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Regulation of kinesin, an intracellular transport motor, by its cargo-binding domain

Author
Friedman, Dara Spatz

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Regulation of Kinesin, an Intracellular Transport Motor,
by its Cargo-Binding Domain

by

Dara Spatz Friedman

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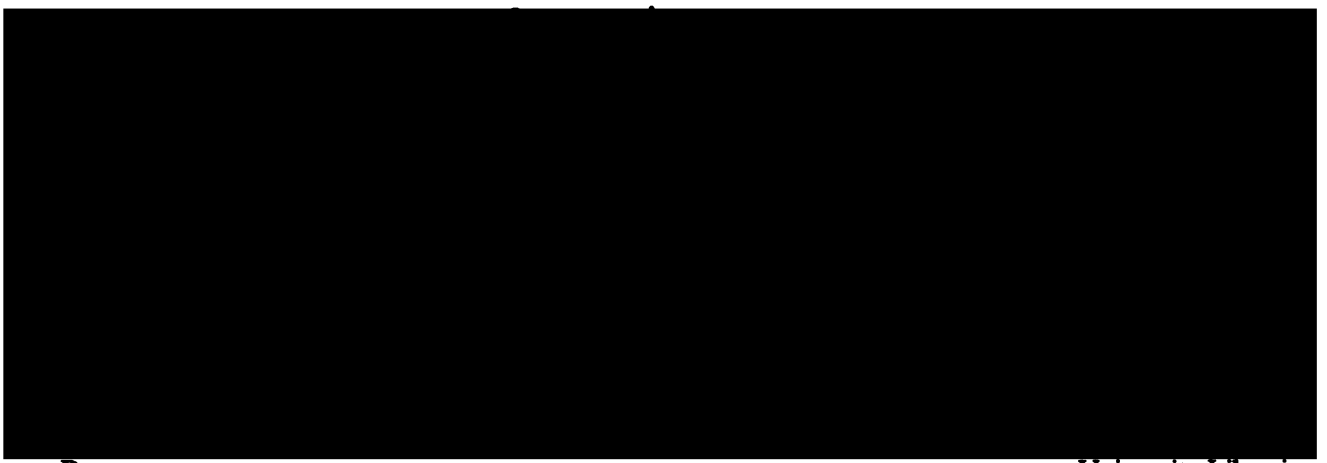

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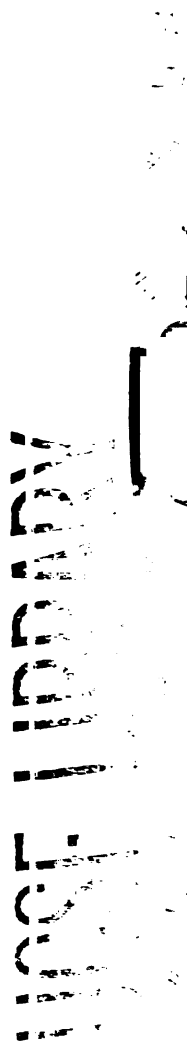

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Valerie Depraetere
Associate Editor
Nature Cell Biology
Porters South
4 Crinan Street
London N1 9XW
UK
Phone: (44/0) 171 843 4743
Fax: (44/0) 171 843 4794
e-mail: v.depraetere@nature.com
(http://cellbio.nature.com/)

Regulation of Kinesin, an Intracellular Transport Motor, by its Cargo-Binding Domain

by

Dara Spatz Friedman

ABSTRACT

Conventional kinesin is an ATP-powered microtubule motor that transports organelles and intermediate filaments. Kinesin is comprised of a motor head, a long stalk, and a cargo-binding tail. Although kinesin can move its cargo processively in vivo, only the minority of kinesin is engaged in this process. Instead, the majority of cellular kinesin is soluble, and the motor activity of this soluble pool may be inhibited until it is recruited to transport cargo. Because it was shown previously that kinesin ATPase activity is low in solution, but elevated when the tail domain is tethered to a bead, inhibition of soluble kinesin may occur through an interaction between the motor and tail domains. To test this idea, I compared a full-length kinesin with a truncated kinesin lacking the tail domain. Both of these kinesins displayed active motility when tethered to a glass surface, which may be due to unfolding and activation the full-length kinesin when surface-bound. However, there were dramatic differences in the motility of full-length and tail-less kinesin in solution. Whereas tail-less molecules bound to and moved along microtubule bundles frequently, full-length kinesin rarely attached to or moved on the microtubules. Thus, the tail may inhibit motor-microtubule binding. Furthermore, the movement of full-length kinesin was slow and discontinuous, in contrast to that of tail-less

kinesin, which was fast and smooth. This suggests that repression by the tail persists even after microtubule attachment, possibly due to inhibition of further microtubule-binding or of ATP hydrolysis. I tested the role of other kinesin domains in tail-mediated motor regulation, and showed that a stalk hinge may be important. I then tested the possibility that the motor domain is regulated also by a tail-independent mechanism, and found that this form of repression may be mediated by a hinge between the motor and stalk. Taken together, I show that kinesin motility is repressed in solution by domains outside of the motor. Furthermore, that tethering the tail to a surface activates kinesin in vitro suggests that cargo-binding may activate kinesin in vivo.

Committee Chair

Advisor

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INTRODUCTION

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Fast and regulated transport of intracellular macromolecules and organelles is vital for many cellular functions. Cells employ molecular motors coupled with a strategically arrayed cytoskeleton to transport some cellular components efficiently. Regulation of a diversity of motor proteins, each with potentially distinct cargo-binding and transport activities, gives the cell control in directing intracellular transport. In particular, the activity of the kinesin motor protein is auto-regulated by the domain that also attaches to cargo, thereby linking its motility with the transport of cargo in vivo.

Places to go, things to do

Moving cellular components from one location to another inside the cell is a requirement for almost every cellular function. Organelles, the subcellular compartments in which most cellular processing occurs, rely on the intracellular transport of membrane components for their biogenesis in newly formed cells, maintenance during interphase, and dynamics in dividing and differentiating cells. In interphase, when protein expression and general cellular housekeeping activity is at its peak, macromolecules move actively between subcellular compartments. During cell division, the movement of chromosomes and other cellular components allows for their segregation into newly forming cells. Thus, the process of intracellular transport enables many of the processes that take place inside the cell.

The cytoplasm is a dense medium in which macromolecules and organelles would move only slowly by diffusion, and then only in an undirected way. This limitation necessitates that the process of intracellular transport be active, not passive; in this regard, molecular motors moving along cytoskeletal tracks play a pivotal role. Although some normally

motor-mediated trafficking can occur in the absence of the cytoskeleton, albeit more slowly (Vallee and Sheetz, 1996), some trafficking events and organelles are almost completely disrupted without an intact cytoskeletal array (for reviews, (see Kelly, 1990, Vallee and Sheetz, 1996). The general mechanism of intracellular transport, in which molecular motors move cargoes along the cytoskeleton, instead of relying on diffusion for movement, is a first step towards efficient movement.

The cytoskeletal array varies among cell types, but functions in each to service the different domains of the cell. In many cell types, microtubules nucleate unidirectionally from the perinuclear region, and emanate outward to the cell periphery (Figure 1). This organization is convenient for allowing transport towards and away from the cell periphery. In neuronal axons, microtubules align unidirectionally from the cell body to the axon buton; in dendrites, the microtubules are of mixed orientation (Sharp et al., 1995). Actin filaments, on the other hand, do not have an organizing center, but exist primarily around the cell cortex and in cell projections (see Titus, 1997)). It is thought that short-range transport occurs along actin filaments, perhaps after delivery via the microtubule network (Kelleher and Titus, 1998). Thus, the cell is well-covered by the cytoskeletal array, giving transport cargoes access, with the help of molecular motors, to the near and far reaches of the cell.

The role of molecular motors in targetting and controlling intracellular transport

It is debatable whether cargoes are indeed targetted to their proper destination, or rather if transport is a stochastic event that achieves targetting only by trapping cargoes once they arrive at the correct

destination. In the latter scenario, motor-driven intracellular transport would serve to accelerate the rate at which cargoes sample the different destinations within the cell and ultimately find the correct one. In the former scenario, which is favored at this time, transport would be directed to cargo-specific destinations; molecular motors, in a number of ways, could help in this regard. First, which cytoskeletal track a motor utilizes can determine the ultimate destination achieved by its cargo, based on actin and microtubule distributions. For example, both kinesin (Marks et al., 1994, Schmitz et al., 1991) and myosin (Fath and Burgess, 1993, Cheney and Mooseker, 1992) localize to trans Golgi network (TGN)-derived vesicles that are secreted by traversing first microtubules to the outskirts of the cell, and then actin to the cell membrane. Second, the polarity of a motor, towards microtubule plus or minus ends, specifies the direction of transport, anterograde or retrograde. In these ways, therefore, molecular motors can begin to direct cellular cargo.

However, the above constraints may not be sufficient to achieve correct placement of cellular cargoes, because cargoes transported toward microtubule plus ends can have different ultimate destinations; an additional degree of targetting may come about through motor-cargo specificity (Coy and Howard, 1994). The enormous diversity of motors and the finding that some motors are membrane-specific make this hypothesis worth considering. Families of kinesins (Hirokawa, 1998), dyneins (Vaisberg et al., 1996, Criswell and Asai, 1998), and myosins (Hammer and Jung, 1996, Mooseker and Cheney, 1995) have been identified, and some members of each family have been localized to different cargoes or parts of the cell. (For summary of localization of kinesins and dyneins, see (Hirokawa, 1998); for myosins, see (Mooseker and Cheney, 1995, Titus,

1997).) Thus, by specifying the cytoskeletal tracks, the direction of movement, and the cargoes moved by each motor, the cell may be able to rely on motors to help direct cargoes to their destinations.

In addition, motor-cargo specificity may allow for more control over the transport of a cargo than just specifying its destination. Another level of control is afforded by the speed of motility; this would influence the time required for certain transport events, particularly for very long-range transport. KIF1A is one of the fastest mammalian kinesins known, moving at ~0.9-1.5 μm/sec (Okada et al., 1995), which makes it an appropriate motor for its axon-traversing cargo, synaptic vesicle precursors. Furthermore, temporal regulation of cargo-association and motor activity, by a developmental or cell cycle clock, or by other stimuli, can dictate when the transport of certain cargoes will occur. For example, dynein is regulated throughout the cell cycle by phosphorylation such that it associates with membranes during interphase and with chromosomes during mitosis (Niclas et al., 1996). Also, the activity of kinesin transport in melanophores is switched on and off by phosphorylation to cause aggregation and dispersion of melanosomes (Reilein et al., 1998). Thus, the cellular transport of distinct cargoes may be controlled by differences in the destinations, speeds, and temporal activities of the motors that move them.

The specification of the motor-cargo interaction

The interactions between motor and cargo necessary to achieve specificity are likely to be defined by the motors themselves, cargo proteins, and free cytoplasmic proteins. These factors may work in concert to dictate specificity in motor-cargo pairing.

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Which domains of the motor protein are required for cargo-binding has been roughly determined. In general, regions outside of the motor's catalytic domain are thought to be responsible for cargo binding, and because these non-motor domains are divergent, they may determine the specificity of this interaction (Coy and Howard, 1994, Mooseker, 1993). The importance of the tail domain and associated subunits in cargo-binding is most understood for conventional kinesins, heterotetramers comprised of two heavy chains (HC) and two light chains (LC) (see Figure 1A, Chapter 1). The heavy chain is divided into four domains: the motor head, neck, stalk, and tail. The motor domain contains the sites for ATP and microtubule binding (Yang et al., 1989). The adjacent neck is divided into two regions: a β-sheet linker involved in motor mechanics and in determining the direction of movement on the microtubule (Case et al., 1997, Endow and Waligora, 1998, Henningsen and Schliwa, 1997) and a coiled-coil whose function is not well understood. Between the neck and stalk domain is a predicted unstructured region that may act as a hinge (hinge 1). The stalk is comprised of two coiled-coil domains separated by a second hinge (hinge 2) that is thought to allow kinesin to fold in half (Hirokawa et al., 1989, Hisanaga et al., 1989, Yang et al., 1989). Finally, the kinesin light chains bind to the carboxyl-terminal end of the second stalk coiled-coil and the tail (Diefenbach et al., 1998, Verhey et al., 1998). Studies of kinesin reveal that the HC C-terminus, and not the motor domain, is involved in the binding of membranes (Skoufias et al., 1994) and intermediate filaments (Liao, 1998).

Both the HC tail and the LCs may specify cargo binding. Even though there is strong homology among the mammalian conventional kinesin HCs thoughout their length, there are some provocative differences among them that may determine membrane specificity. For instance, KIF5A

(neuronal conventional kinesin heavy chain) differs from KIF5B (ubiquitous) in having an additional 69 amino acid domain at the very C-terminal tail. Because this striking difference occurs in the cargo-binding domain, it may play a role in the specification of membrane binding. The significance of this extra domain in neuronal conventional kinesin is explored in Appendix 2 of this thesis.

There are also some less well conserved domains among the different kinesin light chain isoforms that may confer cargo-specificity to the motor proteins with which they are assembled. For instance, the intermediate filament protein vimentin forms a complex with a conventional kinesin heavy and light chain, but the light chain, while reactive with antibodies to a conserved domain, is not reactive with antibodies that recognize at least three known light chain isoforms (Liao, 1998). This result suggests that this motor-cargo complex involves a particular light chain that may specify association. In another example, only one of five light chain isoforms identified in CHO cells localizes to, and is specific for, mitochondria (Khodjakov et al., 1998). Therefore, non-motor domains and subunits of kinesin are responsible for, and may specify, cargo-binding.

Factors besides the motor protein are required for cargo-association in some cases. Cytoplasmic molecules have been implicated in the interaction of dynein, but not kinesin, with its cargo. Dynactin is required for dynein-dependent trafficking, and has been proposed to link dynein to membranes (Burkhardt et al., 1997). Cargo-associated proteins have also been implicated in motor-binding in some cases. Membrane association of myosin V occurs through an interaction with synaptobrevin and/or synaptophysin (Prekeris and Terrian, 1997). Also, rhodopsin has recently been identified as a candidate receptor for dynein (Tai et al., 1999).

Studies of kinesin-membrane binding revealed that integral, but not peripheral, membrane proteins are necessary (Yu et al., 1992). Kinesin-affinity screens identified kinectin as a kinesin-binding integral ER membrane protein (Toyoshima et al., 1992); however, kinectin may not be specific for kinesin binding since anti-kinectin antibodies inhibit both plus- and minus-end directed transport (Kumar et al., 1995). For this reason, the search for a kinesin receptor is ongoing, with research being carried out in many systems in the hope of answering the important question of how kinesin binds to membranes. One such effort to identify a kinesin membrane receptor in rat brain is described in Appendix 2 of this thesis. Thus, even with this last question unanswered, it seems certain that specific motor-cargo associations are accomplished through the contributions of cargo components, cytoplasmic factors, and the extra-motor domains of the motor protein.

Regulation of motor activity

At steady-state, only about a third of kinesin is bound to cargo (Hollenbeck, 1989, Niclas et al., 1994, Verhey et al., 1998); the rest resides free in the cytosol. If this pool of soluble kinesin were enzymatically active, it could theoretically be a sink for unproductive ATP hydrolysis, as well as clog up the cell's microtubules. And yet, kinesin does not co-localize with microtubules in the cell by immunofluorescence; only with overexpression of kinesin is it found localized to the cytoskeleton (Navone et al., 1992, Pfister et al., 1989). Because of this observation, and the deleterious ramifications of unchecked and wasteful energy consumption in the cell, it has been proposed that kinesin that is not associated with cargo is enzymatically silenced in some way.

A compelling analogy can be drawn with conventional myosin II, which functions in filaments in muscle and non-muscle cells. Individual myosin II dimers are comprised of two heavy chains, each with an amino-terminal globular head that contains an actin- and ATP-binding site, and a long coiled-coil rod domain responsible for dimerization of the two heavy chains. Associated with the heavy chains just distal to the heads are two pairs of light chains, one essential and one regulatory. Notably, just as for kinesin, there is believed to be a soluble pool of inactive myosins. It has been proposed that myosins are recruited into filaments from this soluble pool by the activation of individual molecules.

The mechanism for modulating this transition between activation states may rely on a conformational change in myosin induced by the covalent modification of non-motor myosin subunits. Myosin disassembled from filaments with MgATP forms an intramolecular hairpin (Onishi and Wakabayashi, 1982) in which the distal rod comes into contact with the rod adjacent to the motor heads in the region of the light chains (Trybus et al., 1982), as shown by electron microscopy. It is thought that myosins assuming this folded conformation, which act hydrodynamically as compact 10 S molecules, are inactive and unable to form filaments. However, in vitro studies show that phosphorylation of the regulatory light chain (RLC) by the myosin light chain kinase (MLCK) can unfold the myosin into 6 S particles with enhanced motor and assembly activities (Craig et al., 1985, Ikebe and Hartshorne, 1985). In this way, myosin activity may be controlled in the cell by specific activation through non-motor regulatory subunits.

It is tempting to consider a similar mechanism for the regulation of kinesin activity. That is, non-motor elements, brought into contact with

motor domains through a conformational change, may control the motor activities of kinesin in the cell. Furthermore, these conformational changes may be mediated by modification of motor and non-motor domains.

A mechanism of regulation by non-motor domains has been suggested by studies of kinesin conformation. Extended kinesin dimer molecules have been observed by electron microscopy and immunoelectron microscopy: the amino-terminal globular heads are followed by a long (60 nm) stalk domain and a fan-like tail; however, under low (physiologic) salt conditions, a second conformation for kinesin is observed in which the molecule is folded in the stalk, and the head and tail are in contact with each other (Hackney et al., 1992, Hirokawa et al., 1989). In these analyses, kinesin is folded predominantly at the middle of the stalk, though there is another kink near the head as well. In the amino acid sequence, this kink and fold can be identified as disruptions in the coiled-coil domains of the neck and stalk, hinge 1 and hinge 2, respectively. Hydrodynamic studies of kinesin corroborate the existence of a folded and an extended conformation, and demonstrate that the kinesin can shift between them under the control of ionic strength. The folded conformer, which is observed under low ionic strength conditions by EM, is a more quickly sedimenting species (9 S) in velocity gradients than the extended conformer (6 S). As a result, kinesin can form a compact conformer under physiologic conditions in which non-motor domains have the opportunity to regulate the kinesin motor through a physical interaction.

Lending further support to the idea of the auto-regulation of kinesin by its tail, the activity of kinesin in vitro (measured by motor microtubule-stimulated ATPase activity) increases when the tail of the heavy chains and the light chains are removed. Proteolyzed or genetically engineered

kinesin lacking the tail domain has a higher ATPase activity than full-length kinesin (Kuznetsov et al., 1989, Stock et al., 1999), and an endogenous kinesin isolated without light chains has a higher ATPase activity than with light chains (Hackney et al., 1991). While these results convincingly implicated the tail of the heavy chain and the light chain in regulating motor ATPase activity, the importance of these domains in regulating kinesin motility had yet to be addressed before the work of Chapter 1 was completed. In this work, unexpected properties of the regulation of motility by the kinesin tail are elucidated.

Other structural features of kinesins may be needed for the auto-regulation of kinesin activity. First, hinge 2, a break in the coiled-coil region of the stalk, has been proposed to allow the folding required for motor-tail contact (Hirokawa et al., 1989). Second, the neck domain may act as a docking site for the tail (Stock et al., 1999) because it is near the motor and the folded tail and may convey regulatory input from one to the other. Third, the hinge between the neck and stalk is a conspicuous break in what would otherwise be a continuous coiled-coil. There is evidence that this region is involved in motor mechanics (Grummt et al., 1998); and because it may facilitate folding, it may have a role in regulation as well. Such a finding would pioneer an entirely unexplored mechanism of kinesin repression. In conclusion, repression of motor activity would appear to be mediated by potentially many structural components of kinesin; the work described in Chapters 1 and 2 of this thesis elucidates the roles of the neck coiled-coil and hinge domains in motor regulation.

