SITE-DIRECTED MUTAGENESIS OF THE NCD MICROTUBULE MOTOR PROTEIN

by

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(ABSTRACT)

Ncd is a member of the kinesin family of motor proteins. Ncd is involved in the processes of meiosis and early mitosis in D. melanogaster. PCR-mediated site-directed mutagenesis was utilized to introduce specific mutations into pET/MC6, a construct containing the motor domain of ncd. Six mutations were generated, two at glutamic acid residue 656, two at proline residue 649, one at arginine residue 623, and one double mutant at arginine residue 623 and threonine residue 632. Mutants proteins were expressed in bacteria and further characterized. Mutagenesis of the proline or glutamic acid residues resulted in insoluble proteins. The one exception is the mutagenesis of glutamic acid residue 656 into a glutamine, which resulted in a partially soluble protein. Mutagenesis of the arginine residue into an alanine (MC6-A623) resulted in a soluble protein while the double mutation of the arginine and threonine was insoluble. MC6-A623 exhibited a similar S-sepharose ion exchange chromatography binding and elution profile as MC6. Peptide antibodies made to conserved ncd motor domain sequences also recognized MC6-A623. The affinity of MC6-A623 (under the conditions tested) for microtubules was less than MC6. Most interestingly, under the conditions tested, MC6-A623 did not exhibit an increased ATPase rate in the presence of microtubules, a hallmark of the kinesin family of microtubule motor proteins. Analysis of the published ncd crystal structure, other motor protein sequences, and the experimental results of the mutagenesis of arginine residue 623,

suggest that this residue is involved in the binding of MC6 to microtubules.

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Table of Contents

Title Page	 i
Abstract	 ii
Acknowledgments	 iv
Table of Contents	 v
List of Figures	 vii
List of Tables	 viii

Literature	Review	and	Project	Introduction	1
Intr	oduction	to Pro	ject		19

Materials and Methods	25
Site directed mutagenesis and screening	26
Expression of MC6 mutant proteins	31
Western Blotting	32
Motor Purification	32
Preparation of Microtubules	32
Quantitation of Microtubule Binding	33
Steady state ATPase assays	33

Results	Results	35
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Discussion	. 67
Literature Cited	. 73
Curriculum vitae	87

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List of Figures

Figure 1: Schematic of ncd protein	15
Figure 2: Diagram of MC6	22
Figure 3: Amino acid sequence alignment of kinesin-like proteins and dyneins	24
Figure 4: Diagram of site-directed mutagenesis procedure	28
Figure 5: Mutant MC6 protein sequences generated by PCR mediated mutagenesis	38
Figure 6: First round of PCR mediated mutagenesis	40
Figure 7: Second round of PCR mediated mutagenesis	45
Figure 8: DNA sequences of generated mutants	47
Figure 9: Overexpression of MC6 mutant proteins	50
Figure 10: Solubility of MC6 mutant proteins	52
Figure 11: Western blot of MC6 mutant motor proteins	54
Figure 12: S-sepharose purification of mutant proteins	56
Figure 13: Binding profiles of MC6 and MC6-A623	59
Figure 14: Mt-stimulated ATP hydrolysis rate as a function of ATP concentration	61
Figure 15: Mt-stimulated ATP hydrolysis rate as a function of Mt concentration	64
Figure 16: Basal ATP hydrolysis rate as a function of motor concentration	66

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. .

List of Tables

Table	1:	Prime	rs for	site	-directed	mutagene	esis	30
Table	2:	First ro	und	PCR	predicted	product	sizes	42

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Background and Project Introduction

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Introduction to microtubule based motor proteins

Microtubule-based motor proteins convert the chemical energy created by ATP hydrolysis into directional movement along microtubules (Mts). These motors proteins are involved in such cellular processes as axonal transport, cell division, meiosis, mitosis, and organelle transport. Functionally, Mt-based motor proteins must be able to selectively bind cargo, move in the correct direction along Mts, and convert the chemical energy of ATP hydrolysis into mechanical work. There are currently two families of Mt-based motor proteins, dyneins and kinesin-like proteins (KLPs). In order to effectively discuss MT-based motor proteins it is first necessary to examine the microtubule "roadways" these motors utilize.

Microtubules are long polymers composed of head to tail assembled tubulin heterodimers. These 100 kD dimers are composed of α - and β -tubulin subunits, closely related polypeptides of 440-450 amino acids. Mts assemble spontaneously *in vitro* from tubulin heterodimers into 25 nm circumference cylinders typically containing 13 protofilaments of tubulin dimers arranged parallel to the cylinders long axis (Walker and Sheetz 1993). The ordered array of head-to-tail heterodimer assembly confers the Mts with an intrinsic polarity. This polarity is evident in the differing assembly rates for each end of the Mt. The "plus" end of the Mt assembles at a 2-3 times faster rate than the "minus" end (Kirschner and Mitchison 1986). Recently, studies of microtubules using antibodies to α tubulin (Fan, Griffiths et al. 1996) and using fluorescent GTP labeled beads (Mitchison 1993) have determined the plus end of the microtubule to be composed of β -tubulin subunits. Mt-dependent motor proteins can be classified based on their direction of movement along Mts, either "plus" end directed or "minus" end directed.

Within cells, Mts are a component of the cytoskeleton. In the "typical" nondividing cell Mts are arranged with their minus ends anchored to a centrosome and their plus ends radially arranged toward the exterior of the cell. In dividing cells, the Mt composed spindle fibers are arranged with their plus ends overlapping at the mid-line of the cell and their minus ends converging at the spindle poles. Finally, in neuronal cells, the microtubules are ordered in the axon parallel to the axon's long axis and with their minus ends pointing toward the cell body. The morphology of the Mt cytoskeleton is important when considering the function of a given Mt-based motor protein. For instance, all dyneins isolated to date have exhibited minus end directed motility and therefore, could function as retrograde but not anterograde transporters in neuronal cells.

Studies of Mt-associated proteins (MAPs) have indicated that these proteins bind negatively charged residues at the C-terminus of the tubulin subunits (Maccioni, Serrano et al. 1986; Maccioni, Rivas et al. 1988; Henriquez, Cambiazo et al. 1996). Mt-based motor proteins could also utilize these acidic residue charge interactions for Mt-binding.

The dynein family of Mt-dependent motor proteins

Axonal dynein, the first and founding member of the dynein motor protein family, was discovered in 1963 (Gibbons 1963). Axonal dynein is responsible for generating bending movements of cilia and flagella (Porter and Johnson 1989) and is located in the internal flagellar structure, the axoneme. The axoneme is composed of two inner microtubules surrounded in a parallel cylinder by nine doublet microtubules. Dyneins are bound to the outer doublet microtubules and translocate along adjacent microtubules; due to the structural constraints of the axoneme, this translocation results in bending. Each doublet Mt has outer dyneins bound to it near the outside edge of the Mt ring, and inner

dyneins bound to the interior of the Mt ring. Outer dynein has three globular heavy chain domains (>400 kD) while the inner dynein has two or three head domains. The heavy chain domains create the head region and stalk region of the dynein. In association with the heavy chains of the dynein are >2 intermediate chains (55-125 kD) and 4-8 light chains (~20 kD)(Witman 1992).

Cytoplasmic dynein was isolated after axonemal dynein and is involved in retrograde axonal transport, positioning of the golgi apparatus, chromosome segregation during prometaphase, and the movement of daughter nuclei at the end of cell division (Vallee and Shpetner 1990; McIntosh and Pfarr 1991; Skoufias and Scholey 1993; Walker and Sheetz 1993; Asai 1996). It is composed of 2 globular heavy chains (>400 kD), 2-3 intermediate chains (70-74 kD), and 4 light chains (40-60)(Vallee, Wall et al. 1988).

Dyneins have their ATP hydrolysis, ATP binding, and Mt binding regions contained in the heavy chains. (Asai 1996). All dyneins discovered are minus end directed motor proteins (Asai 1996). The N-terminal and central region of the heavy chains are conserved among dyneins while the C-terminal region is not, suggesting the N-terminal and central regions are involved in conserved functions while the C-terminal region could be involved in modulating the functions of each dynein isoform (Schoer 1994). The functions of the light and intermediate chains are not known, although it has been proposed they are involved in cargo interaction and motility regulation.

Kinesin-like Proteins

The second family of Mt-based motor proteins is the kinesin-like proteins (KLPs). These proteins exhibit a 35-40% sequence identity in their motor domains and exhibit microtubule stimulated ATPase activity. Kinesin-like proteins have three major structural

motifs: an approximately 340 amino acid motor/head domain that contains a conserved ATP-dependent microtubule binding region and a nucleotide binding region, a stalk region that is predicted to form an α -helical coiled-coil, and a non-motor tail region (Goodson, Kang et al. 1994). The best studied KLP is, of course, kinesin.

