are tuned for specific cellular functions. Because transmission of mechanical forces through the 14-18 amino acid neck linker domain underlies coordinated stepping (Block, 2007; Hackney et al., 2003; Muthukrishnan et al., 2009; Rosenfeld et al., 2003; Vale and Milligan, 2000; Yildiz et al., 2008), we investigated the role of neck linker length, charge and structure in kinesin-1 and kinesin-2 motor behavior. For optimum comparison with kinesin-1, the KIF3A head and neck linker of kinesin-2 were fused to the kinesin-1 neck-coil and rod. Extending the 14-residue kinesin-1 neck linker reduced processivity, and shortening the 17-residue kinesin-2 neck linker enhanced processivity. When a proline in the kinesin-2 neck linker was replaced, kinesin-1 and kinesin-2 run lengths scaled identically with neck linker length, despite moving at different speeds. In low ionic strength buffer, charge had a dominant effect on motor processivity, which resolves ongoing controversy regarding the effect of neck linker length on kinesin processivity (Hackney et al., 2003; Miyazono et al., 2009; Muthukrishnan et al., 2009; Yildiz et al., 2008). From stochastic simulations, the results are best explained by neck linker extension slowing strain-dependent detachment of the rear head along with diminishing strain-dependent inhibition of ATP binding. These results help delineate how inter-head strain maximizes stepping and suggests that less processive kinesins are tuned to coordinate with other motors differently than the maximally processive kinesin-1.
2.2 Highlights:

- Processivity of both kinesin-1 and kinesin-2 motors scales with neck linker length.
- Positively-charged residues in the neck linker enhance processivity at low ionic strength but have a minor role at higher ionic strength.
- In stochastic simulations, the results can be explained by diminished inter-head strain affecting both trailing-head detachment and ATP binding to the leading head, but not by one mechanism alone.

2.3 Results and Discussion

2.3.1 Extending the Kinesin-1 Neck Linker Decreases Processivity

To investigate the influence of neck linker length on kinesin-1 processivity, homodimeric *Drosophila* conventional kinesin, truncated at residue 559 and fused to a C-terminal GFP were visualized moving along immobilized bovine brain microtubules by single-molecule total internal reflection fluorescence (TIRF) microscopy. To minimize any electrostatic tethering between the motor and microtubule that can complicate the interpretation of mechanical processivity, experiments were performed in 80 mM PIPES buffer. The control Kin1 motor moved at 990 ± 130 nm/s (mean ± SD), with a mean run length of 2.1 ± 0.1 μm (mean ± s.e. of fit) (Figure 2.1A). When the neck linker domain was extended by three residues (Kin1+DAL, corresponding to the last three residues of the kinesin-2 neck linker), the run length dropped by a factor of five to 0.39 ± 0.02 μm and the speed dropped to 600 ± 89 nm/s. Interestingly, inserting only one amino acid (Kin1+L) diminished the run length to 0.89 ± 0.06 μm and the speed to 580 ± 95 nm/s (Figure 1B). These results confirm and extend previous work using full-length kinesin attached to beads
(Muthukrishnan et al., 2009), and they demonstrate that kinesin-1 processivity is significantly reduced when the neck linker domain is extended by even one amino acid.

2.3.2 How Tight is the Kinesin-1 Neck Linker?

For processive kinesin stepping, there is presumably an optimum neck linker length – if the neck linker is too long it will be unable to transmit mechanical tension between the two head domains, but if it’s too short, then the tethered head won’t be able to reach the next binding site on the microtubule. The finding that extending the kinesin-1 neck linker by one residue reduces processivity by half suggests that the length of the native neck linker is an upper limit for maximum head-head coordination. Is it possible to shorten the kinesin-1 neck linker and enhance head-head coordination? To test this question, we deleted the last residue in the kinesin-1 neck linker, Thr344. In high density microtubule gliding assays, Kin1\textsubscript{AT} moved microtubules at 607 ± 86 nm/s. However, no processive events were observed in the single-motor TIRF assay. Dilution profiles of full-length Kin1\textsubscript{AT} motors adsorbed to polystyrene beads were also consistent with a non-processive motor (Supplementary Data; Figure 2.5).
Figure 2.1: Kin1 Run Lengths and Design of Kin1 and Kin2 Constructs

(A) Run length of control Kin1 from TIRF assay. Data were fit to a single exponential. (B) Run lengths of different Kin1 constructs as a function of their neck linker length. Error bars represent the standard error from exponential fits. (C) Amino acid sequence of the kinesin-1 (KHC) and kinesin-2 (KIF3A) neck linkers with the adjacent α6 (last helix in the head domain) and α7 (neck coiled-coil domain). The Kin1 construct includes the entire DmKHC sequence up to residue 559 (in the break between Coil1 and Coil2 of the rod domain (Coy et al., 1999a)) followed by a C-terminal GFP and hexaHis tag. The Kin2 construct includes the KIF3A head and 17 amino acid neck linker domain ending at Leu359 (red sequence) fused to the DmKHC neck-coil and rod, starting at Ala345, the first residue in the neck-coil domain (blue sequence). The cartoon shows the structures of the Kin1 and Kin2 constructs. The neck linker sequences for all constructs used in this study are given in Table 2.1 of Supplementary Data.

Although no Kin1ΔT runs were observed at saturating ATP in the TIRF assay, transient motor binding events were visible at low ATP concentrations. To test whether the Kin1ΔT motors are truly nonprocessive (taking only one step per interaction), the residence time of Kin1ΔT on immobilized microtubules at 2 µM ATP was measured and compared to the microtubule gliding speed. The motor residence times were well fit by a single exponential with mean residence time
of 1.6 s and the gliding speed at 2 µM ATP was 3 nm/s, meaning that during each interaction with the microtubule the motor moves 3 nm/s x 1.6 s = 4.8 nm, less than one 8 nm step (Figure S2; see Supplementary Data for further details). Hence, the kinesin-1 neck linker domain appears to be an optimal length to maximize chemomechanical coordination between the two head domains – extending it by one residue more than halves the run length and shortening it by one residue abolishes processivity altogether.

2.3.3 Shortening Kinesin-2 Neck Linker Enhances Processivity

The intraflagellar transport motor kinesin-2 has a 17 amino acid neck linker, three residues longer than kinesin-1, and in previous work we showed that full-length kinesin-2 is four-fold less processive than kinesin-1 (Muthukrishnan et al., 2009). If the reduced processivity of kinesin-2 results from diminished coordination between the heads due to its longer neck linker domain, then a simple prediction is that shortening the kinesin-2 neck linker domain should enhance processivity. To test this, we made a motor consisting of the head and neck linker of the mouse KIF3A subunit of kinesin-2 (ending in the last residue of the neck linker domain, Leu359) fused to the neck-coil and proximal rod of Drosophila kinesin-1 (starting at the first residue of the neck-coil domain, Ala345) (Figure 2.1C). This kinesin-2 construct was used so that any differences in processivity can be attributed solely to the head and neck linker regions and not to differences such as charge or mechanical integrity of the coiled-coil between kinesin-1 and kinesin-2. Secondly, whereas constructs containing the kinesin-2 coiled-coil were only functional when baculovirus-expressed, these kinesin-1/kinesin-2 chimaeras were functional when bacterially expressed and had similar properties to a baculovirus-expressed KIF3A homodimer investigated previously (Muthukrishnan et al., 2009). These GFP tagged chimaeric constructs are referred to as Kin2 throughout this paper.
The mean run length and speed of control Kin2 were 0.71 ± 0.03 µm and 480 ± 98 nm/s, respectively. To test whether shortening the neck linker enhances processivity, step-wise deletions of one, two and three amino acids were made in the last three residues (DAL) in the kinesin-2 neck linker to create Kin2ΔA, Kin2ΔDA and Kin2ΔDAL. Single-molecule run lengths and velocities were measured in an identical manner to Kin1. Deleting one residue (Kin2ΔA) increased the run length to 1.26 µm and had no effect on the velocity (484 nm/s), supporting the hypothesis. However, deleting two residues (Kin2ΔDA) decreased the mean run length to 0.59 µm, which is less than the control Kin2 motor. Deleting all three residues (Kin2ΔDAL) resulted in no observable processive runs in the single molecule assay (Figure 2.2C), though the motors were functional in the multi-motor gliding assay. A binding experiment at 2 µM ATP, similar to the approach used for Kin1ΔT indicated the motor moves 10.8 ± 1.1 nm per interaction, consistent with the tethered head not being able to reach the next binding site (Figure 2.6). Hence, although the results qualitatively agree with the hypothesis – shortening the kinesin-2 neck linker enhances processivity – there was not quantitative agreement between the kinesin-1 and kinesin-2 results.

Sequence analysis of the kinesin-2 neck linker reveals that both KIF3A and KIF3B contain a proline residue at position 13 of the neck linker domain (Figure 2.1C), and in the only kinesin-2 crystal structure containing the entire neck linker domain (human KIF3B, PDB:3B6U) this proline is in the kinked cis conformation. We used Molecular Dynamics simulations to compare the predicted force-extension profile of kinesin-1 and kinesin-2 neck linkers and found that, while the kinesin-1 neck linker was well fit by a model of a 14 residue worm-like-chain, the 17 residue kinesin-2 neck linker was shorter than predicted by a worm-like-chain model, except at high forces where the proline was forced into the straight trans conformation (Hariharan and Hancock, 2009) (Figure 2.2A). When the proline at position 13 was changed to an alanine and the last three residues of the kinesin-2 neck linker were deleted, the modeled force-extension
curve matched that of kinesin-1 (Figure 2.2B). These simulations predict that if the kinked proline in the kinesin-2 neck linker is replaced by an alanine, then the Kin1 and Kin2 neck linkers should have the same effective length and the inter-head tension that governs head-head coordination should scale similarly with neck linker length.

2.3.4 Kin1 and Kin2 Motor Properties Scale Similarly with Neck Linker Length

When the proline at position 13 of the Kin2 neck linker was substituted with alanine to make Kin2<sub>PA</sub>, the mean run length fell from 0.71 µm to 0.39 µm, consistent with this substitution extending the effective neck linker length. Interestingly, the processivity of this Kin2<sub>PA</sub> construct closely matched that of the equivalent kinesin-1 construct, Kin1<sub>+</sub>DAL (0.39 µm) (Figure 2.2D). More importantly, when the neck linker of this proline-substituted construct was shortened by three residues to match the length of the kinesin-1 neck linker, the mean run length of this Kin2<sub>PA_ΔDAL</sub> construct rose to 1.8 µm, more than twice the native Kin2 processivity and very nearly matching the 2.1 µm run length of wild-type Kin1 (Figure 2.2C). To further examine this correlation, we then made an intermediate length construct, Kin2<sub>PA_ΔDA</sub>, containing a 15 residue neck linker domain and found that its run length closely matched the kinesin-1 construct having a 15 residue neck linker, Kin1<sub>+</sub>L. The striking result here is that when the kinesin-2 neck linker is straightened by removing the <i>cis</i> proline, the processivity of kinesin-1 and kinesin-2 motors match and scale similarly with neck linker length even though motor velocities remain distinct. As an example, the run lengths of Kin1 and Kin2<sub>PA_ΔDAL</sub> are nearly identical, while their motor velocities differ by nearly a factor of two (990 ± 130 nm/s versus 508 ± 71 nm/s).
Figure 2.2: Kin1 and Kin2 Run Lengths Scale with Neck Linker Length

(A) Force extension curves of kinesin-1 and kinesin-2 neck linkers from Molecular Dynamics simulations. Solid lines are predictions of worm-like chain model for 14 and 17 residue polypeptide showing good fit for kinesin-1 (14 residues) and poor fit to kinesin-2 (17 residues).

(B) Predicted force-extension curve when the kinked proline in kinesin-2 is replaced with an alanine and the last three amino acids are deleted (Kin2\_PA\_ΔDAL), compared to kinesin-1. (C) Comparison of run lengths for Kin2 and Kin1 constructs having identical neck linker lengths (# of amino acids). (D) Run lengths following substitution of the \textit{cis} proline in the Kin2 neck linker with alanine (Kin2\_PA). Kin2\_PA constructs containing 14, 15, and 17 amino acid neck linkers are Kin2\_PA\_ΔDAL, Kin2\_PA\_ΔDA, and Kin2\_PA, respectively. Curve for control Kin2 motors is shifted 1.5 amino acids to the left to account for the \textit{cis} proline. All run length and velocity values are given in Table 2.2 of Supplementary Data.

To extend this correlation of processivity with neck linker length, it is possible to estimate the degree to which the proline in the \textit{cis} conformation shortens the kinesin-2 neck linker. Shortening the Kin2 neck linker by one residue increases processivity, while shortening it by two residues diminishes processivity and shortening it by three abolishes processivity altogether (Figure 2.2C). This is consistent with the maximum predicted run length lying
between a deletion of 1 and 2 residues. When the Kin2 curve is shifted to the left by 1.5 amino acids, all three curves fall on top of one another (Figure 2.2D), suggesting that the kinked proline shortens the neck linker domain by the equivalent of 1.5 amino acids (~0.5 nm).

### 2.3.5 Adding Positive Charge in the Neck Linker Enhances Processivity

While it is clear that neck linker length controls processivity, the degree to which charged residues in the neck linker also affect processivity is not clear. Positive charge in the neck-coil domain and in the core head domain have been shown to enhance processivity through favorable electrostatic interactions with the microtubule (Okada and Hirokawa, 2000; Thorn et al., 2000). Understanding the dependence of kinesin processivity on neck linker charge and buffer ionic strength is important for properly interpreting the present data and for resolving disparate results in the literature. Because the DAL sequence introduced into the kinesin-1 neck linker has a charge of -1 at physiological pH, it is possible that the reduced run length for Kin1+DAL is at least partly due to unfavorable electrostatic interactions that limit processivity. To test this, we instead inserted a neutral three-residue insert, Kin1+AAL, and found a similar run length (0.45 µm) (Figure 2.3A). This result confirms that the DAL insert reduces processivity by extending the neck linker length and not by introducing negative charge, consistent with the finding that inserting one neutral amino acid into kinesin-1 also significantly reduces processivity (Kin1+L, Figure 2.1).

The next question was whether introducing a positively charged insert, KAL, leads to a similar fall in processivity. The run length of Kin1+KAL was 1.27 µm, which is more than two-fold greater than Kin1+DAL or Kin1+AAL, but is still considerably less processive than Kin1 (2.1 µm) (Figure 2.3A). Hence, even in 80 mM PIPES buffer, positively charged residues in the kinesin neck linker domain enhance processivity. However, in this case the reduction in processivity due
to lengthening the neck linker still clearly dominates over any electrostatic effects from the positively charged Lysine.

If electrostatic interactions are playing a role in kinesin processivity, then the effect should be magnified in low ionic strength buffers where charge shielding is minimized. To test the effect of ionic strength on kinesin processivity, we measured run lengths of Kin1 and Kin1+KAL in 12 mM PIPES buffer and compared the values to run lengths in 80 mM PIPES buffer. In 12 mM PIPES, the Kin1 run length doubled to 4.2 µm and the Kin1+KAL run length increased four-fold to 4.6 µm, such that the Kin1 and Kin1+DAL run lengths were nearly identical (Figure 2.3B). Hence, in low ionic strength buffers, the reduction in processivity resulting from extending the neck linker was almost perfectly matched by the enhancement in processivity due to electrostatic effects from the positively-charged lysine in the insert.

This finding that charge introduced in the neck linker plays a dominating role at low ionic strength helps to resolve the disparity between the present data and the results of Yildiz et al (Yildiz et al., 2008) who found that inserts as large as 16 residues had no significant effect on processivity. In that work, every insert in the neck linker contained two lysines at its C-terminus – the authors argued that these positive charges compensated for moving the normal positive charge in the neck-coil domain farther from the microtubule. Based on our results, the enhanced electrostatic interactions from these two lysines, which will be amplified in the 12 mM PIPES buffer used, overwhelmed any reduction in processivity resulting from extending the neck linker domains. The simplest explanation is that these positively charged residues enhance processivity by interacting with the negatively charged C-terminus of tubulin (Lakamper and Meyhofer, 2005; Okada and Hirokawa, 2000; Thorn et al., 2000), though other mechanisms cannot be ruled out. We argue that for understanding the chemomechanical coordination between the two head
domains that underlies kinesin processivity, these electrostatic effects should be minimized by using higher ionic strength buffers and minimizing positive charge in any sequence inserts.

![Graph](image.png)

Figure 2.3: Neck Linker Positive Charge and Low Ionic Strength Enhance Processivity.

