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Molecular Motors in Axonal Transport

Cellular and Molecular Biology of Kinesin

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Contents

Abstract

Neurons require a large amount of intracellular transport. Cytoplasmic polypeptides and membrane-bounded organelles move from the perikaryon, down the length of the axon, and to the synaptic terminals. This movement occurs at distinct rates and is termed axonal transport. Axonal transport is divided into the slow transport of cytoplasmic proteins including glycolytic enzymes and cytoskeletal structures and the fast transport of membrane-bounded organelles along linear arrays of microtubules. The polypeptide compositions of

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the rate classes of axonal transport have been well characterized, but the underlying molecular mechanisms of this movement are less clear. Progress has been particularly slow toward understanding force-generation in slow transport, but recent developments have provided insight into the molecular motors involved in fast axonal transport. Recent advances in the cellular and molecular biology of one fast axonal transport motor, kinesin, have provided a clearer understanding of organelle movement along microtubules. The availability of cellular and molecular probes for kinesin and other putative axonal transport motors have led to a reevaluation of our understanding of intracellular motility.

Index Entries: Axonal transport; molecular motors; kinesin; cytoplasmic dynein; membrane-bounded organelles; microtubules; organelle transport.

Introduction

All living cells require protein synthesis followed by transport and correct targeting of these proteins to their proper destinations. In general, protein synthesis occurs on either free polysomes within the cytoplasm or on ribosomes bound to the endoplasmic reticulum. Following translation, polypeptides destined for organelles are properly targeted by either specific amino acid sequences, posttranslational modifications, or structural motifs to a package for translocation. Once these proteins have been properly assembled within the organelle, the organelle itself is often transported throughout the cellular interior to facilitate its role in cellular homeostasis. In neurons, the movement of polypeptides and organelles to their destinations is a formidable task, since axons may be more than a meter long in large mammmals. The morphology of a neuron with its long axonal extension clearly epitomizes the magnitude of a process that translocates large amounts of materials over extended distances. This movement has been termed axonal transport.

Synthesis of axonal proteins, membranes, and the assembly of organelles occurs almost exclusively within the cell body of the neuron. Components destined for the axon or terminals are then translocated down the length of the axon in a specific manner (*see* Grafstein and Forman, 1980; Brady and Lasek, 1982a; Hammerschlag and Stone, 1982; Brady, 1985b; Hammerschlag and Brady, 1989). Soluble proteins, including glycolytic enzymes, and cytoskeletal components, including microtubules and neurofilaments, are cytoplasmically translated, assembled, and transported from the cell body to the synaptic terminus in association with other polypeptides (Black and Lasek, 1980; Tytell et al., 1981; Garner and Lasek, 1982; Lasek and Brady, 1982; Oblinger et al., 1982; *see also* Brady, 1985b; Brady and Lasek, 1982a,b). These cytoplasmic axonal components move at distinctive rates and are denoted slow axonal transport. Conversely, polypeptides destined for synaptic vesicles are synthesized on the rough endoplasmic reticulum and pass through the Golgi prior to packaging for translocation (Hammerschlag and Stone, 1982). The movement of membrane-bounded organelles within the axon is termed fast axonal transport, and includes the movement of mitochondria and vesicles away from the cell body toward the synapse (anterograde transport) and return of material from the synaptic terminus to the cell body (retrograde transport). Both slow and fast axonal transport can be further subdivided into multiple, distinct rate components (Willard et al., 1974; Tytell et al., 1981; Black and Lasek 1980; Brady 1985b).

The polypeptide composition of axonal transport has been thoroughly examined over the past decade, but less is known about the underlying molecular mechanisms of this movement. Recent years have provided a considerable amount of information concerning several molecular motors that are believed to play a role in axonal transport. These motor molecules include kinesin, cytoplasmic dynein, and myosin-like polypeptides. Advances in the cellular and molec-

ular biology of these mechanochemical enzymes have led to a reevaluation of axonal transport. Although these motors are present in neurons, in most instances, a correlation is still lacking between the biochemically characterized proteins and the axonal "cargo" that they are responsible for moving. We will first address an analysis of slow axonal transport and consider candidates for motor molecules involved in this movement. This will be followed by a detailed examination of fast transport and a discussion of the cellular and molecular biology of kinesin, one of the motor proteins known to be involved in the movement of membrane-bounded organelles.