Kinesin was first identified in squid axoplasm as possibly being involved in anterograde transport. Researchers noticed that the addition of AMP-PNP, a nonhydrolyzable analog of ATP, to squid axoplasm induced tight binding of organelles to axonal Mts (Vale, Reese et al. 1985). Native kinesin was isolated by this method. Subsequent screenings have shown kinesin to be present in numerous organisms and cell types (Saxton, P et al. 1988; Hollenbeck 1989).

Molecular characterizations of kinesin have shown it to be composed of a heterotetramer of 2 light chains of 50-80 kD and two heavy chains of 110-135 kD (Bloom, Wagner et al. 1988; Kuznetsov, Vaisberg et al. 1988). Electron microscopy has shown kinesin heavy chains form a homo-dimer mediated by a stalk region α -helical coiled-coil (Hirokawa, Pfister et al. 1989; Scholey, Heuser et al. 1989) and to have the characteristic three domains: a C-terminal tail region, a coiled-coil stalk, and a N-terminal motor domain. (Yang, Laymon et al. 1989). Kinesin light chains are tightly associated with the heavy chains (Kuznetsov, Vaisberg et al. 1988; Gauger and Goldstein 1993) and, like kinesin heavy chains, are highly conserved among kinesins found in different organisms(Cyr, Pfister et al. 1991). Although kinesin light chains are not necessary for motility (Yang, Saxton et al. 1990), they are important for regulation of ATPase activity and therefore, possibly movement (Hackney, Levitt et al. 1991; Hackney 1992). It is assumed that kinesin light chains are involved in kinesin's interaction with cargo.

The discovery of kinesin led to the search for other microtubule motor proteins. Currently, at least sixty kinesin-like proteins have been identified. The methodologies for identification of these proteins range from analysis of mutant phenotypes in the cases of Kar3 (Meluh and Rose 1990) and bimC (Enos and Morris 1990) to the use of conserved kinesin motor domain degenerate primers for PCR screening (Endow and Hatsumi 1991; Stewart, Pesavento et al. 1991). Phylogenic analysis of the motor domains of putative motor proteins have led to the classification of these proteins into 7 subfamilies. Each subfamily is composed of motor proteins with related cellular functions and molecular morphologies (Moore and Endow 1996).

Kinesin and kinesin like proteins have been extensively studied for their roles in neuronal cells. A mature neuronal cell has a long axon that lacks protein synthesis machinery and therefore, most of the proteins present at the distal end of the axon must be transported from the cell body. Kinesin has been shown to be associated with vesicles that accumulate on the proximal side of ligated mouse nerve axons and is therefore suspected of being a anterograde axonal transporter (Hirokawa, Sato et al. 1991). KIF3A and KIF2 have also been shown to be almost exclusively expressed in murine brain tissue and have been linked to anterograde transport in murine neuronal cells (Kondo, Sato-Yoshitake et al. 1994; Noda, Sato-Yoshitake et al. 1995).

Besides neuronal cell functions, KLP's have been implicated in a variety of mitotic and meiotic functions. Immunodepletion experiments and phenotypic analysis in yeast of the bimC subfamily of motor proteins suggest they play a role in the proper separation of spindle poles during prophase as well as early maintenance of the spindle pole during mitosis (Enos and Morris 1990; Hoyt, He et al. 1992; Saunders and Hoyt 1992; Sawin,

LeGuellec et al. 1992; Heck, Pereira et al. 1993). The bimC subfamily of KLPs have been found in a wide variety of organisms, bimC in Aspergillus nidulans (O'Connell, Meluh et al. 1993), CIN8 and KIP1 in Saccharomyces cerevesiae (Roof, Meluh et al. 1992), cut7 in Schizosaccharomyces pombe (Hagan and Yanagida 1992) and KLP61F in Drosophilia melanogaster (Heck, Pereira et al. 1993). All bimC proteins are involved in spindle assembly or function and, of the ones assayed, are associated with the spindle in vivo (Hagan and Yanagida 1992; Hoyt, He et al. 1992; Sawin, LeGuellec et al. 1992). Interestingly, in Saccharomyces cerevesiae, two members of the bimC subfamily, CIN8 and KIP1, play redundant roles; only the absence of both of these motors results in defective spindle pole formation (Roof, Meluh et al. 1992). Further this double mutation is partially restored to a normal phenotype by the inactivation of the KAR3 minus end directed motor protein (Saunders and Hoyt 1992), suggesting the pre-anaphase spindle in Saccharomyces cerevesiae is maintained by a mixture of plus and minus end directed motor proteins. A similar synergy has been found in mammalian cells, spindle pole formation depending upon the actions of plus end directed Eg5, minus end directed cytoplasmic dynein, and minus end directed activity associated with NuMA (nuclear protein that associates with the Mitotic Apparatus) (Gaglio, Saredi et al. 1996)

KAR3, unlike most KLPs, has its motor domain at the C-terminus, making it a member of the C-terminal motor protein subfamily. There are presently 16 members of this subfamily detected in 10 organisms, including 4 motors isolated from *Arabidopsis thaliana* (Moore and Endow 1996). Members of this subfamily are involved in spindle formation and maintenance. The C-terminal family members KAR3, ncd, klpA and CHO2 all have demonstrated MT-crosslinking and bundling activity *in vitro* (McDonald and Goldstein 1990; Meluh and Rose 1990; O'Connell, Meluh et al. 1993; Kuriyama, Kofron et al. 1995)

suggesting they play a role in spindle formation and stability. Interestingly, KAR3 has also been shown to destabilize microtubules at their minus ends *in vitro*, possibly playing a role in microtubule depolymerization during mitosis (Endow, Kang et al. 1994). Phylogenic analysis of the motor domain of all isolated motor proteins with C-terminal heads have placed them in the same subfamily, indicating that all C-terminal KLPs have a common Cterminal motor protein ancestor (McDonald, Stewart et al. 1990; Endow, Kang et al. 1994; Kuriyama, Kofron et al. 1995).

Mechanochemistry of Force Generation

Investigations into the mechanochemistry of force generation among KLPs have focused on kinesin. Kinesin generates movement by a nucleotide dependent crossbridge cycle with Mts (Scholey, Cohn et al. 1989; Cohn 1990). The kinesin head has four states with respect to nucleotide. It has a nucleotide free state, an ATP bound state, an ADP+Pi bound state, and an ADP bound state. Kinesin moves along Mts through a cyclic process of nucleotide binding, nucleotide hydrolysis, and product release (Romberg and Vale 1993). The rate limiting step in this cycle occurs after hydrolysis of the bound ATP, either in the release of the Pi or the release of the ADP (Gilbert, Webb et al. 1995). In the absence of Mts, kinesin hydrolyzes ATP at a very reduced rate.

The affinity of kinesin motor domains for microtubules varies dramatically depending on the bound nucleotide. Without nucleotide the kinesin motor domain has a high affinity for Mts. With the non-hydrolyzable ATP analog AMP-PNP bound, kinesin binds tightly to Mts. When kinesin is bound to ADP, it has a very weak affinity for Mts (Hirose, Lockhart et al. 1995).

Using a construct of kinesin that contains just the motor domain, investigators have discerned by negative stain electron microscopy that a spike projecting from the globular head of kinesin is in different orientations depending on the bound nucleotide. This spike is perpendicular to the Mt when kinesin is bound to a Mt and AMP-PNP and when kinesin is bound to a Mt without nucleotide. The spike is at a ~45° angle toward the plus end of the microtubule when kinesin motor domain is bound to ADP (Hirose, Lockhart et al. 1995).

Recently, the crystal structure of the kinesin head domain and the ncd head domain complexed with MgADP were obtained (Kull, Sablin et al. 1996; Sablin, Kull et al. 1996). Tertiary structure alignments of the kinesin head domain with myosin motor domains have highlighted structural motifs common to the two motors. KLPs and myosins might utilize similar strategies for the conversion of chemical energy to mechanical work. Myosins also has been found to display a spike that switches its angle with respect to its roadway in different nucleotide hydrolysis states (Uyeda, Abramson et al. 1996). However, unlike KLPs, myosins dissociate from their actin roadways upon the addition of AMP-PNP (Trybus and Taylor 1982).

