Involvement of the neck coiled-coil in the velocity of UNC104, a kinesin-related motor protein
by Marcia Rebecca Kary

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University
© Copyright by Marcia Rebecca Kary (2001)

Abstract:
There is considerable debate over the mechanism of motility for kinesin-related proteins. Dimeric motors, such as conventional kinesin, are believed to translocate along microtubules in a hand-over-hand fashion. Some members of the UNC104/KIF1 subfamily maybe monomeric, however, and therefore the mechanism of motility would differ. It is hypothesized that the monomers may operate with a lever-arm mechanism, analogous to myosin. Conventional kinesin and UNC-104 quaternary structures have regions of coiled-coils. In UNC-104 the coiled-coils are found in the neck region, while for conventional kinesin they are found throughout the neck and stalk domain. Coiled-coils are responsible for the dimerization of conventional kinesin. The coiled-coils found in UNC-104 are conserved throughout the subfamily. Since UNC-104 is likely monomeric, the conserved coiled-coils may have another function. Current studies have linked the neck coiled-coil to motor velocity.

This research addresses the possible involvement of the monomeric neck coiled-coil in motor velocity. Specifically, we addressed the possibility of intramolecular coiled-coil formation, resulting in a stiff lever arm, which mediates motor motility. Various neck region truncations of UNC-104 were recombinantly expressed and purified and applied to in vitro microtubule motility assays. The motor velocities were analyzed using a Nikon microscope equipped with differential interference contrast optics.

Three constructs were made; U345/GFP, U360/GFP, and U380/GFP. U345/GFP did not contain the neck linker and showed no movement. U360/GFP and U380/GFP displayed similar velocities, with truncations before the neck coiled-coil and after the neck coiled-coil, respectively.

Neck truncations before and after the neck coiled-coil had an insignificant effect on motor velocity. Alternatively, the elimination of the neck linker had a severe effect on motor velocity, similar to results found for conventional kinesin. These results argue against the possibility of a lever arm, leaving open the possibility that UNC-104 and dimeric conventional kinesin have similar motility mechanisms.

INVOLVEMENT OF THE NECK COILED-COIL IN THE VELOCITY OF UNC104,

A KINESIN-RELATED MOTOR PROTEIN

by

Marcia Rebecca Kary

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

In

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2001

N378
K1499

APPROVAL

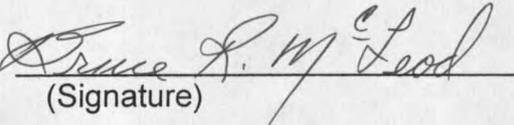of a thesis submitted by

Marcia Rebecca Kary

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.
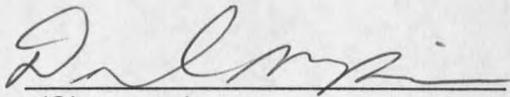
Dr. Daniel W. Pierce _____ 4/23/2001
(Signature)   Date

Approved for the Department of Chemistry and Biochemistry
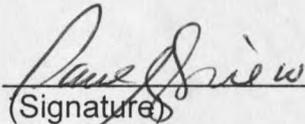
Dr. Paul A. Grieco _____ 4/23/01
(Signature)   Date
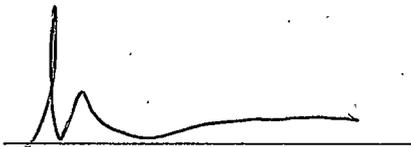
Approved for the College of Graduate Studies

Dr. Bruce R. McLeod _____ 4-23-01
(Signature)   Date

STATEMENT OF PERMISSION TO USE

I dedicate this thesis to my faithful companion Miss Cleopatra, who passed away before seeing me finish. She purred by me during four years of high school, four years of college, two years off, and almost made it the full four years of graduate school. There will be something missing when I receive my degree. I love you kiki.

Thank you daddy for letting me keep her.

TABLE OF CONTENTS

vii

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

ABSTRACT

There is considerable debate over the mechanism of motility for kinesin-related proteins. Dimeric motors, such as conventional kinesin, are believed to translocate along microtubules in a hand-over-hand fashion. Some members of the UNC104/KIF1 subfamily maybe monomeric, however, and therefore the mechanism of motility would differ. It is hypothesized that the monomers may operate with a lever-arm mechanism, analogous to myosin. Conventional kinesin and UNC-104 quaternary structures have regions of coiled-coils. In UNC-104 the coiled-coils are found in the neck region, while for conventional kinesin they are found throughout the neck and stalk domain. Coiled-coils are responsible for the dimerization of conventional kinesin. The coiled-coils found in UNC-104 are conserved throughout the subfamily. Since UNC-104 is likely monomeric, the conserved coiled-coils may have another function. Current studies have linked the neck coiled-coil to motor velocity.