As seen for myosin, covalent modification may play a role in the regulation of the kinesin motor. Both the heavy chains, on serines outside of the motor head, and the light chains, are phosphorylated in vivo

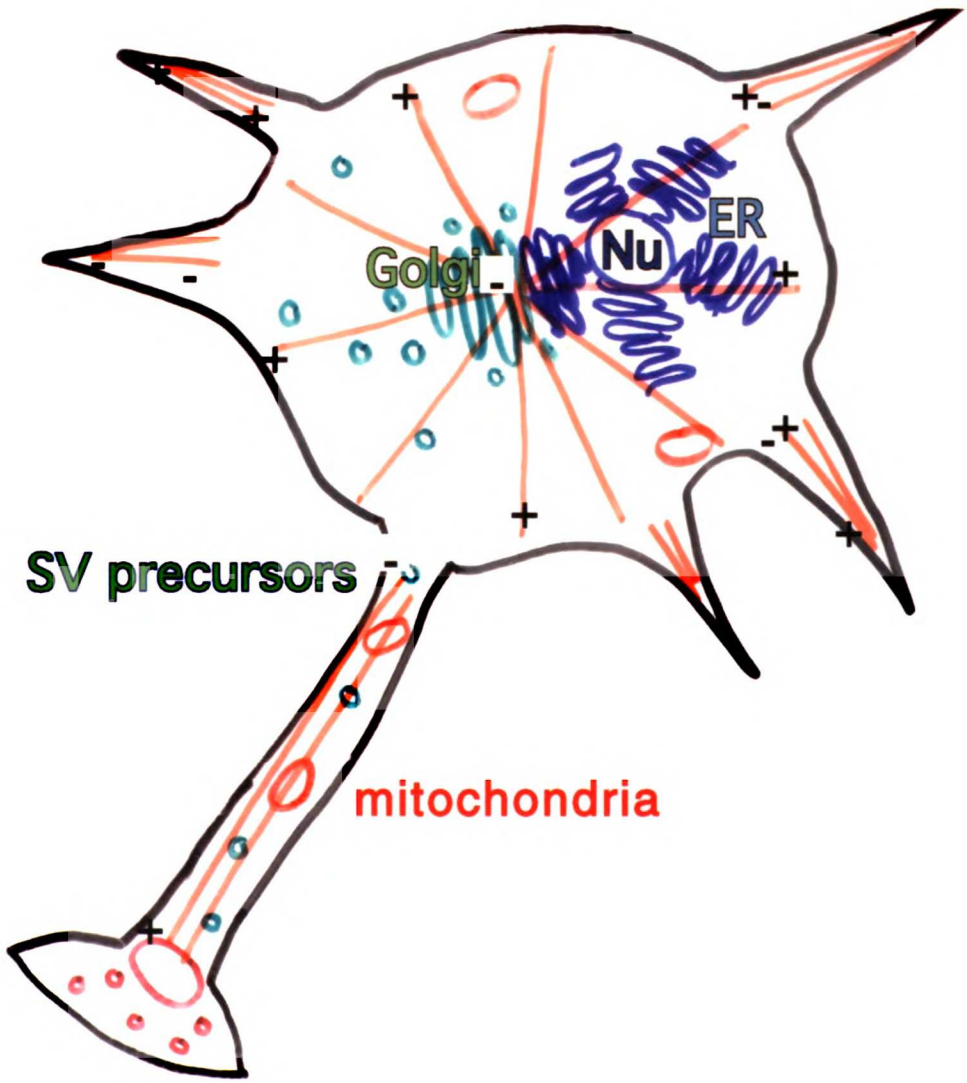
(Hollenbeck, 1993), and their phosphorylation states correlate with motor activity. First, following NGF induction of kinesin-requiring neurite outgrowth in PC12 cells, kinesin phosphorylation increases significantly. Importantly, upon fractionation, mostly phosphorylated, and presumably activated, kinesin associates with membranes (Lee and Hollenbeck, 1995). Second, okadaic acid treatment of T cell extracts causes increased kinesin-mediated microtubule gliding and membrane transport activities in vitro, and there is a corresponding increase in the phosphorylation of kinesin LC (McIlvain et al., 1994). Moreover, a complex of kinesin-associated proteins, including a kinase and phosphatase, has been found to regulate the phosphorylation of KLC in vitro (Lindesmith et al., 1997). Thus, although the data to date is only correlative, modification of non-motor domains and subunits may contribute to the regulation of kinesin activity.

In conclusion, the problem of how to control the activity of soluble kinesin may be managed through an auto-regulatory mechanism in which folding brings the stalk and tail domains into repressive contact with the motor domain. Because the tail domain is also the site of cargo-binding, its role in regulation may provide a direct link between non-cargo bound "off" and cargo-bound "on" states. It is tempting to speculate that cargo-binding disrupts motor repression by displacing the head from the tail and enabling it to interact productively with microtubules and ATP. In support of this hypothesis, binding of kinesin to a glass surface or to beads, which may recapitulate the structural changes experienced by kinesin when it binds to cargo, activates the motor in vitro (Coy et al., 1999, Jiang and Sheetz, 1995, Vale et al., 1985). In this way, then, these cargo-binding and regulatory mechanisms may provide a way for the cell to ensure that only kinesin molecules employed in cargo transport are active.

Figure 1. Highlights of the microtubule array and organelles of the neuron. Microtubules nucleate perinuclearly at the trans Golgi network, and emanate with their plus ends towards the cell periphery. The Golgi and endoplasmic reticulum (ER), as well as other cytoplasmic organelles, rely on microtubules for their maintenance, dispersion, and trafficking. In the axon, microtubules are unidirectional with plus ends at the axon terminus. Anterograde transport of synaptic vesicle (SV) precursors and other organelles rely on microtubules as well.

Figure 1

Highlights of the microtubule array and organelles of the neuron



CHAPTER 1

Single molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain

ABSTRACT

Conventional kinesin transports membranes along microtubules in vivo, but the majority of cellular kinesin is unattached to cargo. The motility of non-cargo bound, soluble kinesin may be repressed by an interaction between the N-terminal and carboxy-terminal cargo-binding tail domains, but neither bead nor microtubule gliding assays have demonstrated such inhibition. Here we have used a single molecule assay that measures the motility of kinesin unattached to a surface. We show that full-length kinesin binds microtubules and moves about ten times less frequently and exhibits discontinuous motion, compared with a truncated kinesin lacking a tail. Mutation of either the stalk hinge or neck coiled-coil activates motility of full-length kinesin, indicating that these regions are important for tail-mediated repression. Our results suggest that the motility of soluble kinesin in the cell is inhibited and that the motor becomes activated by cargo binding.

INTRODUCTION

Conventional kinesin is a motor protein that transports organelles (Hirokawa, 1998, Vale, 1999) and intermediate filaments (Liao, 1998, Prahlad et al., 1998) along microtubules. Native kinesin is composed of two heavy chains (~120 kD) and two light chains (~60-70 kD) (Bloom et al., 1988, Kuznetsov et al., 1988). The heavy chain is divided into four domains: the motor catalytic core, neck, stalk, and tail (Figure 1a). The motor catalytic core contains the sites for ATP and microtubule binding (Yang et al., 1989). The adjacent neck is divided into two regions: a β-sheet linker involved in motor mechanics and in determining the direction of movement on the microtubule (Case et al., 1997, Endow and Waligora, 1998, Henningsen and Schliwa, 1997) and a coiled-coil whose function is not well understood. Between the neck and stalk domain is a predicted unstructured region that may act as a hinge (hinge 1). The stalk is comprised of two coiled-coil domains separated by a second hinge (hinge 2) that is thought to allow kinesin to fold in half (Hirokawa et al., 1989, Hisanaga et al., 1989, Yang et al., 1989). The kinesin light chains bind to the carboxyl-terminal end of the second stalk coiled-coil (Diefenbach et al., 1998, Verhey et al., 1998). Finally, the carboxyl-terminal region of the kinesin heavy chain and the light chain together form a globular tail domain that may bind to cargo (Hirokawa et al., 1989, Skoufias et al., 1994, Yang et al., 1989).

A large proportion of cellular kinesin is not cargo-bound (Hollenbeck, 1989, Niclas et al., 1994, Verhey et al., 1998). The presence of this soluble pool has led to the proposal that non-cargo bound kinesin might be enzymatically inactivated (Hackney et al., 1992). In support of this hypothesis, soluble kinesin is less active in a microtubule-stimulated

ATPase assay than kinesin attached to beads (Jiang and Sheetz, 1995, Moraga and Murphy, 1997). Several results suggest that tail-motor domain interactions may mediate the inhibition of non cargo-bound kinesin ATPase activity (Hackney et al., 1992, Hirokawa et al., 1989). First, electron microscopy reveals that kinesin at physiological ionic strength is folded at hinge 2 such that the head and tail are in close proximity (Hirokawa et al., 1989, Hisanaga et al., 1989). Second, removal of the tail promotes kinesin binding to microtubules (Kuznetsov et al., 1989) and increases microtubule-stimulated ATPase activity (Kuznetsov et al., 1989, Stock et al., 1999). Third, the microtubule binding (Verhey et al., 1998) and ATPase (Hackney et al., 1991) activities of the motor domain have been shown to be repressed further by the light chains bound to the heavy chain tail.

The effect of the tail domain on kinesin motility has been difficult to assess. Native kinesin (Cohn et al., 1989, Hackney et al., 1991, Vale et al., 1985) or bacterially-expressed, tail-less kinesin dimers (Berliner et al., 1995, Yang et al., 1990) bound to glass surfaces or carboxylated beads produce similar motility; however, adsorption of the tail domain onto surfaces may alter kinesin's conformation and de-repress motor activity. To address this problem, we measured processive motility of full-length and truncated kinesin in solution using a single molecule fluorescence assay (Vale et al., 1996).

RESULTS

Expression and activity of kinesin homodimer and heterotetramer

To study the effect of the kinesin tail on motor activity, we prepared three amino-terminally His_6-tagged kinesin constructs: full-length heavy chain with and without kinesin light chains (K963, K963/LC), and a truncated heavy chain that terminates in the hinge 2 region (K560) (Figure 1). We first determined the ATPase activity and motility of the baculovirus-expressed kinesins (Table I). The k_{cat} for microtubule-stimulated ATPase activity of K560 was 21.9 ± 2.1 ATP/sec•head, which is similar to values for a C-terminally histidine-tagged K560 protein expressed in bacteria (Woehlke et al., 1997). The k_{cat} of K963 and K963/LC were 3.4 and 4.9-fold lower than K560, respectively, but similar to those obtained for the native kinesin molecule purified from bovine brain (Hackney et al., 1991, Wagner et al., 1989). These results, as well as others (Kuznetsov et al., 1989), indicate that the tail domain represses the ATPase activity of the motor domain.

In contrast to the ATPase results, however, full-length (K963 and K963/LC) and truncated (K560) kinesins transported microtubules at similar rates in a gliding assay in which the motors were adsorbed onto the surface of a glass coverslip (Table I). Similar numbers of microtubules were moving and the motion was smooth for all three proteins (data not shown). From these results, we conclude that our expressed kinesin constructs have active motor domains and behave similarly to native kinesin (Cohn et al., 1989).

Single molecule analysis of kinesin motility

The finding that full-length kinesin is fully active for motility yet displays a low ATPase rate could result from differences in the conformation of the protein between the two assays. Whereas kinesin in the solution ATPase assay may assume a folded, inhibitory conformation, the adsorption of kinesin onto glass in the gliding assay may abrogate tail-head binding and thereby activate motility. For this reason, we examined kinesin motility in solution using a total internal reflection microscope. In this assay, axonemes are adsorbed onto a slide, and single fluorescently-labeled kinesin molecules from the solution bind to and move along these microtubule substrates.

In the single molecule solution assay, all three kinesin proteins moved, although there were substantial differences in the number of microtubule associations and processive runs observed. The frequencies of single molecule motility events of K963 and K963/LC were 90-99% lower than that observed for K560 (Table II). Once bound to the microtubule, all motors moved but exhibited distinct movement characteristics. Single K560 molecules moved smoothly and continuously (Vale et al., 1996); in contrast, the full-length kinesins displayed discontinuous movement consisting of pauses and bursts of unidirectional motion (Figure 2). Between pauses, the velocities of motion also differed: heterotetrameric and homodimeric kinesin moved 3.3 and 2.3-fold slower than K560 (Table II, Figure 3). To determine if the slower overall velocity of the full-length kinesins was due to discontinuous motion or reflected differences in intrinsic motor activity, we tracked displacement within a motility event. In doing so, we found that full-length kinesin molecules moved at velocities similar to K560 for short time intervals (Figure 2). The wide range of instantaneous velocities suggests that single K963 and K963/LC molecules undergo transitions

between active and less active states, most likely due to reversible inhibition by the tail domain.

Although K963 and K963/LC bound and initiated runs infrequently, once engaged, these full-length kinesin molecules could maintain attachment to and undergo processive movement along the microtubule. The average single molecule run length of K963/LC was the same as K560 (1.03 μm), and the K963 run length was even longer (2.55 μm). As a result of their normal or long run lengths and slow velocities, K963 and K963/LC were bound to the microtubule for ~6- and 3-fold longer time intervals than K560, respectively (Table II). The longer run lengths and association times of K963 compared to K963/LC may be due to a microtubule-binding activity attributed to the naked heavy chain tail (Andrews et al., 1993, Navone et al., 1992), which could maintain kinesin association with the axoneme during times in which the motor domains are detached.

Mutations in the stalk hinge and neck coiled-coil activate motility of full-length kinesin

To understand how the tail represses kinesin motility, we prepared mutations that might interfere with this regulatory mechanism and thereby activate motility of full-length kinesin. To test the idea that tail-head interactions are required for repression (Hackney et al., 1991, Hirokawa et al., 1989), we deleted amino acids 505-610 of the heavy chain to join coil 1 and coil 2 in phase and create a continuous coiled-coil that would eliminate hinge 2 (Figure 1). We tested this mutant as a full-length homodimer (K963 Δ505-610), since the light chains are not required for repression of single molecule motility. The ATPase activity of K963 Δ505-610 was elevated 2.4-fold compared to the wildtype K963 (Table I). In the single molecule

motility assay, K963 Δ505-610 displayed similar frequencies, run lengths, and velocities of movement to the tail-less K560 (Table II). In addition, single K963 Δ505-610 molecules moved smoothly, in contrast to the discontinuous motion of wildtype K963 (Figure 2). These results indicate that deletion of hinge 2 is sufficient to disrupt tail-mediated repression of kinesin motor activity.

Hackney and co-workers recently suggested that the tail may inhibit motor activity by binding to the neck coiled-coil (Stock et al., 1999). To test this idea, we replaced the entire sequence of the native neck coiled-coil (residues 337-370) (Kozielski et al., 1997) with 5 copies of a heptad repeat (AEIEALK) that forms a highly stable coiled-coil (Tripet et al., 1997) (K963 (neck mut)). A K560 protein that had four of the five heptads of the native neck coiled-coil (a.a. 343-370) replaced with this same stable heptad repeat showed nearly wildtype run lengths and velocities in the single molecule assay (Romberg et al., 1998). We found that the properties of K963 (neck mut) (ATPase activity, single molecule velocities, run lengths, association times, and smoothness of motion) were all more akin to the tail-less K560 than to the parent K963 protein (Table II). However, K963 (neck mut) did not appear to be as fully de-repressed as the hingeless K963 Δ505-610, since the frequency of movement of K963 (neck mut) was somewhat lower than K963 Δ505-610. A subset of K963 (neck mut) molecules also appeared to be transiently repressed, as indicated by their occasional pausing (Figure 2) and the bimodal distribution of single molecule velocities (major and minor peaks centered around 22 and 10 μm/min respectively; Figure 3). Thus, tail repression of movement was greatly reduced but not completely eliminated by the neck coiled-coil mutation.

DISCUSSION

In this study, we show that the full-length kinesins are repressed for motility and that the light chains contribute to, but are not essential for this regulation. To explain the fewer processive runs observed for full-length versus tail-truncated motor proteins, we propose that the kinesin tail can inhibit or mask the catalytic core's microtubule binding site. The tail may also inhibit the catalytic mechanism as well, since full-length kinesin molecules display discontinuous motion once they attach to the microtubule. Such tail inhibition may be transitory, since full-length kinesins exhibit short episodes of motion that are of comparable speed to tail-less kinesin. Based on these and other enzymatic results (Kuznetsov et al., 1989, Stock et al., 1999), we conclude that the heavy chain tail represses both the ATPase and motile activities of kinesin.

The analysis of mutant kinesins has allowed us to identify regions that are important for motor regulation by the tail domain. Hinge 2 has been proposed to enable head-tail contact by providing a flexible joint that allows folding. Consistent with this idea, deletion of hinge 2 produces a motor with nearly identical motility properties to a truncated molecule lacking the tail domain. Given this behavior, we anticipated that K963 Δ505-610 would display an extended conformation at low ionic strength, in contrast to a folded conformation for the wildtype heavy chain (Hackney et al., 1992). However, velocity sedimentation analysis showed that K963 and K963 Δ505-610 migrated at similar S values under various salt conditions (see Materials and Methods). This result suggests that K963 Δ505-610 can assume a compact conformation at low ionic strength, as has been seen for a similar hinge 2 deletion in *Syncephalastrum racemosum* kinesin (Grummt

et al., 1998). Folding at hinge 1 may contribute to this compact conformation (Grummt et al., 1998). Although determining the precise conformation of K963 Δ505-610 will require additional studies, our results imply that the head and tail in K963 Δ505-610 are unable to contact one another in the same manner as in wildtype K963. Thus, hinge 2 appears to play an important role in the regulation of motility by the tail.

We have also tested a proposal that tail-mediated repression occurs via an interaction between the tail and the neck coiled-coil (Stock et al., 1999). Consistent with this idea, substituting the native neck coiled-coil with an artificial coiled-coil sequence caused the full-length molecule to behave more like a tail-less molecule and exhibit smooth and continuous motion. Interestingly, the neck coiled-coil mutation can activate some motile properties of a tail-less kinesin molecule as well. The artificial neck coiled-coil in K560 fused to GFP displayed a 2.5- and 1.7-fold activation of ATPase activity and frequency of motility, respectively (data not shown), which agrees with results of a similar construct studied by Romberg et al. (Romberg et al., 1998) Although the exact mechanism of activation is not clear, these results indicate that the native neck coiled-coil can repress motility by tail-independent as well as tail-dependent means.

The sequence of the neck coiled-coil is highly conserved among conventional kinesins (Vale and Fletterick, 1997). However, we find that substitution with an artificial coiled-coil with radically different sequence and stability properties (Tripet et al., 1997) does not alter maximal motility velocity, processivity, or force generation (K. Thorn and R. Vale, unpublished results). Therefore, the neck coiled-coil sequence does not appear to be essential for motor performance, although the coiled-coil structure is important for obtaining maximal velocity and processivity

(Grummt et al., 1998, Romberg et al., 1998). Instead, our results argue that the neck coiled-coil sequence has been selected for and conserved for purposes of motor regulation. The neck coiled-coil contains conserved charged residues asymmetrically positioned on its surface (Kozielski et al., 1997), which may create a docking site for the tail. The neck also contains conserved residues that destabilize the coiled-coil (Tripet et al., 1997), suggesting that partial melting of this structure may be required for tail-mediated repression of motor activity.

Based on our single molecule observations, we would expect that non-cargo bound kinesin in vivo is inhibited from traveling on microtubules and consuming ATP non-productively. The notion that kinesin-microtubule interactions are repressed in the cell is supported by immunofluorescence studies showing that cellular kinesin is not bound to any significant extent to the microtubule cytoskeleton (Navone et al., 1992, Pfister et al., 1989). How, then, is kinesin activated in the cell? Membrane binding occurs through the tail domain of kinesin (Bi et al., 1997, Skoufias et al., 1994), which may prevent the tail from interacting with the motor. Our work further suggests that the catalytic core, neck coiled-coil, stalk hinge, and tail are all potential sites for modifying kinesin activity. Kinesin phosphorylation (Lee and Hollenbeck, 1995, Matthies et al., 1993) or binding of kinesin-associated proteins (Lindesmith et al., 1997, McIlvain et al., 1994) at these sites may activate motility either by changing motor conformation or by facilitating membrane association (Hollenbeck, 1993, Lee and Hollenbeck, 1995).

MATERIALS AND METHODS

Expression constructs

pFastBacHTb (GibcoBRL, Inc.) was used as a vector for baculovirus expression. This vector adds a linker (MSYY), a His_6-tag, a second linker (DYDIPTT), and a tobacco etch virus protease cleavage site (ENLYPQGAMGS) prior to the first codon of the kinesin gene. The histidine tag was chosen, in part, since this positively charged tag is unlikely to bind to the basic C-terminal tail domain. Human ubiquitous kinesin heavy chain (K963) (Navone et al., 1992) was subcloned using a PCR-introduced 5' BamHI site and an endogenous 3' XbaI site. For deleting hinge 2, we first identified amino acids 505-605 of the heavy chain as likely to disrupt the stalk coiled-coil by using the coiled-coil prediction program Paircoil (http://nightingale.lcs.mit.edu). In order to join coil 1 and coil 2 of the stalk and maintain the heptad phase, amino acids 505-610 were deleted by restriction digest using endogenous (nt 1820) and Quik-Change (Stratagene, Inc.) introduced (nt 2138) BclI sites. The neck coil mutation was first made in a bacterial K560-GFP construct in which heptad repeats 1-5 of the neck coiled-coil (a.a. 337-370 in human kinesin) (Kozielski et al., 1997) were replaced with a more stable coiled-coil sequence containing five repeats of the sequence AIEALKA (Tripet et al., 1997). This mutation was subcloned into wildtype K963 using NsiI sites at nt 455 and 2978. A truncated kinesin heavy chain construct (K560) was made by introducing a stop codon after nt1994 (a.a. 560) and 5' BamHI and 3' KpnI restriction sites by PCR. A ubiquitously expressed human kinesin light chain gene (LC) (Cabeza-Arvelaiz et al., 1993), starting at codon 4, was subcloned into

pFastBac using 5' BamHI and 3' SalI restriction sites engineered by PCR. The coding region of all expression constructs was sequenced.