(A) Comparison of Kin1 run length on the charge of the neck linker insert showing that while negative charge does not diminish processivity, adding positive charge does enhance processivity. Experiments were carried out in 80 mM PIPES buffer. (B) Effect of buffer ionic strength on control Kin1 and Kin1+KAL run lengths, showing that in 12 mM PIPES buffer the diminished processivity due to the longer neck linker domain is compensated for by enhanced electrostatic interactions due to the added positive charge in the neck linker domain.

### 2.3.6 How Does Extending the Neck Linker Alter the Kinesin Chemomechanical Cycle?

To understand the mechanism by which extending the kinesin neck linker reduces processivity by up to a factor of five while reducing velocity less than two-fold, we carried out stochastic simulations of the kinesin-1 kinetic cycle (Figure 2.4A) to identify whether
modifications of individual model parameters are sufficient to account for the experimental results. Kinesin processivity is described by two mechanisms – front- and rear-head gating (Block, 2007; Muthukrishnan et al., 2009; Rosenfeld et al., 2003). *Front head gating* holds that when both heads are bound (State 1), ATP binding to the leading head is inhibited by rearward strain, ensuring that the trailing head detaches before ATP binds (State 2). *Rear head gating* holds that detachment from a one-head-bound state (State 5) is slow and forward-directed strain from the second head is necessary to detach the rear head (State 1 to 2 or State 3 to 4) at a rate consistent with the overall cycle time (Block, 2007; Hancock and Howard, 1999).

For wild-type kinesin-1, where front head gating is thought to block State 3 (Rosenfeld et al., 2003), processivity is determined by the relative rates of unbinding of the single attached head (State 5 to State 6) versus rebinding of the tethered head (State 5 to State 1). To test whether changes in $k_{\text{attach}}$ alone can account for the experimental results, simulations were run at a range of $k_{\text{attach}}$ values, while holding all other parameters constant. Depending on the specific parameters used, the experimental results could be accounted for by positing that extending the neck linker reduces $k_{\text{attach}}$ (Figure 2.7 and Table 2.3). However, this attachment step involves tethered diffusion of the unbound head to the next binding site, and the entropic elasticity of the neck linker is expected to significantly constrain this diffusive step (Hariharan and Hancock, 2009; Hyeon and Onuchic, 2007; Muthukrishnan et al., 2009). The fact that a very fast $k_{\text{detach}}$ (>1000 s$^{-1}$) for control Kin1 is required for these simulation results to hold, and the prediction that extending the neck linker slows $k_{\text{attach}}$ (instead of accelerating it by relieving the tethering force of the neck linker) argue against this mechanism.

The next mechanism that was simulated was the possibility that extending the kinesin-1 neck linker disrupts front-head gating by increasing the ATP binding rate in the two-head-bound
state (State 1 to State 3 transition). To test whether a change in ATP binding alone is sufficient to account for the data, $k_{on\_ATP\_2H}$ was varied from 0.02 $\mu$M$^{-1}$s$^{-1}$ (consistent with experimental estimates in the strained state (Rosenfeld et al., 2003)) to 2 $\mu$M$^{-1}$s$^{-1}$ (the unstrained rate (Hackney, 2002; Rosenfeld et al., 2003)). A steep fall in run length was indeed observed, but velocity was unchanged (Figure 2.7). Hence, while this proposed mechanism can account for the effect of neck linker extension on run length, it cannot account for changes in velocity.

The third mechanism that was tested was the possibility that extending the neck linker domain slows strain-induced detachment of the trailing head. This strain-dependent detachment not only underlies the rear-head gating mechanism, it also underlies the front-head gating mechanism – detachment of the trailing head in State 1 must be very fast to prevent ATP binding to the front head and possible detachment from State 3. Depending on the rate constants chosen, it was possible to show that decreasing the strain-induced detachment of the rear head (an expected outcome of lengthening the neck linker) does result in a steep fall in the run length and a moderate decrease in velocity (Figure 2.4B). However, this result was dependent on setting $k_{on\_ATP\_2H}$ to a value 10-fold faster than the experimentally estimated value (Rosenfeld et al., 2003), setting $k_{detach}$ to be very fast (2000 s$^{-1}$), and setting the unbinding rate from the two-head-bound State 3 to be 10-fold faster than the one-head-bound unbinding rate (State 5) (Table 2.3). Hence, changing either the ATP binding rate alone or the strain-dependent detachment rate of the trailing head alone fails to account for the experimental results. Instead, the neck linker extension results are best accounted for by both a slowing of the strain-dependent detachment rate $k_{detach}$ and an increase in the strain-suppressed two-headed ATP binding rate, $k_{on\_ATP\_2H}$.

The striking finding of this study is that virtually all of the difference in processivity between kinesin-1 and kinesin-2 motors results from differences in the length of the neck linker
domain and not from inherent differences in kinetic rates in the heads. Hence, when Kin1 and Kin2 neck linkers are identical lengths (following replacement of the cis proline), their run lengths match despite the fact that motor velocities differ by nearly a factor of two. The results

Figure 2.4: Modeling the Kinesin Chemomechanical Cycle.

(A) Model for the kinesin chemomechanical cycle used to interpret the neck linker extension results. This framework is similar to a previous model (Muthukrishnan et al., 2009), with the difference that motor unbinding from State 3 is combined into one rate constant $k_{\text{unbind,2H}}$ for simplicity. Kinetic parameters are discussed in Supplementary Data and listed in Table 2.3. (B) Experimental Kin1 run length and velocity results plotted as a function of neck linker length. (C) Modeled run length and velocity from stochastic simulations of the model presented in 4A, using rate constant parameters given in Table 2.3. In these simulations, $k_{\text{on,ATP,2H}}$ was set to 0.2 µM$^{-1}$s$^{-1}$ (10-fold above the best estimate from the literature (Rosenfeld et al., 2003)) and both $k_{\text{detach,A}}$ and $k_{\text{detach,T}}$ were varied from 2000 s$^{-1}$ down to 20 s$^{-1}$ to model the effect of reduced strain on the trailing head due to extending the neck linker domain. Hence, for the model to account for the experimental results, extending the neck linker needs to alter two strain dependent mechanisms – detachment of the trailing head and ATP binding to the leading head.
can be accounted for by proposing that extending the neck linker both decreases strain-induced detachment of the trailing head \( k_{\text{detach}} \) and relieves the strain-inhibited binding of ATP to the leading head in the two-head-bound state. Because internal strain between the heads in kinesin-2 motors is less than in kinesin-1, it is expected that kinesin-2 responds differently to external strain such as during bidirectional transport of cargo or when many motors are cooperatively transporting cargo. Sequence predictions indicate that motors in different kinesin families have different neck linker lengths (Hariharan and Hancock, 2009), and they are known to possess different degrees of processivity and work in diverse multi-motor arrangements, hence this correlation of neck linker length with their cellular task may extend across the kinesin superfamily.

### 2.4 Materials & Methods

#### 2.4.1 Motor Constructs and Protein Expression.

Kin1 was made by fusing *Drosophila* conventional kinesin truncated at position 559 to a C-terminus eGFP and His\textsubscript{6} tag. Kin2 was engineered by swapping the head and neck linker of mouse KIF3A into Kin1 (Figure 2.1C). See Supplementary Data for details on cloning procedures and sequences. All motors were expressed in bacteria, as previously described (Hancock and Howard, 1998; Uppalapati et al., 2009).

#### 2.4.2 Motility Assays

Bovine brain tubulin was purified and labeled with Cy5 (GE Healthcare) as described (Hyman et al., 1991; Uppalapati et al., 2009; Williams and Lee, 1982). Taxol-stabilized Cy5-
labeled microtubules were adsorbed onto the surface of flow cells, and the surfaces were blocked with 2 mg/ml casein. Motility solution consisting of ~20 pM motors, 1 mM ATP, 0.2 mg/ml casein, 10 µM Taxol and an oxygen scavenging of 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase and 0.5% v/v β-mercaptoethanol in BRB80 (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) was then introduced. Single-molecule run lengths were visualized by TIRF using Nikon TE2000 microscope (60x, 1.45 NA PlanApo) equipped with a 488 nm Ar ion laser for GFP excitation and a 633 nm He-Ne laser for C5 excitation; experiments were performed at 26°C. Images were captured with a Cascade 512 CCD camera (Roper Scientific, Tucson, AZ) and acquisition and image analysis carried out using MetaVue software (Molecular Devices Corporation, Downingtown, PA); pixel size was 71.0 nm. The duration and distance of single motor runs were recorded manually. To ensure that all events were reliably captured, only events with a minimum run length of 250 nm were analyzed, and this minimum distance was subtracted from all runs (this assumes detachment probability is independent of distance the motor has moved).
2.5 Supplementary Information

<table>
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<tr>
<th>Construct</th>
<th>Neck-Linker Length (Amino Acids)</th>
<th>Run Length (µm) Mean ± SEM (N)</th>
<th>Speed (nm/s) Mean ± SD (N)</th>
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<td>Kin1</td>
<td>14</td>
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<td>580 ± 95 (168)</td>
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<td>600 ± 89 (339)</td>
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<td>Kin1+KAL</td>
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<td>0.45 ± 0.01 (197)</td>
<td>713 ± 127 (197)</td>
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<td></td>
</tr>
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<tr>
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<td>LRYANRA KNKINARINEDAK</td>
<td>AEEWRRL</td>
</tr>
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</table>

The neck-linker domain connects α6, the last helix of the motor domain to α7, the first helix of the dimerization domain. The definitions of the start and end of the neck linker were discussed previously (Hariharan and Hancock, 2009; Muthukrishnan et al., 2009). Kin1 constructs include the control and extensions of 1 and 3 amino acids. Kin2 constructs include the control motor and deletions of 1, 2, and 3 amino acids before and after substitution of Pro355 for Ala.

Table 2.1: Complete neck-linker sequences of motors used in this study

<table>
<thead>
<tr>
<th>Construct</th>
<th>Neck-Linker Length (Amino Acids)</th>
<th>Run Length (µm) Mean ± SEM (N)</th>
<th>Speed (nm/s) Mean ± SD (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kin1</td>
<td>14</td>
<td>2.1 ± 0.10 (263)</td>
<td>990 ± 130 (263)</td>
</tr>
<tr>
<td>Kin1+L</td>
<td>15</td>
<td>0.89 ± 0.06 (168)</td>
<td>580 ± 95 (168)</td>
</tr>
<tr>
<td>Kin1+DAL</td>
<td>17</td>
<td>0.39 ± 0.02 (339)</td>
<td>600 ± 89 (339)</td>
</tr>
<tr>
<td>Kin1+KAL</td>
<td>17</td>
<td>0.45 ± 0.01 (197)</td>
<td>713 ± 127 (197)</td>
</tr>
<tr>
<td>Kin1ΔT</td>
<td>13</td>
<td>Non Processive</td>
<td></td>
</tr>
<tr>
<td>Kin2</td>
<td>336</td>
<td>LRYANRA KNKINARINEDPKDAL</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2ΔA</td>
<td>336</td>
<td>LRYANRA KNKINARINEDPKDL</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2ΔDA</td>
<td>336</td>
<td>LRYANRA KNKINARINEDPKL</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2ΔDAL</td>
<td>336</td>
<td>LRYANRA KNKINARINEDP</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2PA</td>
<td>336</td>
<td>LRYANRA KNKINARINEDAKDAL</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2PA_ΔDA</td>
<td>336</td>
<td>LRYANRA KNKINARINEDAKL</td>
<td>AEEWRRL</td>
</tr>
<tr>
<td>Kin2PA_ΔDAL</td>
<td>336</td>
<td>LRYANRA KNKINARINEDAK</td>
<td>AEEWRRL</td>
</tr>
</tbody>
</table>

* Includes proline at position 13 in the neck linker
** Results from microtubule gliding assays where N is number of microtubules

Table 2.2: Run lengths and velocities of all Kin1 and Kin2 motors used in this study
2.5.1 Details of cloning procedures

To make Kin2 constructs (mouse KIF3A fused to *Drosophila* Kinesin-1), a leucine was inserted into Kin1 at position 364 using Stratagene’s Quick-Change II XL site-directed mutagenesis kit (catalog # 200521) to introduce the restriction site NheI. The KIF3A head and neck-linker regions were then amplified and extended with restriction sites NdeI and NheI at the two ends, and the ends made sticky by digesting with NdeI and NheI. Kin1-NheI plasmid was linearized with NdeI and NheI enzymes and ligated to the KIF3A head and neck linker sequence to yield the Kin2 construct. Kin1 extensions and Kin2 deletions and substitutions were incorporated using Stratagene’s Quick-change II XL site-directed mutagenesis kit.

2.5.2 Bead assays to assess Kin1\(\Delta T\) processivity

Because little information is gleaned from TIRF assays where run lengths are below the level of detection, we explored the processivity of Kin1\(\Delta T\) using a bead assay. Full-length Kin1\(\Delta T\) motors were expressed and adsorbed to casein-coated 0.56 µm polystyrene beads as previously described (Muthukrishnan et al., 2009). Microtubules were adsorbed to flow cells and motility solution consisting motor coated beads, 10 µM taxol, 1 mM ATP, 0.2 mg/ml casein and oxygen scavenging system in BRB80 was

![Figure 2.5: Kin1\(\Delta T\) bead dilution profile.](image-url)

Fraction of beads that move when placed near an immobilized microtubule, plotted as a function of motor dilution. Data were fit best to a Poisson distribution with \(n \geq 3\), consistent with a non processive motor.
flowed in. Beads captured with a weak optical trap were brought in contact with microtubules and bead movements were captured with the CCD camera as described previously (Muthukrishnan et al., 2009).

To assess Kin1ΔT processivity, the fraction of beads that moved when placed next to a microtubule was recorded at a range of motor concentrations (keeping the bead concentration constant). As seen in Figure 2.5, the fraction of moving beads fell steeply with motor dilution. The data were fit to a Poisson distribution as previously described (Block et al., 1990), and the results were poorly fit by a curve with N ≥ 1, and were best fit by a Poisson distribution with n ≥ 3. This result confirms that Kin1ΔT motors are indeed functional, but that they are minimally processive or nonprocessive.

2.5.3 Assessing processivity of Kin1ΔT and Kin2ΔDAL by residence time at 2 µM ATP

While both the TIRF assay and the bead dilution profile of Kin1ΔT suggested that the motors were non-processive, the upper limit of those measurements is approximately 200 nm, so they cannot say whether the motors are minimally processive (taking ~10 steps) or non-processive (taking only one step per interaction). Though motor runs shorter than 200 nm at 1 mM ATP were difficult to record reliably with the TIRF system, transient binding events at 2 µM ATP (where ATP binding is the rate limiting step) were observed. The residence times of these binding events were recorded and fit to an exponential representing the motor off-rate from the microtubule. A mean duration of 1.60 ± 0.05 s was recorded (Figure 2.6A). In parallel, microtubule gliding assays with Kin1ΔT were carried out at 2µM ATP. The measured gliding speed was 3.0 ± 0.1 nm/s.
Based on the 3 nm/s motor speed at 2 µM and the finding that individual motors interact with the microtubule for 1.6 s, the distance a motor moves per interaction is calculated to be $1.6 \text{s} \times 3.0 \text{nm/s} = 4.8 \pm 0.3 \text{nm}$. This is less than one 8 nm step, and from this we conclude that Kin1$_{AT}$ is not processive.

One caveat of this measurement is that the binding time is a single-molecule measurement, while the gliding speed is a manifestation of many motors working together. However, the motor detachment rate when many motors are moving a microtubule is, if anything, expected to be faster than in the single-molecule case due to forced unbinding. The binding duration and consequently the distance per interaction should be therefore be considered an upper limit.

A similar set of measurements were made to assess the processivity of Kin2$_{ΔDAL}$. The measured residence time at 2 µM ATP was $0.6 \pm 0.05 \text{s}$ and gliding speed was $18 \pm 0.4 \text{nm/s}$, giving a predicted distance of $10.8 \pm 1.1 \text{nm}$ per interaction with a microtubule.

Figure 2.6: Kin1$_{AT}$ and Kin1$_{ΔDAL}$ binding events at 2 µM ATP.

Binding times were binned in 1 second intervals and data were fit to a single exponential. Time is reported as fit ± SE of the fit. Motor speeds from microtubule gliding assays at 2 µM ATP are reported as mean ± SEM.
While it can’t be ruled out that Kin2$_{\text{ADAL}}$ takes two steps (16 nm) per interaction, this motor is likely nonprocessive as well. The conclusion from these neck-truncated motors is that shortening the neck linker beyond a critical length either prevents the tethered head from reaching the next binding site or it slows the binding rate to a degree that the motor detaches before the tethered head binds.