This research addresses the possible involvement of the monomeric neck coiled-coil in motor velocity. Specifically, we addressed the possibility of intramolecular coiled-coil formation, resulting in a stiff lever arm, which mediates motor motility. Various neck region truncations of UNC-104 were recombinantly expressed and purified and applied to in vitro microtubule motility assays. The motor velocities were analyzed using a Nikon microscope equipped with differential interference contrast optics.

Three constructs were made; U345/GFP, U360/GFP, and U380/GFP. U345/GFP did not contain the neck linker and showed no movement. U360/GFP and U380/GFP displayed similar velocities, with truncations before the neck coiled-coil and after the neck coiled-coil, respectively.

Neck truncations before and after the neck coiled-coil had an insignificant effect on motor velocity. Alternatively, the elimination of the neck linker had a severe effect on motor velocity, similar to results found for conventional kinesin. These results argue against the possibility of a lever arm, leaving open the possibility that UNC-104 and dimeric conventional kinesin have similar motility mechanisms.

1

CHAPTER 1

BACKGROUND

Cytoskeletal Motor Proteins

The early hypothesis that transport of materials in cells was accomplished by diffusion is very distant from our current understanding. It is now known that cellular transport of materials is primarily accomplished by cytoskeletal motor proteins. Motor proteins attach to cargoes and move them to various locations in the cell. Vesicular organelle transport, flagellar beating, axonal transport, chromosome segregation, and muscle contraction are a few of the motility processes motor proteins perform (Goldstein, 1993). The mechanisms by which motors interact with and move their cargoes are poorly understood.

Cytoskeletal motor proteins use the chemical energy of ATP hydrolysis to generate force and movement along cytoskeletal filaments (Vale and Fletterick, 1997). The three major types of cytoskeletal filaments are f-actin, microtubules, and intermediate filaments. Actin and microtubules are polar polymers of filament-forming proteins with kinetically and structurally distinct "plus" and "minus" ends (Microtubule interactions, http://blocks.fhcrc.org/~kinesin.html). This polarity enables the directional movement of the motor proteins.

Intermediate filaments are not polar (Goldstein, 1993), and hence are not believed to support cellular transport by motor proteins. There are three superfamilies of motor proteins, the kinesins, dyneins, and myosins. Kinesins and dyneins travel along microtubules, while myosin travels along actin filaments (Goldstein, 1993). Actin filaments and microtubules are both involved in the generation of internal cellular movements and structures as well as cellular attachment to and movement on substrates. Actin filaments, for example, participate in the contraction of muscle and unconventional myosin motors are known to transport vesicular cargo along actin filaments (Discovery of Acanthamoeba Myosin-I, the First Unconventional Myosin, http://blocks.fhcrc.org/~kinesin.html). Microtubules are the main structural and force-generating elements in cilia and flagella and form the frameworks of meiotic and mitotic spindles. They additionally participate in organization of the cytoplasm, where in non-dividing cells they align the nucleus, endoplasmic reticulum, Golgi apparatus, and possibly other organelles.

Microtubules are polymerized from specific nucleating complexes within the centrosome. Most microtubules remain attached to the centrosome, but some are released and transported to specific sites within the cell body (Hoenger et al., 1998). Microtubules have two stages of formation; nucleation and elongation. During "nucleation", α/β tubulin heterodimers associate by both lateral and end-to-end interactions to form a fragment of a microtubule (Figure 1). While this process occurs spontaneously in vitro in solutions containing purified tubulin at