Protein expression and purification

Proteins were expressed by recombining the above baculovirus expression constructs individually with baculovirus DNA and infecting Sf9 cells using the Bac-to-Bac expression system (GibcoBRL Inc.). One liter of Sf9 cells was grown in SFM-900 media to a density of ~2 x 10^6 cells/ml and inoculated with virus, to a multiplicity of infection of 0.5, containing one of the heavy chain gene constructs and, in the case of co-expression, the virus containing the light chain gene. After 72 hr, cells were collected at 450xg for 10 min, resuspended in 1.5-fold volume of lysis buffer (50 mM Tris pH 8, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 20 mM imidazole), frozen in liquid nitrogen and stored at -80° C. To prepare protein, the extract was thawed rapidly at 37° C to lyse the cells, fresh PMSF (1 mM) was added, and the lysate was centrifuged at 44,000xg for 40 min. The soluble fraction was bound in batch to 2 ml Ni-NTA agarose (Qiagen Inc.) for 60-90 min, and the resin was loaded into a column and then washed with 60 ml of 20 mM Tris, 0.5 M NaCl, 20 mM imidazole, pH 8 followed by 20 ml of 20 mM Tris, 1 M NaCl, pH 8. His_6-tagged protein was eluted with 20 mM Tris, 0.1 M NaCl, 250 mM imidazole, pH 8. The eluate was diluted with Mono Q buffer (25 mM Hepes pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) until the conductivity was less than that of Mono Q buffer with 100 mM NaCl, and the protein was then applied to a 1 ml Mono Q column (Pharmacia Inc.). Using a 0-1 M NaCl gradient, kinesin heavy chain, with or without light chain, eluted at ~0.37 M NaCl. For heavy and light chain co-expression, the

heavy and light chain heterotetramer was separated away from contaminating heavy chain homodimers on a 5-20% sucrose gradient centrifuged at 68,000xg for 18 hr. All protein preparations were stored in sucrose in liquid nitrogen. Protein concentration was determined as described previously (Woehlke et al., 1997).

Bacterial expression and purification of K560-GFP and K560 (neck mut)-GFP was carried out as described previously (Romberg et al., 1998), using a microfluidizer to lyse the cells. To select for active protein, kinesin was microtubule bound and released as described below.

ATPase and microtubule gliding assays

ATPase activity was measured using a Malachite Green assay(Kodama et al., 1986). Reactions contained 20-100 nM unlabeled kinesin, varying concentrations of microtubules ranging from 0-30 μM, 2 μM taxol, and 0.5 mM ATP in BRB12 (12 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA). Phosphate release was measured 0, 5, and 10 min after the addition of ATP. k_{cat} values were determined using a hyperbolic curve fit of a plot of microtubule concentration versus ATPase rate. Rhodamine-labeled microtubules were used as a substrate for motility by cover glass-adsorbed kinesin, as previously described (Woehlke et al., 1997). The mean motility velocity was determined from measurements of 20 or more gliding microtubules.

Hydrodynamic analysis

S values were determined in different salt conditions (0, 0.15 M, 0.5, 1 M NaCl) by velocity sedimentation on a continuous sucrose gradient, as described previously (Hackney et al., 1992). ~850 ng kinesin was mixed with

standard calibration proteins (BSA, 4.3 S; aldolase, 7.4 S; and catalase, 11.3 S) and loaded onto 7-20% sucrose gradients (Hackney et al., 1992). After centrifugation at 135,000 x g for 14 hr, and fractions were analyzed by SDS-PAGE followed by transfer to nitrocellulose. The calibration proteins were localized by Ponceau S staining and kinesin was detected by immunoblotting using an affinity-purified polyclonal antibody raised against a peptide from the motor domain (a.a. 154-173). K963 and K953 Δ505-610 migrated together under various salt conditions (7.1 S, 0 M NaCl; 6.7 S, 0.15 M NaCl; 5.5 S, 0.5 M NaCl; 4.4 S, 1 M NaCl).

Solution-based motility assays

The motility of single Cy3-labeled kinesin molecules was observed along Cy5-labeled sea urchin axonemes using a total internal reflection microscope. Kinesin was labeled by reacting 75 pmol of kinesin with 2.5 nmol Cy3, a monofunctional NHS-ester that labels free amino groups, for 10 min on ice, and then quenching the reaction with 50 mM glycine pH 7. Cy3-kinesin was removed from free dye by binding the motor to microtubules with 1 mM AMP-PNP and then centrifuging the motor-microtubule complex through a cushion of 40% sucrose in BRB12 at 230,000xg for 5 min. Cy3-kinesin was then released by resuspending the microtubules in 50 µl Mono Q buffer containing 0.2 M KCl and 1 mM ATP for 15 min at r.t.; microtubules were removed by centrifugation as described above. Stoichiometry of labeling was determined by measuring the protein concentration, as described above, and by measuring Cy3 concentration on a fluorimeter using Cy3 standards. Molar stoichiometries ranged from 0.1 to 1.1 Cy3:protein. This labelling procedure did not appear to affect motor activity, since K560 prepared in this way had single molecule velocities and

run lengths that were similar to those of reactive cysteine-Cy3-labeled (Vale et al., 1996) and GFP-tagged (Pierce and Vale, 1998) K560. Preparation(Gibbons and Frank, 1979) and labeling (Pierce and Vale, 1998) of axonemes has been described. Single molecule motility assays were performed as previously described (Pierce and Vale, 1998, Vale et al., 1996) on a custom-built total internal reflection microscope (Pierce and Vale, 1998, Vale et al., 1996). The intensity of the 514 nm argon laser light prior to entering the prism was 5 mW. At least 1 field of 5 or more axonemes were recorded for 4 min for each assay. Since motility was rare for K963 and K963/LC, data were collected for up to 25 min in order to observe more motility events.

Motility was analyzed using an NIH-IMAGE based measuring program developed by Jim Hartman. Segments of videotape were digitally captured at 10 frames/sec, and the run lengths and velocities were determined by marking the binding and dissociation events of a single Cy3-kinesin. Run lengths as short as 0.1 μm could be detected. The mean velocities were determined from at least 14 runs that were greater than 0.5 μm. Runs with pauses (no obvious motion) were measured as only one motility event, but pause times were not included in measurements of velocity. To track single molecule motility, movement of a single fluorescent spot was tracked at 0.5 to 2 sec intervals using a program developed by Kurt Thorn. The run length was determined by non-linear least-squares fitting of the cumulative probability distribution to $1-\exp^{(-x/t)}$, where the cumulative probability distribution at value x is defined as the fraction of runs with lengths shorter than x (fitting program developed by Kurt Thorn) (K560, n=300; K963, n=69; K963/LC, n=20; K963 Δ505-610, n=146; K963 (neck mut), n=145). Association times represent the time interval

between the appearance and disappearance of a moving single fluorescent spot on the microtubule; data points were calculated as run length/velocity for each motility event greater than 0.5 μm. Frequency of movement was determined by counting the number of motility events on an axoneme and normalizing to axoneme length, amount of Cy3-kinesin, and the time of observation.

Accession Numbers: human ubiquitous kinesin heavy chain (U06698) and light chain (L04733).

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Table I. ATPase turnover and microtubule gliding velocities of kinesin proteins

Kinesin Construct	**MT Gliding speed μm/min**	**ATPase turnover (k_{cat}) ATP/s•head**
K560	39.8 ± 3.1	21.9 ± 2.1
K963	46.6 ± 3.3	6.5 ± 0.6
K963/LC	36.9 ± 10.5	4.5 ± 2.4
K963 Δ505-610	42.1 ± 5.3	15.7 ± 4.6
K963 (neck mut)	46.0 ± 2.9	16.4 ± 4.8

Microtubule (MT) gliding assays and MT-stimulated ATPase assays were carried out as described in Materials and Methods. For microtubule gliding, mean velocity ± S.D. are shown for >20 microtubule measurements. The ATPase k_{cat} was derived from a hyperbolic curve fit of ATPase rates at varying microtubule concentration. The mean and standard deviations are shown for 2 protein preparations.

Table II. Single Molecule Motility Measurements

Kinesin Construct	Velocity	Run Length	Association Time	Frequency Prep 1	Frequency Prep 2
	μm/min	μm	min	% K560	
K560	18.3 ± 4.4	1.03 ± 0.01	0.058 ± 0.001	100	100
K963	8.1 ± 3.9	2.55 ± 0.03	0.347 ± 0.006	10.1	2.0
K963/LC	5.5 ± 2.4	1.03 ± 0.04	0.210 ± 0.007	6.9	1.3
K963 Δ505-610	20.8 ± 4.5	1.39 ± 0.02	0.067 ± 0.001	49.0	114.6
K963 (neck mut)	20.4 ± 6.6	1.23 ± 0.01	0.056 ± 0.001	19.7	37.7

Single molecule motility of Cy3-kinesin on sea urchin axonemes in a total internal reflection microscope. For velocity, mean ± S.D. were derived from the data shown in Figure 3. Run lengths and association times (combined data of two independent protein preparations) were determined as described in Materials and Methods. The error of the curve fits are shown. Frequency of binding/movement are shown for two independently prepared protein preparations and are expressed as the percentage of K560, which was analyzed in parallel with the mutants. The absolute K560 frequency values for trial 1 and 2 were 0.53 and 0.15 movements/μm axoneme•nM Cy3-kinesin•min, respectively.

Figure 1. **(a)** Domain organization of wildtype and mutant kinesin constructs. Kinesin heavy chains are solid, and the light chains are grey. All chains have an amino-terminal His_6-tag. (1) K560, homodimer of two kinesin heavy chains truncated at hinge 2. (2) K963, homodimer of two full-length kinesin heavy chains. (3) K963/LC, heterotetramer of two full-length kinesin heavy chains and two light chains. (4) K963 Δ505-610, homodimer of two full-length kinesin heavy chains with hinge 2 deleted. (5) K963 (neck mut), homodimer of two full-length kinesin heavy chains with amino acids 337-370 replaced with artificial highly stable coiled-coil (indicated by an unshaded box). The functions of the heavy chain catalytic core (a.a. 1-322), neck (a.a. 323-371), hinge 1 (a.a. 372-446), stalk 1 (a.a. 447-504), hinge 2 (a.a. 505-605), stalk 2 (a.a. 606-803), and tail (a.a. 804-963) domains are described in the text and elsewhere (Vale and Fletterick, 1997). Ovals represent globular domains, and rectangles indicate predicted α-helical coiled-coils. **(b)** Purified baculovirus-expressed kinesin proteins analyzed by SDS-PAGE. Lanes 1-5 correspond to the numbering of the constructs above. Proteins were prepared as described in Materials and Methods. Velocity sedimentation analysis confirmed that K560 and K963 are homodimers and that K963/LC is a heterotetramer (Hackney et al., 1992) (data not shown). A 1 to 0.93 stoichiometric ratio of the heavy and light chains was determined by Coomassie staining intensity of the two bands on polyacrylamide gels. K963 (neck mut) has the same predicted molecular weight as wildtype K963, but it migrates faster.

Figure 1

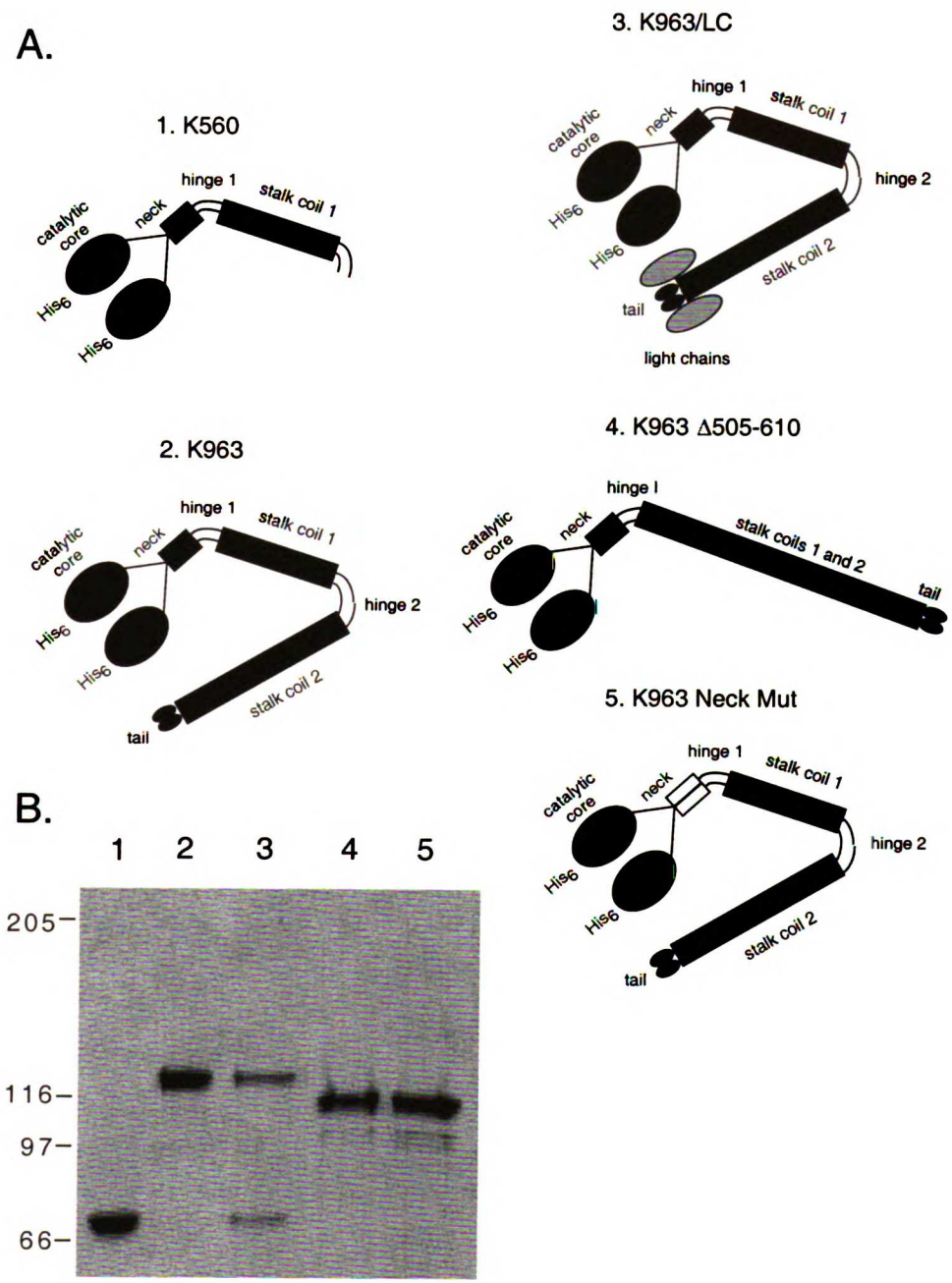

A.
1. K560
catalytic core
neck
hinge 1
stalk coil 1
His6
His6
2. K963
hinge 2
stalk coil 2
tail
3. K963/LC
light chains
4. K963 Δ505-610
hinge I
stalk coils 1 and 2
5. K963 Neck Mut
B.
1 2 3 4 5
205
116
97
66

Figure 2. Tracking movement of single kinesin molecules. Representative runs are shown for each construct. Distance traveled was measured every 0.5-2 sec until the molecule dissociated from the axoneme or photobleached. K560 and K963 Δ505-610 exhibited smooth motion, whereas K963 and K963/LC displayed frequent pauses. K963 (neck mut) runs illustrate both smooth and discontinous movement, although the majority of runs were smooth. Episodes of rapid motion for K963 and K963/LC are highlighted with lines. The highlighted episodes for K963 averaged 14.7 μm/min and ranged from 7.2 to 20.7 μm/min; and for K963/LC, the average 17.1 μm/min and ranged from 11.0 to 27.9 μm/min. Episodic velocities were determined with linear regression to 3 or more consecutive points.

Figure 2

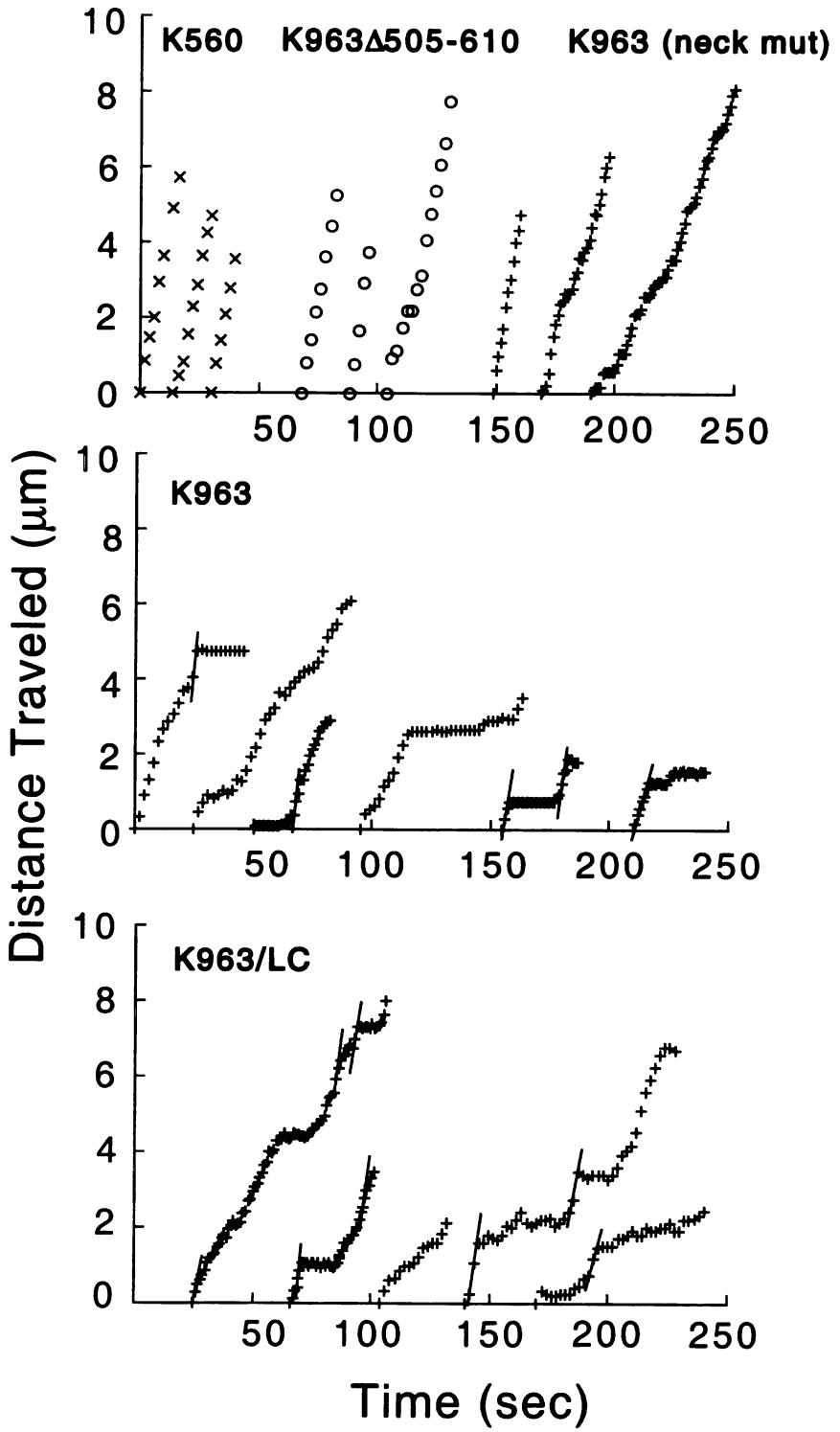
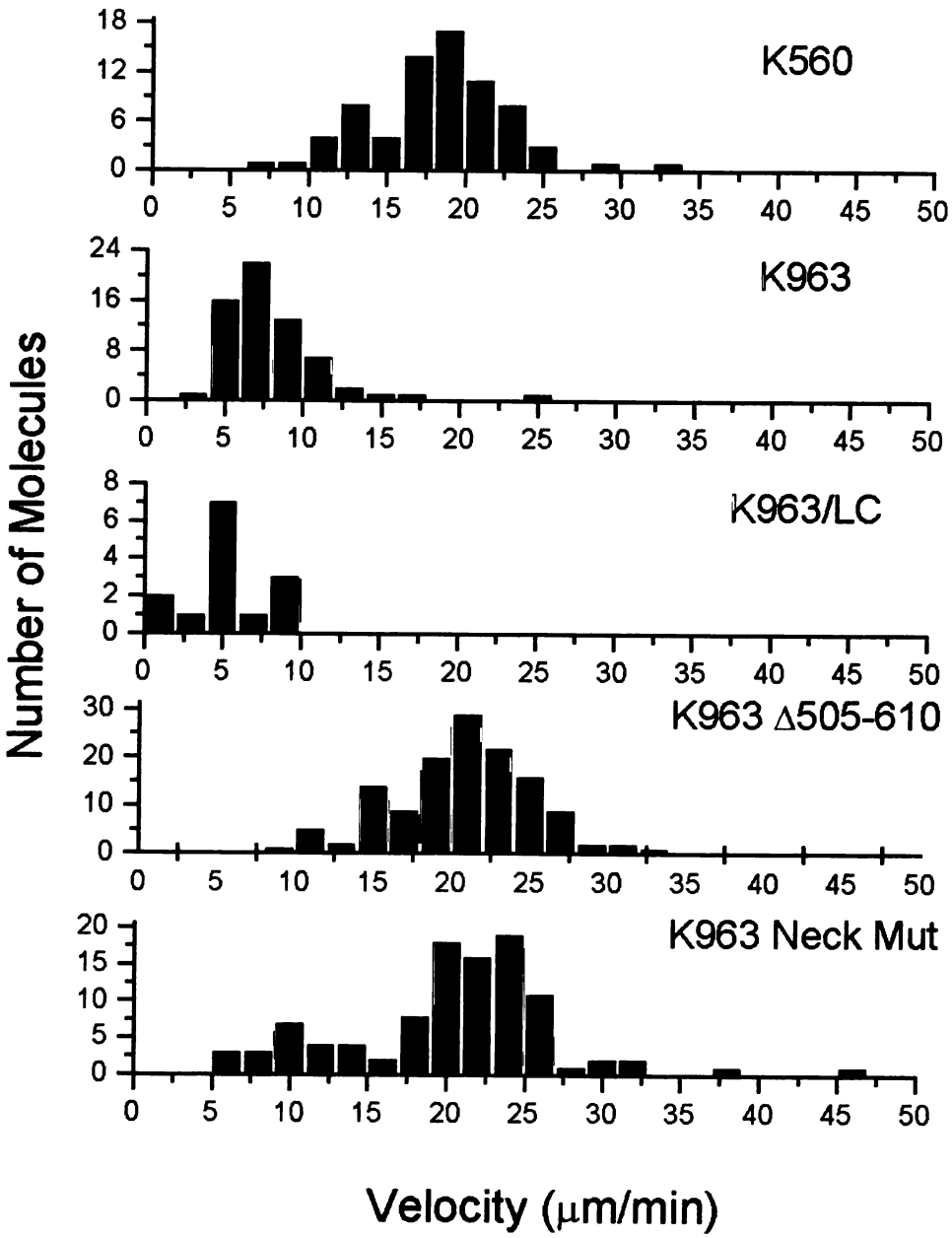

K560
K963Δ505-610
K963 (neck mut)
K963
K963/LC
Distance Traveled (μm)
Time (sec)
0
2
4
6
8
10
50
100
150
200
250

Figure 3. Histograms of velocities for single fluorescent kinesin molecules. Velocities of individual Cy3-labeled kinesin molecules moving on axonemes were measured as described in Materials and Methods. Episodes in which the motor was paused (stationary) on the axonemes were not included in the analysis.