Composition of Slow Transport

Detailed analyses of slow axonal transport have revealed two major rates of movement (*see* Grafstein and Forman 1980; Brady and Lasek, 1982a,b; Brady, 1985b). Slow Component a (SCa) polypeptides move at a rate of 0.1–1.0 mm/d whereas Slow Component b (SCb) polypeptides move at a faster rate, at 2–6 mm/d. Analyses of the polypeptide compositions of SCa and SCb reveal distinct macromolecular complexes that move down the axon in coherent groups (Black and Lasek, 1980; McQuarrie et al., 1980; Tytell et al., 1981; Garner and Lasek, 1982; Lasek and Brady, 1982; Oblinger et al., 1982). SCa includes microtubules and neurofilaments with such constituents as tubulin (Karlsson and Sjostrand, 1971), neurofilament triplet polypeptides (Hoffman and Lasek, 1975), τ proteins (Tytell et al., 1984), and spectrin (Levine and Willard, 1981). This component of slow transport consists of relatively few proteins with five polypeptides accounting for more than 75% of the labeled protein in the ventral motor neuron of rodents (Hoffman and Lasek, 1975). SCb is more complex, with at least 200 polypeptides (*see* Brady and Lasek, 1982b; Lasek and Brady, 1982). The constituents of this rate component include actin (Black and Lasek, 1979; Willard et al., 1979), clathrin (Garner and Lasek, 1981), calmodulin (Erickson and Moore, 1980; Brady et al., 1981), nerve-specific enolase, and creatine kinase (Brady and Lasek, 1981), which comprise cytoplasmic matrix components and microfilaments.

Predicted Properties of a Slow Transport Motor

Some force-producing motor molecule(s) must be involved in the transport of slow-component constituents, but the identity of the force-generating molecule(s) responsible for slow axonal transport remains unknown. In part, this situation stems from the inherent difficulties of studying slow transport. Most experimental approaches are indirect, and the preparations are not easily manipulated for experimental purposes (Brady, 1985b). Based on available information, Lasek and his associates proposed that the constituents of slow transport do not move as individual polypeptides, but as discrete cytological structures, such as microtubules and neurofilaments, commonly referred to as the Structural Hypothesis (Lasek and Brady, 1982; Tytell et al., 1981). Translocation of cytoskeletal polymers and associated "soluble" polypeptides in a macromolecular complex is consistent with observations that slow transport polypeptides move coherently as peaks down the axon (*see* Lasek and Brady, 1982; Cleveland and Hoffman, 1991). In this model, any slow axonal transport motor would need only move the cytological structure through interactions with the substrate and other cytoskeletal structures.

Others have argued that the constituents of slow transport move as individual subunits or small oligomers, which are then inserted into a stationary cytoskeletal network (Nixon and Logvinenko, 1986; Bamburg et al., 1986; Weisenberg et al., 1988). The existence of a stationary cytoskeleton has proven controversial, but discussion of this issue is outside the scope of this article. However, one requirement of this model is germane. If cytoskeletal polypeptides move as monomers or small oligomers, instead of moving as a polymeric complex, then each monomer/

oligomer must be moved individually. The motors responsible for movement of individual polypeptides could be expected to be present at relatively high levels. No such abundant polypeptide motor has been identified in nervous tissue. Two-dimensional gel electrophoresis of slow-component constituents does not reveal an abundant polypeptide that could serve as the molecular motor (Garner and Lasek, 1982; Tytell et al., 1981; *see also* Hammerschlag and Brady, 1989).