The same conserved structures from myosin and the kinesin-like proteins are found in the architecture of G-proteins. G-proteins sense the hydrolysis state of bound nucleotide and adjust their conformation, and therefore their affinities for target proteins, accordingly (Bourne, D.A. et al. 1990; Bourne, D.A. et al. 1991).

The next series of investigations into how KLPs generate motility is at the level of motor interaction with microtubules. Decoration studies with kinesin head domains have established a stoicheometry of one motor head per tubulin heterodimer (Walker 1995; Sosa and Milligan 1996). Crosslinking studies suggest that kinesin motor heads interact with both tubulin subunits (Walker 1995). Mechanical measurements, as well as physical

limitations on how kinesin can walk along a microtubule have concluded that it steps in increments of 8 nm (Svoboda, Schmidt et al. 1993; Howard 1996). This distance corresponds to the span between equivalent positions on tubulin heterodimers.

The dimeric nature of the kinesin heavy chains is the next consideration. Single kinesins has been observed in motility assays to move along microtubules for several hundred steps without detaching (Howard, Hudspeth et al. 1989; Block, Goldstein et al. 1990). This means that the kinesin molecule must remain attached to the microtubule for a large majority of its walk, or else Brownian motion would cause single kinesin molecules to quickly detach from the Mts. This is in contrast to myosin, which stays attached to actin for only a short period of its nucleotide hydrolysis cycle and require numerous myosin molecules to perform the task of translocation along actin polymers (Spudich 1994). An attractive model for keeping kinesin attached to the microtubule is a processive "hand over hand" model. This model states that each head of the kinesin dimer consecutively binds and releases the microtubule, coordinately interacting so a single head is always bound to the microtubule. By biasing the head binding so that each head only binds the tubulin dimer towards the plus end of the Mt, kinesin can achieve directional movement. In one set of experiments that suggest cooperatively between kinesin heads, researchers saturated kinesin dimers with ADP and then added Mts or Mts and ATP. In the first experiment 50% of the ADP was released very quickly following addition of Mts (Hackney 1994). The other 50% of the ADP was released at a rate similar to the rate of release in the absence of Mts. In the second experiment where ATP was added along with Mts, 100% of the ADP was released upon Mt+ATP addition. Further, when monomeric kinesin head constructs were used and saturated with ADP, 100% of the ADP was released immediately upon Mt addition (Hackney 1994). This study, taken with studies that indicate 1) ADP release is

promoted upon Mt binding and 2) in the absence of bound nucleotide kinesin binds Mts tightly (Hirose, Lockhart et al. 1995) suggests the following mechanism for coordinating the binding and release of the two heads: the binding of one of the heads of kinesin to Mts upon loss of ADP prevents the other head from binding Mts (which would promote ADP loss). Further, if ATP can bind to the head attached to the Mt then ADP release from the unattached head is promoted (Howard 1996).

Overall, the current evidence can suggest a model for KLP motility. A kinesin heterodimer walks along the microtubule along a single protofilament in 8 nm step increments. Each head using the energy of ATP hydrolysis to switch between high and low Mt binding affinity state conformations and signaling and biasing the other head's direction of travel. Resulting in a tightly bound processive walk along the microtubule.

Ncd: A minus end directed KLP

The history of ncd can be traced back to investigations of a *Drosophila simulans* mutant, *ca* (claret). This mutant expressed the claret eye color and had a high frequency of meiotic chromosome non-disjunction and loss. Further, maternal chromosomes in early mitotic embryonic divisions showed high degrees of loss (Sturtevant 1929). A *Drosophila melanogaster* mutant produced by X-radiation was recovered with the same phenotype in 1952 (Lewis and Gencarella 1952) and was named claret non-disjunctional (*cand*).

The existence of ca mutants that do not have the non-disjunctional phenotype and the discovery of non-claret disjunctional (ncd), an allele of ca^{nd} that exhibited the chromosomal distribution problems (Lewis and Gencarella 1952) but not the claret eye color, led researchers to look for two separate genes, ca and ncd. Molecular examination of *Drosophila melanogaster* mutants led to the isolation of a 2.2 kb mRNA that was not expressed in the *ncd* mutants (Yamamoto, Komma et al. 1989). Northern blot analysis of this mRNA showed its expression to be ovary-specific in females (Yamamoto, Komma et al. 1989).

Subsequent DNA sequencing of the ncd gene and analysis of the sequence uncovered a high degree of homology to the motor domain of kinesin (Endow, Henikoff et al. 1990). Concurrently, investigators using degenerate primers to conserved regions of kinesin-like protein motor domains isolated ncd from a *Drosophila* embryonic cDNA library (McDonald and Goldstein 1990). Bacterially expressed ncd protein is composed of 700 amino acids (the predicted amino acid length), has a M_r of 77,500, and can be divided into three domains based on the kinesin-like protein model (Endow, Henikoff et al. 1990). The N-terminal 212 amino acids are highly basic with a pI of 12.2 and a 10.4% proline content. The sequence of the central regions suggests a α -helical coiled-coil involved in homo-dimerization of ncd. The C-terminal domain, based on sequence homology to kinesin motor domain, contains the putative ATP-binding and Mt-binding regions (Yang, Laymon et al. 1989; Endow, Henikoff et al. 1990). The C-terminal 320 residues are 41% identical in sequence to the kinesin motor domain (Endow, Henikoff et al. 1990; McDonald and Goldstein 1990) (Figure 1)

Bacterially expressed ncd protein coated on glass coverslips for motility assays translocates Mts with their plus-ends leading, indicating ncd is a minus end directed motor protein (McDonald, Stewart et al. 1990; Walker, Salmon et al. 1990). This result was highly unexpected because of ncd's high homology to the plus-end directed kinesin motor domain. Although other minus end directed KLPs were subsequently identified, but the mechanism by which ncd translocates in the opposite direction to kinesin is still not known. Ncd translocates Mts with a velocity of ~5 μ m min ⁻¹ compared to kinesin's velocity of ~30 μ m min ⁻¹ and axonal dynein's velocity of 200-500 μ m min ⁻¹. Using curved Mts grown from fragments of outer doublets dissociated from axonemes, ncd was observed rotating these Mts at the rate of one complete rotation every 6.1±2.6 s (Walker, Salmon et al. 1990). In the absence of ATP large bundles of microtubules were observed to form. Negative stain electron microscopy of these aggregates suggested the ncd protein was both forming bridges between Mts and forming MT-ncd-ncd-MT crosslinking (McDonald, Stewart et al. 1990).

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Figure 1: Schematic of ncd protein.

Structure of ncd Mt-based motor protein. Structure of ncd based on sequence alignments with other KLP's, bioinformatic sequence analysis (Endow, Henikoff et al. 1990), and rotary shadow electron microscopy (Chandra, Salmon et al. 1993). Ncd can be divided into three domains, a ~200 amino acid highly basic N-terminal tail domain, a central stalk that forms an α -helical coiled-coil, and a C-terminal motor domain containing Mt binding and ATPase domains.





Ncd appears to bind in the same region of the Mt as kinesin (Lockhart, Crevel et al. 1995) Further, decoration studies have shown ncd, like kinesin, has a binding stoichiometry of one motor head per tubulin dimer (Walker 1995; Sosa and Milligan 1996). Chemical crosslinking studies have indicated ncd interacts with both the α - and β -tubulin subunits (Walker 1995).

Truncation studies have shown the 204 residue N-terminal tail of ncd, when bound to a glass coverslip, exhibits ATP-independent binding and bundling of microtubules (Chandra, Salmon et al. 1993). An ncd construct of amino acids 209-700 (containing part of the predicted stalk and the complete motor domains) caused erratic one dimensional diffusion of Mts in a coverslip motility assay. However, upon the addition of a 26 kD glutathione S-transferase (GST) protein to the N-terminal end of this ncd construct, directional translocation of microtubules was restored (Chandra, Endow et al. 1993; Chandra, Salmon et al. 1993) suggesting the inactivation of motility was due to ncd denaturation upon the glass coverslip. The ability of GST-fused N-terminal truncations of ncd to directionally translocate Mts was lost upon removal of amino acids 295-333, a region overlapping the C-terminal end of the stalk and the beginning of the conserved Cterminal motor domain. MC6, a ncd construct containing amino acids 333-700, does not support directional movement when attached to GST. MC6 does exhibit MT-stimulated ATPase activity, a hallmark of kinesin-like proteins. Interestingly, the Mt-stimulated ATPase rate for MC6 is 10 times greater than that of ncd (Chandra, Endow et al. 1993; Chandra, Salmon et al. 1993) suggesting MC6 is lacking the domain necessary for modulation of ATPase activity. Hydrodynamic analysis of C-terminal constructs have correlated ability of proteins to translocate Mts with their existence as dimers (Chandra, Endow et al. 1993).