### 2.5.4 Molecular Dynamics simulations of neck linker force-extension curves

Molecular Dynamics simulations were carried out using Gromacs as described previously (Hariharan and Hancock, 2009). Data in Figure 2.3A were replotted from Ref 1. Simulations were run in constraint mode and included the last residue of $\alpha6$ and the residue of $\alpha7$ as “handles”. Lines in Figure 2.3A and B are worm-like-chain force-extension profiles using a persistence length of 0.5 nm and a contour length of 0.38 nm per amino acid. For the Kin2$_{PA_{\text{ADAL}}}$ simulations in Figure 2.3B, the neck linker structure PDB:3B6U was modified by substituting the proline for an alanine and deleting the last three residues. Force values are mean ± SEM for a 160 ps simulation using 2 fs time steps.

### 2.5.5 Stochastic simulations of the kinesin chemomechanical cycle

To test potential mechanisms by which extending the kinesin neck linker reduces processivity, stochastic simulations of the kinesin chemomechanical cycle were carried out using MATLAB. The structure of the model (Figure 2.4A) and the modeling approach are similar to Muthukrishnan et al (Muthukrishnan et al., 2009), with the difference that motor unbinding from State 3 (following breakdown of front-head gating) is lumped into a single rate constant, $k_{\text{unbind,2H}}$. 
During stepping, transitions between adjacent states were calculated from the first-order rate constant using the Gillespie algorithm \((t = \frac{1}{k} \ln \frac{1}{\text{rand#}})\) (Gillespie, 1977), and the fastest transition was chosen as the dwell time. For each simulation a motor starts in State 1 and steps through the cycle until it detaches from the microtubule, and the cycle time and distance moved (number of steps times 8 nm/step) are converted to run length and velocity. For each parameter set, 3000 motor runs were simulated.

The goal of these simulations was to test whether the observed dependence of run length and velocity on neck linker length can be explained by changes in a single parameter in the kinetic cycle. The governing philosophy is that the kinetic cycle is sufficiently complex that intuition breaks down and it is necessary to simulate the entire cycle to interpret the data. The simulations used the parameters used by Muthukrishnan et al (Muthukrishnan et al., 2009) as a starting point, and specific parameters were adjusted to better fit the data and test hypotheses for the effect of neck linker extension. Simulations focused on three parameters, \(k_{\text{attach}}\), \(k_{\text{on,ATP,2H}}\) and \(k_{\text{detach}}\). To better understand the dynamics of the kinetic cycle, the relative flux through the two-head bound pathway (2H Path, Figure 2.4A) was recorded, as well as the probability the motor unbound from the microtubule through the two-head bound State 3 rather than the one-head bound State 5.

**Varying the attachment rate of the tethered head, \(k_{\text{attach}}\)**

The first set of simulations tested whether the results can be explained by a change in \(k_{\text{attach}}\), the rate that the tethered head binds to the microtubule (transition from State 5 to State 1). This is a key step because it is the primary path of detachment of the motor and the race between
$k_{\text{attach}}$ and $k_{\text{unbind, 1H}}$ determines the run length. Extending the neck linker domain is not expected to alter the motor unbinding rate, $k_{\text{unbind, 1H}}$, but it is plausible that it could alter the rate at which the tethered head reattaches to the microtubule. The most reasonable expectation would be that extending the neck linker domain accelerates $k_{\text{attach}}$ by loosening the tether of the unbound head, enabling its diffusion to the next binding site. However, in simulations where $k_{\text{attach}}$ was increased, the run length increased, opposite of what is seen experimentally. Alternatively, it is formally possible that docking of the native neck linker places the tethered head in the optimal position for binding to the microtubule (despite the fact that the neck linker of the tethered head is expected to remain unstructured (Rice et al., 1999)), and extending the neck linker alters this positioning and consequently reduces the attachment rate. To simulate this possibility, $k_{\text{attach}}$ was set to 1800 s$^{-1}$ (which is necessary to prevent a significant reduction in motor velocity with decreases in $k_{\text{attach}}$, and which is generally consistent with the idea that neck linker docking of the bound head optimally positions the tethered head for attachment). $k_{\text{unbind, 1H}}$ was set to 4 s$^{-1}$ to match observed run length of control Kin1, and $k_{\text{attach}}$ was systematically reduced (entire parameter set given in Table 2.3). As seen in Figure 2.7A, reducing $k_{\text{attach}}$ led to a significant drop in the run length and only a moderate fall in velocity, as seen experimentally. The flux analysis in Figure 2.7C helps to explain this behavior – decreasing $k_{\text{attach}}$ does not alter the relative flux through the two pathways that emerge from State 1, but because it increases the probability of detaching from the one-head bound State 5, the fraction of motors that detach from the two-head bound State 3 falls.
Table 2.3: Rate constants used for modeling simulations

<table>
<thead>
<tr>
<th>Rate Constants</th>
<th>Vary k_{attach}</th>
<th>Vary k_{on_ATP_2H}</th>
<th>Vary k_{detach}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward rate (s(^{-1}))</td>
<td>Backward rate (s(^{-1}))</td>
<td>Forward rate (s(^{-1}))</td>
</tr>
<tr>
<td>k_{detach_ϕ}</td>
<td>250</td>
<td>0.01</td>
<td>250</td>
</tr>
<tr>
<td>k_{on_ATP_2H}[ATP]</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>k_{on_ATP_1H}</td>
<td>2000</td>
<td>20</td>
<td>2000</td>
</tr>
<tr>
<td>k_{unbind_2H}</td>
<td>4</td>
<td>0.02</td>
<td>250</td>
</tr>
<tr>
<td>k_{detach_T}</td>
<td>250</td>
<td>0.02</td>
<td>250</td>
</tr>
<tr>
<td>k_{hydrolysis}</td>
<td>280</td>
<td>3.5</td>
<td>280</td>
</tr>
<tr>
<td>k_{unbind_1H}</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>k_{attach}</td>
<td>1800</td>
<td>1.8</td>
<td>1200</td>
</tr>
</tbody>
</table>

The second set of simulations investigated whether the experimental results can be explained by the breakdown of front head gating – extending the neck linker leads to an increase in $k_{on\_ATP\_2H}$. The definition of front-head gating is that rearward strain on the front head in State 1 slows ATP binding, allowing time for the trailing head to detach (and preventing detachment via State 3). Hence, it is reasonable to propose that extending the length and hence the mechanical compliance of the neck linker domain should reduce the magnitude of this rearward tension and lead to an increase $k_{on\_ATP\_2H}$. To test whether this proposal makes quantitative sense, $k_{on\_ATP\_2H}$ was increased from 0.02 µM\(^{-1}\)s\(^{-1}\) up to 2 µM\(^{-1}\)s\(^{-1}\), which matches the unstrained on-rate $k_{on\_ATP\_1H}$. In these simulations $k_{attach}$ was 1200 s\(^{-1}\), which is similarly fast as for the above $k_{attach}$ simulations, and both motor unbinding rates were set to 4 s\(^{-1}\). Rear head detachment (at 250 s\(^{-1}\)) and hydrolysis (280 s\(^{-1}\)) were co-rate limiting.

Decreasing $k_{on\_ATP\_2H}$ did lead to a sharp reduction in the motor run length, as seen in Figure 2.7B, but there was no change in velocity. The flux analysis in Figure 2.7D shows that
decreasing $k_{on, ATP_{2H}}$ shunted the cycle through the 2H path, resulting in most motors detaching from the two-head bound State 3. Hence the run length data could be quantitatively explained by an increase in $k_{on, ATP_{2H}}$ due to reduced rearward strain, but the fall in velocity could not be explained by this modulation, so a change in $k_{on, ATP_{2H}}$ alone is not sufficient to explain the experimental results.

**Varying the strain-dependent detachment rate of the trailing head, $k_{detach}$**

The final set of simulations investigated whether the results can be accounted for by breakdown of front-head gating through a different mechanism – slowing of rear-head detachment. The definition of rear head gating is that unbinding from the one-head-bound State 5 is slow, and during the normal cycle detachment of this trailing head is significantly accelerated by forward-directed strain caused by binding of the front head (State 1). Hence, it is reasonable to predict that extending the neck linker would slow this strain-dependent detachment. This slowing could reduce processivity because ATP would have time to bind to the leading head (State 3), leading to premature detachment via State 3. To test whether the experimental results can be explained solely by a slowing of $k_{detach}$, simulations were run keeping all parameters constant except $k_{detach}$.

The first problem that arose was that in the parameter sets used above for varying $k_{attach}$ and $k_{on, ATP_{2H}}$ (Table 2.3), rear head detachment is partially rate limiting at 250 s$^{-1}$, and so any decrease in this rate constant reduces the velocity nearly proportionally. To resolve this problem, $k_{detach}$ was increased to 2000 s$^{-1}$ such that it was no longer rate limiting for the wild-type motor, and $k_{attach}$ was reduced to 250 s$^{-1}$ to maintain a similar overall cycle time. While this $k_{detach}$ is quite fast, there are no experiments in the literature that clearly rule this out, and it is possible that the strain in this conformation is significant (Hariharan and Hancock, 2009). Using this parameter
set, a second kinetic problem became apparent – using \( k_{\text{n, ATP, 2H}} \) of 0.02 \( \mu \text{M}^{-1}\text{s}^{-1} \), which is consistent with the best experimental estimates of this parameter (Rosenfeld et al., 2003), the transition from State 1 to State 3 occurs at the relatively slow rate of 20 \( \text{s}^{-1} \) in 1 mM ATP. Hence, although decreasing \( k_{\text{detach}} \) did reduce the run length, it also led to significant slowing of the motor, contrary to what was seen experimentally.

The model was able to account for the experimental results provided: 1) detachment from the two-head-bound State 3 was relatively fast \( (k_{\text{unbind, 2H}} = 10 \text{ s}^{-1}) \), and 2) \( k_{\text{n, ATP, 2H}} \) was increased by a factor of 10 to 0.2 \( \mu \text{M}^{-1}\text{s}^{-1} \). The parameter set for varying \( k_{\text{detach}} \) is presented in Table 2.3 and the results from these simulations are presented in Figure 2.4C of the paper. From the flux analysis in Figure 2.7E, it is clear one reason that \( k_{\text{detach}} \) reduces processivity is by increasing (by five-fold) the probability that the cycle will follow the 2H path. An additional reason that the fraction of motors detaching from State 3 increases so steeply is that following ATP binding to the front head, the rate that the rear head detaches from State 3 also decreases. What these simulations make clear is that using this model structure the observed experimental results cannot be solely explained by a decrease in \( k_{\text{detach}} \) with extensions of the neck-linker domain. Instead, a change in a second strain-dependent parameter \( k_{\text{n, ATP, 2H}} \) is needed to account for the data.
Figure 2.7: Run length, velocity and flux analysis from model simulations.

Simulations used rate constants shown in Table 2.3. (A and C) Run length and velocity results and flux analysis for simulations in which neck linker extension was modeled as a decrease in $k_{\text{attach}}$. (B and D) Run length and velocity results and flux analysis for simulations in which neck linker extension was modeled as an increase in $k_{\text{on_ATP}_2\text{H}}$. (E) Flux analysis for simulations in which neck linker extension was modeled as a decrease in $k_{\text{detach}}$ (run length and velocity results for these simulations are presented in Figure 2.4C of the paper. For run length and velocity data, SEM bars (for 3000 simulated runs per point) are hidden in the symbols. ATP was set to 1 mM in all simulations.
2.6 References


Chapter 3

Inter-head Tension Determines Processivity across Diverse N-terminal Kinesins

3.1 Introduction

N-terminal kinesins are involved in cellular tasks ranging from cargo transport to spindle organization and accordingly, the degree of processivity, defined as the number of steps a motor takes per interaction with a microtubule, varies from 1 to ~1000 (Block et al., 1990; Howard et al., 1989; Valentine et al., 2006; Varga et al., 2006). While positive charge in the neck-coil domain and the strength of dimerization have been proposed to play roles in processivity, differences in processivity between different N-terminal kinesin families are, like differences in motor speeds, generally thought to result from differences in the biochemical properties of their catalytic core domains (Rosenfeld et al., 2009; Valentine et al., 2006).

Processive motor stepping requires that the catalytic cycles of the two motors domains remain out of phase to avoid both motor domains being in a weak-binding or detached state. The inherent biochemical kinetics of each head domain play a role – for instance a slow rate of hydrolysis or detachment from the microtubule should enhance processivity, while fast detachment in the ADP or ADP-Pi state should reduce processivity. However, the key mechanism for synchronizing the cycles of the two motor domains is intramolecular tension generated between the two head domains during phases of the cycle when both heads are bound to the microtubule. This coordination is described by two nonexclusive mechanisms: front head gating – rearward tension on the front head prevents ATP binding until the trailing head detaches;
and *rear head gating*—binding of the leading head to the microtubule accelerates detachment of the rear head (Block, 2007; Hancock and Howard, 1999; Rosenfeld et al., 2003).

![Diagram](image)

Figure 3.1: Roles of the kinesin neck linker.

In N-terminal kinesins, the neck linker plays three important roles. (A) Upon ATP binding, it docks to the catalytic core, producing a conformational change that positions the free head toward the next binding site. (B) During the diffusional search of the free head for the next binding site, the neck linker provides a tethering force, constraining the diffusional search. (C) When both heads are bound to the microtubule, tension between the two heads is transmitted through the neck linker domains and their shared coiled-coil. (D) Model for the kinesin ATP hydrolysis cycle.

In N-terminal kinesins, the neck linker plays three important roles. (A) Upon ATP binding, it docks to the catalytic core, producing a conformational change that positions the free head toward the next binding site. (B) During the diffusional search of the free head for the next binding site, the neck linker provides a tethering force, constraining the diffusional search. (C) When both heads are bound to the microtubule, tension between the two heads is transmitted
through the neck linker domains and their shared coiled-coil. (D) Model for the kinesin ATP hydrolysis cycle.

From stopped-flow experiments, it is clear that isolated motor domains from diverse kinesins possess widely varying catalytic rates for ATPase and microtubule detachment (Rosenfeld et al., 2003; Rosenfeld et al., 2009; Rosenfeld et al., 2005). However, the neck linker domain, which transmits intramolecular tension between the heads, also varies between kinesin families, ranging from 14 to 18 amino acids (Hariharan and Hancock, 2009). The neck linker domain has three important roles in the kinesin chemomechanical cycle (Figure 1). First, neck linker docking to the core motor domain is thought to be the key structural transition driving stepping. Second, binding of the free kinesin motor domain to the next binding site involves diffusion of the head, which is tethered by the entropic spring properties of the neck linker domain. Third, when both heads are simultaneously bound to the microtubule, the forces between the head domains that underlie coordination of their hydrolysis cycles are transmitted through the neck linker domains. The goal of the present work is to identify the degree to which the length of the neck linker controls motor properties for three diverse N-term kinesins: the axonal transport motor Kinesin-3 (Unc104/KIF1A), the mitotic spindle motor Kinesin-5 (KSP/Eg5), and the kinetochore motor Kinesin-7 (CENP-E). This work builds on previous experiments on the transport motors Kinesin-1 (conventional kinesin) and Kinesin-2 (KIF3A/B).

Kinesin-3 motors transport synaptic vesicles to axon terminals in the brain, and mutations in Kinesin-3 result in decreased synaptic vesicles in growth cones and early death (Hirokawa and Noda, 2008). KIF1A monomers were shown to move processively in vitro with mean run lengths of ~0.8 µm (Okada and Hirokawa, 1999), facilitated through electrostatic interactions between the positively-charged loop 12 (K-loop) and the negatively-charged C-terminus of tubulin (Okada and Hirokawa, 2000). Later work showed that Kinesin-3 motors in vivo likely function as dimers,
where dimerization is mediated either by cargo binding (Klopfenstein et al., 2002) or simply through stable dimerization of the motors (Hammond et al., 2009).

Homotetrameric Kinesin-5 motors (Eg5, KSP) (Kashina et al., 1996) are required for proper organization of bipolar mitotic spindles during cell division (Kashina et al., 1997), and small molecule inhibitors of Eg5 show promise as novel anti-mitotic drugs for cancer treatment (DeBonis et al., 2004; Mayer et al., 1999). Biochemical experiments on dimeric Eg5 suggest the motor is minimally processive (Crevel et al., 1997; Lockhart and Cross, 1996). In optical trapping experiments, Eg5 dimers on beads are minimally processive with mean run lengths of ~70 nm (Valentine et al., 2006), though GFP-tagged Eg5 tetramers under no load move 0.6 µm per interaction, assisted by diffusional tethering of the motor to the microtubule (Kwok et al., 2006). This moderate processivity is consistent with Kinesin-5 motors working in groups to slide antiparallel overlapped microtubules, a function quite different from long-distance transporters like Kinesin-1.