Recent experiments from the Kirschner laboratory (Reinsch et al., 1991; Tanaka and Kirschner, 1991; Sabry et al., 1991) provide striking evidence that tubulin can be transported in the form of polymeric microtubules, rather than as individual subunits. Reinsch et al. (1991) utilized a photoactivatable caged-fluorescent form of tubulin to label the microtubules and total tubulin pool of embryonic *Xenopus* neurons in primary culture. When a small patch of the caged-fluorescent tubulin in a neurite is activated, the fluorescent patch moves in the anterograde direction down the neurite at rates consistent with slow axonal transport. The fluorescent patch moves as a discrete patch consistent with an earlier report from Keith (1987), and much like the coherent movement of radioactively labeled polpeptides in slow transport (Garner and Lasek, 1982; Tytell et al., 1981). The companion studies (Tanaka and Kirschner, 1991; Sabry et al., 1991) utilize the fluorescent tubulin to follow the movements of microtubules in the axons and growth cones of both *Xenopus* and grasshopper axons. These studies provide graphic evidence that cytoskeletal elements can move without giving any hint of a stationary axonal cytoskeleton.

The interaction of a slow component motor(s) with its corresponding "cargo" is likely to be complex. It is not known whether both rates of slow transport are mediated by the same force-generating protein or if multiple motors are involved. Consideration of the properties of candidate slow transport motors must account for transport rate variations during injury, regeneration, and grafting experiments (*see* Brady, 1988,1992), which implies that the in vivo rates are locally variable. All mechanochemical ATPases identified to date produce similar rates, movements, and force production from the hydrolysis of a single ATP molecule, so these different rates are likely to result from the frequency of movements or the amount of resistance (Brady, 1991). One might expect a slow transport motor to have a potential to interact with several cytoskeletal elements either directly or indirectly. The nature of these interactions and any indirect coupling of these motors to the substrate or other cytoskeletal elements remains to be determined. In recent years, several mechanochemical enzymes have been proposed to play a role in slow transport. Three of these molecular motors and their possible roles in slow transport will be discussed below.

Dynamics of Dynamin, Dynein, and Other Postulated Slow Transport Motors

The mechanochemical enzyme dynamin has been proposed to play a role in slow transport (Shpetner and Vallee, 1989; Vallee and Bloom, 1991), but no direct evidence supports this hypothesis. In fact, recent molecular biological data suggest that dynamin is unlikely to be the slow axonal transport motor (*see* Brady, 1991; Bloom, 1992). Dynamin is a ~100-kDa polypeptide that was originally reported to have microtubule-activated MgATPase activity in the presence of a soluble cofactor. This putative neuronal motor molecule appeared to mediate microtubule bundling and sliding in vitro (Shpetner and Vallee, 1989). Cloning and sequencing of the cDNA encoding this polypeptide revealed primary sequence homology with the MX family of GTP-binding proteins (Obar et al., 1990) and the yeast vacuolar sorting protein SPO15 (Yeh et al., 1991). These data initiated a reevaluation of the nucleotide specificity of the dynamin polypeptide, which was subsequently found to possess GTPase activities in the absence of the soluble cofactor necessary for reported MgATPase activities (Obar

et al., 1990). The soluble cofactor is now thought to be a nucleoside diphosphokinase that rephosphorylates GTP by the hydrolysis of ATP (*see* Bloom, 1992). A putative function of dynamin can be inferred from cloning and sequencing of the *Drosophila* dynamin homolog, responsible for the *shibire* mutation (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). *Shibire* mutants possess a defect in the recycling of endocytic vesicles that suggests a role for the dynamins in endocytic events. At present, dynamin appears to be a regulatory protein that may be important in vesicle trafficking but is probably not a motor molecule.