Evidence from truncation studies suggests the domains responsible for MT binding and ATP binding are located in the 333-700 C-terminal predicted motor domain of ncd. The ability to translocate microtubules requires part of the stalk region of ncd between amino acids 295-333. This region is also required for ncd to exist as a dimer (Chandra, Endow et al. 1993).

Recently, the motor domains of both ncd and kinesin complexed to MgADP have been examined by X-ray crystallography (Kull, Sablin et al. 1996; Sablin, Kull et al. 1996). A remarkable structural similarity was discovered between the catalytic domain of myosin and the motor domains of kinesin and ncd. Seven out of eight core β -sheet strands and all six core α -helices of the kinesin and ncd motor domains overlap with corresponding elements in myosin (Kull, Sablin et al. 1996). The structural relatedness of myosin and kinesin proteins had evaded non-spatial comparisons of the motors because of the presence of large inserts of amino acids in myosin not present in KLPs. The ncd-MgADP crystal structure shows an eight stranded β sheet flanked on both sides by three α -helices. Separate from this core is 3 antiparallel β sheets that form the nucleotide binding lobe (Sablin, Kull et al. 1996). This structure is almost identical to the structure obtained for the kinesin motor domain, the largest differences being in the surface loops surrounding the nucleotide-binding cleft and other regions of the protein. Electrostatic analysis of ncd and kinesin crystal structures reveal these motors to have two charged surfaces, a mostly negative face in the nucleotide binding region and a positively charged face that could be involved in Mt binding. The distribution of positively charged clusters on the putative Mtbinding face is conserved between kinesin and ncd, suggesting the two motors sit on the Mt in the same manner. The location of conserved residues, the spatial similarity with the actin binding site in myosin, and the conserved structures between kinesin and ncd are

strong evidence that amino acid residues 483-493 and 620-623 are involved in Mt-binding (Sablin, Kull et al. 1996).

The ATP binding sequence identified from truncation studies contains four sets of conserved residues. Crystallographic studies show these loops are involved in interactions with the adenine ring and the nucleotide phosphates (Kull, Sablin et al. 1996; Sablin, Kull et al. 1996). Comparison of the nucleotide binding pocket of ncd and three proteins representative of different G-protein classes reveals spatial alignment of four conserved motifs among the G-proteins and ncd. The functional similarities between G-proteins and ncd suggest these residues are important in sensing the hydrolysis state of the bound nucleotide and modifying ncd's conformational state with respect to the nucleotide's state (Kull, Sablin et al. 1996). Unfortunately, the C-terminus of ncd was too disordered in the crystal structure for analysis.

The crystal structures of kinesin and ncd strongly bolsters claims of functionality assigned to residues for microtubule binding and ATP binding. As well as further outlining a possible area of investigation for how ncd uses ATP to translocate along Mts.

Ncd in vivo

The hunt for the role of of ncd during meiosis and early mitosis started with examination of *Drosophila melanogaster* mutants. Ncd deficient mutants exhibit diffuse/irregular early metaphase I spindles. Mid-metaphase I and anaphase I spindles are diffuse, broad, or multipolar. In mutant oocytes the defective spindle randomly segregates chromosomes, accounting for the frequent loss of chromosomes during meiosis in these mutants (Hatsumi and Endow 1992).

Polyclonal peptide antibodies have been raised against ncd using non-conserved Cterminal and N-terminal sequences. Early *D. melanogaster* embryo cells were fixed and the spindles isolated. Antibodies to ncd reacted with the spindle in all stages of meiosis and to early mitotic spindles. Mitotic spindles from 24 hour old embryos did not react with the antibodies (Hatsumi and Endow 1992).

Interestingly, researchers found that a *D. melanogaster* cell line reacted with ncd antibodies during mitosis even though the cell line was derived from cells of the late embryo which do not react with the antibodies. Punctate staining of these cells were observed during prometaphase, localized over the chromosomes. The spindle stained during metaphase and anaphase. During telophase the midbody region stained. The antibody stain became diffuse over the cytoplasm in early post cytokinesis. During interphase the anti-ncd antibody did not bind (Hatsumi and Endow 1992). The pattern of staining in the *Drosophila* cell line indicates there is both control of expression of the ncd protein and control of ncd's targeting.

Site-directed Mutagenesis of ncd construct MC6

In order to further characterize ncd we have employed site-directed mutagenesis techniques to alter specific residues in the ncd motor domain. Site-directed mutagenesis of the kinesin heavy chain (KHC) was successfully used to produce mutations in the ATP binding motif of KHC which resulted in a kinesin mutant that bound Mts in a rigor-like manner *in vivo* (Nakata and Hirokawa 1995). Ncd⁴, a naturally occurring mutant of ncd, was shown to have a point mutation in its putative Mt binding domain that changes a highly conserved valine into phenylalanine (Komma, Horne et al. 1991). Analysis of ncd⁴ has

shown it to have significantly different Mt affinities and a reduced Mt stimulated ATPase rate when compared to wild type ncd (Moore, Song et al. 1996).

In order to facilitate manipulations of the ncd head domain sequence for mutagenesis, a truncated ncd sequence was used. MC6, which was discussed earlier in the text, contains amino acids 333-700 of the full length ncd sequence (Figure 2). Previous studies have shown that MC6 has a similar basal ATPase rate to ncd but has a >10 times higher MT stimulated ATPase rate. MC6 has also been shown to tightly bind Mts in the presence of AMP-PNP (Chandra, Salmon et al. 1993).

Three residues in MC6 were chosen for site-directed mutagenesis. The first one is glutamic acid residue 656. This residue is within 40 amino acids of the C-terminus of MC6 and is believed to be incorporated in an α -helix located in the ATP-binding lobe region (Sablin, Kull et al. 1996). Motor sequence alignments of various KLPs and dyneins have shown glutamic acid residue 656 of ncd to be part of the sequence PxxxxxExxxL which is highly conserved among KLPs (Figure 3). This residue was changed into a positively charged lysine and an uncharged glutamine.

The next residue, proline 649 is another member of the conserved PxxxxxExxxxL sequence. It is believed to reside in a loop connecting a β -strand and an α -helix also located in the nucleotide binding lobe region (Sablin, Kull et al. 1996). This reside was changed to a methionine and to an alanine.

The final residue, arginine 623, is part of a highly conserved HIPYR sequence located within 100 amino acids of the C-terminus of MC6. Tertiary alignments of MC6 with the head domain of myosin also suggest arginine 623 is located in the Mt binding region of MC6 (Kull, Sablin et al. 1996; Sablin, Kull et al. 1996). Figure 2: Diagram of MC6.

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MC6 protein consists of amino acids 333-700 of the full length ncd. It contains the putative Mt binding and ATP binding domains.

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MC6 (aa 333-700)



Figure 2

Figure 3: Amino acid sequence alignment of kinesin-like proteins and dyneins.

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Alignment performed on motor domains of 16 kinesin-like proteins and 8 dyneins. Boxed amino acids PxxxxxExxxxL are conserved in amino acid type and location among both dyneins and KLPs. Site-directed mutagenesis was performed on the conserved proline and glutamic acid shown.

A	Chlamydomonas alpha dynein (fragment) Chlamydomonas beta dynein (2017-2042) Sea urchin ($A.c.$) beta dynein (1950-1975) Sea urchin ($T.g.$) beta dynein (1950-1975) Yeast cyto. dynein (1894-1919)	A A I V	F F F F F		T T T T	M M M L	NNNN	P P P P P	G G G G G	Y Y Y Y Y	I P A A N	66666	RRRR	A A T T S	E E E E E	L L L	EEEEE	S S Z Z Z	L L L L	K K K K K	A A A A K	LLLS	F F F F	R R R R R R	P P P E	I V C C F
В	Drosophila KHC (306-331) Ncd (628-653) Nod (293-318) klpA (729-754) Kif3 (318-343) Kar3 (696-721) Unc-104 (320-345) Eg5 (325-350) bimC (389-414) KatA (745-770) KRP85 (315-340) KRP95 (fragment)		VFLFCFL-IFCV	\square	C Z C M Z Z A H H Z Z Z	C > L > M M	\$ \$ \$ \$ \$ G \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$ \$		AFHLASAAADAA	SQQQDSDSRPES	FDCAYSIISTYY	Z Z O Z Z Z Z Z Z D Z Z	EFLLYIFLLAYF	S Q S S D N D E E G D D		T S T T T T T T S T T	SKSTSNSSSCST	T S T S T S T T T S T T		DRRKRRRDDRRR	人人と人人と人とととと	GAGAAAAAAAAA	RATTNSDNFANN	RSSKRKRRRRRR	A V A V A A V A A	X X X X X X X X X X X X X X X X X X X
Ċ	Dictyostelium cyto. dynein (2068-2093) Drosophila cyto. dynein (1999-2024) Rat cyto. dynein (2012-2037)		F F	V 1 1	T T T	M M	Z Z Z	P P P	G G G	Y Y Y	A A A	G G G	R H R	s s s	N N N	L L	D D D	Z Z Z	L L	K K K	к к к	L L L	F F F	R R R	s s s	M L L
D	Kip2 (466-491) cut7 (382-407) MKLP1 (407-432) CENP-E (304-329)	Т М І		C V V C	T T C T	V V V I	D S N T	T S P	R T K V	N N A S	D T E F	A N D D	A L Y E	A E E T	E E L	T T N T	N S Q L	Т Т Q	L L F	R E R A	F Y F S	А А А Т	S A E A	R R V K	A A T Y	K K Q M