The kinetochore-associated motor Kinesin 7 (CENP-E) is required for proper alignment of chromosomes during metaphase (Schaar et al., 1997; Wood et al., 1997), and single-molecule experiments have demonstrated that CENP-E motors are processive kinesins that are capable of generating substantial forces (Espeut et al., 2008; Kim et al., 2008; Yardimci et al., 2008). A kinetic analysis of CENP-E found that while the rate of neck linker docking in the monomer is relatively slow like Eg5, suggesting a less processive mechanism, the rear head detachment rate in the dimer was only 1.5 times faster than the overall hydrolysis rate, suggesting the dimer spends most of its cycle in a two-head bound state, which should enhance processivity (Rosenfeld et al., 2009). Complicating these interpretations, half-site reactivity and turbidity experiments suggested that upon introduction of ATP many motors take one or only a few steps before dissociating, conflicting with the 250-step run lengths measured in single molecule experiments.
Measured differences in the biochemical properties of isolated kinesin motor domains support the argument that variations in motor processivity result from differences in the properties of the catalytic core. A simple prediction is that engineered Kinesin-3 motors should be highly processive due to their positively charged loop 12, Kinesin-5 motors should be minimally processive due to their inherent biochemistry, and Kinesin-7 engineered without the native neck-coil domain may be minimally processive due to removal of electrostatic tethering with the microtubule. On the other hand, we showed previously that processivity differences between Kinesin-1 and Kinesin-2 motors can be fully explained by differences in the lengths of their neck linker domains (Shastry and Hancock, 2010). This finding suggested that, while motor speed is determined by properties internal to the motor domain, processivity is controlled by mechanical communication between the motor heads, transmitted through the neck linker domains.

In the present work, we ask: *does the length of the neck linker domain control processivity across diverse N-terminal kinesin families?* While Kinesin-1 has a 14 amino acid neck linker, Kinesin-2 and Kinesin-3 have 17 residue neck linkers, and Kinesin-5 and Kinesin-7 motors have 18 residue neck linker domains (Hariharan and Hancock, 2009). In the literature there is significant dispersion in reported run lengths due to different buffer conditions and experimental techniques, but Kinesin-3 and Kinesin-7 motors have similar processivity as Kinesin-1, Kinesin-5 motors are minimally processive, and Kinesin-2 motors are somewhere in between. If altering the neck linker domain results in similar changes in processivity across diverse kinesins, this argues that intramolecular strain between the two motor domains is the key determinant of processivity as opposed to the biochemical properties of the core motor domain.
Our approach was to fuse the head and neck linker domains of Kinesin-3, 5 and 7 to the neck-coil and proximal coiled-coil region of Kinesin-1 (Figure 2), and measure motor run lengths and speeds in a single-molecule fluorescence assay. Dimerizing every motor using an identical neck-coil domain avoids any uncertainty due to unwinding of the coiled-coil during stepping. Further, using a standard neck coil domain and carrying out all experiments in 80 mM PIPES buffer minimize any influence of nonspecific electrostatic interactions on processivity. To test the influence of neck linker mechanics on kinesin stepping, the neck linker domain of each motor was systematically altered and the motor speed and processivity compared.

<table>
<thead>
<tr>
<th>DmKHC (Kinesin-1)</th>
<th>α6: LDFGRRK TVKKNVCVEELT ...</th>
<th>Neck-Linker</th>
<th>α7: AEEWKRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MmKIF3A (Kinesin-2)</td>
<td>LRYANRA KNIKNKARINEPKDAL ...</td>
<td>LRQFOKE</td>
<td></td>
</tr>
<tr>
<td>CeUNC104 (Kinesin-3)</td>
<td>LRYADRA QIVCQAVNEDPNKL ...</td>
<td>IRELNEE</td>
<td></td>
</tr>
<tr>
<td>X1KSP (Kinesin-5)</td>
<td>LDYNARA KSNMKEVNQKLTKAL ...</td>
<td>IKETEE</td>
<td></td>
</tr>
<tr>
<td>X1CENP-E (Kinesin-7)</td>
<td>LQFASTA KHVRNTPHVNEVLDDEAL ...</td>
<td>LKRYRKE</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.2: Design of N-term kinesin constructs.

Amino acid sequences of Kinesin-1, 2, 3, 5 and 7 motors with adjacent α-6 (catalytic core) and α-7 (neck-coil) sequences. The catalytic core and neck linker of CeUnc104, X1KSP and X1CENP-E were fused to the neck-coil and rod of DmKHC to make Kin3, Kin5 and Kin7, respectively.
3.2 Results and Discussion

3.2.1 Kinesin-3 processivity scales inversely with neck-linker length.

When the Unc104 head and 17-residue neck linker were fused to the Kinesin-1 coiled-coil and GFP, the resulting Kin3_{17} construct moved processively with a mean run length of 0.60 ± 0.03 μm (mean ± s.e of fit) and a speed of 2.77 ± 0.52 μm/s (mean± s.d). This run length is somewhat less than the 1.5 μm measured for Unc104 dimerized by a Leu zipper downstream of its coiled-coil domain (Tomishige et al., 2002) and the 1.2 μm measured for a naturally dimeric KIF1A (Hammond et al., 2009), but both of those measurements were performed in low ionic strength buffers, which enhance electrostatic interactions with the microtubule. In 12 mM PIPES buffer, Kin3_{17} had a run length of 2.7 ± 0.2 μm, in reasonable agreement with these previous results.

When the neck-linker of Kinesin-3 was shortened to 14 amino acids (Kin3_{14}) to match the Kinesin-1 neck-linker length, the run length increased three-fold to 1.87 ± 0.09 μm (mean ± s.e of fit), very similar to the Kinesin-1 run length of 2.1 μm measured previously (Shastry and Hancock, 2010). This result is consistent with a highly processive dimer created previously by Tomishige et al, which consisted of the head and first 12 neck linker residues of Unc104 fused to the last two neck linker residues and coiled-coil of Kinesin-1 (Tomishige et al., 2002). To test the hypothesis that motor processivity scales with neck-linker length, we made an intermediate construct, Kin3_{15}, whose run length was measured to be 0.95 ± 0.03 μm. Hence, like Kinesin-1, processivity of Kinesin-3 motors scales inversely with the length of their neck-linker domain.
When Unc104 neck linker was further shortened to 13 residues, the run length decreased to 0.67 ± 0.06 µm. This contrasts with Kinesin-1, where shortening the neck linker to 13 residues abolished processivity (Shastry and Hancock, 2010), presumably because the tethered head is no longer able to stretch the 8 nm to the next binding site. One possibility is that the positively charged K-loop in Kin3\textsubscript{13} enables processivity in a similar manner to monomeric Kinesin-3 (Okada and Hirokawa, 1999). The fact that Kin3\textsubscript{14} has similar processivity as Kinesin-1 implies that the K-loop in Kinesin-3, does not significantly enhance the unloaded processivity of Kinesin-3 dimers at physiological ionic strength.

### 3.2.2 Kinesin-5 processivity

To systematically characterize the influence of neck-linker length on Kinesin-5 motor processivity, the core motor domain and neck linker of XIKSP were fused to DmKHC coiled-coil and GFP (Kin5\textsubscript{18}), and the neck linker length was varied from 18 to 14 amino acids. No single-motor runs were observed for Kin5\textsubscript{18} or Kin5\textsubscript{17} in 80 mM PIPES buffer, although the motors were active in a multi-motor microtubule gliding assay (Table 3.2). As the lower limit of detection for processive events is approximately 250 nm, these results are consistent with processivity values for dimeric and tetrameric Kinesin-5 reported previously (Kwok et al., 2006; Valentine et al., 2006). In low ionic strength buffers, some processive runs were observed, but the high fluorescence intensity of the moving spots suggests these are motor aggregates and not single molecule events.

When the neck linker domain was shortened to 14 residues by deleting last four amino acids (ΔKKAL to make Kin5\textsubscript{14}), processivity increased dramatically to 1.77 ± 0.20 µm, while the speed remained slow at 0.08 ± 0.02 µm/s (mean ± s.d of mean). This result is similar to results
from Lakamper et al (Lakamper et al., 2010) who studied a similar construct. This result demonstrates that, to the degree that there are any unique kinetic characteristics of Kinesin-5 that result in low processivity in the wild-type motor, they can be completely compensated for by shortening the neck linker to enhance head-head coordination. Lengthening the neck linker by one amino acid (Kin513) halved the run length to 0.95 ± 0.13 µm, in agreement with Kinesin-1 containing a 15 residue neck (Shastry and Hancock, 2010), while having little influence on motor speed (0.09 ± 0.02 µm/s (mean ± s.d ).

3.2.3 Kinesin-7 processivity

In single-molecule fluorescence experiments, Xenopus CENP-E has been shown to be as processive as Kinesin-1 and to generate comparable stall forces (Kim et al., 2008; Yardimci et al., 2008). Disagreements in measured CENP-E motor speed and processivity have been explained by suggesting that the CENP-E coiled-coil region may possess a weak propensity for dimerization, may interact electrostatically with the microtubule to enhance processivity, or may sterically inhibit the motor domains in a phosphorylation-dependent manner (Espeut et al., 2008; Kim et al., 2008; Sardar et al., 2010; Yardimci et al., 2008). Hence, fusing the Xenopus CENP-E head and neck linker domain to the stable and well-characterized Kinesin-1 coiled-coil is an ideal approach for clearly defining the motile properties of CENP-E.

From sequence analysis of the CENP-E neck region, the coiled-coil either starts at Asp342 or Leu346 positions a and d of the heptad repeat. Because Asp never occupies the d position in kinesin neck-coils of other well characterized coiled-coils, we conclude that CENP-E has an 18 residue neck linker domain (Hariharan and Hancock, 2009; Walshaw and Woolfson, 2001). When the entire 18 residue neck linker was included, these Kin718 motors had a run length of 1.31
± 0.06 µm (mean ± s.e of fit) and moved at a speed of 0.46 ± 0.07 µm/s. This run length is within 15% of the value measured by Kim et al in a comparable ionic strength buffer and it is half of that measured by Yardimci et al at low ionic strength (10 mM PIPES). We also made a similar construct using the head and neck linker of human CENP-E and found the motor moved at 10 nm/s, consistent with results from Gilbert lab (Sardar et al., 2010), suggesting the slow speeds of human CENP-E are not a result of misregistration of the coiled-coil as has been suggested (Rosenfeld et al., 2009).

To test whether shortening the CENP-E neck-linker enhances processivity, the run lengths of Kinesin-7 motors with shorter neck-linkers were characterized. Shortening the neck linker from 18 to 17 amino acids reduced the run length to 0.8 ± 0.06 µm (mean ± s.e of fit), and shortening it further to 15 or 14 residues further reduced processivity to 0.49 ± 0.02 µm and 0.55 ± 0.02 µm, respectively (Figure 3d). Motor speeds increased from 0.46 ± 0.07 µm/s for Kin7\textsubscript{18} to 0.57 ± 0.11 µm/s for Kin7\textsubscript{14} (Figure 4b). In contrast to the other N-terminal kinesins tested, Kinesin-7 processivity decreased when its neck-linker was shortened.
Figure 3.3: Processivity scales with neck linker length.

(A) Histogram of single-molecule runs of Kin3$_{14}$ from TIRF assay. The mean run length was calculated from a single exponential fit. (B) Run lengths of Kin3 constructs as a function of their neck linker length. Error bars represent the standard error from exponential fits and asterisk denotes putative wild-type neck linker length. (C) Run lengths of different Kin5 constructs as a function of their neck linker length. Run lengths below 250 nm could not be reliably detected by our TIRF system and hence run lengths for Kin5$_{17}$ and Kin5$_{18}$ are estimates (upper limit of 250 nm) and shown in grey. (D) Run lengths of Kin7 constructs as a function of neck linker length. Kin7$_{TR}$ constructs have Thr$_{333}$ mutated to Arg as described in text.

### 3.2.4 Alpha-6 instability

In an effort to understand the divergent behavior of CENP-E, we analyzed family-specific sequences at the interface of the catalytic core and the neck linker. Our underlying hypothesis was that sequences that destabilize the end of helix $\alpha_6$, thus extending the neck linker domain at its N-terminal end should have a similar effect as extending the neck linker at its C-terminal end, at least for states in which the neck linker is undocked. Notably, the neck linker is
undocked in the free head as it undergoes tethered diffusion to its next binding site and in the leading head when both heads are bound to the microtubule (Figure 1). The sequence RAK at the junction between the end of α-6 and the beginning of the neck-linker, is conserved across most N-terminal kinesin families, with a notable exception being Kinesin-7, where both Xenopus and human CENP-E contain the sequence TAK (Figure 2). If Thr_333 in XICENP-E destabilizes the end of α_6, this could lead to an effectively longer neck linker domain in Kinesin-7. To test this prediction, Thr_333 was mutated to Arg in Kin7_14, the motor for which tightening the neck linker should have the strongest effect on processivity. While the speed of Kin7_14_TR (0.55 ± 0.08 µm/s) was unaltered, the run length increased by a factor of three to 1.7 ± 0.24 µm, matching the processivity of other motors having 14 amino acid long neck-linkers. Furthermore, the run lengths of Kin7_15_TR and Kin7_17_TR varied inversely with neck linker length, matching the results for Kinesins 1, 2, 3 and 5 (Figure 3d).

If Thr_333 is indeed destabilizing the end of α_6 and functionally extending the Kinesin-7 neck linker, then converting RAK in Kinesin-1 to TAK would be predicted to reduce processivity in a similar manner. To test this prediction, Arg_329 was mutated to Thr_329 in Kin1 to create Kin1_RT. This motor was functional in a microtubule gliding assay with a velocity of 483 ± 17 nm/s, but no single molecule runs were observed in the TIRF assay, suggesting the motor is nonprocessive. Extending the neck linker of Kin1_RT to 17 residues did not rescue processivity, suggesting that the effect of the T→R substitution is not simply to shorten the neck linker. As this mutation is in the core motor domain, it is perhaps not surprising that it strongly alters motor behavior; one interpretation is that terminating α_6 with RAK represents an “optimal” structure and there are other compensating amino acid substitutions in Kin7 that aren’t present in Kin1.
3.3 Implications for the Kinesin Hydrolysis Cycle

Why does processivity depend so strongly on neck linker length? Below, we analyze the three potential mechanisms by which altering the neck-linker may regulate processivity: neck linker docking, tethered diffusion, and transmission of inter-head tension.

Following ATP binding, the kinesin neck linker is thought to transition from an unstructured coil to a beta-strand conformation, which is stabilized by the N-terminal “cover bundle” (Hwang et al., 2008; Khalil et al., 2008; Rice et al., 1999; Vale and Milligan, 2000). Stabilizing interactions between conserved residues in the neck linker and the core motor domain have been worked out from structural studies on Kinesin-1 (Case et al., 2000; Hwang et al., 2008; Khalil et al., 2008), and there is no evidence from existing crystal structures of other motors having longer neck linkers that the extra residues in the C-terminus of the neck linker participate in neck linker docking. The fact that deleting residues beyond the 14 present in Kinesin-1 does not substantially alter motor velocities and actually enhances processivity supports the argument that these “extra” neck linker residues are not involved in docking with the core motor domain. Hence, we discount the hypothesis that altering the neck linker length at the C-terminus controls processivity by altering docking to the catalytic domain.

The role of the neck linker domain in tethered diffusion of the free head was recently investigated by our group using Brownian Dynamics simulations (Kutys et al., 2010). If the neck linker is modeled as a worm-like chain, the neck linker is expected to strongly hinder the diffusion of the tethered head to the next binding site, and a large force between the heads (>30 pN) is expected in the two-head bound state. Extending the neck linker is expected to relieve this constraint and thus accelerate the rate that the tethered head binds to the next binding site.
Because this one-head bound state is vulnerable to detachment, which will end a processive run, extending the neck linker domain is expected to, if anything, enhance processivity. Hence, we discount the hypothesis that altering the neck linker controls processivity by altering the tethered diffusion step in the hydrolysis cycle.

In contrast to these other mechanisms, extending the neck linker length is expected to reduce the mechanical coordination (or “gating”) between the head domains. The “rear head gating” model holds that detachment of the trailing head is slow until the lead head binds and exerts a force that accelerates its detachment, while the “front head gating” model holds that rearward tension in the two-head bound state prevents ATP binding until the trailing head detaches (Figure 1C). Both mechanisms work to maximize the number of steps the motor takes before detachment, and each involves inter-head tension transmitted via their neck-linkers and their shared coiled-coil. Thus for both gating mechanisms, extending the neck linker domain is expected to decrease the tension between the motor domains and thus decrease processivity.