Figure 3


K560
K963
K963/LC
K963 Δ505-610
K963 Neck Mut
Number of Molecules
Velocity (μm/min)

CHAPTER 2

A novel regulatory mechanism of kinesin motor activity

ABSTRACT

Conventional kinesin is a processive microtubule motor protein that powers the transport of intracellular cargoes. Each of the two identical heavy chains has an amino-terminal head domain followed by neck, hinge, stalk and tail domains. Although the neck domain has been proposed to couple the motor heads, previous experiments have shown that the neck coiled-coil is not essential for processivity. Because the neck coiled-coil sequence is highly conserved among conventional kinesins, it likely plays an important role in kinesin motility. The recent finding that the neck coiled-coil sequence is important for tail-mediated motor repression suggests that it may convey regulatory information from non-motor domains of kinesin to the motor head. To explore the interaction of the neck coiled-coil with the stalk domain, we examined kinesin folding in a tail-less construct. We find that kinesin without a tail domain can undergo salt-dependent folding as detected by hydrodynamic analysis. This may occur by bending at the hinge that separates the neck and stalk domains, possibly bringing the motor head and stalk coiled-coil into contact. To test the role of the neck coiled-coil sequence in folding and in motor activity, we analyzed a tail-less kinesin mutant with an altered neck sequence. We find that folding is disrupted in this molecule and that some of its motor properties are enhanced, suggesting that folding, mediated by the endogenous neck sequence, may have an important motor regulatory function.

INTRODUCTION

Conventional kinesin is a heterotetrameric motor protein, comprised of two heavy and two light chains, that can move on microtubules for long distances during a single encounter. This processivity may be the result of chemomechanical and physical coupling between the two amino-terminal heavy chain catalytic cores (amino acids 1-322) and the domains that follow. Carboxy-terminal to the motor catalytic core are the neck, hinge, stalk and tail domains. The neck is subdivided into an amino-terminal β sheet motif (amino acids 323-337) that may specify motor directionality (Case et al., 1997, Endow and Waligora, 1998, Henningsen and Schliwa, 1997) and a coiled-coil subdomain (amino acids 338-371). The hinge, called hinge 1, comprises ~75 amino acids with a high content of glycines and prolines which are predicted to form a flexible region. This domain separates the neck from the stalk domain. The stalk is divided into two coiled-coil regions (amino acids 447-504 and amino acids 606-803) separated by another flexible region, hinge 2. The tail is a globular, light chain-associated region that binds cargo. Our understanding of the role of the non-catalytic core domains in processivity is limited.

The high degree of sequence conservation (~65%) in the neck coiled-coil among the conventional kinesins suggests it has an important role in kinesin motility. It has been postulated that the coiled-coil acts as a zipper, opening and closing to faciliate the continual movement of the heads relative to each other during motility. In fact, deletions of this region demonstrate involvement of the neck coiled-coil in motor processivity (Romberg et al., 1998) and in basic motor function (Grummt et al., 1998). However, a mutant "stable coil" kinesin (replacement of the carboxy-

terminal four heptad repeats of the neck coiled-coil (amino acids 342-370) with a more stable heterologous coiled-coil of equal length) has a run length similar to wildtype (Romberg et al., 1998). Thus, this neck domain appears to be involved in, but not completely required for, processivity, suggesting that there may be other functions of this region.

The neck coiled-coil also has been proposed to have a role in tail-mediated motor repression. The tail will bind in trans to a kinesin head fragment containing the head and neck, but not to a fragment lacking the neck coiled-coil (Stock et al., 1999), and a variant of the "stable coil" mutant (in a full-length kinesin construct) reverses inhibitory effects of the tail (Chapter 1). These results suggest that the neck coiled-coil may provide a docking site for the inhibitory heavy chain tail.

A third function of the neck coiled-coil may be understood by considering the phenotype of the "stable coil" mutant in a kinesin lacking the tail domain (K560). Not only does K560-stable coil move processively, but its frequency of motility and rate of microtubule-stimulated ATP hydrolysis are both elevated relative to wildtype tail-less kinesin. These differences point to a potential role for the coiled-coil in motor regulation that is unrelated to the one described for the tail. To explore this possibility, it is essential to understand the structure of the kinesin molecule around the neck coiled-coil. The motor and neck structures have been studied extensively, however, the structure of domains immediately downstream of the neck coiled-coil have not received as much attention.

In order to gain insight into the significance of the neck coiled-coil and its structural environment, we examined the conformation and motility of a neck coiled-coil mutant, "K560 (neck mut)," in which the heterologous stable coiled-coil is extended to include the first heptad. This

extension is based on the dimer crystal structure (Kozielski et al., 1997), which was not available when the "stable coil" mutant was constructed. The findings from these studies suggest a mechanism of motor regulation by the neck mediated by folding at hinge 1.

RESULTS

Another structural element besides hinge 2 may produce a salt-dependent conformational change

Velocity gradients have been used by David Hackney (Hackney et al., 1992, Stock et al., 1999) to corroborate with EM (Hirokawa et al., 1989, Hisanaga et al., 1989) and gel filtration (Hackney et al., 1992) data that kinesin forms a compact conformation under low ionic strength and an extended conformation at high ionic strength. Specifically, Hackney and co-workers determined that kinesin migrates further in velocity gradients under low ionic strength conditions, in which it is expected to be compact (9 S for the heterotetramer, HC_2LC_2; 6 S for the homodimer, HC_2), than in high ionic strength, when it is expected to be extended (6.7 S for HC_2LC_2; 5.1 S for HC_2). By EM analysis, this folding is most obvious in the middle of the kinesin molecule that corresponds to the predicted position of hinge 2 (Hirokawa et al., 1989).

However, kinesin may fold independently of hinge 2. Schliwa and co-workers have found that a hinge 1-deleted fungal kinesin has different hydrodynamic properties from wildtype kinesin (Grummt et al., 1998). To confirm and extend these findings, we tested the ability of human kinesin molecules lacking hinge 2 and the tail to fold. "K560" terminates at hinge 2 after the first stalk coiled-coil, "K560-GFP" is the same construct fused to the green fluorescent protein, and "K963 Δ505-610" is a full-length protein with the second hinge region deleted. We find that the mobility of all of these proteins is retarded similarly in high ionic strength buffer relative to low ionic strength (Table I, Figure 1). These results imply that these molecules fold in a hinge 2-independent way, and this is responsible for the salt-

dependent hydrodynamic changes observed. One possibility for the shift in velocity sedimentation is that the two motor heads splay apart under high salt (Stone et al., 1999). Another possibility is that a flexible region besides hinge 2 allows a folding event in the wildtype full-length and truncated kinesin molecules. A likely candidate is hinge 1.

Mutations in the neck coiled-coil increase motor activity

Romberg et al (1998) replaced the last four heptads of the neck coiled-coil with a heterologous and much more stable coiled-coil, and found that this K560-stable coil has normal processivity, but an increased ATPase rate and frequency of motility relative to wildtype K560. To confirm these earlier findings, we analyzed a mutant in which the heterologous stable coiled-coil was extended to include the first heptad, "K560 (neck mut)." This constitutes a complete replacement of the neck coiled-coil seen in the crystal structure (Kozielski et al., 1997). Similarly to K560-stable coil, the ATPase activity of K560 (neck mut)-GFP was increased compared with wildtype K560-GFP (Figure 2, Table II). In single molecule motility assays, K560 (neck mut)-GFP also displayed similar velocities to wildtype K560 (Table II). This finding is surprising given the tight coupling between ATP hydrolysis and movement expected for kinesin (Coy et al., 1999, Schnitzer and Block, 1997), which would predict faster velocities of motility for motors with higher rates of ATP hydrolysis. This result suggests that the ATPase and motility cycles of this mutant are partially uncoupled. Furthermore, run lengths for K560 (neck mut) and wildtype K560 were similar to each other (data not shown). Finally, the frequency of motility is elevated in the (neck mut) protein (Table II). Overall, K560 (neck mut) behaved similarly to K560-stable coil. However, in the analysis of K560-stable coil and wildtype K560

(Romberg et al., 1998), the difference in frequencies was more dramatic than seen for K560 (neck mut). This discrepancy may be due to differences in the method used to measure frequency. Thus, relative to wildtype K560, some motor activities of K560 (neck mut) are increased.

Kinesin folding is disrupted in the (neck mut) mutation

Because folding has been shown to be a critical parameter in motor regulation (Chapter 1, Stock et al., 1999), we wondered if proper folding was disrupted in K560 (neck mut). To pursue this possibility, we performed hydrodynamic analysis to determine whether the molecule folds under low ionic strength conditions. As shown in Table I, we find that for K560 (neck mut)-GFP, the S value does not shift with salt. In fact, in both high and low salt conditions, the molecule migrates slowly by velocity sedimentation, as if it were an extended molecule under both low and high salt conditions. Wildtype K560-GFP also does not show a shift under some conditions, but it always migrates faster than K560 (neck mut)-GFP, suggesting that it is more compact. These results suggest that K560 (neck mut)-GFP may not be folding correctly at hinge 1.

DISCUSSION

The (neck mut) gain-of-function phenotype is noteworthy, because it arises from alterations in a highly conserved motor domain believed to be involved in motor mechanics. That ATPase activity and frequency of motility in the mutant are increased over wildtype tail-less kinesin brings into question the normal activation state of wildtype kinesin. Specfically, these findings point to the possibility that even the wildtype tail-less kinesin molecule is repressed in some way. A long-standing observation that lends support to this hypothesis is that kinesin-powered microtubule gliding velocities typically exceed what would be expected from its rate of ATP hydrolysis. This disparity is most conspicuous for full-length kinesin, but is also apparent for the tail-less kinesin. This difference may reflect distinct kinesin conformations between the two assays. In the gliding assay, kinesin may unfold when it attaches to the glass slide, thus freeing the head from tail binding and enabling it to interact freely with microtubules (Figure 3). In the solution-based ATPase assays, however, proper protein conformation is preserved, and tail-mediated repression is in effect. By analogy, it is possible that an inhibitory structure exists in tail-less kinesin that is disrupted upon binding to glass, or once a kinesin successfully initiates motility. If so, the (neck mut) mutant may owe its gain-of-function phenotype to its ability to alter an inhibitory interaction that exists within the tail-less molecule.

These results bring attention to a hinge region that resides between the neck and stalk domains of kinesin. A folding at hinge 1 may mediate a salt-dependent conformational change which could have important ramifications for motor activity. Specifically, this hinge may allow for an

inhibitory interaction between the neck coiled-coil domain and the first coiled-coil of the stalk. This interaction may be inhibitory either because it interrupts the neck from performing its catalytic role, or because it brings the stalk domain in close contact with the catalytic core. It is tempting to speculate, therefore, that the (neck mut) phenotype is due to a disruption of a neck-stalk interaction that depends both on hinge 1 and the neck coiled-coil amino acid sequence.

Further experiments on kinesin mutants could test whether the neck and stalk were involved in such a regulatory scheme together. One important construct to test this idea is a stalk coil 1 deletion. Such a construct has been made in a fungal kinesin, and it produces an active motor, as determined by microtubule gliding analysis (Grummt et al., 1998). However, it should be determined whether deletion of the stalk coil 1 alters salt-dependent conformational change or affects motor performance in solution-based assays.

In conclusion, a potential model for repression of a tail-less K560 molecule involves an inhibitory interaction between the stalk domain and the motor head. The tail-dependent (Chapter 1) and independent mechanisms of repression may be linked, since both mechanisms involve the conserved neck coiled-coil domain.

MATERIALS AND METHODS

Protein expression constructs and purification

The construct used to express wildtype K560-GFP has been described (Woehlke, 1997). To make the "neck mut" mutation, PCR and QuikChange mutagenesis (Stratagene) were used to replace heptad repeats 1-5 of the neck coiled-coil (a.a. 337-370 in human kinesin) (Kozielski et al., 1997) with a more stable coiled-coil sequence containing five repeats of the sequence AIEALKA(Tripet et al., 1997). This new sequence was then subcloned into the wildtype K560-GFP expression construct.

Bacterial expression and purification of K560-GFP and K560 (neck mut)-GFP was carried out as described previously (Romberg et al., 1998), using a Microfluidizer (Microfluidics International Corporation) to lyse the cells. To select for active protein, kinesin was microtubule bound and released. Motor was bound to microtubules with 1 mM AMP-PNP and then the motor-microtubule complex was centrifuged through a cushion of 40% sucrose in BRB12 at 230,000 x g for 5 min. Kinesin was then released by resuspending the microtubules in 50 µl buffer containing 12 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 M KCl, 1 mM ATP, and 20% sucrose for 15 min at r.t.; microtubules were removed by centrifugation as described above.

ATPase assays

Microtubule-stimulated ATPase rates were measured in a specrophotometric coupled enzyme assay (Catterall and Pederson, 1971). For Trial 1, k_{cat} was estimated as the mean turnover rate of two or more points at microtubule concentrations between 10 and 15 µM, which is near kinesin

saturation. For Trial 2, k_{cat} was determined by a hyperbolic curve fit of ATPase rates at varying microtubule concentrations.

Single molecule motility analysis

Single molecule motility assays were performed as previously described (Pierce et al., 1997, Vale et al., 1996) on a custom-built total internal reflection microscope (Pierce et al., 1997, Vale et al., 1996). The intensity of the 488 nm argon laser light prior to entering the prism was 5 mW. At least 1 field of 5 or more axonemes were recorded for 4 min for each assay. Motility was analyzed using an NIH-IMAGE based measuring program developed by Jim Hartman. Segments of videotape were digitally captured at 10 frames/sec, and the run lengths and velocities were determined by marking the binding and dissociation events of a single K560-GFP. The mean velocities were determined from at least 50 runs that were greater than 0.5 μm. Frequency of movement was determined by counting the number of motility events on an axoneme and normalizing to axoneme length, amount of K560-GFP, and the time of observation.

Hydrodynamic analysis

S values were determined in different salt conditions (0, 0.15 M, 0.5, 1 M NaCl) by velocity sedimentation on a continuous sucrose gradient, as described previously (Hackney et al., 1992). ~850 ng kinesin was mixed with standard calibration proteins (BSA, 4.3 S; aldolase, 7.4 S; and catalase, 11.3 S) and loaded onto 7-20% sucrose gradients in 40 mM Tris, 15 mM HCl, 2 mM $MgCl_2$, 1 mM K-EGTA, 0.1 mM K-EDTA, pH 8.3, containing the above salt. After centrifugation at 135,000 x g for 14 hr in a TLS-55 rotor, fractions were analyzed by SDS-PAGE and transferred to nitrocellulose. The calibration

proteins were localized by Ponceau S staining and kinesin was detected by immunoblotting using an affinity-purified polyclonal antibody raised against a peptide from the motor domain (a.a. 154-173).

Table I. S values for kinesin heavy chain constructs under different salt conditions.

Construct	S Value			
	0 M NaCl	**0.15 M NaCl**	**0.5 M NaCl**	**1 M NaCl**
K963 Wildtype	7.1 (*n*=3)	6.7 (*n*= 2)	5.5 (*n*= 2)	4.4 (*n*=3)
K963 Δ505–610	7.1 (*n*= 1)	6.7 (*n*=2)	5.5 (*n*=2)	4.4 (*n*= 1)
K560	7.4 (*n*= 2)	6.7 (*n*= 2)	5.7 (*n*=2)	5.2 (*n*= 2)
K560 GFP	6.3 (*n*=1)	6.7 (*n*=1)	5.9 (*n*=1)	6.5 (*n*=1)
K560 (neck mut) GFP	5.1 (*n*=1)	N.D.	N.D.	5.5 (*n*=1)

Hydrodynamic analysis of proteins lacking hinge 2 but containing hinge 1. S values were calculated from standard calibration proteins, as described in Materials and Methods.

Table II. Motor properties of wildtype and (neck mut) K560-GFP.

Construct	ATPase turnover (k_{cat})		Velocity	Frequency	
	Trial 1	Trial 2		Trial 1	Trial 2
	(ATP/sec-head)		(μm/min)	(fold wildype K560)	
K560 wildtype	34 ± 6	31 ± 7	22 ± 5	1.0	1.0
K560 (neck mut)	96 ± 20	47 ± 3	18 ± 5	1.5	2.0

Microtubule-stimulated ATPase and single-molecule motility assays were carried out as described in Materials and Methods. For ATPase measurements, Trial 1 and 2 were carried out on two independent protein preparations. ATPase values in Trial 1 are the mean ± S.D. for ATPase rates at 12 μM microtubules, a concentration expected to saturate the kinesin; values in Trial 2 are the hyperbolic curve fit ± error in the fit of ATPase rates at varying microtubule concentrations (see Figure 2). Velocity values are the mean ± S.D. of 50 or more single molecule velocities. The absolute frequency for K560 (neck mut)-GFP for both trials was 3.9 movements/μm axoneme•nM K560-GFP•min.

Figure 1. Sucrose density gradient sedimentation of K963 and K963 Δ505-610. Shown are fractions from a 7-20% sucrose gradient loaded with K963 and K963 Δ505-610 (analyzed by SDS-PAGE followed by immunoblot with an anti-kinesin antibody). Gradients were prepared as described previously (Hackney et al., 1992), containing the indicated amount of salt, and centrifuged for 14 hr. at 135,000 x g. Fraction 1 is the top of the gradient, and the positions of the calibration standards are marked above the lanes. Lanes labeled "WT" and "Δ505-610" were loaded with K963 and K963 Δ505-610, respectively, to show their migration on SDS-PAGE.

Figure 1

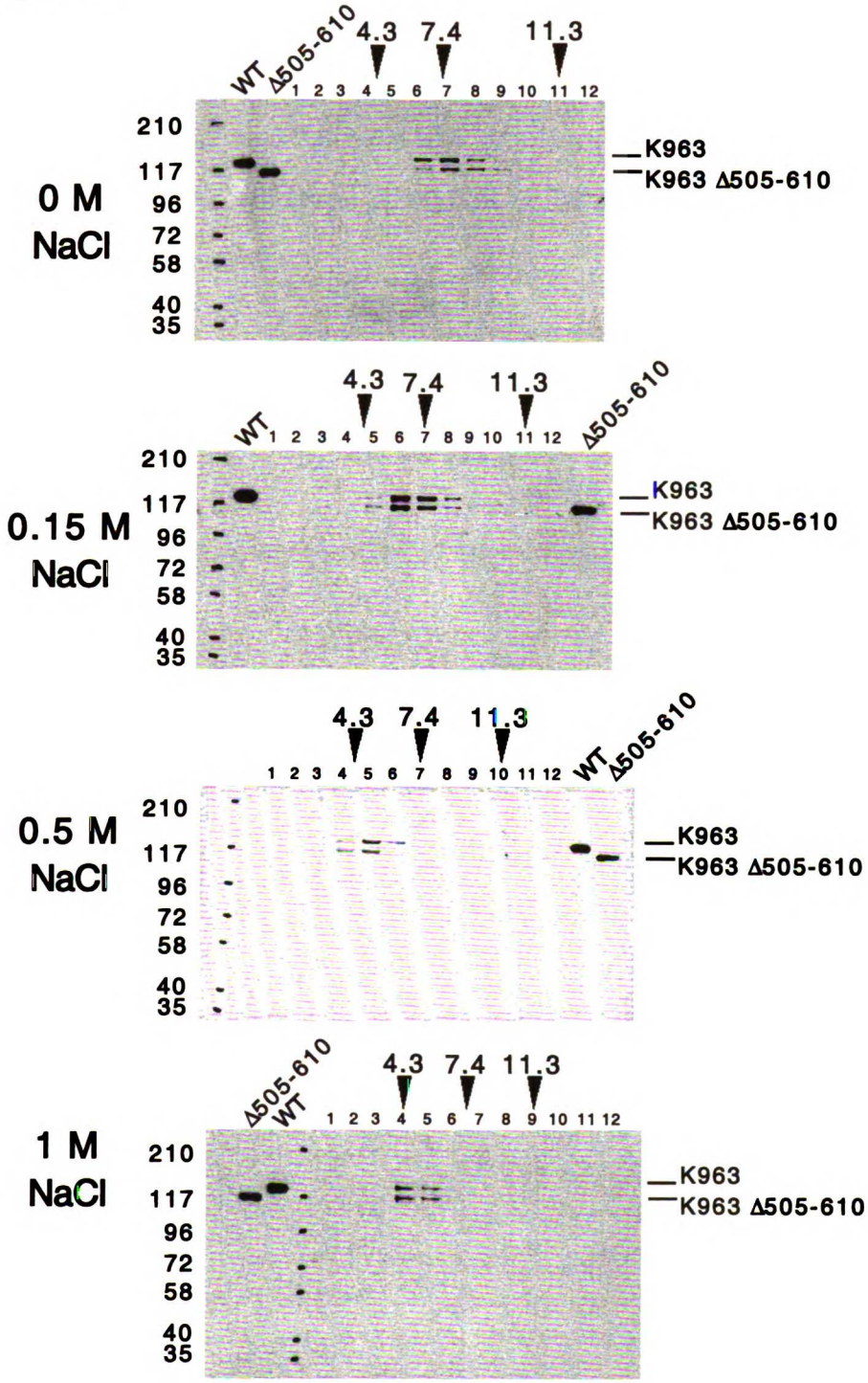

0 M NaCl
WT
Δ505-610
4.3
7.4
11.3
1 2 3 4 5 6 7 8 9 10 11 12
210
117
96
72
58
40
35
K963
K963 Δ505-610
0.15 M NaCl
0.5 M NaCl
1 M NaCl

Figure 2. Microtubule-stimulated ATPase activity of wildtype and (neck mut) K560-GFP. ATPase rates were determined as described in Materials and Methods. The k_{cat} for K560 (neck mut) (47 ± 3 ATP/sec-head kinesin) is elevated relative to wildtype K560 (31 ± 7 ATP/sec-head). The Km for microtubules is 9.8 ± 5.7 and 2.0 ± 0.6 μM tubulin, for wildtype and (neck mut), respectively. The wildtype K560 Km value is higher than usual in this particular experiment.