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Figure 3

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Materials and Methods

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Site-directed mutagenesis and mutant screening

Each MC6 mutant was generated using overlap extension PCR (McPherson 1991). The procedure used two rounds of PCR to generate a double stranded mutant cassette that was ligated into a truncated pET/MC6 (Chandra, Endow et al. 1993) named pET/MC6 Δ BamH I, creating the MC6 sequence with the desired mutation (Figure 4).

The first round of mutagenesis consisted of two reactions. Reaction A used forward primer 4599 which annealed to upstream MC6 sequences and a reverse mutagenic primer containing the desired nucleotide mutation (Table I). Reaction B consisted of a forward mutagenic primer containing the desired nucleotide mutation and a reverse T7 terminator primer. The products of these reactions were 1% agarose gel purified (Geneclean II by Bio 101 using method of Lewis et al, 1995) and used as template in the second round of PCR with 4599 and T7 terminator as the primers. The forward strand of product A and the reverse strand of product B share the mutagenic sequence and, in the second round PCR reaction, anneal to one another. The final PCR product created contained the desired mutation on both strands. All PCR reactions were carried out with Taq DNA polymerase (Boehringer Mannheim) using 35 cycles (1 min 94°C, 1 min 55°C, 2 min 72°C) with a final 5 min extension at 72°C.

The mutant fragments generated were 1% agarose gel purified, digested with BamH I (Promega) at 37°C, and ligated into pET/MC6ΔBamH I. pET/MC6ΔBamH I was created by digestion with BamH I and subsequent re-ligation with T4 Ligase (Boehringer Mannheim) followed by transformation and gel purification of the appropriate plasmid Figure 4: Diagram of site-directed mutagenesis procedure.

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The procedure used two rounds of PCR to generate a double stranded mutant cassette that was ligated into a truncated pET/MC6 (pET/MC6 Δ BamH I), creating the MC6 sequence with the desired mutation. M1 and M2 represent mutagenic primers from Table 1. Solid black dot represents desired point mutation.


Clone into pET/MC6∆BamH I

Figure 4

Table 1: Primers for site-directed mutagenesis

Custom primers (Gibco BRL) for introducing point mutations into MC6. All primers written in 5' to 3' direction. Bold letters in nucleotide sequences indicate introduced mutations. Last two primers indicated are 4599 and T7 terminator which are used for PCR-mediated site-directed mutagenesis but do not contain nucleotide mutations.

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Mutation site	Nucleotide Sequence	Primer Name
MC6-A656	TGTTTCCAAGCGTCCGTCAAGTCG	A656F
	CGACTTGACGGACGCTTGGAAACA	A656R
MC6-K656	TGTTTCCAAAAGTCCGTCAAGTCG	K656F
	CGACTTGACGGACTTTTGGAAACA	K656R
MC6-Q656	TGTTTCCAACAGTCCGTCAAGTCG	Q656F
	CGACTTGACGGACTGTTGGAAACA	Q656R
MC6-A649	AACGTCTCGGCGTTCCAAGAC	A649F
	GTCTTGGAACGCCGAGACGTT	A649R
MC6-M649	AACGTCTCGATGTTCCAAGAC	M649F
	GTCTTGGAACATCGAGACGTT	M649R
MC6-A623	ATCCCGTACGCGAACTCCAAG	A623F
	CTTGGAGTTCGCGTACGGGAT	A623R
N/A	TCGAATGGCCAAGAACAACAA	4599
N/A	GCTAGTTATTGCTCAGCGG	T7Terminator

Table 1

containing the BamH I deletion. The mutant sequence was ligated into the BamH I site of pET/MC6ΔBamH I and transformed into NovaBlue cells (Novagen-Heat shock transformation using manufacturer's suggested protocol). Resultant colonies were screened by colony PCR using MC6-A656F and T7 terminator primers (Table 1). Colony PCR was performed by boiling 50µl suspensions of single bacterial colonies, centrifuging the suspensions (1 min 12,000 g) and using 10 µl of the supernatant as template for 100 µl PCR reactions. The PCR was performed under standard conditions: [4 min 94°C, 34 cycles (1:10 min 94°C, 1:15 min 50°C, 2:15 min 72°C), 5 min 72°C] using Taq DNA polymerase. Positive plasmids were isolated by Wizard miniprep kit (Promega) and sent to UVA Biomolecular Research center for sequencing.

Expression of MC6 mutant proteins

Mutant plasmids were transformed into BL21(DE3) pLys cells (Novagen). Cells were grown in Luria-Miller Broth at 37°C to OD₅₅₀ of 0.6-0.8 cooled to 22°C and induced with 0.2 mM isopropyl b-D-thiogalactopyranoside (Amersham Life Sciences) for 4 hrs. with rapidly shaking. Cells were pelleted at 3840x g and frozen at -70°. Pellets were lysed by resuspension in 3.5ml /gram cells of AB (20 mM NaPIPES, 1 mM MgSO₄, 1 mM EGTA) supplemented with 0.5 mM DTT (Boehringer Mannheim), 1 mM MgATP (Sigma), and 1 mM PMSF (Sigma). Lysates was supplemented with DNAse I (40 μ g/ml) (Boehringer Mannheim) and 10 mM MgCl2 and incubated on ice for 30 minutes. Lysate samples were centrifuged in a TL-100.3 rotor at 16,000X g (20,000 rpm, 15 min, 4°C) and the supernatants collected and centrifuged again in the TL100.3 at 100,000X g(50,000 rpm, 15 min, 4°C). The high speed supernatant (HSS) were collected and frozen at -70°C. Mutant protein solubility and expression were assessed by running lysate and HSS samples on a 10% SDS-PAGE.

Western Blotting

HSS prepared above were separated by SDS/PAGE. Proteins were transferred to a nitrocellulose membrane by electrophoresis at 100 V for 30 minutes. Membrane was incubated with blotto (5% w/v nonfat dry milk in TTBS(0.3 M NaCl, 20 mM Tris-Cl, 0.05% v/v Tween-20, pH 7.4)) for 1 hour at 22° C with vigorous shaking. Membrane was probed at 22° C with 1:1000 dilution of 1° polyclonal peptide rabbit antibodies against conserved KLP sequences HIPYR and LAGSE (Sawin, Mitchison et al. 1992). Blot was then rinsed with TTBS and incubated for 1 hour at 22° C with a 1:10000 dilution of horseradish peroxidase conjugated anti-rabbit antibody (Pierce). Blot was developed using 1.5 mls each luminol/enhancer solution and Stable peroxide solution (Pierce, following manufactures suggested protocol).

Motor Purification

High speed supernatants prepared above were purified on a 1 ml s-sepharose column (Pharmacia) equilibrated with AB supplemented with 1 mM DTT and 0.5 mM MgATP. The HSS was passed over the column and the motor protein eluted in 3 ml of 0.25 M NaCl dissolved in AB supplemented with 1 mM DTT and 0.5 mM MgATP. Eluted fractions were screened on 10% SDS-PAGE and motor protein concentrations measured by Biorad protein assay reagent using manufacture's protocol.

Preparation of Microtubules

Tubulin was prepared from porcine brains by phosphocellulose chromatography (Walker, O'Brien et al. 1988). For quantitation of microtubule binding assays, microtubules were assembled by incubation at 37°C with MgGTP (Sigma) and taxol. The microtubules were then pelleted at 67,000g and resuspended in AB supplemented with 2X molar concentration of taxol. Microtubule protein concentrations were determined by Biorad protein reagent assay. Concentration measurements were corrected by measuring tubulin concentrations in 6M guanidine hydrochloride. The tubulin extinction coefficient was calculated using the method of Gill and von Hippel (1989).