Although dynamin is unlikely to be responsible for the force production in slow transport, several motor molecules that are present in the axon may function in this capacity. One molecular motor that could play a role in slow transport is cytoplasmic dynein, previously called MAP1C (Paschal and Vallee 1987). Cytoplasmic dynein is a complex of polypeptides consisting of two heavy chains (~410 kDa) and several associated polypeptides (ranging in size from 150–53 kDa). Cytoplasmic dynein is a microtubule-stimulated ATPase that has been demonstrated to mediate gliding of microtubules in vitro (Shpetner et al., 1988). If microtubules of known polarity are used in such assays, this motor protein generates microtubule gliding with the microtubule minus ends trailing (Schnapp and Reese, 1989; Schroer et al., 1989). Since axonal microtubules are oriented with their plus ends distal to the cell body, such data have been interpreted to suggest that cytoplasmic dynein is responsible for retrograde movement of membrane-bounded organelles. However, this directionality of microtubule gliding is also consistent with a role in slow transport. These two functions are not mutually exclusive, since the composition of smaller subunits for cytoplasmic dynein appears variable (Vallee et al., 1989).

The proposed role of cytoplasmic dynein in fast axonal transport has somewhat obscured its possible function in slow transport. It is possible that this motor protein functions to move cytoskeletal constituents in the anterograde direction as slow transport and may also move retrograde moving organelles. The differences in rate of the anterograde slow component and retrograde fast component may be attributable to "pauses" in the movement of cytoplasmic elements for variable periods of time, resulting in an overall slower rate. The putative roles of cytoplasmic dynein in both slow and fast transport warrant further examination.

Another class of motor molecules that has been identified in the axon is myosin-like proteins (Willard, 1977; Kuczmarski and Rosenbaum, 1979). No axonal functions have been identified as yet, but it is possible that these proteins are also candidates to play a role in slow transport. Unfortunately, unlike fast transport, slow transport has not been amenable to direct manipulations. The most obvious difficulty in directly studying slow transport results is the fact that most of the movements of cytoskeletal structures cannot be visualized. However, the use of photoactivatable fluorescently tagged tubulin has recently enabled the visualization of microtubule polymer movement in neuronal cells (Reinsch et al., 1991). Such methods may provide the necessary accessibility of slow transport to experimental manipulations.

Fast Axonal Transport Motors

The most thorough studies of axonal transport motors to date focus on fast transport, where the molecular mechanisms underlying organelle motility are beginning to be understood. The last decade of research has generated substantial data toward understanding the mechanisms of fast axonal transport. Two separate motor molecules, kinesin and cytoplasmic dynein, have been implicated in the movement of organelles along axonal microtubules (*see*: Bloom et al., 1989; Vallee et al., 1989; Brady, 1991; Vallee and Bloom, 1991). Although data indicate that kinesin generates movement in the anterograde direction (Vale et al., 1985b), cytoplasmic dynein is thought to generate force unidirectionally in the retrograde direction (Paschal and Vallee, 1987; Schnapp and

Reese, 1989; Schroer et al., 1989). Although cytoplasmic dynein has been implicated as the retrograde motor, this designation is still equivocal (*see* Brady, 1991). Cytoplasmic dynein is a complex protein, containing multiple polypeptide subunits. The cellular and molecular biology of this probable retrograde motor has not been as thoroughly characterized as its anterograde counterpart, kinesin. Recent molecular genetic studies have provided the primary sequence for two of the subunits of cytoplasmic dynein: the 150-kDa associated polypeptide (Holzbaur et al., 1991) and the heavy chain of flagellar dynein (Gibbons et al., 1991; Ogawa, 1991). Primary sequence analysis of the 150-kDa associated polypeptide of cytoplasmic dynein revealed homology to the gene product of the *Drosophila Glued* dominant mutation. *Glued* mutants display a variety of developmental defects that should facilitate determination of the roles for cytoplasmic dynein in development. Flagellar dynein heavy chains display a number of interesting features, including multiple nucleotide binding sites, but future work will be required to determine which of these features are also characteristic of cytoplasmic dyneins. Numerous laboratories are attempting to isolate cDNA clones for all of the cytoplasmic dynein polypeptides. However, the number of polypeptides involved and the large size of the heavy chains suggest that this work may be expected to take several years.