Figure 1:
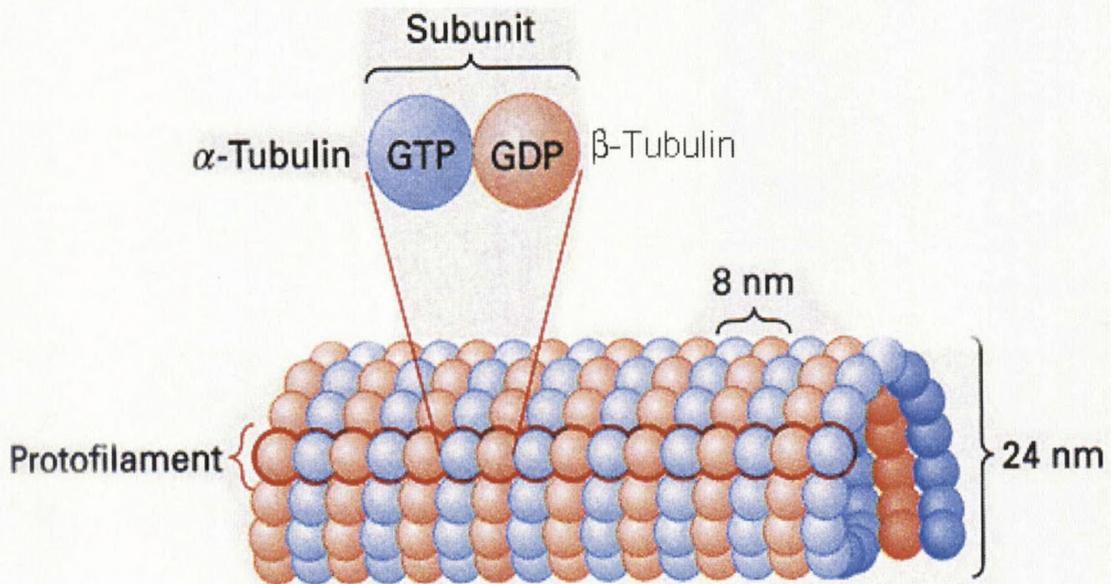Organization of Microtubule Subunits

Figure1:
The organization of α/β tubulin subunits, or dimers, in a microtubule. Each dimer measures 8nm in length. Microtubule formation involves the stacking of tubulin dimers end to end, which form a protofilament. The protofilaments are packed side by side, forming the walls of the microtubule.
(Song & Mandelkow, 1993)

high concentrations, in the cell most microtubules are nucleated by a specific centrosomal complex (the γ-TuRC ring complex) that caps the microtubule minus end and thus causes a microtubule array to form in which the plus ends are distal. Microtubules that release from the centrosome are generally transported with their positive ends leading; thus maintaining the plus-end-distal polarity orientation within the cell (Wang *et al.*, 1996). Structurally, microtubules are composed of 13 protofilaments that associate laterally to form a hollow cylinder, with the protofilaments running parallel to the cylinder axis. Each protofilament is a string of α/β tubulin heterodimers. While 13 protofilaments microtubules are more typical, microtubules with 12 to 16 protofilaments have been observed in various organisms and cell types (Goldstein, 1993).

Movement of motor proteins along microtubules is unidirectional. All known dynein motors have minus-end directed movement. Most kinesin motors have plus-end directed movement, although there are some exceptions such as the minus-end directed kinesin Ncd (McDonald *et al.*, 1990).

Motor proteins have a role in many cell biological processes. Because of this, there is much interest in the research their mechanisms and cellular functions. Current studies have proven that motor proteins are linked to diseases. Familial amyotrophic lateral sclerosis (FALS) is a neurodegenerative disease that affects middle-aged people (Dupuis *et al.*, 2000). This disease involves the accumulation of neurofilament proteins, which impairs axonal transport by retarding motor protein movement. With the onset of this disease there is an

increase in the expression of KAP3, a non-motor KIF3A associated subunit. This compensatory mechanism delays the degenerative process by sustaining part of axonal transport through increased motor availability. These findings compound the need for a further understanding of kinesin structure and function.

In the 1960's dynein was discovered in cilia and in the 1980's the first kinesin (now called conventional kinesin) was isolated from squid giant axons, sea urchin eggs, and chick brain (Brady, 1985; Scholey et al., 1985; Vale et al., 1985). In 1990 genes from *S. Cerevisiae* and *Aspergillus nidulans* were found to contain a region of ~350 amino acids which was 30-40% identical to the catalytic core of conventional kinesin, proving the existence of a kinesin superfamily. Each subfamily within the superfamily was found to contain a relatively well-conserved core motor domain linked to highly divergent non-motor domains, reflecting the functional diversity of their cellular roles. The structure of kinesin superfamily members is described in reference to that of the conventional kinesin heavy chain (KHC), which is the motor-contain polypeptide of the conventional kinesin holoenzyme.