Quantitation of microtubule binding

Selected motor protein concentrations $(1 \ \mu\text{M}, 2 \ \mu\text{M}, 3 \ \mu\text{M}, 4 \ \mu\text{M}, 5 \ \mu\text{M}, 6 \ \mu\text{M})$ were incubated in 100 μ l reactions containing AB, 1.35 mg/ml bovine serum albumin (BSA)(Biorad), 25 μ M Taxol (CalBiochem), 2 mM MgAMP-PNP (Sigma), 62.5 mM NaCl, and 5 μ M taxol stabilized porcine microtubules (TMTs) for 20 minutes at room temperature. A 10 μ l sample was removed, and the remaining reaction carefully overlaid on a 500 μ l of 25% sucrose AB cushion supplemented with 2.5 μ M taxol and 0.2 mM MgAMP-PNP. The samples were centrifuged at 67,000 X g at 25°C for 10 minutes, the supernatant and sucrose cushion gently washed with 2.5 M NaCl solution to remove nonspecific protein binding, and the supernatant and cushion carefully removed. The 10 μ l of 2X SDS-PAGE loading buffer (SB) was added to the 10 μ L total protein sample and 25 μ l of 1X SB was added to the pellet. Both samples were vigorously vortexed, boiled for 5 min, and 10 μ l of each fraction loaded on a 7.5% SDS-PAGE. The resultants commassie stained gels were scanned while wet on a Microtek MRS 600ZS scanner using Adobe Photoshop software. Known motor protein amounts were loaded on each gel and used as a standard curve. Motor protein concentrations in each sample were determined by analysis with NIH Image software.

Steady state ATPase assays

ATPase activity was measured using a coupled pyruvate kinase-lactate dehydrogenase assay (Huang and Hackney 1994). Assays were performed at 22°C in 500 μ l reactions of 50 mM Tris-Acetate pH 7.4, 2 mM MgCl₂, 3 mM Phospho(enol) pyruvate (Sigma), 0.05-1 mM MgATP, 20 μ M Taxol, 2-5 μ M GTP depleted TMTs, 7.5 U pyruvate kinase (Sigma), and 12.5 U lactate dehydrogenase (Sigma). Reactions were initiated by the addition of 25-100 nM motor protein. The decreased absorbence caused by the conversion of NADH to NAD+ (Sigma) was monitored continuously at 340 nm. Results

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PCR-mediated site-directed mutagenesis was used to create specific mutations in pET/MC6 (Figure 5). The resulting mutant plasmids were sequenced and transformed into bacterial expression cells. Mutant proteins were assayed for their solubility, ability to be recognized by antibodies, ability to be purified by ion exchange, ATPase activities, and their affinity for Mts.

Generation of Site-specific mutations in pET/MC6

PCR was used to incorporate primers containing desired point mutations into both strands of pET/MC6. The PCR reactions were performed in two rounds. The first round involved creating two PCR products containing single strand point mutations in an overlapping region of pET/MC6. The second round involved using the first round products as template to create a PCR product with the desired mutation on both strands.

The first round of PCR-mediated mutagenesis involved two reactions per desired mutant. Each product of the two reactions had the desired mutation, present in one of the primers, incorporated into one end of the PCR product (Figure 6). For each reaction, the first round of mutagenesis produced a major band with few secondary products. The mobilities of the PCR products match the predicted mobilities for the desired products (Table 2). The bands were isolated and purified, generally yielding about 20 ng/ μ l of DNA per 100 μ l PCR reaction.

Figure 5 Mutant MC6 protein sequences generated by PCR mediated mutagenesis.

PCR-mediated site directed mutagenesis was utilized to introduce specific point mutations into MC6. Mutations were generated at three highly conserved residues in the putative Mt binding region of MC6, arginine 623, proline 649, and glutamic acid 656.

Box at top of page representative of MC6 domains. Identified functional domains are labeled. Arrows begin at region of MC6 mutated and point to the region of the amino acid sequences involved in mutagenesis. Bold letters indicate residues altered by mutagenesis. The heavy underline indicates putative Mt binding domain. Directly beneath the amino acid sequence of MC6 are the generated mutants. To the right of each mutant is its designation.

	MC6	
HIPY <u>R</u> NSKLTHLLMPSLGGNSKTLM	IFINVS <u>P</u> FQDCFQ <u>E</u> SVKSL	Construct Name
HIPY <u>A</u> NSKLT		MC6-A623
HIPY <u>A</u> NSKLTHLL <u>T</u> PSL		MC6-A623 /T632
	INVS <u>A</u> FQDC	MC6-A649
	INVS <u>M</u> FQDC	MC6-M649
	CFQ <u>K</u> SVK	MC6-K656
	CFQ Q SVK	MC6-Q656

Figure 5

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Figure 6 First round of PCR mediated mutagenesis.

PCR was used to incorporate primers containing the desired point mutation into single stands of two overlapping PCR products. Figure shows agarose gel electrophoresis of the first round of mutagenesis PCR products. Lane headings indicate primer set used for each reaction (see table 1). Arrow indicates position of 600 base pair marker.



Figure 6

Table 2 First round PCR predicted product sizes.

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Sizes of PCR products from the first round of mutagenesis were determined by agarose gel electrophoresis. All PCR reactions used pET/MC6 as template. Lane heading refers to gel shown in Figure 6. Primer sequences can be found in Table 1.

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Lane	Primer Pairs	Predicted Band Size (bp)	Actual Band Size Match?
1	K656R : 4599	477	Y
2	K656F : T7 terminator	262	Y
3	M649R : 4599	455	Y
4	M649F : T7 terminator	278	Y
5	Q656R : 4599	477	Y
6	Q656F : T7 terminator	262	Y
7	A649R : 4599	477	Y
8	A649F : T7 terminator	262	Y
9	R623R : 4599	375	Y
10	R623F : T7 terminator	361	Y

Table 2

The second round of mutagenesis used the products of the first round reaction pairs as template. In the PCR reaction these products, containing the incorporated mutant primers, anneal to each other and produce the template for the polymerase. Detectable PCR products for MC6-A472, MC6-A621, MC6-A623, MC6-A649, MC6-M649, MC6-K656, and MC6-Q656 were obtained (Figure 7). The major PCR products were approximately 700 bp, the predicted size for the mutant sequences. Few secondary PCR products were observed.

The major products were purified from the gel, digested with BamH I and cloned into pET/MC6 Δ BamH I. Ligation products were screened for the insert by colony PCR, with successful ligations being detected in about 10% of screened colonies (data not shown). Plasmid DNA was isolated from positive colonies and sent to UVA Biomedical Research Center for sequencing.

Mutant Sequence Information

To confirm that the point mutations were present, each plasmid was sequenced from primer 4599 on the leading stand and T7 terminator on the lagging strand. Point mutations were confirmed by their presence on both strands. The mutants generated are shown (Figure 8). MC6-A623/T632 arose from generation of MC6-A623 with a spontaneous mutation present at residue 632. Figure 5 shows the location of the generated mutants and their corresponding amino acid substitutions.

Overexpression and solubility of generated mutants

Confirmed mutants were transformed into *E. Coli* strain BL21 (DE3) pLys expression cells and induced to overexpress the MC6 mutants using IPTG. Room

Figure 7: Second round of PCR mediated mutagenesis.

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The second round of PCR used products of the first round as template and resulted in the incorporation of the desired point mutation into both strands. Figure shows agarose gel electrophoresis of the second round of mutagenesis PCR products. All reactions used 4599 and T7 terminator as reaction primers (Table 1). Lane headings indicate mutation in the template used for each reaction.

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Figure 7

Figure 8: DNA sequences of generated mutants.

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To confirm the presence of point mutations isolated mutant plasmids were sequenced from primer 4599 in the leading strand and T7 terminator primer in the lagging strand. Sequencing was performed by UV a Biomolecular Research Center. Each point mutation confirmed by sequencing both strands. The top strand is the wild type sequence, the bottom strand is the mutant sequence.