Although the cellular and molecular biology of cytoplasmic dynein remains in its infancy, the past seven years have provided a substantial amount of information concerning the characteristics of the anterograde fast axonal transport motor protein kinesin. The cellular and molecular biology of kinesin have provided a more thorough understanding of the mechanisms of fast axonal transport. A description of the advances in microscopy that led to the discovery of kinesin, and the subsequent biochemical, pharmacological, and enzymatic characteristics of this mechanochemical enzyme will be presented. Finally, we will provide current information on the molecular genetics of kinesin and discussion of how these data have provided the most detailed understanding of an axonal transport motor to date. The area of molecular motors has recently been reviewed in detail (Brady, 1991; Vallee and Bloom, 1991; Bloom, 1992). The citations presented here provide illustrative examples and are not intended to be a thorough review of the literature.

Visualization of Organelle Movement

The rapid advances made in the fast axonal transport field over the past several years stem directly from the ability to visualize organelle movement along microtubules. Advances in video microscopy in the early 1980s enabled the "cargo" of fast transport to be visualized and subsequently manipulated. Initial reports of the direct visualization of rapid bidirectional particle movement within neurons were made in 1964 with the use of time-lapse differential interference contrast cinemicrography (Burdwood, 1964). Such microscopic techniques provided investigators with a first-hand view of fast transport in living neurons, but these techniques could not resolve cellular structures. In 1981, Allen et al. introduced video-enhanced contrast differential interference contrast (VEC-DIC) microscopy, which enabled the detection of cellular components as small as single microtubules with diameters of 25 nm. The resulting images of living cells were unequaled by previous methodologies. Soon after, methods were developed using VEC-DIC to allow the direct manipulation of fast axonal transport (Brady et al., 1982,1985).

Fast Axonal Transport in Extruded Axoplasm

Although VEC-DIC was capable of detecting the movements of individual 30–50nm vesicles in cultured vertebrate neurons (Breuer et al., 1981; Smith and Kendal, 1984), this experimental system was not ideal because of the presence of an

intact plasma membrane and poor optical properties. This permeability barrier prevented direct experimental manipulation of the motile processes being studied. The development of the squid giant axon extruded axoplasm preparation circumvented this obstacle and enabled analysis of effects from externally administered agents (Brady et al., 1982,1985).

The giant axon from the squid *Loligo pealei* is an unmyelinated axon typically reaching diameters of ~500 μm (Gilbert et al., 1990). Using a relatively simple technique, a cylinder of axoplasm can be mechanically extruded from the axon, thereby removing it from the plasma membrane and surrounding connective tissue. On extrusion, the axoplasm retains its cylindrical shape and can be easily manipulated in vitro. The extruded axoplasm is mounted between microscopic coverslips to produce a perfusion chamber accessible during imaging by VEC-DIC (Brady et al., 1982,1985). Rapid transport of three organelle classes, based on size, can be seen in such preparations. These organelles can be observed moving both anterograde and retrograde along linear arrays of microtubules (Brady et al., 1982,1985; Allen et al., 1985). In fact, video microscopy in conjunction with electron microscopy has determined that a single microtubule can support organelle motility (Hayden and Allen, 1984; Allen et al., 1985) in both directions (Hayden and Allen, 1984; Schnapp et al., 1985) and in a variety of cell types (Schliwa, 1984). Under proper buffer conditions, the axoplasm will maintain its integrity (Morris and Lasek, 1982) and support movement for several hours (Brady et al., 1982). This system, devoid of permeability barriers, has enabled direct pharmacological, biochemical, and immunological manipulations of fast axonal transport (Brady et al., 1985).