Using a Monte Carlo modeling approach and parameter sets developed previously (Muthukrishnan et al., 2009; Shastry and Hancock, 2010), we tested whether the experimental results could be explained by an effect of neck linker length on front or rear head gating. The neck linker was modeled as a worm-like chain and inter-head tension in the two-head bound state calculated as the force required for stretching each neck linker by 4 nm. Front head gating was modeled by incorporating a force dependent $k_{on,ATP_{2H}}$ such that longer neck linkers led to faster ATP binding rates (see Figure 1D). Rear head gating was modeled by making $k_{detach}$ force dependent such that longer neck linkers reduced the detachment rate. As seen in Figure 4C, both mechanisms were able to account for the steep dependence of run length on neck linker length. Furthermore, both gating mechanisms were consistent with neck linker length having only a
minor effect on velocity (Figure 4D). Thus, the results can be quantitatively accounted for by neck linker extension diminishing either front or rear head gating.

Figure 3.4: Summary of run length and velocity data.

(A) Results from Figure 3 combined with results from Shastry 2010 (Shastry and Hancock, 2010), demonstrating that run lengths for diverse N-terminal motors scale inversely with neck-linker length. (B) Motor velocity plotted as a function of run length for constructs having different neck linker lengths. Results show that, while changing neck linker length has dramatic effects on run length, it has a minimal influence on unloaded motor velocity. Table of all run length and velocity results is presented in Table 3.2. (C and D) Kin1 and Kin5 simulation results using model shown in Figure 1 and incorporating force-dependent rates for rear head (RH) or front head (FH) gating. Experimental results are shown as solid points. Details of simulations and parameter sets used are given in Supplementary information, section 3.6.
3.4 Implications for Diverse N-terminal Kinesins

The most significant finding from this study is that when the neck linkers are shortened to 14 residues and fused to the Kinesin-1 coiled-coil, Kinesin-3, Kinesin-5 and Kinesin-7 (following Thr->Arg substitution in alpha-6) quantitatively match the processivity of the canonical Kinesin-1. This result suggests that differences in unloaded processivity between diverse kinesins is primarily due to differences in the lengths of their neck linker domains (which presumably controls inter-head tension) rather than specific tuning of rate constants in their ATP hydrolysis cycles. While differences in kinetic rates clearly lead to different motor speeds and may result in different responses to external loads, the present results impact a number of studies in the literature and provide an important constraint for ongoing studies of diverse kinesins.

One unique feature of Kinesin-3 motors is a positively-charged insert (the K-loop) into the microtubule-binding loop 12 of the motor domain, which may be expected to enhance the processivity of Unc104 dimers (Okada and Hirokawa, 1999). However, when the Unc104 head and its entire 17 residue neck linker were fused to the Kinesin-1 neck-coil, the run length was 0.6 µm in 80 mM PIPES. While this run length is 50% greater than Kinesin-1 containing a neutral or negatively charged three residue insert, however, it is only half that of a Kinesin-1 containing a positively charged insert (Kinesin-1_KAL), that matches the net charge of the last three residues (AKL) in the Unc104 neck linker. Furthermore, when the Unc104 neck linker was shortened to 14 residues, the run length was within 15% of Kinesin-1. Hence, the K-loop appears to play a minimal role in processivity of Kinesin-3 dimers in moderate ionic strength buffers, contrary to expectations.
While Kinesin-5 containing its native neck linker was less processive than other motors with similar length neck linkers, when the neck linker was shortened to 14 residues, Kinesin-5 was just as processive as Kinesin-1. It has been proposed that the minimal processivity of human Eg5 is due to some combination of slow docking of the neck linker to the core motor domain, slow or gated ATP binding to the head, and rapid dissociation of the head in the ADP state (Rosenfeld et al., 2009; Rosenfeld et al., 2005). At a minimum, the robust processivity for Kin5_{14} argues that shortening the neck linker to enhance head-head communication can completely override these properties of the core motor domain.

The outlier in the kinesin motor families tested in this work is Kinesin-7. Both Xenopus and human CENP-E have been shown to be processive in vitro, but there has been some debate as to precisely how processive the wild-type motor is and what role the coiled-coil domains play in electrostatic tethering, dimerization, and motor regulation (Kim et al., 2008; Sardar et al., 2010; Yardimci et al., 2008). In our hands, dimeric wild-type Xenopus CENP-E containing a wild-type neck linker is more processive than other motors with similar length neck linkers, but it is less processive than wild-type Kinesin-1. As the Kinesin-7 neck linker (particularly the distal portion) has a net negative charge, and since Kinesin-7 motors do not contain a K-loop, this result cannot be explained by electrostatic tethering to the microtubule by the head or neck linker domains. A recent comparison of the kinetic properties of monomeric CENPE and KHC found that while some characteristics, like slow dissociation in the ADP state, may provide an explanation for enhanced processivity of CENP-E, other characteristics such as the 10-fold slower neck linker docking rate suggests that CENP-E should be minimally processive like Eg5 (Rosenfeld et al., 2009); hence there is no clear indication from CENP-E kinetics that it should be more processive than an analogous Kinesin-1 construct. Further, it is not clear from structural or kinetic data why
shortening the Kinesin-7 neck linker should not enhance processivity as it did for all other motors tested.

We found that changing one residue at the head:neck interface reduced processivity of Kinesin-7 with a wild-type neck linker but enhanced processivity of constructs with shorter neck linker domains. The hypothesis motivating this mutation was that the divergent TAK sequence at the head:neck interface destabilizes the end of alpha-6, extending the neck linker at its proximal end. If this were the case, then we would expect that shortening the neck linker at its distal end should enhance processivity similar to the other families tested; hence the explanation cannot be that simple. One possible interpretation is that the TAK sequence destabilizes the end of alpha-6 such that when the neck linker is shortened, resulting in higher inter-head forces in the two-head bound state, the end of the helix unfolds. This would explain the lack of processivity enhancement upon shortening the neck linker, but it still does not explain the design advantage that leads to enhance processivity of the 18 residue NL construct. The fact that introducing this TAK sequence into Kinesin-1 destroys processivity highlights the importance of this “pivot point” in head-head coordination.

3.5 Conclusions

These experiments demonstrate that the length of the kinesin neck linker domain is the primary determinant of unloaded processivity across five different N-terminal kinesin families. This result suggests that the range of run lengths between these diverse motors results not from tuning of biochemical rate constants in their motor domains, but rather from differences in head-head communication due to their neck linker domains. While the extent to which the motor properties diverge when the motor is moving against an external load is still being defined, these
results demonstrate a striking commonality in mechanism across N-terminal kinesin families and provide important constraints for designing kinetics experiments to identify key rate constants that determine their diverse behavior.

3.6 Methods

3.6.1 Motor Constructs and Protein Expression

Kin1 was made by fusing Drosophila conventional kinesin truncated at position 559 to a C-terminus eGFP and His$_6$ tag, as previously described (Shastry and Hancock, 2010). To generate other Kin3, Kin5 and Kin7 constructs, the motor domains and associated neck linker sequences were amplified by PCR, digested with restriction enzymes and ligated into the Kin1 plasmid, replacing the Kinesin-1 head. Neck linkers were shortened and lengthened using the Stratagene Quick-change II XL site-directed mutagenesis kit, and all final constructs were confirmed by sequencing. All motors were expressed in bacteria, and purified by Ni column chromatography as previously described (Shastry and Hancock, 2010).

3.6.2 Motility Assays

Bovine brain tubulin was purified, labeled with Cy5 (GE Healthcare), polymerized, and taxol-stabilized as previously described (Shastry and Hancock, 2010). Single molecule TIRF experiments were performed at 26°C, images were captured with a Cascade 512 CCD camera (Roper Scientific, Tucson, AZ) and acquisition and image analysis carried out using MetaVue software (Molecular Devices Corporation, Downingtown, PA) with a 71 nm pixel size (Shastry and Hancock, 2010). To ensure that all events were reliably captured, only events with a
minimum run length of 250 nm were analyzed, and this minimum distance was subtracted from all runs (this assumes detachment probability is independent of distance the motor has moved).

3.7 Supplementary Information

Stochastic simulations of the kinesin chemomechanical cycle

To test potential mechanisms by which extending the kinesin neck linker alters processivity, stochastic simulations of the kinesin chemomechanical cycle were carried out using a model developed previously (Muthukrishnan et al., 2009; Shastry and Hancock, 2010). The neck linker was modeled as a worm like chain:

$$f_{WLC}(x) = \frac{k_B T}{L_p} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right)^2 - \frac{1}{4} + \frac{x}{L_c} \right]$$

with a persistence length of 0.7 nm based on previous Molecular Dynamics simulations (Hariharan and Hancock, 2009) and a contour length equal to 0.364 nm multiplied by the number of amino acids in the neck linker domain (14 to 18) (Kutys et al., 2010; Pauling et al., 1951). The two-head bound state (8 nm separation between the heads) was modeled as a 4 nm extension of each neck linker. Because the force necessary to extend a worm-like chain rises steeply near its contour length ($L_c = 5.1$ nm for a 14 residue neck linker), lengthening the neck linker significantly reduces the predicted inter-head tension (from 35 pN for a 14 residue NL down to 13 pN for a 18 residue NL).

To model rear head gating, $k_{\text{detach} \_R}$ and $k_{\text{detach} \_T}$ (see Figure 1D in manuscript) were modeled as force-dependent off-rates with:
\[ k_{\text{detach}}(F) = 30s^{-1} * e^{\frac{F*0.5\text{nm}}{k_BT}} \]

where 30 s\(^{-1}\) is the unstrained off-rate, 0.5 nm is the characteristic bond length, and \(k_BT = 4.1\) pN-nm. To model front head gating, force dependence was incorporated into \(k_{\text{on,ATP,2H}}\) as:

\[ k_{\text{on,ATP,2H}}(F) = 2\mu M^{-1}s^{-1} * e^{\frac{F*0.6\text{nm}}{k_BT}} \]

The different velocities of Kin1 and Kin5 were modeled by setting \(k_{\text{hydrolysis}}\) to 280 s\(^{-1}\) for Kin1 and 10 s\(^{-1}\) for Kin5. The complete list of rate constants under experimental conditions (1 mM ATP) is presented in Table 3.1 below.

The goal of this modeling work was to establish that either front head gating or rear head gating can quantitatively account for the experimental results. There is a large parameter space to explore, and extending the neck linker would be expected to affect both \(k_{\text{detach}}\) and \(k_{\text{on,ATP,2H}}\). Hence, many other combinations of parameters and mixtures of front and rear head gating are possible, and the model is not tightly constrained.

<table>
<thead>
<tr>
<th>Rate Constants</th>
<th>Vary (k_{\text{on,ATP,2H}})</th>
<th>Vary (k_{\text{detach}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{\text{detach,\phi}})</td>
<td>250 0.01</td>
<td>2060 to 141 0.01</td>
</tr>
<tr>
<td>(k_{\text{on,ATP,2H}*}[\text{ATP}])</td>
<td>12 to 312 20</td>
<td>200 20</td>
</tr>
<tr>
<td>(k_{\text{on,ATP,1H}*}[\text{ATP}])</td>
<td>2000 20</td>
<td>2000 20</td>
</tr>
<tr>
<td>(k_{\text{unbind,2H}})</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>(k_{\text{detach,2H}})</td>
<td>250 0.01</td>
<td>2060 to 141 0.01</td>
</tr>
<tr>
<td>(k_{\text{hydrolysis}})</td>
<td>280 for Kin1 3.5</td>
<td>280 for Kin1 3.5</td>
</tr>
<tr>
<td>(k_{\text{unbind,1H}})</td>
<td>10 for Kin5 3</td>
<td>10 for Kin5 3</td>
</tr>
<tr>
<td>(k_{\text{attach}})</td>
<td>1200 1.2</td>
<td>280 0.28</td>
</tr>
</tbody>
</table>

Table 3.1: Rate constants used for modeling simulations.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Neck-Linker Length (Amino Acids)</th>
<th>Run Length (µm)</th>
<th>Speed (nm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SE of Fit (N)</td>
<td>Mean ± SD (N)</td>
</tr>
<tr>
<td>Kin1&lt;sub&gt;14&lt;/sub&gt;</td>
<td>14</td>
<td>2.1 ± 0.10 (263)</td>
<td>990 ± 130 (263)</td>
</tr>
<tr>
<td>Kin1&lt;sub&gt;15&lt;/sub&gt;</td>
<td>15</td>
<td>0.89 ± 0.06 (168)</td>
<td>580 ± 95 (168)</td>
</tr>
<tr>
<td>Kin1&lt;sub&gt;17&lt;/sub&gt;</td>
<td>17</td>
<td>0.39 ± 0.02 (339)</td>
<td>600 ± 89 (339)</td>
</tr>
<tr>
<td>Kin2&lt;sub&gt;14&lt;/sub&gt;</td>
<td>14</td>
<td>1.78 ± 0.08 (398)</td>
<td>508 ± 71 (398)</td>
</tr>
<tr>
<td>Kin2&lt;sub&gt;15&lt;/sub&gt;</td>
<td>15</td>
<td>0.95 ± 0.05 (165)</td>
<td>480 ± 88 (165)</td>
</tr>
<tr>
<td>Kin2&lt;sub&gt;17&lt;/sub&gt;</td>
<td>17</td>
<td>0.39 ± 0.03 (105)</td>
<td>526 ± 92 (105)</td>
</tr>
<tr>
<td>Kin3&lt;sub&gt;13&lt;/sub&gt;</td>
<td>13</td>
<td>0.67 ± 0.06 (127)</td>
<td>2680 ± 332 (186)</td>
</tr>
<tr>
<td>Kin3&lt;sub&gt;14&lt;/sub&gt;</td>
<td>14</td>
<td>1.87 ± 0.09 (186)</td>
<td>2680 ± 332 (186)</td>
</tr>
<tr>
<td>Kin3&lt;sub&gt;15&lt;/sub&gt;</td>
<td>15</td>
<td>0.82 ± 0.08 (254)</td>
<td>2550 ± 490 (254)</td>
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<tr>
<td>Kin3&lt;sub&gt;17&lt;/sub&gt;</td>
<td>17</td>
<td>0.6 ± 0.03 (264)</td>
<td>2770 ± 520 (264)</td>
</tr>
<tr>
<td>Kin5&lt;sub&gt;14&lt;/sub&gt;</td>
<td>14</td>
<td>1.77 ± 0.2 (79)</td>
<td>81 ± 21 (79)</td>
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<tr>
<td>Kin5&lt;sub&gt;15&lt;/sub&gt;</td>
<td>15</td>
<td>0.95 ± 0.13 (76)</td>
<td>93 ± 22 (76)</td>
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<tr>
<td>Kin5&lt;sub&gt;17&lt;/sub&gt;</td>
<td>17</td>
<td>&lt; 0.2</td>
<td>55 ± 6 (51)*</td>
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<tr>
<td>Kin5&lt;sub&gt;18&lt;/sub&gt;</td>
<td>18</td>
<td>&lt; 0.2</td>
<td>64 ± 6 (49)*</td>
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<tr>
<td>Kin7&lt;sub&gt;14&lt;/sub&gt;</td>
<td>14</td>
<td>0.55 ± 0.02 (177)</td>
<td>570 ± 114 (177)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;15&lt;/sub&gt;</td>
<td>15</td>
<td>0.49 ± 0.02 (111)</td>
<td>526 ± 125 (111)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;17&lt;/sub&gt;</td>
<td>17</td>
<td>0.8 ± 0.06 (168)</td>
<td>447 ± 68 (168)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;18&lt;/sub&gt;</td>
<td>18</td>
<td>1.31 ± 0.06 (120)</td>
<td>466 ± 75 (120)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;14&lt;/sub&gt;_TR</td>
<td>14</td>
<td>1.7 ± 0.24 (187)</td>
<td>558 ± 88 (187)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;15&lt;/sub&gt;_TR</td>
<td>15</td>
<td>1.10 ± 0.08 (151)</td>
<td>549 ± 88 (151)</td>
</tr>
<tr>
<td>Kin7&lt;sub&gt;17&lt;/sub&gt;_TR</td>
<td>17</td>
<td>0.60 ± 0.04 (187)</td>
<td>494 ± 103 (187)</td>
</tr>
</tbody>
</table>

* Speeds determined from ensemble microtubule gliding assays.
Note: Kin1 and Kin2 values were taken from Shastry & Hancock 2010.