MC6 Vs. MC6-Q656

GACTGTTTCCAA**GAG**TCCGTCAAGTC GACTGTTTCCAA**CAG**TCCGTCAAGTC

MC6 Vs. MC6-K656

GACTGTTTCCAA**GAG**TCCGTCAAGT GACTGTTTCCAA**CAG**TCCGTCAAGT

MC6 Vs. MC6-A623

CATATCCCGTAC**AGG**AACTCCAAGC CATATCCCGTAC**GCG**AACTCCAAGC

MC6 Vs. MC6-A623/T632

CATATCCCGTACAGGAACTCCAAGCTGACGCACCTGCTGATGCCCTC CATATCCCGTACGCGAACTCCAAGCTGACGCACCTGCTGACGCCCTC

MC6 Vs. MC6-A649

CATCAACGTCTCG**CCG**TTCCAAGACTG CATCAACGTCTCG**GCG**TTCCAAGACTG

MC6 Vs. MC6-M649

GACCACATCCCGTACAGGAACTCCAAGCTG GACCACATCCCGTACGCGAACTCCAAGCTG

Figure 8

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temperature inductions for four hours resulted in levels of protein expression visually similar to the wild type MC6 expression levels (Figure 9). The solubility of the motors were assessed by consecutive low and high speed centrifugations. Low speed centrifugation of bacterial cell lysates (16,000X g) resulted in undetectable levels of motor proteins, MC6-A623/T632 and MC6-A649 in supernatant fractions. After high speed centrifugation (100,000X g), supernatants had detectable levels of soluble protein for MC6-A623 and MC6-Q656 (Figure 10). MC6-M649 had very low levels of protein that were detected by antibody probes (Figure 11). MC6-Q656 protein solubilities were preparation dependent. The preparation of MC6-Q656 that was purified by centrifugation was soluble in the HSS fraction, but the preparation used for western blotting was not soluble.

S-sepharose Ion exchange chromatography of mutants

MC6 characteristically binds S-sepharose and can be eluted off in 250 mM NaCl, resulting in >95% pure MC6 protein suitable for further protein analysis. S-sepharose purification was utilized to characterize the mutant proteins expressed and to purify mutant protein for further analysis (Figure 12).

MC6 bound S-sepharose and eluted in 250 mM NaCl. MC6-A623 also bound S-sepharose and eluted at 250 mM NaCl. MC6-M649 was not detected in the fractions.

Approximately 1.25 mg of MC6-A623 per gram of cell pellet was obtained from Ssepharose ion exchange purification. The eluted protein contained less than 5% contaminant proteins of various molecular weights (Lane F1 of MC6-A623 gel, Figure 12).

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Figure 9: Overexpression of MC6 mutant proteins.

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Sequenced mutant plasmids were transformed into expression cells and induced to overexpress proteins for four hours. Mutant protein expression is similar to MC6 expression levels. The figure shows SDS/PAGE of lysates of expression cells containing mutant plasmids. Mutant designations are above each gel. Zero and four hour induction times are indicated. Mobility of MC6 is indicated by arrow.

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Induction Times (Hrs)

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Figure 9

Figure 10: Solubility of MC6 mutant proteins.

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To assay the solubility of the mutant proteins, expression cell lysates were subjected to centrifugation at 16,000X g followed by supernatant centrifugation at 100,000X g. Figure shows SDS/PAGE of expressed mutant protein solubility. Constructs designations are above each gel. Lanes: LYS- bacterial pellet lysate; LSS- low speed centrifugation supernatant; HSS- high speed centrifugation supernatant. MC6 standard is indicated by arrow.



Figure 10

Figure 11: Western blot of MC6 mutant motor proteins.

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Mutant proteins were tested for their ability to be recognized by antibodies against KLPs. HSS Supernatants of expression cell lysates were probed with polyclonal peptide antibodies against conserved KLP sequences, HIPYR and LAGSE. Lane headings indicate proteins probed. HIPYR and LAGSE antibodies detected MC6, MC6-M649, and MC6-A623 in the HSS fractions.



Figure 11

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Figure 12: S-sepharose purification of mutant proteins.

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Mutants were assayed for their ability to bind S-sepharose ion exchange resin and elute off in the presence of NaCl. Figure shows SDS/PAGE of S-sepharose purification of soluble mutants. Constructs designations are above each gel. Lanes: H- High speed centrifugation supernatant; FT- column flow thru; W- column wash; F1-3- consecutive 1 ml fractions collected of 0.25 M NaCl column elution buffer; F4-6 consecutive 1 ml fractions collected of 0.5 M NaCl column elution buffer. MC6 standard is indicated by arrow.



H FT W F1 F2 F3 F4 F5 F6

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MC6-A623



H FT W F1 F2 F3 F4 F5 F6

MC6-M649



H FT WF1 F2 F3 F4 F5 F6



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Binding Profiles of MC6 and MC6-A623

Wild type MC6, in the presence of AMP-PNP, a non-hydrolyzable analog of ATP, has a high affinity for Mts. Binding profiles of MC6 and MC6-A623 were generated and compared to determine the relative affinity of MC6-A623 for Mts.

Mt binding assays were performed on MC6-A623 and compared to the wild type MC6. Briefly, motor proteins were incubated with Mts in the presence of AMP-PNP which induces tight binding of MC6 to Mts. The reaction mixtures were then pelleted and motor protein amounts in the pellet determined. By incubating motor proteins with excess amounts of Mts it was determined that only 40% of MC6 and MC6-A623 proteins were capable of binding the microtubules. It is not known why such large fractions of both motors were incapable of binding the microtubules. Samples of the supernatant obtained after centrifugation demostrated that 60% of the Mts were polymerized and thus would pellet.

Motor proteins were assayed for affinity to Mts at a range of concentrations and the resulting data plotted as μ M motor protein in the pellet versus μ M motor protein in the supernatant. Data points were fitted to a rectangular hyperbole and the K_d for each protein obtained. The K_d for MC6 was 0.2 ± 0.1, the K_m for MC6-A623 was 0.3 ± 0.1. Binding saturated at ratios of 0.9 ± 0.1 MC6:1 tubulin heterodimer and 0.6 ± 0.1 MC6-A623:1 tubulin heterodimer(Figure 13).

ATPase assays of MC6 and MC6-A623

ATPase assays were performed using a coupled lactate dehydrogenase-pyruvate kinase reaction (Huang and Hackney 1994). The assays were used to detect the response of MC6-A623 to different concentrations of ATP and microtubules in comparison to wild Figure 13: Binding profiles of MC6 and MC6-A623.

Binding profiles of MC6 and MC6-A623 to Mts in the presence of AMP-PNP were generated in order to characterize MC6-A623's affinity for Mts.

Mt pelleting assays were performed on MC6 and MC6-A623. Data fit to curves to obtain dissociation constants (K_d). MC6 curve is the solid line; MC6-A623 curve is the dashed line. MC6 (solid circular data points) has a K_d of 0.2 ± 0.1 . MC6-A623 (hollow square data points) has a K_d of 0.3 ± 0.1 . Binding saturated at ratios of 0.9 ± 0.1 MC6:1 tubulin dimer and 0.6 ± 0.1 MC6-A623: 1 tubulin dimer.



Figure 13

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Figure 14: Mt-stimulated ATP hydrolysis rate as a function of ATP concentration.

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S-sepharose purified MC6 and MC6-A623 were assessed for their rate of Mtstimulated ATPase activity in various concentrations of ATP. 50 nM of MC6 (black bar) and 50 nM MC6-A623 (gray bar) were assayed for ADP production in the presence of 2 μ M Mts. Bar height indicates motor hydrolysis rate over a 20 min assay period. Standard error is indicated.

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Figure 14

type MC6. In the presence of 2 μ M TMTs, MC6 hydrolyzed ATP at 6.31 ADPmotor⁻¹sec⁻¹ while the rate of MC6-A623 was 0.129 ADPmotor⁻¹sec⁻¹. At 4 μ M TMTs the rate of ATP hydrolysis for MC6 was 5.36 ADPmotor⁻¹sec⁻¹ and for MC6-A623 was 0.115 ADPmotor⁻¹sec⁻¹. The further increase of ATP concentration up to 6 μ M resulted in an ATPase rate of 3.22 ADPmotor⁻¹sec⁻¹ for MC6 and 0.132 ADPmotor⁻¹sec⁻¹ for MC6-A623 (Figure 14).

Increasing levels of TMT concentrations were also assayed while keeping the ATP concentration at 1 mM. The increasing concentrations resulted in increased ATPase rates for MC6 but no detectable rate increase for MC6-A623. For both the variable ATP concentrations and the various TMT concentrations the MC6-A623 concentrations were doubled and assayed for ATPase rate. In all cases the rate of hydrolysis slightly decreased (Figure 15).