The extruded axoplasm preparation has been heavily exploited for the past decade to determine the underlying molecular mechanisms of fast axonal transport. Perfusion of a variety of agents into this preparation enabled direct evaluation of the effects on organelle motility (Brady et al., 1985). Initial experiments determined a requirement for oxidative phosphorylation and ATP in transport (Brady et al., 1982,1985). The introduction of oxidative phosphorylation inhibitors or agents that deplete ATP concentrations results in the complete cessation of movement (Brady et al., 1982,1985). Additional studies examining the role of cytoskeletal elements in fast axonal transport revealed the requirement for microtubules that serve as tracks for movement. Microfilaments are not necessary for organelle transport, but their role in maintaining proper axoplasmic organization facilitates efficient organelle motility (Brady et al., 1984,1985). One of the most provocative pharmacological findings utilizing this system was related to the nucleotide dependence of fast axonal transport.

Because of the ATP dependence on transport, an analysis of the effects of ATP analogs was undertaken (Brady et al., 1985). Adenyl-5'-*yl*-imidodiphosphate (AMP-PNP), a nonhydrolyzable ATP analog, was tested on organelle transport in the extruded axoplasm. Perfusion of the axoplasm with buffer containing 0.5–10 m*M* AMP-PNP blocked organelle movement in the periphery of the axoplasm, where one can observe transport on individual "splayed" microtubules. The observed inhibition was interesting, because the organelles were "frozen" in place on the microtubules giving the appearance of a "string of pearls" (Lasek and Brady, 1985). This attachment is reversible, as demonstrated by subsequent perfusion of ATP into the chamber, which was followed by a recovery of organelle motility. The observed organelle binding in the presence of AMP-PNP was presumably via the motor protein, thus providing a means to identify and purify this novel ATPase.

Identification and Purification of Kinesin

The stable interaction of organelles and microtubules in the presence of AMP-PNP was utilized in the identification of the fast axonal transport motor (Brady, 1985a; Vale et al., 1985a). Based on axoplasm studies, one would predict that the motor protein would remain bound to microtubules

in the presence of AMP-PNP, but not ATP. Brain homogenates were incubated in the presence of microtubules and either AMP-PNP or ATP. The microtubules were harvested by centrifugation, and the associated proteins were examined by SDS-PAGE. In chick brain, the patterns of bands sedimenting with microtubules under these conditions were virtually identical with the exception of an ~130-kDa polypeptide (Brady, 1985a). This polypeptide sedimented with microtubules in the presence of AMP-PNP, but not ATP. The 130-kDa-enriched pellet exhibited elevated ATPase activities, which was consistent with transport studies demonstrating a requirement for ATP. Concurrently, polypeptides of 110–120 and 60–70 kDa were isolated from the squid optic lobe and bovine brain with similar characteristics (Vale et al., 1985a). This new class of motor molecules was termed kinesin from the Greek *kinein*, to move.

Although originally described and characterized from neuronal tissues, kinesin has been found in a variety of cell types (Pfister et al., 1989) and is thought to play a role in microtubule-based motility in most higher eukaryotic cells. The biochemical, biophysical, pharmacological, and enzymatic properties of this motor protein have been extensively examined since its identification in 1985. As expected for a molecular motor responsible for the movement of organelles along microtubules, kinesin has been shown to be a microtubule-stimulated ATPase. In addition, this motor protein is capable of force generation in vitro (Cohn et al., 1987,1989; Porter et al., 1987; Saxton et al., 1988; Howard et al., 1989), and is localized to membrane-bounded organelles as demonstrated by quantitative immunoblotting and electron microscopic (EM) analyses (Leopold et al., 1992).

Biophysical and Biochemical Properties of Kinesin

The kinesin holoenzyme is a heterotetramer consisting of two heavy chain (~124 kDa) and two light chain (~64 kDa) subunits (Kuznetsov and Gelfand, 1986; Bloom et al., 1988; Murofushi et al., 1988; Wagner et al., 1989) with a native mol wt of ~380 kDa (Bloom et al., 1988; Kuznetsov et al., 1988) (Fig. 1). Multiple isoforms, differing in apparent mol wt and isoelectric point, exist for both the heavy chain and light chain polypeptides (Wagner et al., 1989). Recent evidence indicates that light chain heterogeneity is generated from posttranslational modifications (Elluru et al., 1991) in combination with multiple polypeptide species differing in primary sequence (Cyr et al., 1991). The mechanisms for generating multiple heavy chain species are unknown, although phosphorylation of the kinesin heavy and light chains has been demonstrated in vivo (Murphy et al., 1989; Elluru et al., 1991; Buster et al., 1990; Hollenbeck, 1991).