Table 3.2: Run Lengths and speeds of all motors studied in this work
3.8 References


Chapter 4

Labeling Strategy for FRET Experiments and Explorations into Kinesin Structure-function

4.1 Introduction

Molecular motors including kinesins, myosins and dyneins drive essential cell movements and their behavior must be understood to gain insight into many biological processes. As the sizes of these motors are in the order of a few tens of nanometers, studying single molecules poses a challenge. Although many microscopic and optical trapping methods have been employed to study motor properties, there is a need for developing novel techniques with better resolution. This study explored novel labeling strategies to engineer fluorescent kinesins in order to resolve motor transitions at the nanometer scale. Flexible loop regions of kinesin were mutated by introducing six-residue fluorophore-binding inserts, and the resulting activity of these motors was assessed with multi-motor microtubule gliding assays and single-molecule fluorescence assays. While the original goal of creating a motor whose fluorescence is quenched when binding to microtubules was not realized, the activity of some of the mutant kinesins generated in this study was regulated by the addition of external reagents. Hence, this study identified flexible loops that are sensitive to labeling and other loops that can be labeled without compromising motor function. In addition, this project helped to establish a non-lethal kinesin phenotype whose activity could be regulated, useful for studying cell biological roles of specific kinesins.
Chemical energy from ATP hydrolysis is converted into mechanical energy during kinesin’s processive stepping along microtubules. Though extensive biochemical and biophysical studies have contributed greatly towards understanding the mechanism motor function, questions regarding the chemomechanical cycle (figure 1) still remain unanswered. It is still unresolved whether sub steps occur during the motor’s 8nm stepping cycle. Another important unanswered question is: what is the rate limiting step?

![Kinesin chemomechanical cycle adapted from Muthukrishnan et al 2009.](image)

Conventional methods of microscopy and kinetic measurements are not sufficient to resolve the sub steps of the hydrolysis cycle and their kinetics. Forster resonance energy transfer (FRET) between a donor-acceptor pair of dyes is a phenomenon that is very sensitive over a distance of 1-10nm (Lakowicz, 1999), and is an ideal probe to delineate the conformation changes that a kinesin motor undergoes as it takes 8 nm steps along a microtubule. FRET between motor catalytic domains and microtubules would enable measuring the dynamics of
kinesin-microtubule interactions at nanometer spatial resolution. Some of the challenges in labeling motor domains include finding a site on the motor domain for a good FRET signal and a labeling chemistry that does not impair motor function.

To achieve selective bioconjugation of proteins, maleimide chemistry (labeling reactive thiol groups) remains one of the most widely used techniques. This approach includes mutating cysteines in the native protein to serines and introducing reactive cysteines for site-specific labeling with sulphhydryl groups. Previously, motor conformations in different nucleotide states have been investigated by measuring FRET between the two motor domains of a cysteine-lite kinesin labeled with donor and acceptor pair of dyes (Mori et al., 2007; Rosenfeld et al., 2003; Tomishige et al., 2006). Two drawbacks of this experimental set up are: 1) cysteine-lite kinesins exhibit ~3 fold diminished processive function and the rate of ADP release is 20 fold slower than wild-type (Rosenfeld et al., 2003). 2) FRET between the motor heads is interpreted to determine kinesin interaction with microtubules. At any time during the hydrolysis cycle, at least one of the heads must remain bound to the microtubule and identifying if the rate limiting step occurs in the 1H bound state or the 2H bound state places constraints on possible candidates for the rate limiting transition.

Our approach was to measure FRET between donor labeled motor heads and acceptor labeled microtubules. Donor-labeled motors are expected to exhibit maximum fluorescence in solution; when both heads are bound to the microtubule, maximum FRET occurs and donor fluorescence is quenched. In a single head bound state (figure 4.2 A), the fluorescence signal is expected to be intermediate. The FRET signal in the two head bound state is then expected to be twice that in the 1H bound state. A major challenge is the site-specific labeling of kinesin heads without affecting motor function. FlAsH is a fluorescein arsenic hairpin binding dye that binds with a high specificity and affinity to a tetracysteine motif CCXXCC, where X is not a cysteine
and is often PG (Griffin et al., 1998). The FlAsH dye does not fluoresce until it binds to the tetra-cysteine motif and is cell permeable making it useful for labeling intracellular proteins. The CCXXCC motif is not a common endogenous motif in proteins and our reasoning was that the 6 amino acid insertion and the small size of the dye would not be expected to affect motor function significantly. FlAsH based FRET with mantATP, a fluorescent analogue of ATP, has previously been measured to study the dynamics of the upper 50kDa Domain of myosin V (Sun et al., 2006). A FlAsH based FRET approach has also been used to determine G-protein coupled receptor activation in cells (Hoffmann et al., 2010). Introducing a CCPGCC sequence in processive Myosin V did not affect motor function and by analogy the expectation was that a CCPGCC insertion in the kinesin motor will not adversely affect its properties. A CCPGCC motif was introduced at various sites on the catalytic domains and motors were labeled with TC-FlAsH II In-cell Tetracysteine tag detection kit (catalogue no T34561, Invitrogen). Motor function post-mutagenesis and post-labeling was tested and results interpreted to define effects of mutations and labeling on motor activity. Purified bovine brain tubulin dimers were labeled with acceptor dye rhodamine-NHS (catalog no C1171, Invitrogen) and FRET between FlAsH tagged motors and polymerized rhodamine microtubules was measured.
Figure 4.2: FRET strategy and FlAsH dye chemistry
A) Schematic representation of motor in solution, 1H bound and 2H bound FRET. B) Chemical composition of FlAsH reagent and schematic of tetracysteine labeling.

4.2 Methods

4.2.1 Molecular Biology

Mutations were introduced into kinesin head domain using Agilent Technologies’ QuickChange XL II kit or by using Splice by Overlap Extension (SOE) PCR (Ho et al., 1989; Horton et al., 1989). Motors were expressed in bacteria as previously described (Uppalapati et al., 2009).
4.2.2 FlAsH labeling

The CCPGCC-kinesin motors (~10-20 μM) were incubated with 1 mM TCEP (*tris*(2-carboxyethyl)phosphine) for 5 minutes in elution buffer (50 mM NaPO₄, 300 mM NaCl, 500 mM Imidazole, 1 mM MgCl₂, pH 7.0) to reduce the thiol groups in the cysteines. Reduced motors were then incubated with approximately 5 fold excess TC-FlAsH II (catalog no T34561, Invitrogen) for 2 hours at 4 ºC. Protein concentration and labeling stoichiometry was measured using a UV absorption spectrophotometer. Excess dye was not separated out because FlAsH fluoresces only when bound to CCXXCC motif and hence no background is expected from excess free dye.

4.2.3 Motility assays

Bovine brain tubulin was purified and labeled with Cy5 as described (Hancock and Howard, 1998; Hyman et al., 1991; Uppalapati M. et al., 2009; Williams and Lee, 1982). Anti-His C-term antibody (Invitrogen catalog no R930-25) (20 μg/ml) was adsorbed on to the surface of glass flow cells. Histidine-tagged kinesin motors at a concentration of ~0.5 μg/ml was introduced into the chamber and allowed to bind to the antibody. Motility solution containing 40 nM Taxol stabilized Cy5 microtubules and an oxygen scavenging system of 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase and 0.5% v/v β-mercaptoethanol in BRB80 (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) was then flowed into the chamber. Video images were captured with a GenWac camera and microtubule gliding speeds were recorded and analyzed manually.
4.3 Identifying the sites to introduce CCPGCC mutagenesis

To obtain a good FRET signal, the distance between the donor and acceptor fluorescent dyes must be comparable to the Forster Radius. In addition to the distance constraint, adding the tetracysteine-motif or the FlAsH dye to the motor must not significantly impair motor function. From the crystal structure of the kinesin motor, flexible loops on the motor surface were identified. Our reasoning was that introducing the tetracysteine motif into these flexible regions and incorporating the FlAsH dye would be expected to have less dramatic effect on the motor function than modifications on the more rigid α-helices or β-sheets. Seven sites were targeted on the motor domain for introducing the tetracysteine motif: N-terminus, Loops 1, 2, 3, 5, 8 & 14.

Recent studies have focused on uncovering the role of the N-terminus of kinesin in motor function. The N-terminus forms a cover bundle with the neck-linker by forming a β-sheet and this feature is thought to fold forward during the force generation transition (Khalil et al., 2008). For a good FRET signal, a fluorophore at this site must be within the Forster radius and though it may be the most benign site for introducing the mutation, it may not be the best site for optimum FRET signal.

From aligning crystal structures of kinesin-1 and ncd, it was observed that the largest structural differences in the two motor domains were in their surface loops (Sablin et al., 1996). Loops 1 & 5 surround the entrance to the nucleotide binding cleft, loop 2 is a short lobe directed closer to the microtubule, loop 8 is thought to be involved in microtubule binding. Loop 2 in ncd is 10 residues longer than in kinesin-1 and has been shown to be involved in microtubule depolymerization in kinesin-8 and kinesin-14 (Ogawa et al., 2004; Peters et al., 2010; Sablin et al., 1996; Shipley et al., 2004). Monastrol, an inhibitor of kinesin-5 and a potential anti cancer
drug, binds in a hydrophobic pocket between loop 5 and α-helix 3 (Maliga and Mitchison, 2006). Understanding how these structural elements define motor function is important for understanding structure-function relationships of kinesins. Building on existing knowledge of the different loops, characterizing changes in motor function when the 6 amino acid sequence is introduced should help to shed light on the specific functions of these loops.

Figure 4.3: Crystal structure of kinesin-1 superimposed on ncd structure showing the different loops (Sablin et al., 1996).

4.4 Effect of introducing CCPGCC sequence on kinesin motility

The various engineered tetracysteine motors were assayed in a microtubule gliding experiment described in the methods section. The N-terminus of the kinesin motor is expected to be the least affected by introducing six amino acid residues. In a gliding assay, the Kin1Cys4N-term exhibited robust motility with wild type speeds (Table 1). From the crystal structure of a kinesin bound to microtubule, Loops 1, 2 & 14 are in close proximity to the microtubule surface and
introducing a donor in this region is expected to produce maximum FRET signal. Kin1Cys4L₁,₁ and Kin1Cys4L₂ were functional in a gliding assay with speeds similar to wild type. Kin1Cys4L₁₄ showed reduced speeds in a gliding assay with 113 ± 10 nm/s, significantly lower than wild type speed of 593 ± 21 nm/s.

Loops 3 and 5 are adjacent to the nucleotide binding P-loop and perturbations in these loops are expected to significantly alter motor function. In microtubule gliding assays Kin1Cys4L₃ and Kin1Cys4L₅ both bound microtubules to the surface, but did not exhibit any microtubule gliding activity. This result suggests that nucleotide binding or hydrolysis is affected by these mutations, since both the φ and T states are high affinity states. Kin1Cys4L₈ also bound microtubules in a gliding assay but did not show any motility, again suggesting that ATP binding or hydrolysis events are affected.

4.5 Effect of FLAsH labeling on motility

To test motor activity in the presence of the arsenic hairpin binding dye, functional Kin4Cys motor constructs were labeled with FLAsH dye as described in the methods section and their microtubule gliding speeds were measured post-labeling. Though motors are functional pre-labeling, it is possible that addition of the dye changes protein structure and perturbs its function. Labeled Kin1Cys4N-term and Kin1Cys4L₁₄ were functional in gliding assays with speeds of 618 ± 15 nm/s and 106 ± 9 nm/s, similar to their speeds prior to labeling, suggesting that labeling with the dye did not affect motor function.

Labeled Kin1Cys4L₁ and Kin1Cys4L₂ did not show any microtubule binding events in a gliding assay, suggesting that in the presence of the dye the inserted mutation inhibits
microtubule binding. While the mechanistic basis of this result is not clear, these constructs provide a novel tool for investigating ligand dependent motility of kinesins. Because these dyes are cell-permeable, these constructs can be used as a tool to study the role of different kinesin motors in cells by transiently inhibiting their action with the use of the FlAsH dye. Collaborations are in place with the Verhey lab at University of Michigan to investigate kinesin-1 function in vivo by introducing Kin1Cys4L1 and Kin1Cys4L2 into cells depleted of native kinesin-1 by transient transfection and observing changes in cargo transport and motor localization following addition of FlAsH dye. This strategy is an alternative to making siRNA knockouts which can be lethal to the cell, and can potentially be applied to other kinesin family motors.

<table>
<thead>
<tr>
<th>Mutation site</th>
<th>Putative function</th>
<th>Mutation location</th>
<th>Gliding speed “+”FlAsH</th>
<th>Gliding speed “−”FlAsH</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminus</td>
<td>Force generation</td>
<td>MCCCCG…</td>
<td>608 ± 10 nm/s</td>
<td>618 ± 15 nm/s</td>
</tr>
<tr>
<td>Loop1</td>
<td>ATP binding</td>
<td>ACCPCCSK</td>
<td>597 ± 12 nm/s</td>
<td>No microtubule binding</td>
</tr>
<tr>
<td>Loop2</td>
<td>Microtubule depolymerization</td>
<td>GCCPCCKV</td>
<td>582 ± 8 nm/s</td>
<td>No microtubule binding</td>
</tr>
<tr>
<td>Loop3</td>
<td>ATP binding/hydrolysis</td>
<td>PKCCPGCCN</td>
<td>Microtubules bind but no gliding</td>
<td>N/A</td>
</tr>
<tr>
<td>Loop5</td>
<td>ATP binding/hydrolysis</td>
<td>VICPGCCD</td>
<td>Microtubules bind but no gliding</td>
<td>N/A</td>
</tr>
<tr>
<td>Loop8</td>
<td>Microtubule binding</td>
<td>GCCPCCA</td>
<td>Microtubules bind but no gliding</td>
<td>N/A</td>
</tr>
<tr>
<td>Loop14</td>
<td>Microtubule binding</td>
<td>SCCPGCA</td>
<td>113 ± 7 nm/s</td>
<td>106 ± 9 nm/s</td>
</tr>
</tbody>
</table>

Note: Wild type kinesin speed is 593 ± 21 nm/s.

Table 4.1: Motor constructs with the labeling site, putative functions of the loops, microtubule gliding speeds before and after labeling with the FlAsH dye. Genbank ID: M24441.
4.6 FRET results

The efficiency of energy transfer (E) from donor to acceptor is a function of the distance between the two fluorophores and is expressed in terms of the distance between the two dyes and the Forster radius. The Forster radius (R₀) is the distance at which efficiency of energy transfer is 50%.

\[ E = \frac{R_0^6}{R_0^6 + r^6} \]  

(1)

The Forster radius is a function of quantum yield (Q_D) of the donor, the overlap integral of donor emission and acceptor absorption (J), refractive index of the solution (n) and the orientation parameter of the donor – acceptor pair (κ) (Lakowicz, 1999).

\[ R_0^6 = 8.79 \times 10^{-5} \left[ \kappa^2 n^{-4} Q_D J(\lambda) \right] (\text{Å}) \]  

(2)

The overlap integral (J) is calculated according to

\[ J(\lambda) = \frac{\int_0^{\lambda_2} F_D(\lambda) \varepsilon_A(\lambda) \lambda^2 d\lambda}{\int_0^{\infty} F_D(\lambda) d\lambda} \]  

(3)

where \( F_D \) is donor fluorescence intensity, \( \varepsilon_A \) is the acceptor molar absorption coefficient at wavelength \( \lambda \) (Lakowicz, 1999). Quantum yield of CCPGCG-FlAsH reported in literature is 0.7 (Adams et al., 2002); orientation parameter value and refractive index of solution are assumed to be 2/3 and 1.4 (Lakowicz, 1999) respectively. The spectral overlap of FlAsH motors and Rhodamine-NHS microtubules is shown in figure 4.4 and overlap integral according to equation (3) is estimated to be \( 1.76 \times 10^{15} \text{ nm}^3 \text{M}^{-1} \). With the values for all the quantities, the Forster radius from equation (2) is calculated as 4.83 nm. Hence, for a good FRET signal, the distance between the FlAsH dye and Rhodamine-NHS must be ≤ 5 nm.
FlAsH labeled Kin1Cys\textsubscript{N-term} and rhodamine labeled microtubules were used to obtain the absorption and emission spectra.

To test for energy transfer between labeled kinesins and microtubules, FlAsH-tagged Kin1Cys\textsubscript{4N-term} and Kin1Cys\textsubscript{4L14} were incubated with rhodamine microtubules in the presence of AMP-PNP, a non-hydrolysable analog of ATP. In the presence of AMP-PNP kinesins bind to microtubules with both heads on the microtubule (Kawaguchi and Ishiwata, 2001). In this conformation maximum FRET between FlAsH dye on both heads and the rhodamine on the microtubules occurs and maximum signal is expected. FlAsH labeled motors (100 nM) were mixed with a 10 fold excess of microtubules in the presence of 0.5 mM AMP-PNP, incubated for 20 minutes, excited at 470 nm in a PTI fluorimeter and emission spectrum recorded. Donor quenching was used to measure FRET. With Kin1Cys\textsubscript{4N-term} bound to microtubules, no donor quenching was observed in the emission spectrum. With both heads of Kin1Cys\textsubscript{4L14} bound to microtubules in the presence of AMP-PNP, donor quenching was observed (Figure 4.5) and FRET efficiency was estimated as 10%. In the presence of ADP, motors bind to microtubules with one head (Hackney, 1994). The maximum FRET signal in the 2H bound is only 10% and this signal in the 1H bound state is expected to be 5% which is in the noise range of the signal.
The low FRET signal indicates that the distance between the FRET dyes is much greater than their Forster Radius and with the motor speed reduced by a factor of 5, the FlAsH labeling site is not ideal for probing the conformation changes occurring during the hydrolysis cycle.