Conventional Kinesin

The conventional kinesin holoenzyme is an elongated tetramer that consists of two heavy chains and two light chains (Cole and Scholey, 1995; Sablin, 2000) (Figure 2). The heavy chain of kinesin is comprised of four distinct regions; the catalytic core (amino acids 1-322), neck (amino acids 323-414), stalk (amino

6

Figure 2:
Conventional Kinesin Heavy Chain Domains

Neck linker
323-336
Neck hinge
371-414
Catalytic
core 1-322
Neck
337-370
C-terminal
915-963
Stalk I
415-563
Stalk II
591-914
Hinge 564-590→
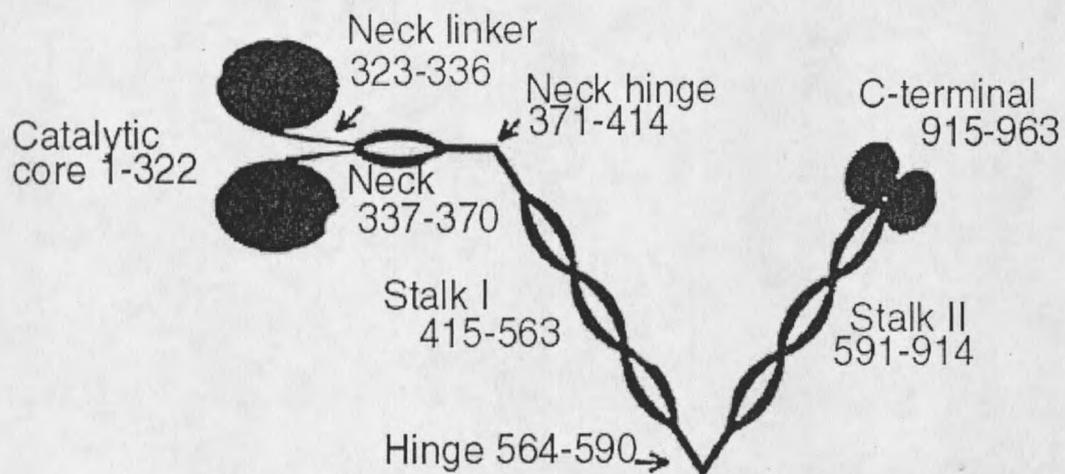
Figure 2:
Conventional kinesin heavy chain consists
of four distinct regions; catalytic core, neck,
stalk, and tail domains. The catalytic core is
a globular domain, which includes the ~350
amino acid conserved region. The neck
domain sequence is subfamily specific. It is
divided into three regions; neck linker,
coiled-coil, and hinge. The stalk domain
forms two coiled-coils separated by a
flexible hinge. The tail domain is globular
and is thought to bind cargo.
(Pierce, D. 1999)

acids 415-914) and tail domains (amino acids 915-963). The catalytic core is a globular domain that generates force and binds ATP and microtubules. The kinesin superfamily may be divided into three main groups depending on position of the catalytic core within the polypeptide (Sablin, 2000). Kin C proteins are minus-end directed kinesins with the motor domain at the C-terminus, Kin N are plus-end directed and have N-terminal motor domains, and members of the Kin I have central (intermediate) motor domains and do not demonstrate traditional motor activity but may rather function as microtubule depolymerizing factors; their functions are still largely unknown (Desai et al., 1999). My focus will be on the Kin N kinesins.

Adjacent to the catalytic core is the neck domain. This domain has been shown to be crucial to the mechanical functioning of the motor and determines movement direction (Case et al., 2000). The first 10 amino acids of the neck are slightly conserved within all the Kin N motors and highly conserved within individual subfamilies. This region is termed the neck linker. Underlining its critical role in the movement mechanism, motor velocity decreases 200-500 fold when it is replaced with a random polypeptide sequence (Case et al., 2000).

The neck linker is followed by a α-helical region made up of 20-30 residues. These amphipathic α-helices contain a heptad repeat $(abcdefg)_n$, where positions a and d are generally nonpolar amino acids (Thormahlen et al., 1998). When these residues align a hydrophobic seam is generated along one side of the helix. When two amphipathic α-helices come in contact they wind around

another, interfacing hydrophobic/nonpolar residues, resulting in formation of a coiled-coil dimer. Dimerization of synthetic peptides corresponding to this part of the neck domain of a number of kinesins via coiled-coil formation has been proven via CD spectroscopic analysis (Tripet et al., 1997). The neck coiled-coil region is conserved within subfamilies.

Recently, the Vale lab at UCSF discovered that by duplicating the first heptad repeat in the neck coiled-coil of K560, a construct from human conventional kinesin truncated at amino acid 560, the processivity of the motor increased by threefold (Romberg et al., 1998). These results along with others confirm the necks involvement in motor function.