Figure 2

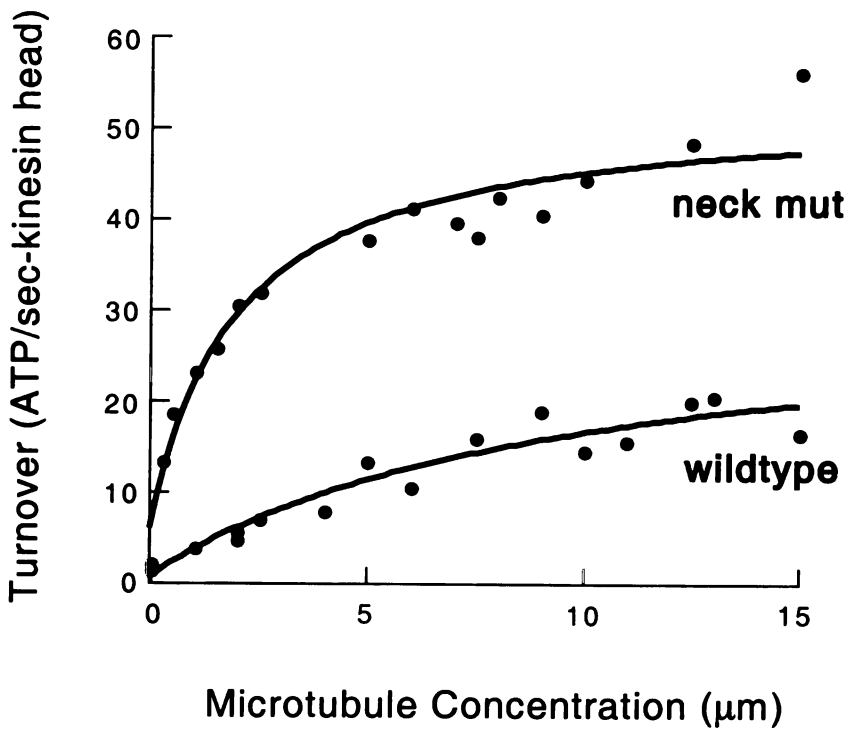
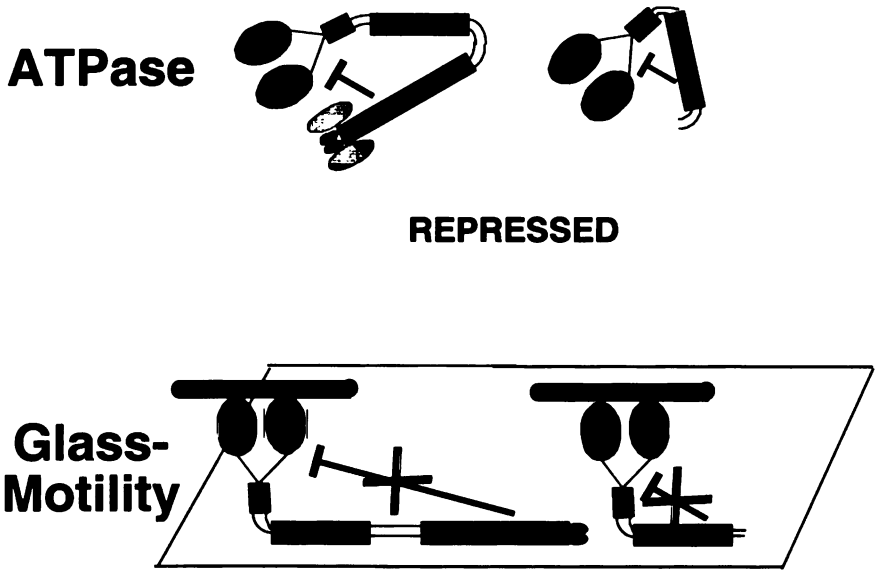

Turnover (ATP/sec-kinesin head)
60
50
40
30
20
10
0
0
5
10
15
neck mut
wildtype
Microtubule Concentration (μm)

Figure 3. Model for the conformational change-induced activation of full-length and tail-less kinesins. In a solution based ATPase assay, the tail of full-length kinesin and the first coiled-coil of the stalk is able to make inhibitory contact with the motor. Binding to glass in a microtubule gliding assay unfolds the molecule and prevents motor inhibition.

Figure 3



APPENDIX 1

Assaying the conformation of wildtype and mutant K963

The finding described in Chapter 1 that deletion of the stalk hinge of kinesin heavy chain is sufficient to de-repress the full-length motor protein suggests a structural requirement for folding in motor inhibition. The region deleted in that study includes a predicted disruption of the stalk coiled-coil; therefore, one would expect the resulting deletion, K963 Δ505-610, to be unable to fold at this hinge into a conformation that allows tail-head interactions.

While proper folding at hinge 2 may be inhibited, conformations besides an extended molecule are not ruled out. As described in Chapter 2, a hinge 2-deleted molecule may still be able to bring the head and tail in close proximity, by bending at hinge 1 and over the length of the stalk domain. Alternatively, if hinge 2 were not completely deleted, the heavy chain could fold, but with an altered line-up between the head and tail domains, such that the tail came into contact with the first half of the stalk or completely overshot the stalk and head regions altogether. In any of these scenarios, however, it is unlikely that the head and tail are oriented properly for repression.

In order to determine the conformation of K963 Δ505-610, and in particular whether it folds like wildtype K963 in low salt despite the deletion, I compared its structure to that of wildtype K963 in a number of ways. I tried velocity gradients, FRET, anisotropy, analytical ultracentrifugation, and electron microscopy. The hydrodynamic analyses are described in Chapter 2. Here I will briefly describe my attempts at distinguishing between mutant and wildtype kinesin heavy chains using FRET and anisotropy.

FRET Analysis

One idea for assaying folding was to label the head and tail with a fluorescence energy donor and acceptor, respectively, and measure transfer between the two. To this end, Nora Hom-Booher made bacterial and baculovirus constructs for expressing BFP-K963 (wt or Δhinge 2)-GFP, and I tried expressing them in bacteria and in Sf9 cells. From bacteria, I managed to obtain full-length protein, along with proteolysis products. In particular, since the His tag was on the C-terminus, there was a lot of cleaved GFP-His, and some of this species may have remained even after enrichment for motor-containing species through Mono Q chromatography or microtubule bind and release. In the final preparation, I had a mixture of BFP-K963-GFP-His, K963-GFP-His, which was corroborated by Western blotting using K963 (purified K963 wt or Δhinge 2) as a standard, and GFP-His. I did not see any full-length protein expression in Sf9 cells from either of two independent bacmid preps from one pFAST-Bac construct for either of the two species (wt or Δhinge 2).

The amount of bacterially expressed BFP-K963-GFP and K963-GFP was too little to quantitate, but enough to give fluorescence signal. We hoped to see transfer between BFP (excitation, 380 nm; emission 440 nm) and GFP (excitation, 488 nm; emission, 507 nm), as had been seen in a study of Ca^{2+}-calmodulin binding (Miyawaki et al., 1997). Instead, what I think we saw was direct GFP excitation at 380 nm: the scans of K560-GFP are nearly identical to those of BFP-K963-GFP (Figure 1, see also Figure 2). I tried exciting BFP-K963-GFP at another wavelength, 330 nm, that is also supposed to excite BFP, but then emission at both 440 nm and 507 nm was eliminated. This would suggest that there was not enough BFP for detection, although there should be equal amounts of BFP and GFP in this microtubule bound-and-released sample. Even if the the scans were reading BFP and GFP

emission, the spectra of BFP-K963$_{WT}$-GFP was no different from that of BFP-K963$_{\Delta 505\text{-}610}$-GFP (Figure 3), and no different between high and low salt (Figure 4). It would seem, then, that GFP is not usable in this assay because of its excitability at 380 nm. It is possible, however, that this assay could be applied to the question of kinesin folding if a different acceptor and donor pair were used, such as CFP and YFP.

Anisotropy

Folding of K963 at hinge 2 halves the molecule's length. Because of this large effect on shape, I tried using anisotropy to determine if the shape of K963 WT differed, between low and high salt, and from K963 Δ505-610. I tried both Cy3-labeled purified baculovirus K963 and GFP-labeled protein described above. I found no difference in anisotropy between low and high salt for Cy3 labeled WT and Δ505-610 protein. However, I found that both WT and Δ505-610 GFP-labeled protein had increased anisotropy in high salt (Figure 5). This result, while preliminary, may suggest that anisotropy can be used as an assay for kinesin folding. If so, it would appear that K963 Δ505-610 has the same folding characteristics as K963 WT.

Figure 1. Emission spectra of K560-GFP-His and BFP-K963$_{WT}$-GFP-His (excited at 380 nm). A bacterial prep of K560-GFP was diluted 1:1000, and a BFP-K963-GFP microtubule bind and releasate from a NTA-Ni^{2+} column eluate peak was diluted 1:20, in Mono Q (Hepes) Buffer A (no salt), and scanned on a fluorimeter. We would expect the BFP of BFP-K963$_{WT}$-GFP-His to be excited by 380 nm light, and emit at 440 nm, or at 507 nm if there were transfer between the BFP and GFP. Contrary to what we see, the GFP of K560-GFP-His is not expected to be excited by 380 nm light; and if it were, we would expect it to emit at 507 nm, not at 440 nm. However, because it is excited by 380 nm light and emits at 440 nm, we cannot say whether the emission peak we see for BFP-K963$_{WT}$-GFP-His at 440 nm is due to BFP or GFP fluorescence. Because of this ambiguity, we are unable to measure transfer of BFP fluorescence energy in BFP-K963$_{WT}$-GFP-His.

Figure 1

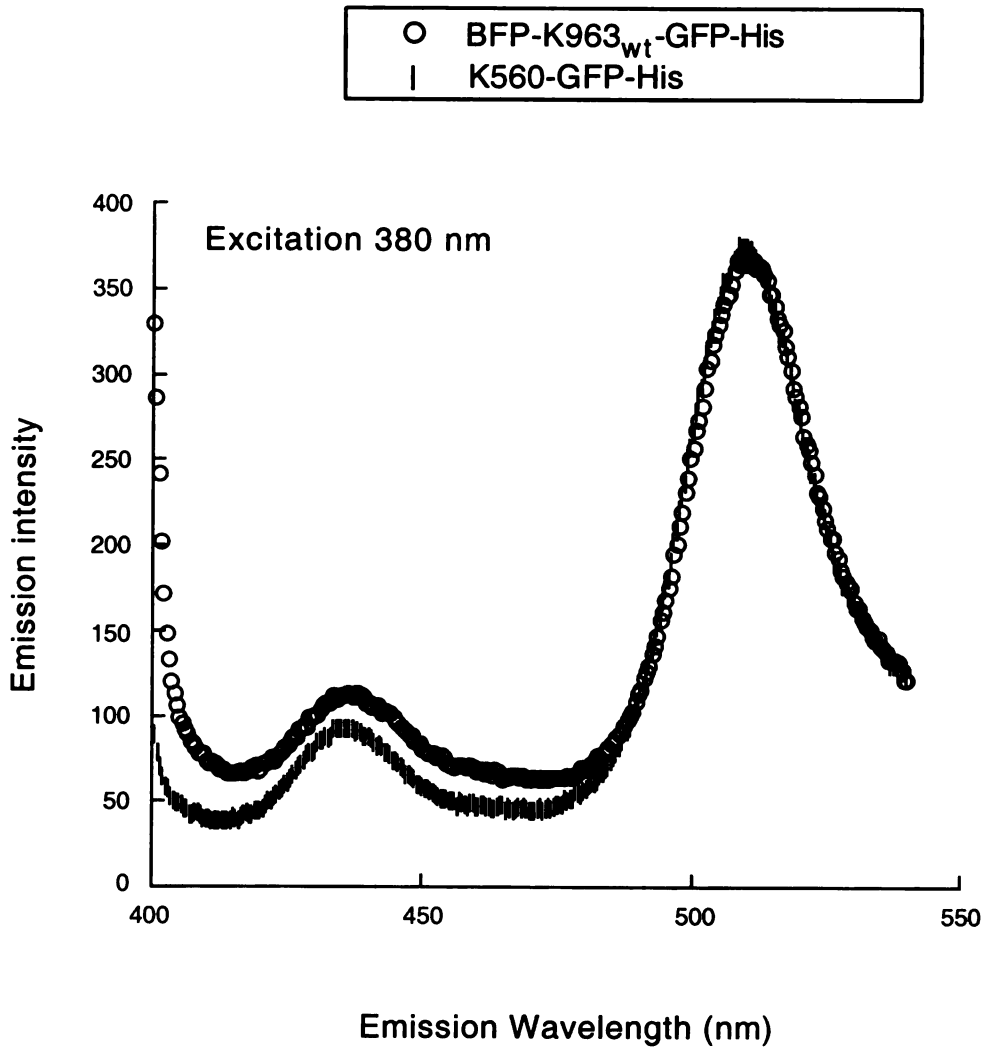

BFP-K963wt-GFP-His
K560-GFP-His
Excitation 380 nm
Emission intensity
400
350
300
250
200
150
100
50
0
400
450
500
550
Emission Wavelength (nm)

Figure 2. Excitation spectrum of K560-GFP (emission at 507 nm), diluted 1:1000. Peak emission is expected at excitation of ~488 nm, which is what is seen here. There is a relatively small amount of excitation at 380 nm, the wavelength used to excite BFP, however this signal is very apparent in scans of K560-GFP and BFP-K963-GFP at 380 nm excitation.

Figure 2

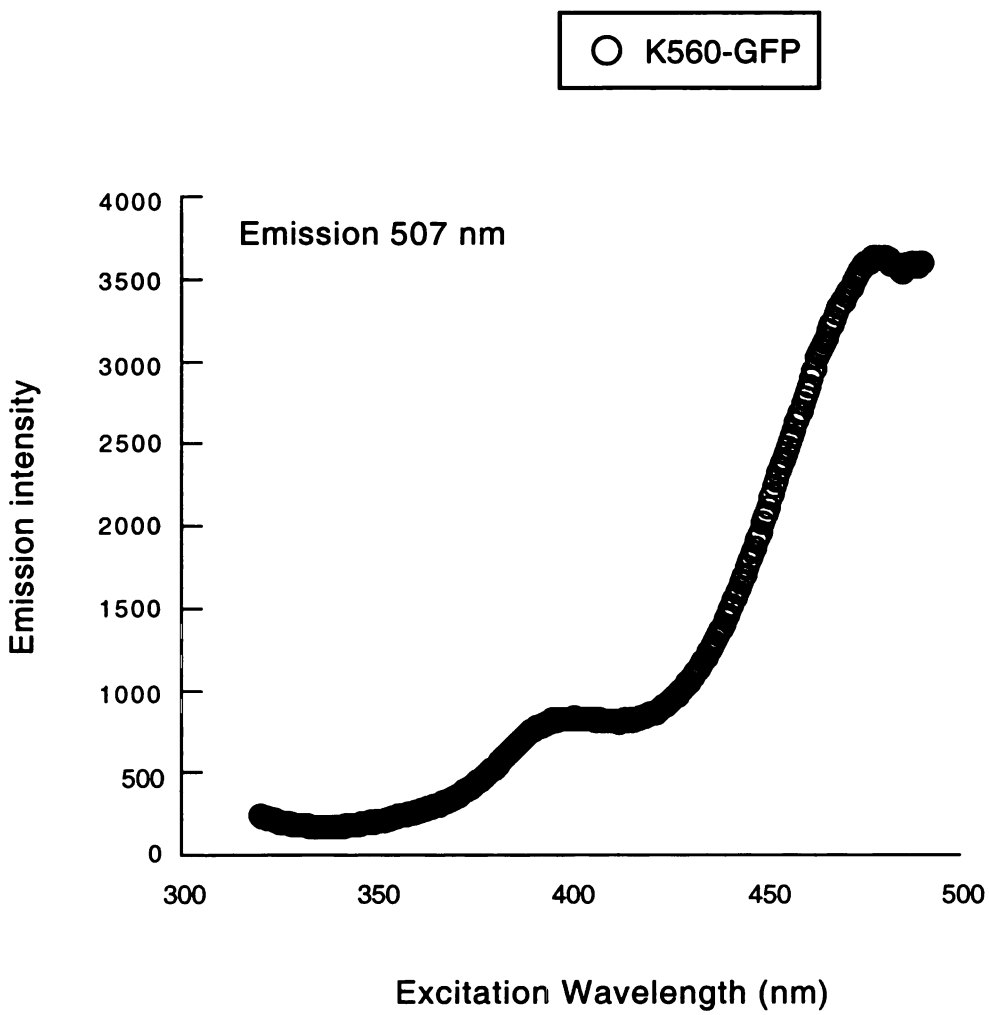

K560-GFP
Emission 507 nm
Emission intensity
4000
3500
3000
2500
2000
1500
1000
500
0
300
350
400
450
500
Excitation Wavelength (nm)

Figure 3. Emission spectra of BFP-K963$_{WT}$-GFP-His and BFP-K963$_{\Delta 505\text{-}610}$-BFP-His (excited at 380 nm) at low and high ionic strength. A green NTA-Ni^{2+} column flow through was run over a Mono Q column. The 350 mM NaCl Mono Q fractions were diluted 1:20 in H_2O (low salt) or in 0.5 M NaCl (high salt). The profiles for WT and Δ505-610 are similar, although WT may have more of what might have been thought to be BFP signal (emission at 440 nm). That there is no inversely proportional difference in GFP fluoresence (emission at 507 nm), it is unlikely to be FRET that is causing the decrease in emission at 440 nm.

Figure 3

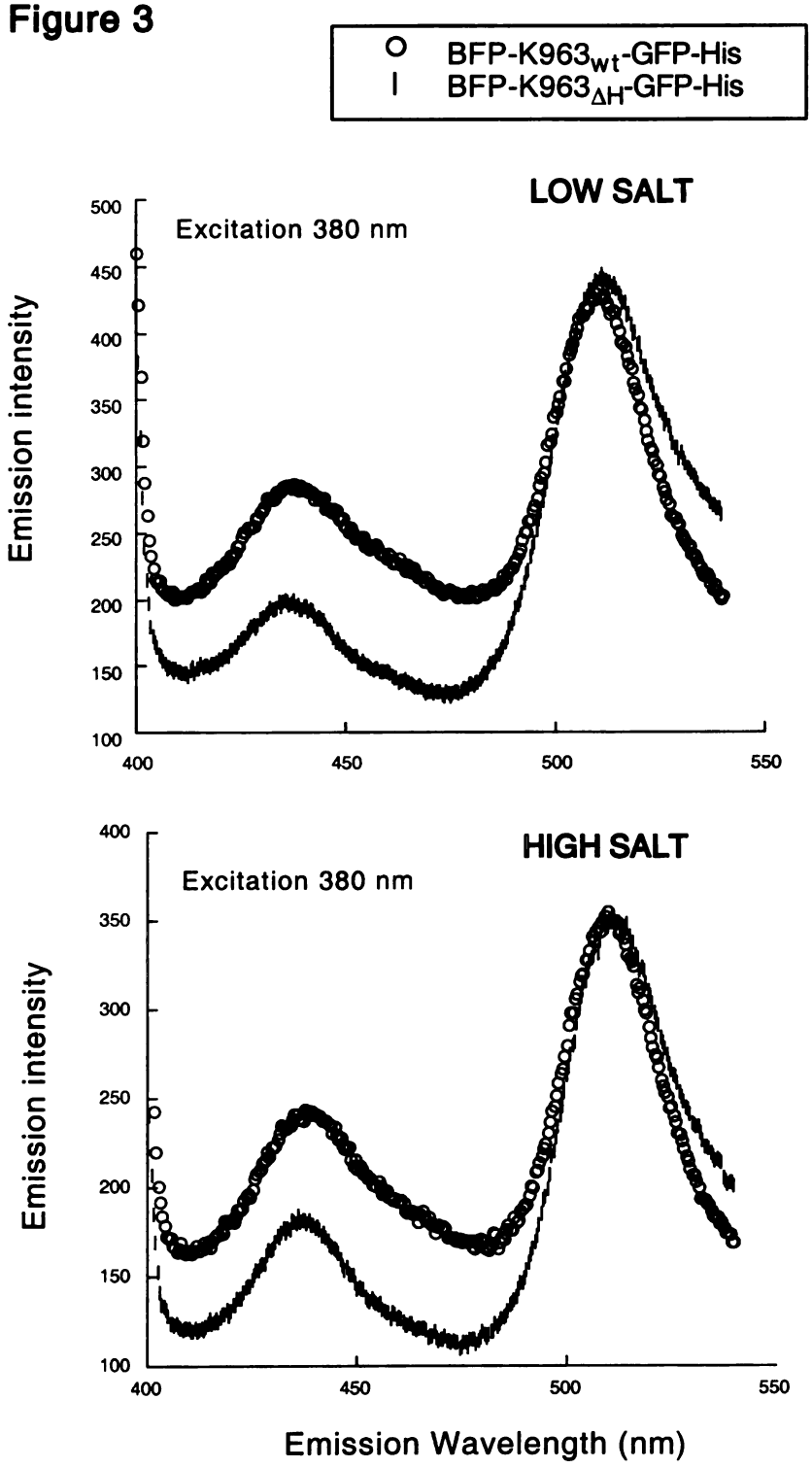

BFP-K963wt-GFP-His
BFP-K963ΔH-GFP-His
LOW SALT
Excitation 380 nm
Emission intensity
500
450
400
350
300
250
200
150
100
400
450
500
550
HIGH SALT
Excitation 380 nm
Emission intensity
400
350
300
250
200
150
100
400
450
500
550
Emission Wavelength (nm)

Figure 4. Emission spectra of BFP-K963$_{WT}$-GFP-His and BFP-K963$_{\Delta 505\text{-}610}$-BFP-His (excited at 380 nm) at low and high ionic strength. This is the same as shown in Figure 3, except with different pairings for comparison. Salt may dampen emission at both 440 nm and 507 nm, but there is no qualitative change in the ratio of acceptor/donor fluorescence with salt.