Basal rate ATPase assays were performed for MC6 and MC6-A623. Reactions mixtures were heated to 37° C before addition of motor proteins and then allowed to cool to room temperature over the course of the reactions. 100 nM MC6 hydrolyzed ATP at a rate of 0.303 ADPmotor⁻¹sec⁻¹; 200 nM MC6 hydrolyzed ATP at a rate of 0.245 ADPmotor⁻¹sec⁻¹; 100 nM MC6-A623 hydrolyzed ATP at a rate of 0.089 ADPmotor⁻¹sec⁻¹; 200 nM MC6-A623 hydrolyzed ATP at a rate of 0.121 ADPmotor⁻¹sec⁻¹ (Figure 16).

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Figure 15: Mt-stimulated ATP hydrolysis rate as a function of Mt concentration.

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MC6 and MC6-A623 were assessed for their rate of Mt-stimulated ATPase activity in various concentrations of Mts. 50 nM of MC6 (black bar) and MC6-A623 (gray bar) were assayed for ADP production in the presence of 1 mM ATP. Bar height indicates motor hydrolysis rate over a 20 min assay period. Standard error is indicated.

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Figure 15

Figure 16: Basal ATP hydrolysis rate as a function of motor concentration.

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Basal ATPase rates of MC6 and MC6-A623 were determined. MC6 (black bar) and MC6-A623 (gray bar) were assayed for ADP production in the presence of 1 mM ATP and in the absence of Mts. Bar height indicates average motor hydrolysis rate over a 20 minute assay period. Standard error is too small for representation on graph.



Figure 16

Discussion

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We have utilized PCR-mediated site-directed mutagenesis to examine the roles that three different residues play in the motor domain of ncd. This technique has allowed us to specifically create point mutations in the nucleotide sequence of ncd. The resultant proteins were expressed and characterized.

Mutagenesis of glutamic acid 656 into a lysine resulted in insoluble protein. Changing this residue into a glutamine resulted in a protein that was partially soluble. However, a subsequent resulted in no soluble protein. Further analysis of this mutant is needed.

Mutagenesis of proline residue 649 into a methionine resulted in a very slightly soluble protein that was recognized by antibodies to conserved KLP sequences. However, not enough protein was obtainable for further study. Mutagenesis of the proline into an alanine resulted in insoluble protein and no further characterization was possible.

Finally, the mutagenesis of arginine 623 into an alanine resulted in a mutant MC6-A623 protein and a unintentional double mutantion MC6-A623/T632. The double mutantion was not soluble, however MC6-A623 was. MC6-A623 was recognized by conserved KLP antibodies and had a similar S-sepharose binding and elution profile to MC6. Further characterization of MC6-A623 revealed it to have less affinity for Mts than MC6 and did not exhibit Mt-stimulated ATPase activity.

Mutagenesis of glutamic residue 656

Glutamic acid residue 656 is within 50 residues of the C-terminus of MC6. Crystallography has indicated that this residue is part of an α -helix that is located in the nucleotide binding lobe of MC6. This residue is highly conserved among KLPs. Garnier-Robson and Chou-Fasman algorithms applied through the DNASTAR program Protean

68

both indicate that this region is an α -helix as well. The existence and importance of the α helix containing glutamic 656 can further be inferred from the conservation of its residues among both KLPs and dyneins (Figure 3). The Garnier-Robson algorithm predicted the complete disruption of the α -helix with the substitution of a lysine (MC6-K656) and a partial disruption of the α -helix with a glutamine substitution (MC6-Q656). Unfortunately, bacterially over-expressed MC6-K656 was not soluble enough to allow for purification and further study. MC6-Q656, in one preparation, did have about 50% solubility upon purification by high speed centrifugation. No further characterization of MC6-Q656 has been performed. The results of this mutagenic study suggest that the α -helix containing glutamic acid residue 656 is important for solubility of bacterially-expressed MC6 protein.

Mutagenesis of proline residue 649

Proline residue 649 is within 60 residues of the C-terminus of MC6. Crystallographic studies have indicated this residue is involved in a loop connecting a β -strand and an α -helix. Proline residue 649 is absolutely conserved in the head region of over 40 KLP and dynein microtubule motors. The structure of proline makes it an ideal amino acid for hairpin turns. The amino and carboxyl terminus of proline are at 90° to each other, compared to other amino acids which have 180° separation. MC6-M649 has a methionine, a long chain hydrophobic sulfur-containing residue at this position. Presumably this substitution would destroy any structural role served by the highly conserved proline. The same is true of MC6-A649; the alanine substitution also would not be able to serve the same structural role as the proline. It is therefore not suprising, although disappointing, that MC6-A649 was completely insoluble and MC6-M649 was mostly insoluble. It is also interesting to note that the next structural element in the C- terminal direction from the conserved proline is an α -helix that contains the highly conserved glutamine 656 mutated above, as well as numerous other conserved residues. It could be the case that the cause of insolubility created by the proline residue substitution is the same as for the glutamic acid substitution, that is, disruption of the α -helix, either by mutagenesis of the glutamic 656 or by "repositioning" the α -helix by mutagenesis of proline 649.

Mutagenesis of Arginine 623

Arginine 623 is a very polar hydrophilic residue within 80 amino acids of the Cterminus of MC6. This arginine residue is absolutely conserved among 40 KLPs motor domains. Crystallography of the MC6 places arginine 623 in loop 12, which connects α helixes α -4 and α -5. Loop 12 also contains a highly conserved proline at residue 621. According to Kull et al.(1996) tertiary structural alignment of kinesin with myosin showed that all 6 major α -helixes and seven of the eight β -strands that make up the bulk of the kinesin and ncd motor heads align with analogous elements in the crystal structure of myosin. Formerly, sequence alignments of ncd with myosin did not find these similarities because of the presence of two large inserts in the myosin molecule relative to KLPs. One of these inserts corresponds to the placement of loop 12 in the MC6 head domain. The myosin insert is part of the actin binding interface. Furthermore, analysis of the MC6 crystal shows it has two charge faces, a negative charge face involved in nucleotide binding and a positive charge face that could be involved in Mt binding. Other proteins that bind microtubules bind by use of positive charges. The clusters of positive charges on the positive charge face of ncd are spatially conserved with kinesin and can be found in loop 8, loop 9, and loop 12. The structures of ncd and kinesin, along with the similarity to myosin, strongly suggests that loop 12 is involved in Mt binding. Further arginine 623 is, by virtue of its positive charge, conservation between KLPs, and its proximity to a highly conserved proline likely to be involved in Mt binding.

This speculation above is supported by study of MC6-A623. MC6-A623 binds Mts with a less affinity than MC6, which is to be expected with the loss of a positively charged residue in loop 12. More interestingly, however, is the apparent loss of Mt stimulated ATPase activity of MC6-A623. MC6-A623's ATPase rates were not significantly different in the presence of 2, 4, or 6 μ M of Mts or in the total absence of Mts. In contrast, MC6's ATPase rate was stimulated approximately 35 fold in the presence of 6 μ M tubulin versus no tubulin.

An explanation of this result can be gleaned by once again using other proteins as a model. Helix α -4 is connected to loop 12, the site of arginine 623, and is analogous to a structure in myosin that has been shown to change position in different nucleotide binding states. This conformational change might cause the masking of the actin binding interface. Further, α -4 and its N-terminal partner loop 11 have analogous structures in G proteins. These structures, known as switch II, are involved in translating the nucleotide hydrolysis state into changes in the binding affinity of the G-protein for its target proteins. It has been proposed that loop 12, by its spatial association with α -4 is affected by the conformational change that α -4 might undergo in different nucleotide binding states (Kull et al. 1996).

Using all the above arguments I propose the following: Loop 12 is exposed upon ATP binding to MC6, which increases the binding affinity of MC6 for Mts by increasing the exposure of positive nucleotides to the Mt. Then the tight binding of loop 12 to the Mt

71

allows for the either the hydrolysis of ATP into ADP-Pi or for the release of the ADP nucleotide. In the case of MC6-A623 the binding of ATP causes the exposure of loop 12 to the Mt. But, the replacement of the positively charged arginine with a neutral alanine does not leave loop 12 with enough positive charges to tightly bind the Mt, thus the lower affinity of MC6-A623 for Mts. Further, the lack of tight binding prevents either the release of ADP or the hydrolysis of ADP-Pi. In either case the result would be a lack of Mt stimulated ATPase activity.

Site directed mutagenesis was successfully used to empirically confirm the significance of three residues in MC6. Most significantly, this technique and subsequent protein characterizations have suggested arginine 623 is involved in microtubule-ATP binding lobe interactions.

The future course of this research includes cloning MC6-A623 into a full length ncd sequence and testing its motility, further characterization of bacterially expressed MC6-M649 and MC6-Q656, and expression of insoluble MC6 mutants in eukaryotic expression systems.

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