The neck linker and catalytic core combined are termed the "motor domain". The motor domain includes the ~350 amino acid conserved region mentioned earlier. Several crystal structures exist for kinesin motor domains including human conventional kinesin (Kull et al., 1996), Drosophila Ncd (Sablin et al., 1996), and rat conventional kinesin (Sack et al., 1997) (Figure 3). Lower resolution crystal structures have also been obtained for conventional kinesin dimers (Kozielski et al., 1997). The core motor domain consists of a central eight-stranded β-sheet sandwiched between six α-helices, three on either side. Comparison of myosin and kinesin crystal structures reveal that the larger myosin motor domain contains within its active site the same eight-stranded β-sheet sandwiched between six α-helices as conventional kinesin (Kull et al., 1996; Rayment et al., 1993). Within the motor domain there are several highly

conserved regions with distinct functions; including the P-loop, L12, L11 and Switch II α4-helix regions (Case *et al.*, 2000).

The P-loop participates in binding of the β and γ phosphates of ATP (Sack *et al.*, 1999). The Switch II α4-helix is involved in communication between the bound nucleotide and the MT binding site. These two regions are analogous to similar motifs in myosins and G proteins, which also share a similar fold. In G proteins the analogous region detects whether GTP or GDP is bound to the active site (Sack *et al.*, 1997). Depending on which form of the nucleotide is bound the switch region undergoes a conformational change. This initiates a chain reaction resulting in alterations in target protein binding affinity. The L12 region is the main MT binding site, although other regions contribute as well. This short highly conserved loop follows the Switch II α4-helix (Vale and Fletterick, 1997). L11 is a large 15 amino acid β-ribbon loop. It is directed away from the nucleotide-binding site and is thought to change its conformation during the ATP cycle.

There are three segments of (predicted) coiled-coil in conventional kinesin. Following the first region, in the neck domain, is a proline-glycine rich "hinge" region that separates the neck and stalk domains. The stalk domain is largely α-helical coiled-coil but is interrupted in the middle by another hinge. The stalk is not essential for motility, but does perform important functions. It acts as a spacer, conveys forces, and is responsible (along with the neck coiled-coil) for dimerization, which is the most common quaternary structure arrangement

among kinesins. Kinesin heavy chain dimerization is a result of the coiled-coil formation by the stalk and neck. The coiled-coils enable a kinesin heavy chain to bind with the corresponding region of its partner heavy chain, forming a long α-helical coiled-coil, which in turn forms a dimeric motor protein (Sack *et al.*, 1999).

The C-terminal end of the heavy chain folds to form a second globular region, termed the tail domain. Important differences among kinesins are found in this domain. The tail domain comes in various sizes, secondary structure and location with in the primary sequence. Like the stalk, the tail is not essential for motility but does perform other important functions. Since these two domains are not involved in motility they have not been studied as extensively as the head and neck domains. Hypotheses have been extended to the function of the tail for cargo binding, cargo regulation, and light chain binding (Cole and Scholey, 1995).

The light chain of KHC is involved in cargo binding and regulation (Cole and Scholey, 1995). It participates with the tail domain in the formation of a conformation of kinesin in which the tail domains fold back and interact with regions in the neck (and possible core) domains. In this conformation, the ATPase activity of the motor domains is inhibited (Verhey *et al.*, 1998). One hypothesis is the binding of cargo interferes with this head-tail interaction, releasing the motor domains and allowing kinesin to be motile. Transgenic mice lacking KHC displayed defects in axonal transport and other functions. Transgenic mice lacking kinesin light chains display the same defects, proving

light chains play an essential role in motor function (Rahman *et al.*, 1999). There are several light chain isoforms generated by alternative splicing, although their distinct functions have not been determined (Wedaman *et al.*, 1993).

Five motor quaternary configurations have been found: heterotetrameric ($\alpha_2\beta_2$), homotetrameric (α_4), homodimeric (α_2), heterotrimeric ($\alpha_2\beta$) and monomeric (α) (Figure 4). Movement is defined as either processive or multiple-motor nonprocessive. Processive movement occurs when an individual motor is able to translocate some distance along a microtubule. Multiple-motor nonprocessive movement is an additive effort made by many motors attached to a microtubule. Motor movement can be confirmed using microscopy or ATPase assays. Microtubule gliding and/or single molecule motility assays are used to visualize and quantitate microtubule velocity using differential interference contrast (DIC) or total internal reflection (TIR) microscopy. Three factors determine observed rates of motility in multiple motor gliding assays: (1) ATPase rate, (2) Step size, (3) Processivity or not. For processive motors, velocity is ATPase rate times step size. For nonprocessive, motors can make additive contributions to velocity, so observed velocity depends on how many motors are driving movement and is faster than the ATPase x step size. *In vitro* processive movement has only been observed for KHC and UNC104/KIF1 subfamily motors, although controversy exists as to whether the UNC104/KIF1 movement should be termed processive.