Figure 4

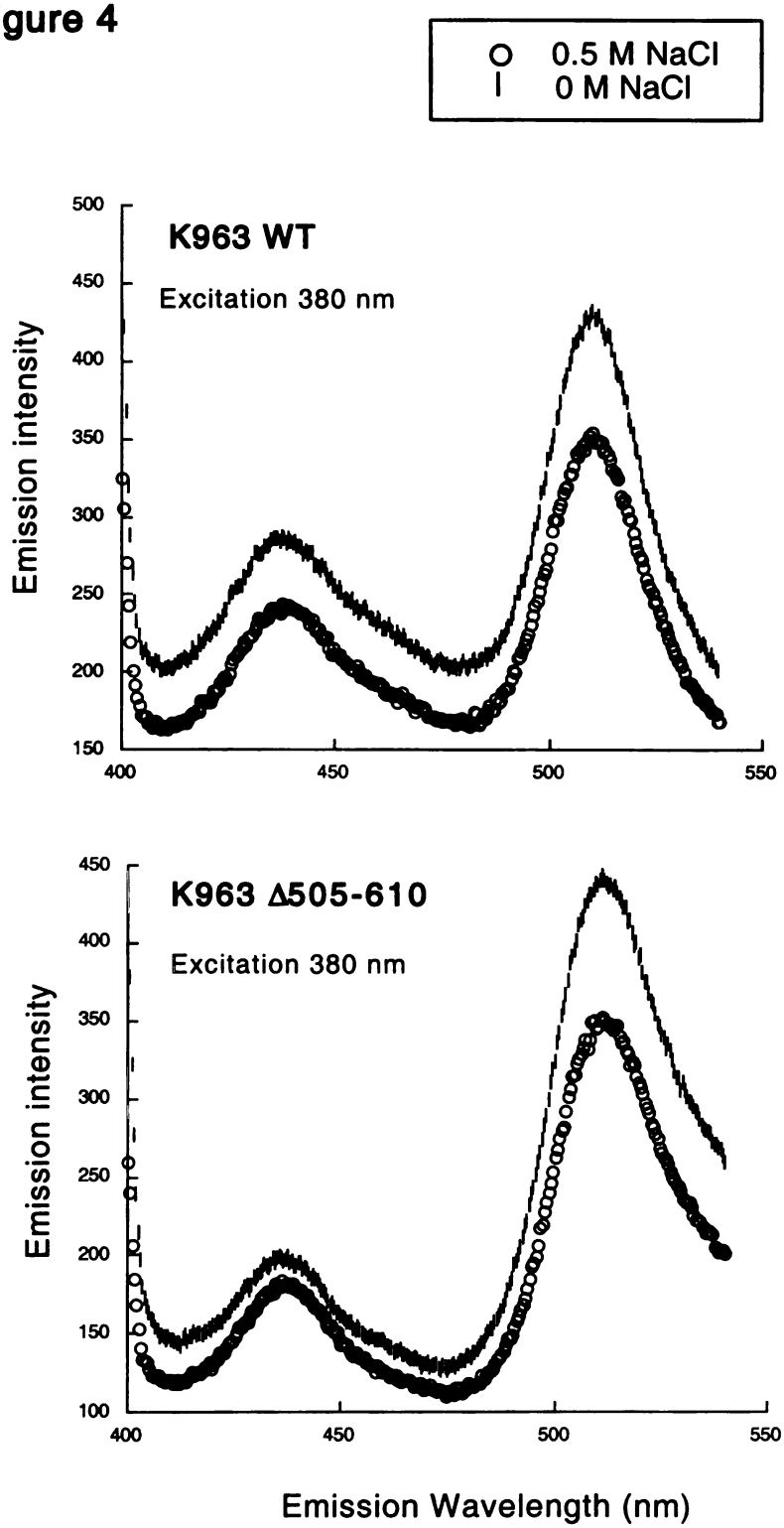
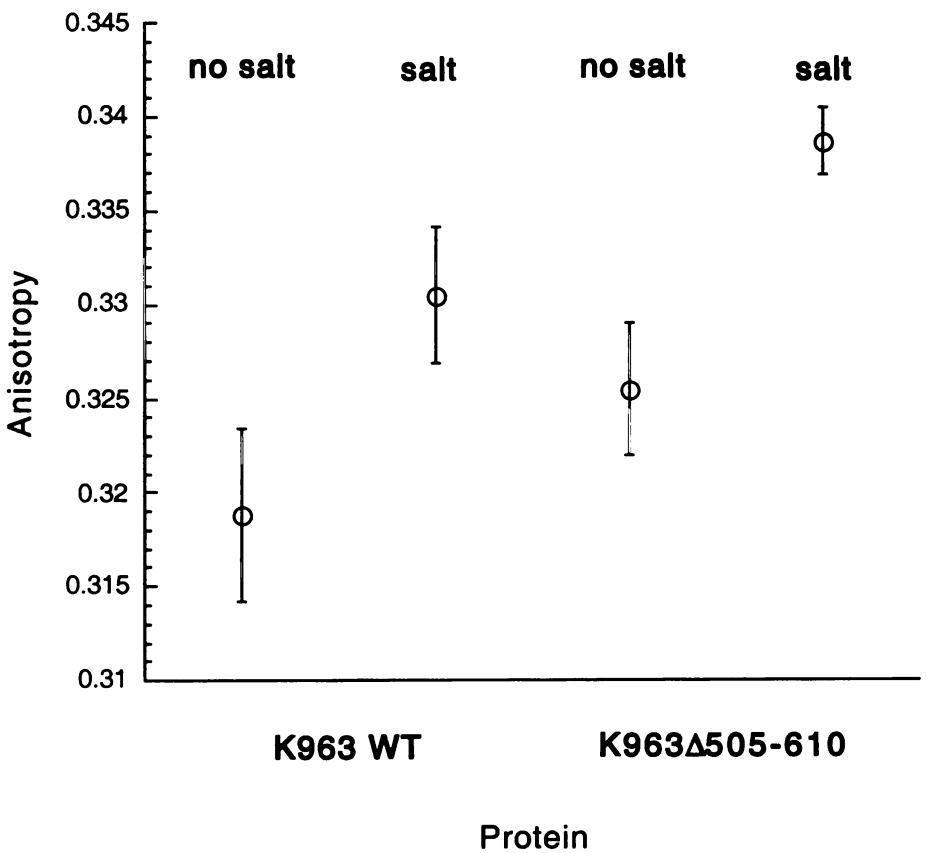

0.5 M NaCl
0 M NaCl
K963 WT
Excitation 380 nm
Emission intensity
500
450
400
350
300
250
200
150
400
450
500
550
K963 Δ505-610
Excitation 380 nm
Emission intensity
450
400
350
300
250
200
150
100
400
450
500
550
Emission Wavelength (nm)

Figure 5. Anisotropy measurements of GFP-tagged kinesin. Bacterially expressed BFP-K963-GFP-His was purified over NTA-Ni^{2+} and through microtubule bind and release. The proteins were sequentially diluted 1:4 in Mono Q Buffer A (no salt) and 1:2 in Mono Q Buffer A or B (1 M NaCl). Anisotropy of the GFP molecule attached to full-length kinesin was read using a fluorimeter at excitation 482 nm, emission 509 nm. Changes in anisotropy would indicate that the molecule to which the fluorophore is attached has experienced a change in the rotational correlation time, which is inversely related to the speed of rotation. For larger-shaped molecules, therefore, rotational correlation time and anisotropy are large. Anisotropy values for a spherical molecule range from 0 to 0.4, with lower values reflecting more rotation during fluorescence lifetime. In the case of kinesin, the folded, more compact molecule would be predicted to rotate more quickly and have a lower anisotropy than that of the extended molecule. Because GFP is attached to kinesin through a short linker (Gly-Thr), we can monitor kinesin's rotation through the rotation of GFP. For this reason, the decrease in anisotropy in low salt for both $K963_{WT}$-GFP and $K963_{\Delta 505\text{-}610}$-GFP may reflect folding of both molecules.

Figure 5


0.345
0.34
0.335
0.33
0.325
0.32
0.315
0.31
Anisotropy
no salt
salt
no salt
salt
K963 WT
K963Δ505-610
Protein

APPENDIX 2

The kinesin-membrane interaction

ABSTRACT

Intracellular membrane traffic is a complex system of transport vesicles traveling back and forth between many subcellular organelles. Many of these trafficking events are microtubule-dependent, and a diversity of molecular motors move vesicles in either direction along microtubules. Dispersion of the endoplasmic reticulum (ER), lysosomes and pigment granules, fusion of early and late endosomes, and anterograde axonal transport are microtubule-dependent processes that involve movement towards the plus ends of polarized microtubules (Hoyt, 1994, Vallee and Sheetz, 1996). Even though there is a diversity of plus-end directed motors of the kinesin family that are thought to transport these membrane vesicles (Hirokawa, 1998), it is not understood how a vesicle is directed to its particular destination in the cell. One possibility is that the motors themselves can specify the route of the vesicles they carry. If this is true, the motor must bind only particular membrane vesicles and the interaction between the motor and its membrane must be specific. My aim was to characterize the interaction between motors and the membranes they transport.

INTRODUCTION

A multitude of kinesin-like motors have been identified (for review, see (Hirokawa, 1998). One purpose of this motor diversity may be to enable the transport of different cargoes to specific targets in the cell (Coy and Howard, 1994). There are some examples of specific motor-membrane pairs in the kinesin family: KIF1A transports synaptic vesicles in mice (Okada et al., 1995) and C. elegans (Hall and Hedgecock, 1991), KIF1B transports mitochondria in vitro (Nangaku et al., 1994), conventional kinesin is responsible for ER (Lippincott-Schwartz et al., 1990) and lysosome (Nakata and Hirokawa, 1995) movement, and kinesin-II moves intracellular lipid rafts anteriorly in cilia(Cole et al., 1998). These examples demonstrate the potential of membrane-specific motors to specify the transport of cargo to specific cellular targets.

I was most interested in learning how motors might recognize and bind specific cargo. I decided to study conventional kinesins because of the lab's cloning and experience with them. About a third of conventional kinesin in the cell is membrane-associated at steady state (Hollenbeck, 1989, Niclas et al., 1994, Verhey et al., 1998), and very little is known about how kinesin associates with membranes. Cargo-binding is believed to occur through the tail domain (Skoufias et al., 1994) which is comprised of the C-termini of homodimerized heavy chains and two light chains (Bloom et al., 1988, Kuznetsov et al., 1988) (Appendix 2, Figure 1a). Studies in sea urchin suggest that the tail of kinesin heavy chain is sufficient for membrane-binding, although light chains enhance this interaction (Skoufias et al., 1994). Furthermore, soluble proteins, besides kinesin, are thought to be neither necessary for binding to membranes (Skoufias et al., 1994), nor for

interactions with a putative membrane receptor, kinectin (Toyoshima et al., 1992). Moreover, binding studies carried out after alkaline extraction and proteolysis treatment of membranes suggest that integral, but not peripheral, membrane proteins are necessary for binding (Yu et al., 1992).

As mentioned above, there is a candidate membrane receptor for kinesin, kinectin. Kinectin was described as a potential receptor based on its affinity for kinesin (Toyoshima et al., 1992), but its antibody inhibition of both plus- and minus-end directed microtubule transport (Kumar et al., 1995) suggests that it may bind kinesin non-specifically, perhaps through its coiled-coil domains. Because of this result, kinectin may not be a kinesin membrane receptor.

My approach to understanding the motor-membrane interaction was to compare two kinesins of the conventional kinesin family. Mammals have at least two conventional kinesins; ubiquitous (Navone et al., 1992) (KIF5B) and neuronal (Niclas et al., 1994) (KIF5A) kinesin heavy chains were cloned from a human placental or hippocampal cDNA library, respectively. Neuronal-specific KHC (nKHC) is 65% homologous to ubiquitously-expressed KHC (uKHC) overall (Figure 1B), which is expressed in many cell types, including neurons. Most studies of membrane-association have examined ubiquitous, but not neuronal, kinesin. However, while these two heavy chains would be predicted to share the same basic structure, they are strikingly different in their carboxyl-termini. Neuronal KHC has an additional 69 amino acids that ubiquitous KHC lacks. Though the function of these additional amino acids is unclear, because they occur in the membrane-binding domain, they may distinguish u- and n-KHC's membrane partners.

Though the membranes transported by u- and n-kinesin are not known, there is some evidence to suggest distinct functions for the two kinesins. The localization of u- and n-KHC differ in newborn rat hippocampal neurons (Niclas et al., 1994). Furthermore, two rat kinesin heavy chains that likely correspond to neuronal and ubiquitous KHC were found to travel in optical nerves with distinct cargoes (Elluru et al., 1995). Finally, the expression of nKHC, but not that of uKHC, is upregulated in cultured neurons upon induction with NGF (Vignali et al., 1995). These studies indicate that ubiquitous and neuronal kinesin may have different cellular functions and therefore be found on different membranes.

My plan was first to determine kinesin's membrane localization and the nature of its binding to membranes. Secondly, I wanted to develop an assay to look at the membrane binding and transport of kinesin. Finally, I hoped to identify kinesin tail-interacting proteins, and then test the role of these identified proteins in kinesin function.

RESULTS

Ubiquitous and neuronal kinesin bind the same membranes

I first wanted to identify a membrane preparation in which to investigate the localization of conventional kinesins (Protocol 1). By differential centrifugation of a crude rat brain lysate, I found the kinesins to be enriched in a fraction populated by "small" transport vesicles. While previous membrane preparations in the lab included an initial low-speed spin of 800-1000 x g after homogenization to pellet unbroken cells and large membranes, I saw that both kinesins were more concentrated in subsequent membrane preparations if the initial spin was faster (3,700 x g) (Figure 2). This information is valuable because it hones in on which types of vesicles (by virtue of their size) the kinesins may bind and transport.

I then wished to determine whether u- and n-KHC transported different cargo from one another, and possibly to define the membranes transported by each. This information would be important for assessing the authenticity of a candidate kinesin receptor by whether it co-localized with its putative motor ligand. In addition, knowing whether u- and n-KHC transport the same membranes would allow one to speculate on the need for common and motor-specific binding mediators. If they bound different membranes, finding distinct binding factors would seem likely.

To determine whether ubiquitous and neuronal kinesin transported distinct membranes, I immunoisolated kinesin-attached vesicles using one kinesin as an immunoaffinity handle, and then looked for the presence of the other kinesin on the immunoisolated vesicles. This protocol was modified from one developed previously to isolate KIF1A-containing vesicles (Okada et al., 1995) (Protocol 2). The result from these experiments

was that u- and n-kinesin heavy chain were detectable in both u- and n-kinesin heavy chain immunoisolated membranes (Figure 3a). This result suggests that ubiquitous and neuronal kinesin bind some membranes in common; however, there may still be some non-overlapping membranes binding partners (Figure 3b). An informative experiment would be sequential depleting immunoisolations to determine whether one kinesin was depleted from the membrane fraction after immunodepletion of the other.

A unique carboxy terminus may target neuronal kinesin heavy chain to membranes

In some of my blots looking at nKHC in rat brain extracts, I see a doublet of ~120 and 130 kD. Josh Niclas and Francesca Navone also saw these two cross-reacting species. Notably, when I look at the distribution of these two bands in low-speed and high-speed supernatants, and in high-speed pellets, I see that the lower band is absent from membranes (Figure 4). Because the lower band has the same molecular weight as uKHC, it is possible that it is simply missing the 69 C-terminal amino acids that distinguish nKHC from uKHC. If this were true, it would suggest that the C-terminal 69 amino acids of nKHC are required for its targetting to membranes.

We do not yet know whether the 120 kD band is a C-terminal truncation of nKHC. It needs to be determined whether the upper and lower bands contain the 69 amino acids. An antibody recognizing the C-terminal 69 amino acids would be helpful in resolving this issue. To this end, I expressed and purified from inclusion bodies (because of insolubility) the C-terminal 69 amino acids fused to GST (GST-n69). After inoculating

rabbits with the solubilized inclusion bodies, the rabbits' serum began to recognize a band co-migrating with nKHC. However, I was not able to affinity purify the antibody with His-tagged n69 that was expressed and purified on a nickel column under denaturing conditions. In testing the bleeds again to make sure of their recognizing nKHC, I saw that they in fact did not. I discovered this by immunodepleting nKHC (with the stalk antibody) from rat brain LSS, and probing the supernatants and pellets with the stalk antibody and with the bleeds. Whereas the stalk antibody recognized nKHC in the pellet and nothing in the supernatant, the bleeds recognized a band in the supernatant and nothing in the pellet. So without this reagent we still do not know what piece of nKHC is missing in the smaller isoform.

Motility still not accomplished with brain extracts

An assay would be necessary to test any assertion I might make about the requirements of kinesin-membrane binding. The first assay one might try is a membrane motility assay; however, there has been very limited success with brain motility. Josh Niclas was able to get rat liver Golgi vesicles to move with the addition of Xenopus or clam, but not rat brain, high-speed supernatants. Furthermore, I was unsuccessful at reconstituting motility using crude brain extract. I tried to do so with Nira Pollock using her Dicty extract preparation (except for lysis conditions) and assay, and by preparing my membranes by microtubule affinity purification (Schnapp et al., 1992). I was able to purify microtubule-bound membranes, but I did not in any case see movement upon addition of supernatant.

Kinesin is not stripped from membranes with alkaline, salt, or nucleotide treatment

The next assay I tried to develop was a binding assay between kinesin and kinesin-stripped membranes. The purpose of a binding assay would be to test the role of candidate proteins in kinesin's binding to membranes. The general idea is first to strip motors from rat brain membranes and then rebind native or recombinant KHC. I would then be in a position to identify the KHC membrane binding domain and to test candidate mediators of KHC binding either by antibody inhibition or by biochemical complementation.

Josh Niclas stripped dynein from Xenopus membranes using 0.3 M KI; Jon Scholey stripped sea urchin egg kinesin using 0.5 M KCl followed by 0.1 M Na_2CO_3, pH 11 (Skoufias et al., 1994), and Mike Sheetz stripped chick brain kinesin with 0.1 M Na_2CO_3, pH 11.3 (Yu et al., 1992). I tried many conditions for stripping kinesins off rat brain membranes, but I was unable to completely strip kinesin in any consistent way. Although 0.6 M KI, 0.1 M Na_2CO_3 pH 11, 2 M NaCl, and GTPγS all stripped 80% or more of the motors, the success rate for each hovered below 50%.

Ubiquitous and neuronal kinesins are peripheral membrane proteins

The inability of such caustic measures to strip kinesin off membranes suggested that rat brain kinesin might be a membrane protein. Because kinesin lacks a transmembrane domain, it is unlikely to be integral; however, it could be GPI-linked. To test this possibility, I looked at the partitioning of kinesin after Triton-X114 treatment. Using a protocol from the Red Book (Ausubel et al., 1995), I found that both u- and n-kinesin heavy chains segregated with peripheral membrane proteins, as shown by

Western (Figure 5). In long exposures, there is some uKHC in the 37 °C hydrophobic and amphipathic membrane protein pellet, but this may be due to insufficient washing of the pellet before resuspension. Therefore, these results confirm that kinesin is a peripheral membrane protein.

No other proteins co-sediment with kinesin solubilized from membranes

Because kinesin appears to be very tightly anchored to the membrane, but not by direct attachment to the membrane, it may be in a tight complex with other membrane proteins. To isolate this potential kinesin complex, I solubilized and released kinesin from membranes and ran sucrose gradients to size the resulting kinesin complexes.