Figure 4.5: FRET between FlAsH tagged Kin1Cys_{L14} and rhodamine tagged microtubules. FlAsH tagged Kin1Cys_{L14} motors were incubated with rhodamine microtubules in the presence of AMP-PNP. Emission Spectra of motors alone in solution, microtubules alone in solution and motors and microtubules in AMP-PNP was obtained for an excitation wavelength of 470 nm.

Loops 6, 7 and 9-13 that have not been targeted will be investigated for their proximity to the microtubule using existing crystal structures and CCPGCC tags introduced into these loops and their function and FRET signals with microtubules will be recorded as potential probes. Our experimental approach thus far has been to target the flexible loops and will be extended to labeling the rigid beta sheets also.

An alternate strategy is to label exposed cysteine residues on the motor heads with a donor and measure FRET between motors and acceptor labeled microtubules. Cysteine residues in the neck-linker and neck-coil of kinesin-1 were mutated to serine and motor speed and run length was characterized to ensure wild type behavior. Motors were incubated with maleimide
Alexa-488 and dye : motor ratio and labeling time were empirically controlled to obtain a labeling ratio of ~ 1 dye per motor head. When Alexa-488 kinesin motors were incubated with rhodamine microtubules in the presence of AMP-PNP and solution excited at 470 nm, Alexa-488 quenching corresponding to a FRET signal of ~18% was observed (figure 4.6). This is the best FRET signal that has been observed in this study and these probes are being developed in the lab for further FRET studies to delineate the sub-steps in the chemomechanical cycle.

![FRET between Alexa 488 labeled kinesin-1 and rhodamine labeled microtubules.](image)

Figure 4.6: FRET between Alexa 488 labeled kinesin-1 and rhodamine labeled microtubules. Alexa 488 tagged Kin1Cys4L14 motors were incubated with rhodamine microtubules in the presence of AMP-PNP. Emission Spectra of motors alone in solution, microtubules alone in solution and motors and microtubules in AMP-PNP was obtained for an excitation wavelength of 470 nm.

4.7 Conclusions and future directions

This study has shown that introducing a 6 amino acid sequence into loops 3, 5 & 8 in the kinesin motor domain abolishes motor function, insertions in the N-terminus and loops 1, 2 generates a functional motor with wild type characteristics and insertion in the loop 14 produces a motor with diminished speed. On labeling with the FIAsh tag, Kin1Cys4L1 and Kin1Cys4L2 do
not bind to microtubules while function of Kin1Cys4\textsubscript{N-term} and Kin1Cys4\textsubscript{L14} are not changed compared to their pre-labeling speeds. Hence, the N-terminus and loop 14 are good sites for labeling kinesins. A FRET signal for motors interacting with microtubules, is observed only for Kin1Cys4\textsubscript{L14} motors and this low value of 10\% is not suitable for distinguishing between 2H bound state and 1H bound. Though this construct is not an ideal probe for delineating the sub-steps in the kinetic cycle, the motor may be useful for other studies, for example to investigate the conformation of the neck-linker relative to the motor core domain. Kin1Cys4\textsubscript{L1} and Kin1Cys4\textsubscript{L1} are ideal candidates for probing ligand mediated inhibition of kinesins, and a collaborative study is being conducted to understand the effects of kinesin inhibition in cells. These phenotypes are better than kinesin gene knockouts, which are often embryonic lethal. In future studies, FlAsH tags on other loops will be investigated and alternate labeling strategies using cysteine-maleimide chemistry will be explored for obtaining motor-microtubule FRET.
4.8 References


Chapter 5

Collective Transport Behavior of Fast and Slow Motor Ensembles

5.1 Introduction

Motor-driven cargo transport along cytoskeletal filaments is one of the best studied mechanisms of active cellular transport. Kinesins are one class of microtubule-associated motors that are involved in many of these transport activities including axonal transport, intraflagellar transport and organelle transport (Hirokawa and Noda, 2008). As these remarkable protein machines are involved in various essential cellular tasks, their activity has been linked to many disease states including neurodegenerative diseases and infertility (Goldstein, 2001). Understanding how kinesins carry out their transport activities is important for uncovering the progress of the diseases and for developing potential therapeutics.

Investigations of single kinesin motor properties has revealed great details about their structure (Kull et al., 1996; Turner et al., 2001), biophysics (Block, 2007) and biochemistry (Mori et al., 2007). Individual molecules of the kinesin-1 family are processive motors capable of walking ~2 μm per interaction with a microtubule (chapter 2 results) and can pull loads of up to 6 pN (Svoboda and Block, 1994; Svoboda et al., 1993). To carry out long distance transport, for example in neuronal transport, in which the distances far exceed the distance that a single kinesin can walk (Goldstein and Yang, 2000), clusters of motors can bind to the same cargo, enabling it to move large distances and will allow the motors to pull large cargo particles. Though single motor behavior has been studied in great detail and much is understood about the mechanism of operation of kinesins, not much is understood about collective motor transport.
One of the challenges in analyzing cargo transport by multiple motors is knowing precisely how many motors are attached to the particle being transported. Simulation studies of cargo trafficking by multiple motors have served to provide a framework for mechanistic analysis of motor assemblies and set parameters that can be tested by experiments (Klumpp and Lipowsky, 2005). DNA and protein polymer based scaffolds have recently been engineered to precisely conjugate a known number of motors in order to facilitate quantitative analysis of multiple motor movements (Diehl et al., 2006; Schweller et al., 2008). Analyzing how motor assemblies work in concert requires a knowledge of their mechanical compliance and how mechanical forces affect motor unbinding forces.

Cryo-electron microscopy studies have demonstrated that molecular motors work in groups to transport cargo (Ashkin et al., 1990). Observations of ‘saltatory movements’ and step-like changes in velocity during cargo transport in *in vivo* studies support the idea of transport by multiple motor systems (Hill et al., 2004; Kural et al., 2005). Kinesins and dyneins have opposite polarity on microtubules and bidirectional transport using groups of these motors is a very important physiological mechanism responsible for intracellular transport of mitochondria, pigment granules and lipid droplets (Welte, 2004). Bidirectional motion can be realized by either a tug of war mechanism between the two classes of motors or by a coordinated mechanism where motors receive cellular cues to move towards plus ends or minus ends of microtubules. Understanding the basis of this motion will vastly enhance our knowledge of many cellular processes.

In addition to bidirectional movements, cooperative behavior by multiple plus-end directed motors has been observed during the transport of frog melanocytes (Levi et al., 2006). In *C. elegans*, two kinesin-2 motors, which move at different speeds, regulate each other’s activity
to shuttle intraflagellar transport particles to build the organism’s sensory cilia (Snow et al., 2004). Slow kinesin-5 motors are thought to act as brakes and control rate and direction of migration of neurons (Falnikar et al., 2011). This idea of fast and slow moving motors working together to modulate transport of organelles and cargo in cells may be a more widely present phenomenon than is currently known. However, to interpret these cellular phenomena, it is important to study how different motors coordinate in a well defined in-vitro motility assay.

In this study, motility of motor constructs generated in chapter 3 were assayed in a microtubule gliding assay. Motor speeds of kinesins 1, 2, 3, 5 & 7 vary over a 25 fold range and mixtures of fast and slow motors in all combinations were adsorbed on to a glass flow cell. Two key characteristics of kinesin motors are speed and processivity. To control for processivity, only motors with 14 amino acid neck-linkers that are processive to the same degree were utilized. Microtubule gliding speeds were characterized for uniform populations of motors and effect of motor mixing on overall gliding was then quantified. To describe the mechanism by which fast and slow kinesin-2 motors transport IFT particles, Pan et al proposed two models, the alternating action model and the mechanical competition model (Pan et al., 2006). In the alternating action model, fast and slow motors sequentially move cargo, with instantaneous speeds of the component motors. This model inherently assumes that the motors are nonprocessive and is not applicable to the class of motors investigated in this study. In the mechanical competition model, both motors simultaneously work to move the cargo such that the slow motor exerts a drag on the fast motor and the fast motor pulls on and accelerates the slow one. This model is simple in its scope and is a starting point for making predictions of stall forces. To understand intracellular transport, it is important to understand the mechanical compliance and the detachment kinetics of the motors involved. By developing more complex models, and inputting experimentally determined parameters, the unknown values can be extracted.
Figure 5.1: Microtubule gliding assay

The motors generated in chapters 2 and 3 were characterized for their unloaded processivity and speed in single molecule assays. The motivation of this study is to compare the mechanical performance of these various motors. The advantages of the approach used in this work are: 1) It is much easier than using optical tweezers to manipulate motor clusters, 2) It assesses motors in a single assay thus controlling for variables between experiments, and 3) The geometry of the experimental setup is very relevant to intracellular transport. This assay provides insights into behavior of diverse motors, for which little mechanical data exist.

5.2 Methods

5.2.1 Motor constructs, purification, and concentration determination

The various GFP-tagged motor constructs were engineered as described in chapters 2 and 3 and motors were expressed in bacteria and purified as described previously (Hancock and Howard, 1998). Motor concentrations were estimated relative to kinesin-7 using a spectrofluorimeter and measuring the GFP emission peak. Motors of the kinesin-1, 2, 3, 5 & 7
families were diluted 100 fold in 80 mM PIPES buffer, excited in a spectrofluorimeter at 475 nm and their peak emission at 510 nm was recorded. Fluorescence intensity is dependent on the concentration of the fluorophores and hence the concentrations of the various motors were assessed relative to the concentration of kinesin-7 intensity.

Because the proteins can undergo denaturation during the purification step or subsequent storage conditions, motor activity needs to be quantified. Active motor concentrations were assayed using a microtubule pelleting assay as follows. Motors were incubated with microtubules and AMP-PNP for 20 minutes and then pelleted in a Beckman airfuge. The supernatant was collected, the pellet was resuspended in fresh buffer, and the GFP emission was recorded for both. The ratio of motors in pellet to the total motors in supernatant and pellet gives the ratio of active motors that can bind to microtubules.

With the motor concentrations known relative to kinesin-7 and the percentage of active motors, each of the motors were diluted to the same stock concentration in 80 mM PIPES buffer with 1 mM ATP and 0.2 mg/ml casein. For example, 4 μl of kinesin-7 motor, containing 50% active motors were diluted in 100 μl of buffer. The volume (V) of kinesin-1 motors is then calculated based on the fluorescent intensity (I) of the motors and their activity ratio (α) as

\[ V_{kin1} = \frac{V_{kin7} \cdot I_{kin7} \cdot \alpha_{kin7}}{I_{kin1} \cdot \alpha_{kin1}} \]

\[ V_{kin1} = \frac{4 \cdot 35.7 \cdot 0.5}{24.3 \cdot 0.87} = 3.4 \mu l \]

From the stock solutions thus obtained motors were further diluted by a factor of 10 in ratios of 20%, 40%, 60% and 80% of fast and slow motors for microtubule gliding assays. To get a ratio of 20% kinesin-1 and 80% kinesin-7, 2 μl of kinesin-1 and 8 μl of kinesin-7 were diluted in 90 μl of 80 mM PIPES buffer containing 1 mM ATP and 0.2 mg/ml casein.
5.2.2 Microtubule gliding assay

Microtubule gliding assays were performed as described in chapter 4. Fast and slow motors diluted to the same concentration were mixed in ratios of 20%, 40%, 60%, 80%, 100% and adsorbed on to the glass surface of the flow cells. Cy5 labeled microtubules were flushed in with an oxygen scavenging system as described in chapter 4 and microtubule gliding was observed and captured with a Cascade 512 CCD camera (Roper Scientific, Tucson, AZ). Videos were acquired using MetaVue software (Molecular Devices Corporation, Downingtown, PA) with a 71 nm pixel size and gliding speeds were analyzed manually. At least 30 different microtubules were assayed for each data point over a minimum distance range of 2 μm.

5.3 Results

Speeds of the different kinesins used in this study vary over a 25-fold range. For these experiments, fast and slow motors were mixed together in different ratios and the resulting microtubule gliding speeds were measured. Kinesin-2 and kinesin-7 have similar speeds and were not assayed together. The relative speeds of the motors are,

\[ \text{Kin3} > \text{Kin1} > \text{Kin2} = \text{Kin7} > \text{Kin5} \]

If the ensemble speeds of a mixture of fast and slow motors lies along the unity line, above the unity line or below it, then they will be described as proportional, super-proportional or sub-proportional, respectively.
5.3.1 Kinesin-1 speeds dominate over kinesins-2, 3 & 5 but speeds are averaged with kinesin-7

Kinesin-1 motors were mixed with varying ratios of kinesins 2, 3, 5 & 7, adsorbed on to a glass flow cell and microtubule gliding speeds were measured. To compare how ensembles of fast and slow motors move microtubules, motor speeds were normalized between 0 and 1 and speeds were plotted as a function of the fraction of faster motors. When normalized speeds of kinesin-2 were plotted against the fraction of the faster kinesin-1, all the values lay above the unity line indicating that microtubule gliding is more influenced by kinesin-1 even in the presence of 80% kinesin-2 (figure 5.2). Kinesin-3 motors behaved in a manner similar to kinesin-2 motors, when mixed with kinesin-1. Even though kinesin-3 motors are 3 fold faster than kinesin-1, in the presence of just 20% kinesin-1, microtubule gliding speed was closer to kinesin-1 than kinesin-3 (figure 5.2A). In the presence of 20% kinesin-1, kinesin-5 speeds dominated in multi-motor gliding assays, while at higher fractions kinesin-1 motors dominated. When kinesin-7 and kinesin-1 motors were mixed together, microtubule gliding speeds observed were the weighted average of the two motor speeds, indicating that their speeds are proportional.

Figure 5.2: Microtubule gliding speeds of mixtures of kinesin-1 with other N-terminal kinesins. A) Microtubule velocity plotted as a function of kinesin-1 fraction. B) Normalized speeds of motors plotted as function of the faster motor speed.
5.3.2 Kinesin-2 motor mixing experiments

Kinesin-2 motors were mixed in different ratios with 3 other kinesins and microtubule gliding speeds were recorded. Microtubule gliding speeds were plotted as a function of kinesin-2 fraction and normalized speeds were plotted as a function of the faster motor. Kinesin-7 and kinesin-2 speeds are very similar and hence motor mixing experiments were not carried out for this motor pair. When mixed with kinesin-1 motors, microtubule gliding was dominated by kinesin-1 indicating that speeds are super-proportional. From the normalized velocity plot, when kinesin-3 and kinesin-2 mixing speeds are plotted against fast motor kinesin-3 fraction, the speeds for all ratios were below the unity line (sub-proportional), indicating that the slower kinesin-2 dominates. For kinesin-5 mixing experiment, speeds plotted as a function of kinesin-2 showed that the values were all below the unity line indicating that kinesin-5 dominates.

Figure 5.3: Microtubule gliding speeds of mixtures of kinesin-2 with other N-terminal kinesins. A) Microtubule velocity plotted as a function of kinesin-2 fraction. B) Normalized speeds of motors plotted as function of the faster motor fraction.

5.3.3 Fast kinesin-3 motors are overwhelmed by all the other motors

To understand how fast kinesin-3 motors behave in the presence of the other slower kinesins, kinesin-3 motors were mixed in varying ratios with kinesins 1, 2, 5 and 7. When the
microtubule gliding speeds were measured for the different motor concentrations, it was observed that kinesin-3 speeds were sub-proportional to all the other motors even in the presence of 60-80% kinesin-3. From the normalized velocity curves, except for the single data point of 20% kinesin-5, all the speeds lie below the unity line. Kinesin-3 motors are slowed down in the presence of the other slower N-terminal kinesins to different degrees, kinesin-1 having the most effect, then kinesin-7, kinesin-2 and kinesin-5 in that order.

Figure 5.4: Microtubule gliding speeds of mixtures of kinesin-3 with other N-terminal kinesins.
A) Microtubule velocity plotted as a function of kinesin-3 fraction. B) Normalized speeds of motors plotted as function of the faster motor speed.

5.3.4 Kinesin-5 motors work very well at high concentrations

The mitotic motor kinesin-5 was mixed in varying ratios with other N-terminal kinesins and adsorbed on to a glass surface. Motor behavior was investigated in a microtubule gliding assay and motor speeds were plotted against the fraction of kinesin-5 motors. To understand the relative effective of each kinesin family member on kinesin-5 speeds in the multi-motor assay, normalized velocities were plotted against fast motor concentration, with kinesin-5 being the slowest of the five motors in this study. At high concentrations, kinesin-5 motors dominated over the other motors and speeds of the motor mixtures are representative of kinesin-5 motors as
evidenced by the 80% kinesin-5 data point which is below the unity line. At higher concentrations, kinesin-1 and kinesin-7 dominate. Kinesin-2 and kinesin-3 speeds on the other hand are sub-proportional, indicating that kinesin-5 dominates in these cases.