Figure 4:
Kinesin Superfamily Quaternary Structures

KHCs

KLCs

Heterotetramer: Kinesin

KHCs

Homodimer: Ncd

85kDa

115 kDa

Heterotrimer: KRP
85/95

95kDa

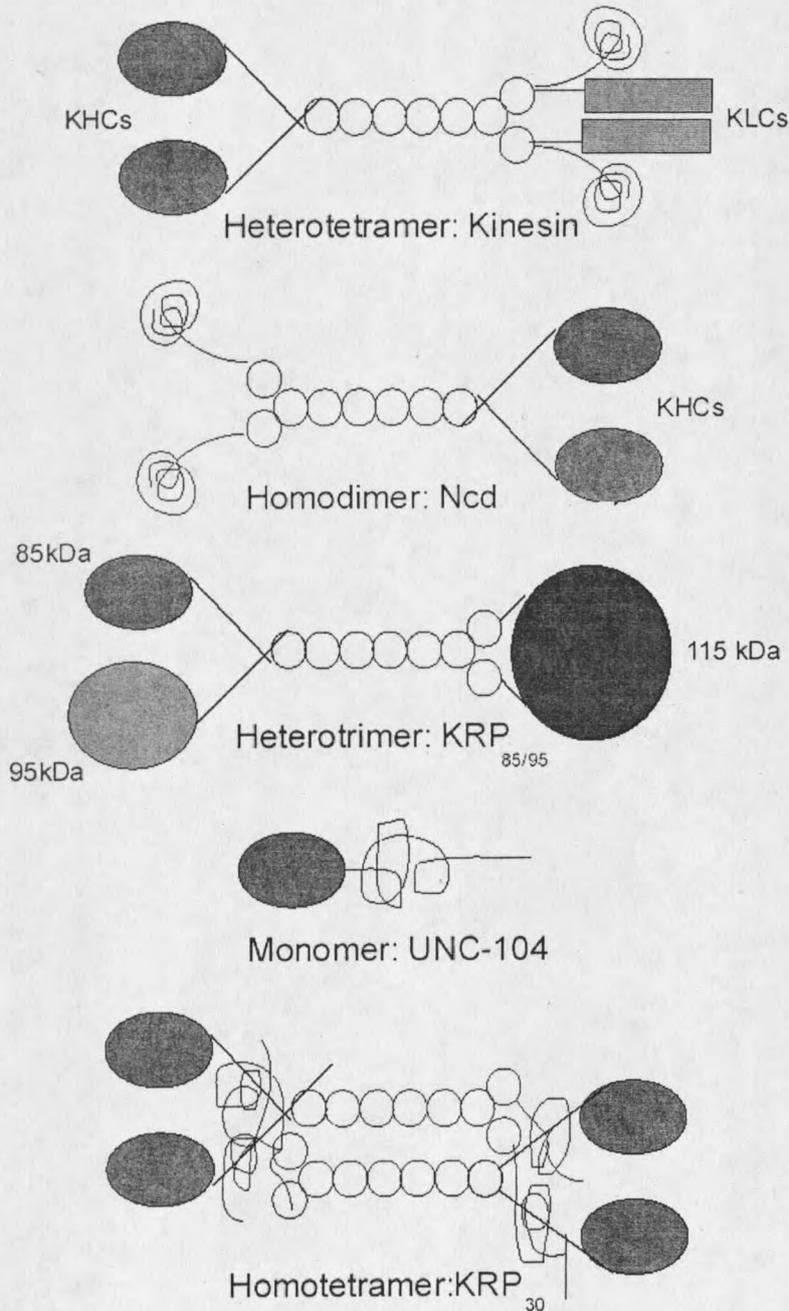Monomer: UNC-104

Homotetramer:KRP 30

Figure 4: Quaternary Structures of the kinesin superfamily. Schematic depicts kinesin heavy chanins (KHCs) and kinesin light chains (KLCs). All kinesins are dimeric with the exception of the monomeric UNC104/KIF1 subfamiliy (Cole, *et.al.*, 1995).