I used a number of detergents to release kinesin from membranes. Because I wanted to preserve protein complexes, I concentrated on non-ionic detergents; triton-X100, octylglucoside, and digitonin solubilized kinesin out of membranes well (Table 1, Protocol 3). I found that uKHC from solubilized membranes and detergent-treated high-speed supernatants ran the same on sucrose gradients, peaking ~9 S, which is the sedimentation of soluble kinesin (Hackney et al., 1992). I also tracked other kinesins (with a pan kinesin antibody, anti-HYPR) and myosins (with a pan myosin antibody, anti-LEAF), and found no differences in mobility on gradients between the soluble and membrane-released motor proteins. Therefore, a motor complex was not preserved during these solubilization procedures.

CONCLUSIONS

Reflections on the experiments

I spent a fair amount of time trying to discern if u-and n-KHC were on distinct organelles: they appeared not to be. However, this result is not completely unexpected. nKHC is developmentally regulated, with highest expression seen in embryonic and early postnatal brain (Vignali et al., 1997). n-kinesin may be double-teaming with u-kinesin during early development when there is high demand for organelle transport. It is also possible that n-kinesin transports a specific membrane population during development and then reverts to transporting the "regular" vesicles (those transported by u-kinesin) in adult brain. Because of this possibility, it may be worthwhile to look in embryos or differentiating PC12 cells to find n-KHC-specific membranes.

The role of the carboxy-terminal 69 amino acids of nKHC (n69) in membrane association is still unknown. That n69 may be required for membrane association of nKHC is suggested by the observation of an anti-nKHC reactive protein band that co-migrates with uKHC only in non-membrane fractions. However, that this smaller band lacks n69 is a big assumption that has yet to be tested. Even if it were true that the smaller band were a C-terminal truncation of nKHC, it is unclear why this species would be unable to bind membranes if uKHC, which also lacks this C-terminal domain, is membrane-associated. A complicating factor is that a n-specific KHC has been cloned by Larry Goldstein (KIF5C) which lacks the C-terminal 69 amino acids. If this protein were recognized by our anti-nKHC antibodies, the subband may be a non-membrane-transporting kinesin

motor. If so, it would be interesting to compare this non membrane-bound truncated nKHC with the membrane-binding uKHC.

The mystery of how kinesin attaches to membanes remains. Even though kinesin partitions as a peripheral membrane protein, it has been unremovable by non-denaturing conditions. Furthermore, the affinity of kinesin for membranes, 14 nM (Skoufias et al., 1994), would not by itself predict such a long-lasting association. However, the resistance to salt extraction shown here has also been seen previously. Doug Murphy's group found that a variety of ionic strengths and alkaline and acid pH did not appreciably remove kinesin from membranes (maximum 50% removal with 0.5 M NaCl). They also were unable to remove kinesin with a mixture of phospholipases, phosphokinase A, or ATP. And yet, in spite of kinesin's tenacity in membrane-binding, its apparent tight association with some membrane factor is not maintained through the gentlest of solubilization procedures.

It is possible, however, than kinesin was not removable from the membranes because it was denatured. Denaturation could be caused by the stripping procedures. This seems unlikely, however, since a low to high range of each stripping agent was tested. However, denaturation could also be caused by pelleting the membranes out of a high speed supernatant. It might be worth trying to collect membranes on sucrose cushions, rather than pelleting them to the bottom of the tube and resuspending them in buffer.

Another possibility is that there are two distinct pools of kinesins distinguished by their ability to bind cargo. Because only a portion of cellular kinesin is membrane-associated at a given time, it is possible that a particular subset of kinesin molecules is ever and always membrane-bound.

However, this is not thought to be the case. That activation of transport in vivo causes reversible kinesin phosphorylation and membrane-association suggests that kinesins are recruited to membranes from a soluble, less-phosphorylated pool (Lee and Hollenbeck, 1995). Furthermore, the work presented in Chapter 1 suggests that individual kinesin molecules are capable of a range of activity states. Since kinesin molecules are in equilibrium between active and inactive catalytic states, there may also be a balance between solubility and cargo-association for each kinesin molecule. If this were true, we would expect kinesin to be competent of attachment and detachment from membranes. The work presented here suggests that the regulation of the exchange between solubility and membrane-association may by quite complex.

Reflections on the theory of membrane-specific motors

A current hypothesis is that cargo-specific motors enable transport of each cargo to its appropriate destination. However, it is not known whether there is a motor dedicated to every cargo type, and vice versa. There may be some overlap, in which the same motor transports more than one cargo, or in which more than one motor transports the same cargo. However, rather than compromising the efficiency and regulation of intracellular transport, such overlap may serve a useful purpose in this regard. For instance, having the same motor transport different cargoes the same cellular destination is more efficient than having a separate motor for each. Moreover, if more than one motor can transport a single cargo, the activities of the different motors may be alternately regulated. As a result, either the presence or absence of a unique motor distribution would have interesting ramifications for the control intracellular transport.

A second part of the hypothesis that cargo-specific motors are responsible for targetting cargoes is that motors can guide their cargoes appropriately. This may be possible if motors were sensitive to the cellular environment and able to recognize the different domains of the cell. For instance, we are beginning to understand how the directionality of the microtubule is read by motors by identifying the domains responsible for motor polarity (Case et al., 1997, Endow and Waligora, 1998, Henningsen and Schliwa, 1997). Motors may also discern microtubule domains in addition to microtubule polarity. This may be accomplished through region-specific coating of microtubules with the various microtubule-associated proteins (MAPs). For instance, in the neuron, microtubules of the dendrites are coated with MAP2 (Hirokawa, 1993), whereas tau associates with axonal microtubules (Binder et al., 1985). It has been shown that overexpression of certain MAPs can inhibit motility by a specific classes of motors (Ebneth et al., 1998, Heins et al., 1991, Lopez and Sheetz, 1993); perhaps endogenous levels of MAPs guide transport motors to different parts of the cell. To this end, it would be interesting to test the sensitivity of neuronal kinesin, which localizes mostly to the cell body rather than the axon (Niclas et al., 1994), in its motility on MAP or tau-coated microtubules. In some way, then, differences among motor domains may enable kinesins to discern local microtubule environments in the cell.

Table I: Detergents used to solubilize kinesin from membranes.

DETERGENT	CRITICAL MICELLE CONCENTRATION	CONCENTRATION TO RELEASE KINESIN
TRITON-X100	0.02%	0.5%
OCTYLGLUCOSIDE	0.7%	2.5%
DIGITONIN	<0.01%	0.75%
BIG CHAPS	0.3%	>2.5%
DEOXYCHOLATE	0.2%	0.5%

Solubility was determined by a 15 min speed at 100,000 x g. The concentration required to solubilize kinesin from a 10 mg/ml membrane preparation was determined by titration. Although more than the critical micelle concentration was necessary to solubilize kinesin, the amounts required for digitonin, big CHAPS and deoxycholate are within limits recommended by Manu Hegde and others for isolating non-micellar protein. Also, the released proteins ran as sharp peaks on velocity gradients, suggesting that they were not in micelles, which would run non-uniformly.

Protocol 1: Your basic membrane prep.

Rat brains were available from the Tessier-Lavigne lab. They typically sacrificed 1-3 rats at a time for their experiments. All dissection was carried out in their hood. (Try to think about something else during the first few steps.)

1. Sever heads from body with a guillotine.
2. Crack open the skull using the Friedman Rongeur (That's really what it's called. Fine Science Tools sells these (cat. 16000-14, ph. 800-521-2109). Also, Roboz has a fine selection of Bone Rongeurs and Bone Cutting Forceps (ph. 800-424-2984).
3. Spoon out brains with a spatula into BRB80* or acetate buffer** on ice.
4. Mince tissue in a petri dish with a razor in ~2 volumes (~5 ml/brain) of the above buffer, with 150 mM sucrose and a protease inhibitor cocktail of aprotinin, PMSF, leupeptin, pepstatin, and chymostatin. Homogenize using a hand dounce.
5. Pellet at 800-8,000 x g for 10 min to remove unbroken cells and larger membranes.
6. Layer 2 ml of the low-speed supernatant over a 1 ml 300 mM sucrose cushion in a fixed angle tube. Pellet at 100,000 x g, 30 min. Save supernatant (high-speed supernatant). Resuspend high-speed pellet in 50 µl buffer.

*BRB80 - 80 mM K-Pipes, pH 6.8, 5 mM $MgCl_2$, 1 mM EGTA

**acetate buffer - 100 mM KOAc, 5 mM EGTA, 3 mM MgOAc, 10 mM K-Hepes, pH 7.4

Protocol 2: Immunoisolation of kinesin-containing vesicles

The most important thing is that kinesin-containing membranes are too heavy to remain in the supernatant of any immunoprecipitation. I tried using sucrose cushions that would let beads through and block membranes, and even though I tried different types of beads and pellets, I could not find an adequate cushion. Finally I found that I could avoid these problems by using magnetic beads. I coupled anti-kinesin antibodies to Dynabeads, and these worked well at isolating only kinesin-containing membranes.

1. Prepare membrane fraction (Protocol 1, 3,700 x g low-speed spin).
2. Add 140 µg membranes to 2 mg dynabeads coupled with 15 µg anti-kinesin. (Coupling protocol is in the Dynal manual (800-638-9416).) Bring to 2 mM EDTA. Incubate at 4 °C, 1 hr. Wash beads with PBS using magnet (Dynal MPC-E-1) to suck out beads. Release kinesin and membranes from beads by boiling in gel sample buffer.

Protocol 3: Sizing kinesin solubilized from membranes

1. Prepare membranes from a 8,000 x g, 10 min low-speed supernatant.
2. Resuspend membranes in 150 mM sucrose in acetate buffer + 0.5 M KCl to get rid of easily removed peripheral membrane proteins.
3. Dilute with 250 mM sucrose/acetate buffer and pellet over 525 mM sucrose/acetate cushion at 100,000 x g, 10 min.
4. Resuspend membranes to 5-15 μg/μl in solubilization buffer. I tried a number of different buffers:
    - 350 mM KOAc, 50 mM Hepes, 12 mM MgOAc, 15% glycerol, pH 7.5 (for determining initial solubility)
    - same as above with 100 mM KOAc
    - 10 mM Hepes, 2 mM $MgCl_2$, 1mM EGTA, pH 7.5
5. Add equal volume solubilization buffer + 2X detergent (See Table I for detergents used). Incubate on ferris wheel in cold room 15 min.
6. Pellet unsolubilized material at 100,000 x g, 15 min.
7. Load supernatant (solubilized membrane proteins) onto a 5-20% sucrose gradient in solubilization buffer. Spin at top speed in the TLS55, 4 hrs.
8. Collect 200 μl fractions from the top. Standards ran as follows: BSA (4.3 S) - fraction 3, aldolase (7.4 S) - fractions 4-5, catalase (11.3 S) - fraction 6, thyroglobulin (19.4 S) - fraction 10.

Clones used:

uKHC (Navone, 1992) Accession number X65873

nKHC (Niclas, 1994) Accession number U06698

Antibodies used:

anti-uKHC and anti-nKHC(Niclas et al., 1994) are isoform-specific antibodies raised against the stalk domain.

anti-LAGSE and anti-HYPR (Sawin et al., 1992) are peptide antibodies that recognize a conserved motif in all known kinesins. The LAGSE motif is also found in some myosins.

anti-LEAF is a peptide antibody from Christine Fields that recognizes a conserved motif in myosins.

Figure 1. Models of kinesin structure and sequence. **(a)** Kinesin is a heterotetramer of two heavy chains (solid) and two light chains (white). The tail, comprised of the C-terminus of the heavy chains and the light chains, functions in membrane-binding. **(b)** uKHC and nKHC share significant amino acid homology. nKHC also encodes for an additional 69 amino acids at its C-terminus. Numbers indicate residue number.

Figure 1

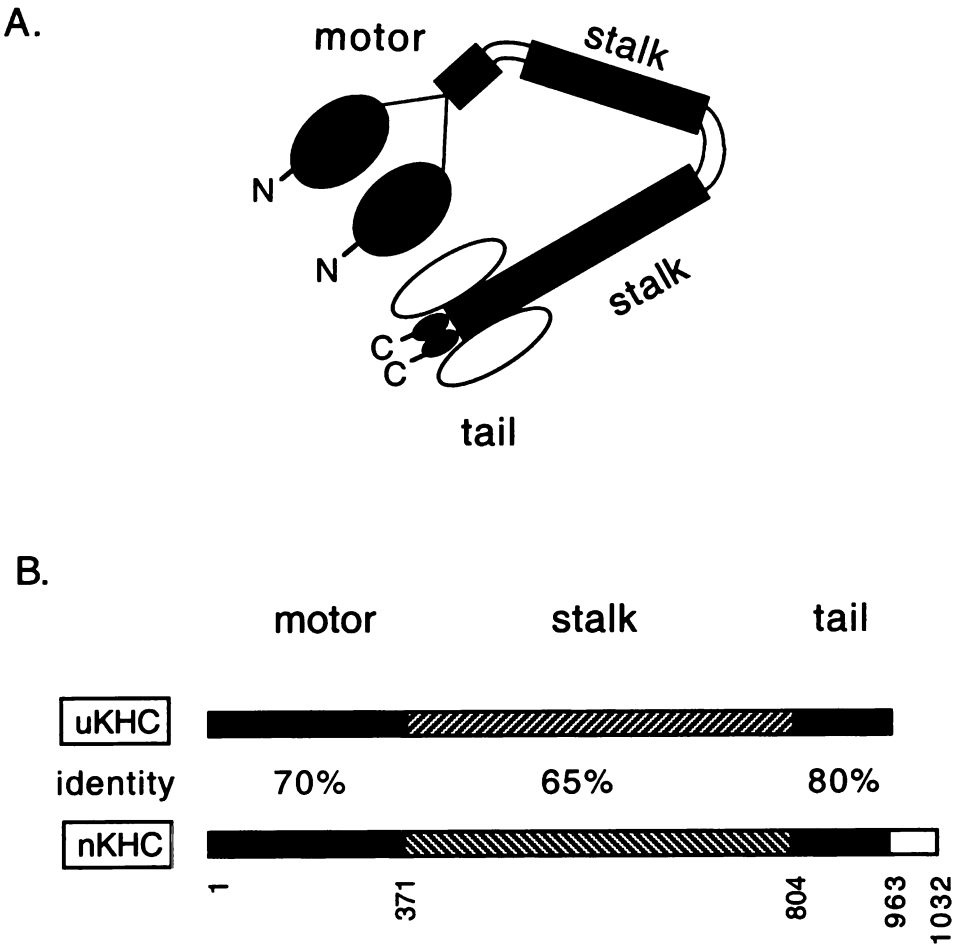

A.
motor
stalk
N
N
stalk
C
C
tail
B.
motor
stalk
tail
uKHC
identity
70%
65%
80%
nKHC
1
371
804
963
1032

Figure 2. Enrichment of kinesin by differential centrifugation. Rat brain homogenate (~10mg/ml) was centrifuged at 800, 1,700, or 3,700 x g for 10 minutes, the supernatant of which was then layered over sucrose and pelleted 30 minutes at 100,000 x g. The pellet was resuspended in buffer and 25 μg in 10 μl was dot blotted to nitrocellulose. Blots were probed either for nKHC or uKHC. Both kinesins were enriched in the 3700 x g spin.

Figure 2

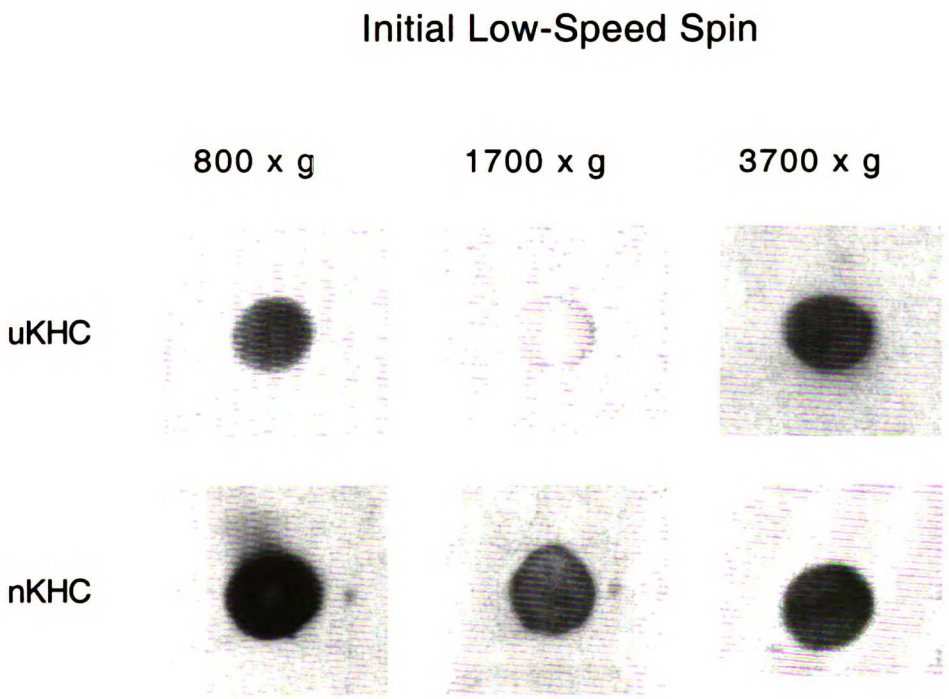


Figure 3. Immunoisolations of kinesin-containing membrane vesicles. **(a)** Vesicles were immunoisolated from a membrane fraction with magnetic beads coupled with random rabbit IgG, affinity-purified anti-nKHC, or affinity-purified anti-uKHC. The supernatant from boiling the beads in sample buffer was run on a protein gel, transferred to nitrocellulose, and probed with anti-nKHC or anti-uKHC. **(b)** Ven diagram models depicting the distribution of u- and n-kinesin on membranes; there may be partial or complete overlap.

Figure 3

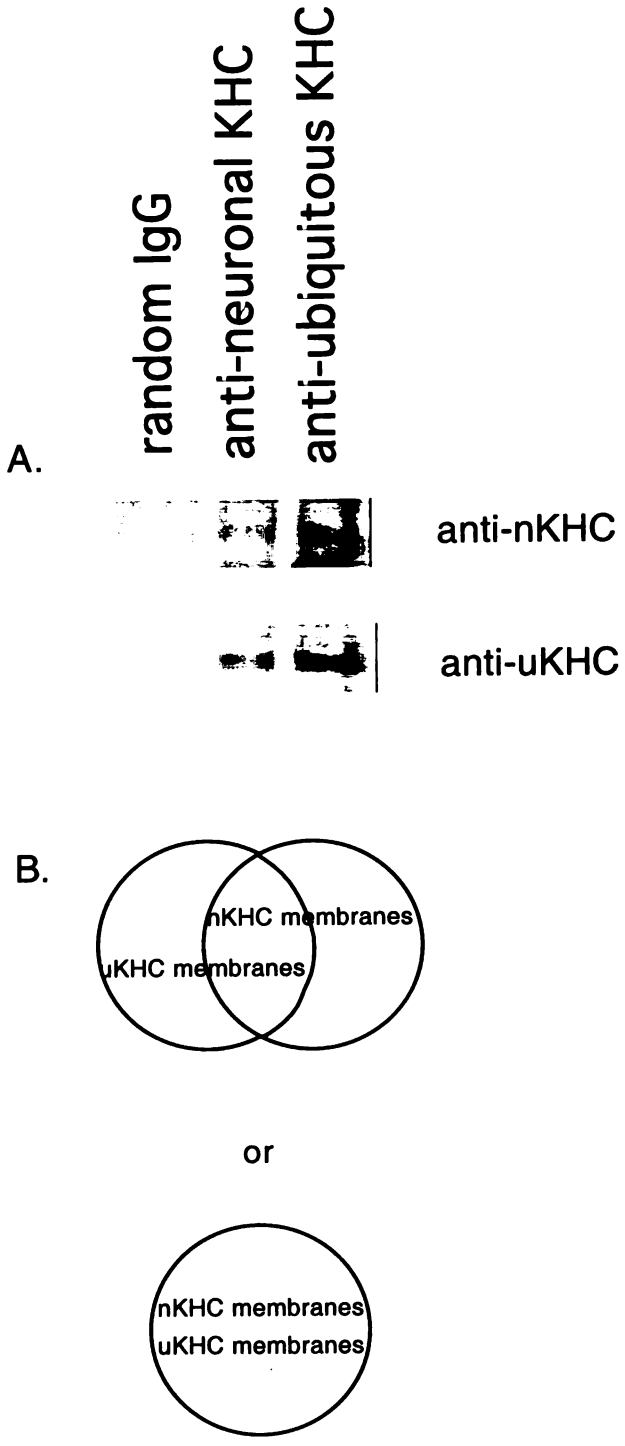

A.
random IgG
anti-neuronal KHC
anti-ubiquitous KHC
anti-nKHC
anti-uKHC
B.
nKHC membranes
uKHC membranes
or
nKHC membranes
uKHC membranes

Figure 4. Visualization of the nKHC doublet. Crude rat brain homogenate was pelleted for 10 min at 800 x g to make a low-speed supernatant (LSS) and pellet (LSS). The supe was layered over 300 mM sucrose and centrifuged 30 min at 100,000 x g to produce a high-speed supernatant (HSS) and pellet (HSP). 85 μg of each fraction was run on a 5-15% gradient polyacrylimide gel and transferred to nitrocellulose. The blot was probed with anti-nKHC. The lower (120 kD) band of the nKHC doublet is absent in the membrane fraction (HSP).