Figure 5.5: Microtubule gliding speeds of mixtures of kinesin-5 with other N-terminal kinesins. A) Microtubule velocity plotted as a function of kinesin-5 fraction. B) Normalized speeds of motors plotted as function of the faster motor speed.

### 5.3.5 Kinesin-7 motors are as strong as kinesin-1 and overpower kinesins 2, 3 and 5.

In a multi motor mixing assay the behavior of CENP-E kinesin-7 was characterized by measuring microtubule gliding speeds in the presence of processive kinesins 1, 3 and 5. The relative behavior of kinesin-2 and kinesin-7 was not investigated as the two motors move at very similar speeds. From the normalized velocity curves, it is observed that when mixed with kinesin-1 the speeds lie close to the unity line, indicating that they mixture speeds are proportional. Kinesin-3 and kinesin-5 motors on the other hand are dominated by kinesin-7. Kinesin-3 motors are 5-fold faster than kinesin-7 and in the presence of kinesin-7 are slowed down to a high degree. At high concentrations of kinesin-5, kinesin-7 is slowed down and this trend reverses as the motor ratio increases beyond 20% of the total as seen in the normalized velocity plot.
Figure 5.6: Microtubule gliding speeds of mixtures of kinesin-7 with other N-terminal kinesins. A) Microtubule velocity plotted as a function of kinesin-7 fraction. B) Normalized speeds of motors plotted as function of the faster motor speed.

5.4 Discussion

This study investigated the motility of collective motor assemblies to gain insights into the mechanistic properties of motor complexes necessary for cellular functions like long distance cargo transport and microtubule sliding in mitotic spindles and neurons. Axonal transport by microtubular motors is one of the well known examples of long distance transport and this occurs over distances much larger than the distance moved by a single motor (Goldstein and Yang, 2000). By being attached to many motors, a single cargo element can increase the probability that it stays attached to a microtubule or actin filament, enabling it to cover the large distances it needs to move without detaching. To carry out any transport activity the motor must be processive and it must be able to pull the cargo against the viscous drag imposed by the cytoplasm.

Simulations of long distance cargo transport by multiple kinesins have demonstrated that for motors like kinesin-1 that bind strongly to microtubules, the average walking distance
increases exponentially as the number of motors increases (Klumpp and Lipowsky, 2005) given by the equation

\[ <x> = \frac{v}{N\pi} \left( \frac{\pi}{\varepsilon} \right)^{N-1} \]

where \( <x> \) is the average walking distance, \( v \) is velocity of a single motor, \( N \) is the number of motors, \( \pi \) is the binding rate and \( \varepsilon \) is unbinding rate. Because transport distances by multiple motors exceed the length of microtubules in-vitro, this model has been difficult to quantitatively verify experimentally.

Motor velocities are force dependent, and from force-velocity characteristics of kinesin-1, the velocity decreases approximately linearly with applied force (Schnitzer et al., 2000; Svoboda and Block, 1994) according to the equation,

\[ v = v_0 \left( 1 - \frac{F}{N F_s} \right) \]

where \( v_0 \) is the unloaded velocity, \( F \) is the force applied on the motors and \( F_s \) is the stall force and \( N \) is the number of motors.

Rewriting the equation, the force \( F \) experienced by a motor can be expressed as,

\[ F = N F_s \left( 1 - \frac{v}{v_0} \right) \]

If the stall forces of the fast and slow motors are \( F_{\text{stall,f}} \) and \( F_{\text{stall,s}} \), and the force exerted by each type of motor is \( F_f \) and \( F_s \), and the number of fast and slow motors are \( M \) and \( N \), then the force balance equation for the ensemble is

\[ M F_f + N F_s = 0 \]

Writing the force exerted by each motor, in terms of its velocity and stall force, the intermediate velocity can be represented as

\[ v = \frac{v_f v_s [\alpha v_f + (1 - \alpha)]}{v_f (1 - \alpha) + n \gamma v_s} \]
where $\alpha$ is the mole fraction of the fast motors (M/M+N) and $\gamma$ is defined as

$$\gamma = \frac{F_{\text{stall},f}}{F_{\text{stall},s}}$$

The mechanical competition model is a simple analytical model for explaining the mechanical behavior of a motor ensemble. One of the caveats in this model is that it does not consider the number of motors bound to the cargo and does not account for unbinding and binding of motors. The transition rate model accounts for the association and dissociation of motors as they walk, thus allowing for a different number of motors attached at any time (Klumpp and Lipowsky, 2005). Motor unbinding rates are dependent on the force exerted by each motor and can be calculated by using the collective force exerted on the ensemble. Binding rates can be calculated by knowing the number of motors and with known values of the binding and unbinding rates, a motor binding probability distribution and thus the number of bound motors at any point of time can be estimated. This allows for calculation of the forces exerted by the fast and slow motors, and predictions of speeds can then be compared with experimental evidence.

Mechanical properties of multi-motor transport was investigated computationally by Kunwar et al using a stochastic model (Kunwar et al., 2008). The influence of thermal fluctuations and viscosity on motor dynamics was studied and theoretical predictions were compared to experimentally observed step size distributions of a cargo moved by two kinesins. Mechanical compliance between motors in an ensemble is very important for propagating mechanical tension and Kunwar et al show that in the presence of external load, motors share the force unevenly which leads to longer run lengths (Kunwar et al., 2008).
Using an engineered two motor system, Jamison et al showed that geometry of motor binding dictates how multiple motors transport cargo and reported that motors move cargo primarily by the action of one motor (Jamison et al., 2010). From the experimental results of single kinesin stepping, a computational model of a two motor system was developed assuming that load is equally distributed between the two kinesins. The probabilities of different microstates assumed by a two kinesin system was computed and used to estimate the detachment forces, which were higher for a two kinesin system than for a single kinesin carrying load (Jamison et al., 2010). The analysis presented in this study is a preliminary investigation into multiple-motor behavior. A more detailed analysis of the mechanical properties requires a more complex computational model and collaborations are underway with Erkan Tuzel, WPI for developing this model and interpreting the experimental data in the context of these models.

Different kinesins have different mechanical properties, characteristic of their diverse cellular roles. Kinesin-1 motors are the most processive of the transport kinesins and are among the strongest kinesins with a single motor capable of pulling loads up to 6 pN force (Meyhofer and Howard, 1995; Svoboda and Block, 1994), while still being able to step processively. When mixed with kinesin-2 and kinesin-3 motors, even at a low ratio of 20%, the kinesin-1 motors speed up the slower kinesin-2 and slow down the faster kinesin-3 motors. This is consistent with kinesin-1 being stronger than kinesin-2 or kinesin-3. At low concentrations kinesin-1 does not affect kinesin-5 motility significantly, though at ratios greater than 20%, motor speeds are super-proportional. Single kinesin-5 motors do not stall, but dissociate from microtubules at high forces (Valentine et al., 2006) and hence at higher concentrations they can resist being accelerated by kinesin-1 motors. CENP-E (kinesin-7) motors are involved in the transport of kinetochores during mitosis and the motors in addition to being very processive also need to be able to support substantial loads. The stall force of kinesin-7 at ~6 pN (Yardimci et al., 2008) is comparable to
kinesin-1 and thus the speed of an ensemble of faster kinesin-1 and slower kinesin-7 is the weighted average of speeds at the two different motor ratios. Substituting for $\gamma=1$ in the velocity equation, experimental results agree well with the predicted velocities for a mixture of kinesin-1 and kinesin7. From kinesin-7 speeds in the presence of the other kinesins, it is evident that kinesin-7 overwhelms the faster kinesin-3 by slowing it down and speeds up kinesin-5. For experiments where kinesin-1 and kinesin-7 were mixed with the other motors, alternating fast and slow events were not observed. When the data was fit to a mechanical competition model with $\gamma=1$, from experimental results for kinesin-1 and kinesin-7 stall forces, the data fit the model well.

Figure 5.7: Experimental and simulation results of kinesin-1 and kinesin-7 mixing.

Kinesin-2 motors in conjunction with cytoplasmic dynein are involved in bi-directional transport of intraflagellar transport (IFT) particles (Scholey, 2008). Two different kinesin-2 family members work in cooperation to build sensory cilia in C. elegans neurons (Snow et al., 2004). In both these cases, collective assemblies of kinesin-2 motors are involved in transporting and motor velocities and mechanical properties are modulated by their partner motors. In optical trapping assays, single kinesin-2 motors were shown to pull external loads and interestingly like
kinesin-5 motors these motors dissociated from microtubules at loads ≥ 4 pN without stalling (Andreasson, 2010). From the motility experiments it is observed that kinesin-2 motors are accelerated by kinesin-1 motors, which are stronger than kinesin-2, and are slowed by kinesin-5 motors. This indicates that the kinesin-5 are sub-proportional to speeds of kinesin-2. Kinesin-3 motors on the other hand are slowed down by kinesin-2 and in the absence of force-velocity characterization for kinesin-3, the result indicates this motor is likely to be able to pull lower forces than kinesin-2. For kinesin-1 and kinesin-2 mixing experiments, the velocity data was fit to the mechanical competition model assuming γ = 1, 2 and 10. From the simulation of the mechanical competition model curve it is evident that the data fit best when the ratio of stall forces γ = 10. Experimental results show that kinesin-2 does not stall even at 6 pN forces, the stall forces of kinesin-1 and the model does not agree well with observed results, though it is a good fit for kinesin-7 and kinesin-1 behavior. To address the discrepancies between experimental and mechanical competition models, a more rigorous analysis using a more complex model is necessary. Collaborations are in place to develop a more detailed model of multi-motor transport and to interpret experimental results in the framework on the model.

Figure 5.8: Experimental and simulation results of motor mixing for kinesin-1 and kinesin-2.
In the presence of a small fraction of other motors, kinesin-5 motors dominate and slow down the faster motors. This may relate to their cell function roles of microtubule sliding in mitotic spindles where many molecular motors are expected to be present and kinesin-5 motors may crowd and regulate the speed of microtubule sliding. Kinesin-5 motors have been shown to regulate neuron migration by slowing neurons in developing cells (Falnikar et al., 2011) and is thought to be overexpressed at the sites where a neuron has reached its destination. This may act as a brake for axonal transport and may be a common mechanism of slowing and eventually stopping cargo and organelle transport.

5.5 Conclusions and future directions

During cargo transport by multiple motors, different motors exert different forces and our experiments indicate that kinesin-1 and kinesin-7 motors are the strongest motors in that their speeds are the least affected when working together with other motors. At high concentrations kinesin-5 motors slow down all the other faster motors, indicating that this property may be exploited in cells to slow down and reverse cargo movements. Kinesin-3 motors are the weakest motors and are slowed down by all of the other kinesins. A more complex model of multi-motor cargo transport needs to be developed and collaborative efforts are in place to carry out simulations of the microtubule sliding experiments. Kinesin-1 motor constructs with shorter stalk and tail domains slowed down each other in microtubule gliding assays, compared to constructs with longer non-motor domains (Bieling et al., 2008). Mechanical coupling may be another mechanism for regulating the cargo movement in cells and will be a next logical step in analyzing the effects of mechanical signaling in kinesin motor assemblies.
5.6 References


Chapter 6
Conclusions and Future Directions

Kinesin motors perform essential cellular transport activities like organelle transport, chromosome movements, maintaining mitotic spindle during mitosis, regulating the dynamics of microtubules (Desai et al., 1999; Hirokawa and Noda, 2008). Understanding the structural and biochemical basis of their function is important for understanding their in-vivo behavior, to target these motors for therapeutic uses. Although kinesin-1 has been the most studied of the 14 classes of kinesins, recent work on other kinesin family members has enhanced our understanding of how these molecular machines function. Single molecule biophysical characterization has been employed to study individual motors and multi motor assays have been designed to understand how they function in groups as applicable to their cellular tasks.

6.1 Insights into kinesin processivity

The overarching aim of this thesis was to determine the role of the neck-linker domain in kinesin processivity and to elucidate the precise mechanism of motor stepping cycle using single molecule and ensemble studies. Run lengths of neck-linker mutants with varying lengths for 5 different kinesin families were characterized using single molecule total internal reflection microscopy and the results indicated that processivity scaled inversely with the neck-linker length. Despite a 25-fold range of motor speeds, when the neck linkers were shortened to 14 amino acids, kinesins 1,2,3,5 & 7 all moved the same distance on microtubules. This finding is profound as it suggests that the ability of a kinesin motor to walk along microtubules depends only on the tension generated between the two heads and not on its inherent biochemical
properties, thus tuning each processive kinesin family member for its specific cellular role. From stochastic simulations of the kinetic cycle, either front head gating or rear head gating can account for processivity of kinesins and results place constraints on the possible candidates for the rate limiting step. From state 1 of the cycle in figure 6.1, two of the rate constants \( k_{\text{detach}} \) and \( k_{\text{on,ATP,2H}} \) are influenced by inter-head tension and are force dependent rates. For kinesins with longer neck-linkers strain between heads in the two head bound state is lowered, opening the nucleotide pocket allowing ATP to bind and be hydrolyzed. This allows both heads to go into the low affinity ADP state, leading to dissociation and termination of the processive cycle. In the second scenario, inter-head tension for longer kinesins is reduced decreasing the detachment rate and shunting the cycle along the other pathway and again leading to low affinity states for both motor heads and unbinding from the microtubule. These pathways are not mutually exclusive and differentiating between the two pathways is a challenging task. Different kinesin family members have different speeds even though their run lengths are identical implying that neither detachment of rear head nor ATP binding to the front head is the rate limiting step. One possibility that can explain for the variations in motor velocities is that the hydrolysis rate is different for different motors. Novel biochemical experiments need to be designed to probe the kinesin chemomechanical cycle in greater detail to understand the precise mechanism of motor processivity and to pinpoint the rate limiting step.
Ensemble studies of motor mixtures were performed to gain mechanical insights into motor behavior when working cooperatively in groups. Motor velocities of mixtures of fast and slow moving kinesins showed that stronger motors dominate over faster motors and helped to estimate relative strengths of motors when they work in groups, useful for cell biology studies of organelle transport, chromosome dynamics, and bi-directional trafficking. From labeling motors for single molecule kinetic studies, sites suitable and unsuitable for labeling were mapped. In addition motor constructs whose activity can be controlled by addition of specific ligands were generated, useful for investigating kinesin inhibition and its implications for cell biology studies.
6.2 Future directions

The fact that kinesin processivity is controlled by internal strain between the two heads in dimeric kinesins calls into question any interpretations of motor processivity made from monomer kinetic studies on kinesins (Rosenfeld et al., 2009; Rosenfeld et al., 2005). To unearth if either front head gating or rear head gating alone can account for kinesin processivity, stop flow kinetic experiments need to be designed to measure biochemical properties of dimeric motor constructs. To test if neck-linker affects the ATP binding rate $k_{on_{ATP}}$, stop flow experiments measuring pre-steady state kinetics of kinesins with different neck-linker lengths need to be designed. Using fluorescent ATP analogs, the binding kinetics can be determined as shown previously (Rosenfeld et al., 2003) and will shed light on the chemomechanical cycle. Neck-linker docking is the most important structural transition that occurs during motor stepping. Investigations into neck-linker structure have shown that ATPase rates are affected by even slight modifications to the neck-linker (Case et al., 2000; Kalchishkova and Bohm, 2008). Deleting the neck-linkers in single headed different kinesins and comparing the ATPase rates with single headed motors that contain the whole neck-linker will provide insights into the role of the neck-linker in motor mechanochemistry. Kinesin-8 motors have been shown to be very processive with run lengths averaging over 10 µm (Varga et al., 2006) and are possibly the most processive kinesins. Another avenue of research is to study the processivity behavior of the remaining N-terminal kinesins including kinesins-4, 6, 8 & 9 and examine the role of the neck-linker in their processivity. The neck-linker of kinesins is analogous to the lever arm of myosins, another class of molecular motors and testing the role of the lever arm might shed light on the mechanism of myosin processivity and help establish a common mode of operation of processive motors. Collaborations are in place for computational modeling of multi-motor trafficking studies to gain insight into mechanistic details and correlate with experimental results from this study.
Experiments using optical tweezers to trap and study cargo attached to multiple motors can be designed and will provide details of stepping behavior of an ensemble of motors. For optical trapping experiments involving motor clusters, it is important to know the mechanical compliance of the motors. Motors are attached to their cargo via their stalk region and the length of this stalk region is not precisely known. NMR techniques can be used to determine the exact length of the motor stalk and tail region.
6.3 References

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PUBLICATIONS

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