Figure 4

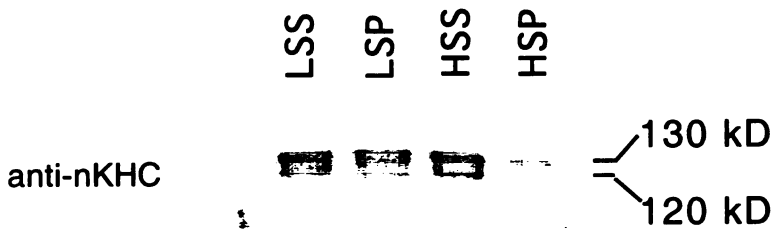
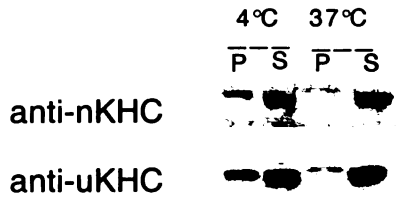


Figure 5. Kinesin is a peripheral membrane protein. Membranes prepared from a 8,000 x g, 10 min supernatant were resuspended to 4 μg/μl in TBS, and were incubated with one-fifth volume of pre-equilibrated Triton X-114 for 15 min on ice. The "4 °C supe" was separated from the "4 °C pellet" by a 10 min 10,000 x g spin in a refrigerated microfuge. The supe was heated to 37 °C and pelleted 10 min at 1,000 x g to give a "37 °C pellet" and "37 °C supe." These fractions were run on a polyacrylimide gel, transferred to nitrocellulose, and blotted with either anti-uKHC or anti-nKHC. Transmembrane and membrane-linked proteins partition to the "37 °C pellet," and peripheral membrane proteins partition to the "37 °C supe."

Figure 5



CONCLUSIONS

Because kinesin's cargo- and microtubule-binding domains are on opposites ends of the molecule, their roles in cargo motility have been studied independently. The work presented here brings us closer to understanding how the activities taking place at the two ends of kinesin are linked and affect one another.

The interaction of kinesin with its membrane cargo

The question of how kinesin associates with its membrane cargo is as important as it is long-standing. Our attempts to manipulate membrane-bound kinesin suggest that its interaction with membranes is complex and interesting. This work, described in Appendix 2, was carried out using two conventional kinesins expressed in the neuron, defined by the neuronal (Niclas et al., 1994) (KIF5A) and ubiquitous (Navone et al., 1992) (KIF5B) kinesin heavy chains (KHC). We show in rat brain that the kinesin-membrane interaction is resistant to a number of treatments, as has also been seen in bovine adrenal gland (D.B. Murphy, unpublished data) and squid axoplasm (Schnapp et al., 1992). In contrast, kinesin has been stripped from membranes in sea urchin egg (Skoufias et al., 1994) and embryonic chick brain (Yu et al., 1992). This disparity suggests that the mechanism of kinesin-membrane binding may vary in some fundamental way among different systems. One possible factor could be differences in the putative cargo-binding domains, however, the heavy and light chains are very highly conserved across species. Another explanation may be that the binding affinity or mechanism of attachment could vary among the different membrane cargoes or cell types. We addressed one aspect of this possibility and confirm that ubiquitous and neuronal KHC are peripheral,

not integral, membrane proteins. However, this result does not rule out other possible mechanistic differences in membrane binding.

Because of the importance of the tail in cargo-binding, the difference in neuronal- and ubiquitous-KHCs of an nKHC-specific addition in the tail prompted us to look for differences in their membrane targetting. Our studies revealed that they overlapped to a significant extent in their membrane distribution, and leave open the possibility for both common and isoform-specific binding interactions. The latter would suggest that the very carboxy-terminus of kinesin directs specific binding, but the former would suggest that another part of the tail domain that is similar between the two kinesins would have this role. Light chains may be responsible for directing these two kinesins to the same membranes, as two closely related light chain genes have been identified in brain that associate indiscriminately with both ubiquitous and neuronal heavy chains (Rahman et al., 1998). Thus, in future experiments aimed at identifying binding partners of the mammalian kinesins, it will be important to assess the exact regions and subunits involved in the membrane interaction.

The control of motor activity by auto-regulation and cargo-binding

Our work has shown that the motor activity of ubiquitous conventional kinesin is repressed when it is free in solution. Specifically, as described in Chapter 1 of this thesis, we find that full-length kinesin heavy chain displays lower ATPase activity, slower, more staggered motility and lower frequency of motility due to inhibition by the tail. Light chains have a very small effect on motility properties, which suggests that they play a minor role in kinesin auto-regulation. In addition, in Chapter 2, we describe another possible regulatory system that is tail-independent. In both

cases, repression is thought to occur through intramolecular folding that brings the tail or stalk domain into contact with the motor head of soluble kinesin.

The mechanism by which motility is repressed, therefore, likely involves interference with the normal functioning of the kinesin motor. Two obvious activities that may be disrupted are microtubule binding and ATP hydrolysis. Because a decrease in the frequency of motility probably reflects a decrease in the ability to make productive encounters with the microtubule, microtubule-binding may be affected in repressed motors. It is interesting to note that while k_{cat} is depressed in full-length kinesin, its K_m value for microtubules is similar to that of K560, based on our preliminary data (not shown); this result is at odds with long-standing values (Kuznetsov and Gelfand, 1986, Wagner et al., 1989), but is supported by more recent work (Stock et al., 1999). Because this parameter is enzyme concentration-independent, it is unaffected by individual kinesin molecules that are completely unreactive with the microtubules. For this reason, the K_m results are consistent with the hypothesis that a steady state population of motors is completely repressed for microtubule-binding. There may also be interference with motor ATPase activity, however, this process is difficult to separate from microtubule binding since ATP hydrolysis is microtubule-stimulated.

Furthermore, we make the important observation that inhibition is transitory. That is, an individual motor can switch from active to repressed, even after initiation of movement, as demonstrated by the discontinuous motion of the full-length motors. This phenomenon suggests that these motors are in an equilibrium between active and inactive motor states. In the active state, a motor can initiate movement along microtubules; in the

inactive state, it cannot. Most full-length motors, then, are in an inactive state most of the time, relative to the tail-truncated motors, and prevented from associating with the microtubule. However, a transiently active motor traveling along the microtubule can be inhibited in situ, probably by the tail's coming back into contact with the motor, and prevented from its walk along the microtubule (Figure 1). Therefore, soluble full-length kinesin can be repressed for motility even after microtubule-association.

In the cell, organelle transport is renowned for its processivity and speed, which would appear to be at odds with its in vitro stop-and-go phenotype. For this reason, there may be a scheme for over-riding auto-repression and maintaining kinesin activation during its time in transport. In vitro, assays in which kinesins are activated by tethering them to a glass surface point to a potential mechanism for stabilizing kinesin in its active state. In the cell, cargo-binding may turn kinesin "on" and keep it in an active state while bound. This may be possible if cargo, in binding to the kinesin tail, unfolds kinesin and prevents an inhibitory interaction with the motor head. In Chapter 1, we analyze the activity of heavy chain mutants that may recapitulate the conformation of a cargo-bound kinesin and find that they are activated to almost tail-less levels (Figure 2). From these results, we speculate that cargo-induced unfolding of repressed cytoplasmic kinesin results in the stabilization of kinesin motor activity in the cell.

However, tail-repression coupled with cargo-activation may not alone account for the regulation of kinesin in the cell. First, because our results suggest that the association time with microtubules is similar for full-length and tail-less kinesins, it is possible that other cellular mechanisms are used to repress soluble kinesin. These may work either directly by interfering with the motor or the cargo-binding tail, or indirectly

by stabilizing the folded conformation. Furthermore, it is possible that another mechanism, either separately or in coordination with cargo-binding, unfolds and activates kinesin. Phosphorylation is a likely candidate to play this role (Figure 3). For these reasons, kinesin auto-regulation most likely coordinates with other regulatory mechanisms in the cell to control the transport activity of kinesin. Understanding this potentially complex scheme is one of the next steps towards understanding how intracellular transport is regulated.

Future directions

Studies of membrane trafficking and motor mechanics have identified non-motor domains as important sites for the modulation of motor activity in response to cellular cues such as cargo binding. Specifically, the tail domain may both mediate and specify interaction with cellular cargo, and modulate motor activity in response to cargo occupancy. Our work, while allowing some of these conclusions to be drawn, points in multiple directions for future research.

Our findings on the nature of the kinesin-membrane interaction highlight persistent questions about kinesin's and docking proteins' roles in cargo-association. Because the rat brain system is biochemically challenging, future work in this area might be carried out in genetically and biochemically tractable systems. In particular, S. pombe offers some advantages in studying membrane motility. First, pombe utilizes microtubules for intracellular transport (Ayscough et al., 1993), and therefore would likely be a rich biochemical source for membrane-bound kinesins. Secondly, some kinesins have already been identified and characterized genetically, and appear to function in membrane transport

(Cande, personal communication). Development of in vivo and in vitro assays for microtubule- and kinesin-based motility would be a first step towards identifying the motors and accessory factors involved in intracellular transport.

Another outstanding question is whether cargo-binding activates kinesin, and if this level of control is important for regulating transport in the cell. Because a cause-and-effect relationship between membrane binding and kinesin activation has yet to be determined, these are important issues to address. To address the role of cargo-binding and unfolding in kinesin activation, a number of in vitro and in vivo experimental avenues can be followed. The influence of phosphorylation or cargo-binding on kinesin activity can be tested in vitro by assaying their effects on kinesin ATP hydrolysis and solution-based motility. Phosphorylation, if stimulatory, might cause an increase in basal and/or microtubule-stimulated ATPase rates, as well as mask repression of motility. Cargo-binding, if activating, would also be expected to enhance motor activity. One challenge, however, may be in reconstituting a productive interaction with an endogenous kinesin cargo. A potential alternative would be to use the cytoplasmic portion of a kinesin receptor, if identified, as a ligand. Using a minimal cargo domain in this way may also decrease background in ATPase assays due to cargo-associated ATPases. If activation were achieved by either modification or cargo-binding, it would be interesting to look for kinesin unfolding, if such an assay becomes available.

Manipulation of kinesin in vivo can be used to determine if kinesin folding is integral to the regulation of transport in the cell. If this regulation were important for control of cellular transport, cells in which kinesin folding is disrupted might display transport defects. Specifically, we would

expect kinesins for which auto-regulation has been compromised, such as the (neck mut) and hinge 2-deleted isoforms, to show increased transport of their cargoes in vivo. S. pombe, N. crassa, and D. melanogaster may be potential systems for carrying out this work, especially because a good deal is known about the role of conventional kinesin in the later two. These would also be systems in which other kinesin regulatory pathways could be explored.

Figure 1. Model for kinesin activity in vitro. Kinesin repression can occur either before or during microtubule association. **(a)** Repression of soluble kinesin prevents its association with the microtubule and leads to low frequency of productive interaction. **(b)** An equilibrium between active and inactive motor states causes transient repression of a motor traveling along the microtubule, resulting in discontinuous motility that is marked by episodes of fast motion characteristic of tail-less kinesin.

Figure 1

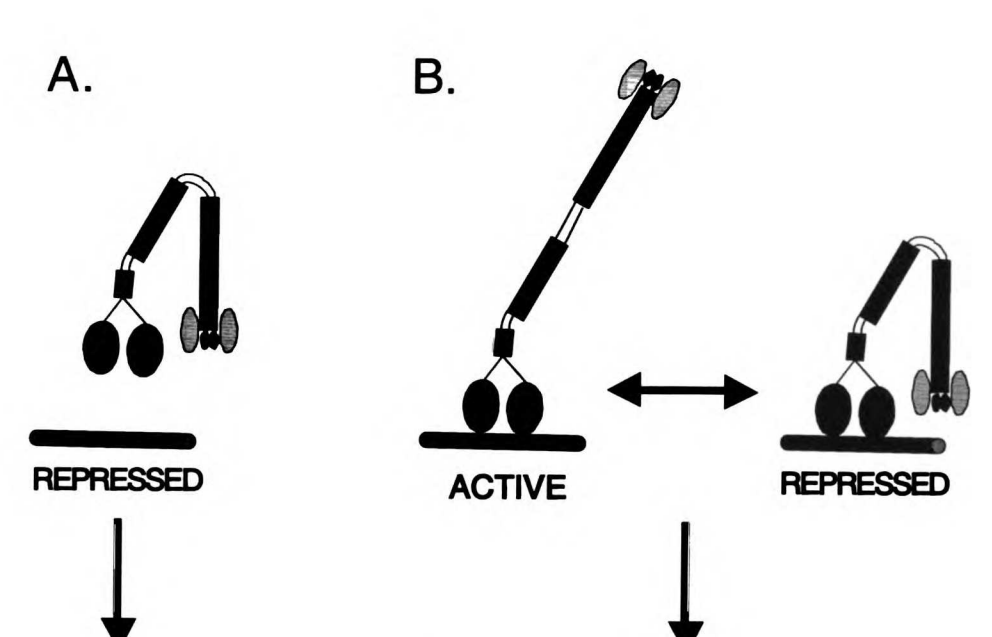

A.
B.
REPRESSED
ACTIVE
REPRESSED
low frequency
discontinuity and slow velocity

Figure 2. Model for cargo-activation of kinesin, and for the auto-activation of the kinesin mutants. Cargo-binding stabilizes kinesin in an extended conformation, in which the tail-head, and possibly stalk-head, interaction is disrupted, that is recapitulated by K963 Δ505-610 and K963 (neck mut).

Figure 2

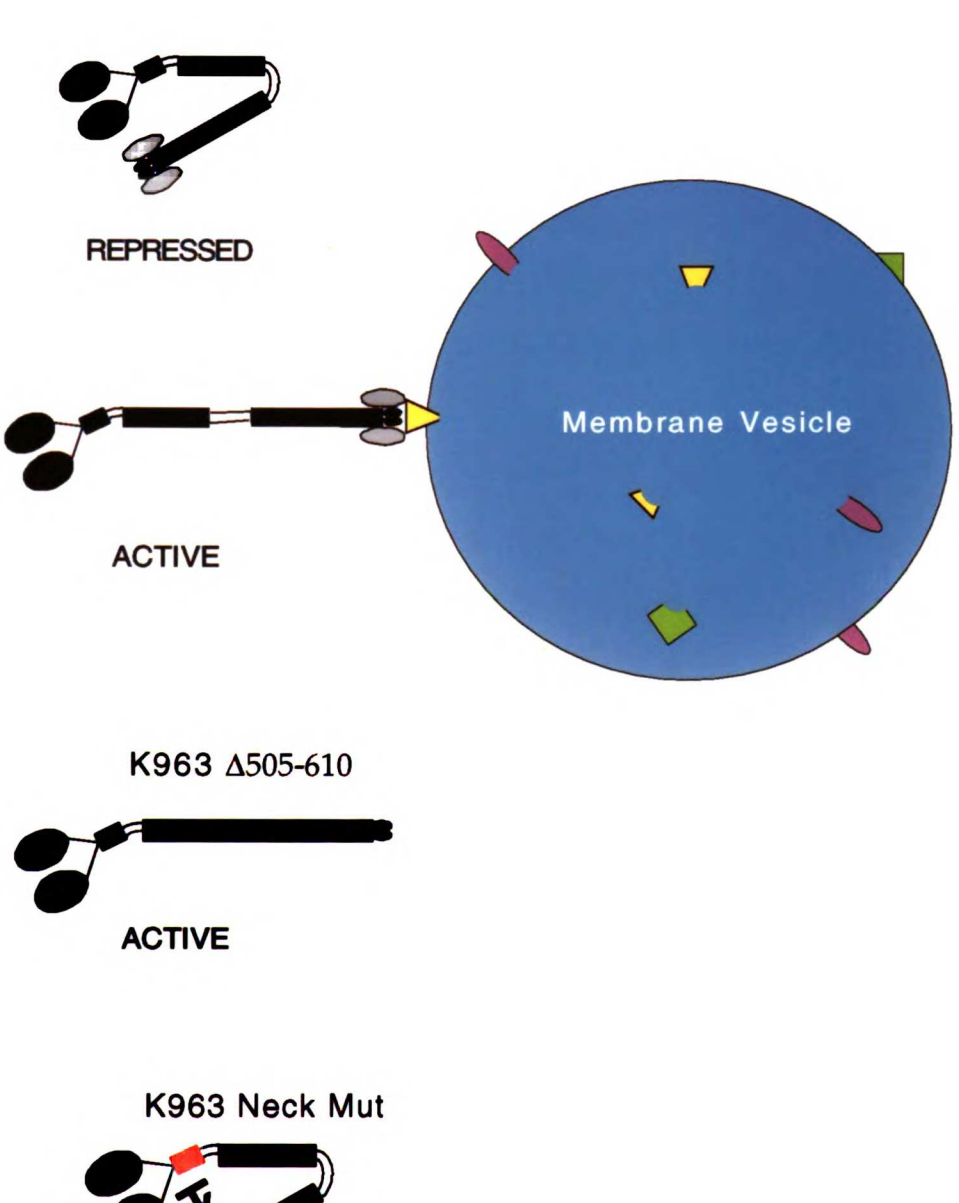
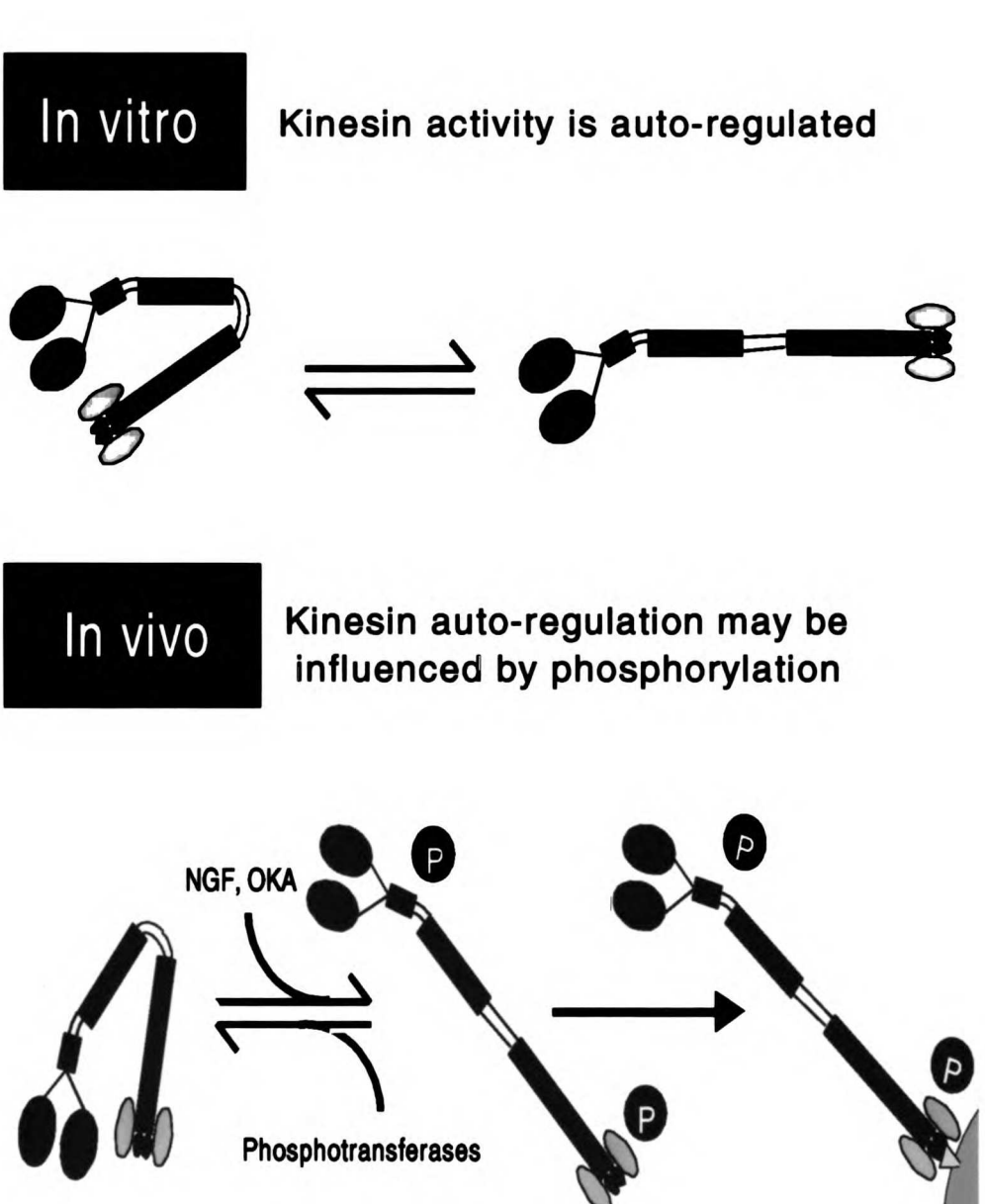

REPRESSED
Membrane Vesicle
ACTIVE
K963 Δ505-610
ACTIVE
K963 Neck Mut
ACTIVE

Figure 3. Model for other cellular inputs into the activation of kinesin. In the cell, the folded-to-unfolded transition may be mediated by cues other than, or in concert with, cargo-binding. Phoshorylation of heavy and/or light chains may mediate kinesin activity. (abbreviations: OKA-okadaic acid)

Figure 3


In vitro
Kinesin activity is auto-regulated
In vivo
Kinesin auto-regulation may be influenced by phosphorylation
NGF, OKA
P
P
P
P
Phosphotransferases

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