Rotary shadow EM analyses of purified kinesin provided a more thorough understanding of the quaternary structure of the kinesin heterotetramer. Kinesin is an elongated, rod-shaped molecule of ~80 nm in length with a distinctive structure (Hirokawa et al., 1989; Scholey et al., 1989). Two globular heads are located at one end of the molecule and connected by an extended shaft to a diffuse "fan-shaped" tail. The shaft region resembles an α-helical coiled-coil domain analogous to that seen in myosin molecules. Frequently, a "kink" is observed within the kinesin rod ~35 nm from the head domains (Hirokawa et al., 1989; Scholey et al., 1989).

The "kink" observed in the rod is believed to add flexibility to the kinesin molecule and facilitate organelle binding. An examination of kinesin-coated microspheres bound to microtubules reveals crossbridges of ~25–30 nm (Hirokawa et al., 1989). These data are consistent with crossbridges between vesicles and microtubules in vivo (Hirokawa, 1982; Miller and Lasek, 1985). Although the crossbridges are demonstrated to be ~25–30 nm long, the kinesin molecule is ~80 nm in length. Thus, it is postulated that these crossbridges represent the distance from the kinesin heads to the "kink" in the rod.

The functional architecture of the holoenzyme was mapped by immunolocalization with monoclonal antibodies to kinesin heavy chains and

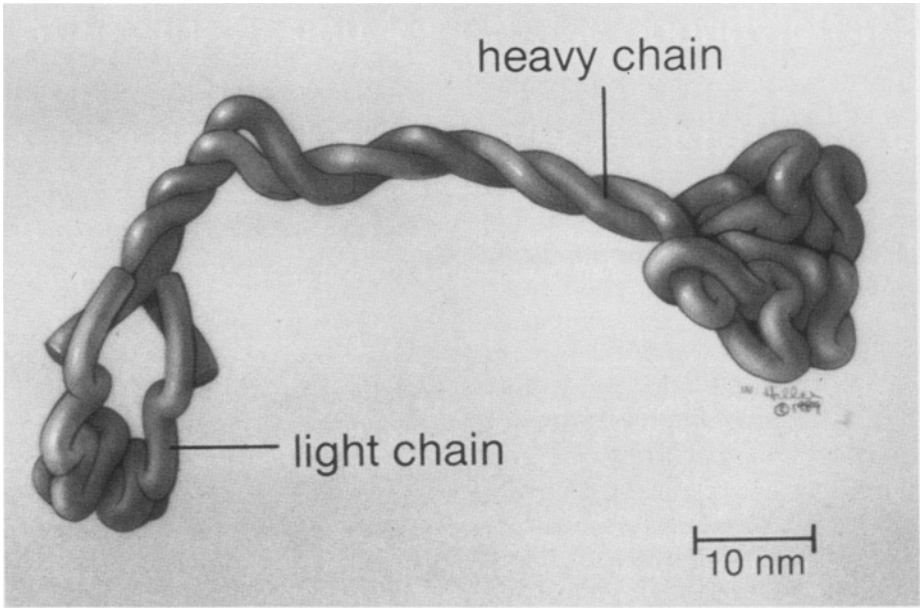

Fig. 1. Proposed model of the kinesin heterotetramer. Based on immunoelectron microscopic data, a model for the microtubule-stimulated ATPase has been derived (Hirokawa et al., 1989). Subsequent molecular genetic analyses have provided supporting evidence for this heterotetrameric structure (Yang et al., 1989; Kosik et al., 1990;Wright et al., 1991; Cyr, Clendening, and Brady, unpublished data). Two kinesin heavy chains interact in a parallel manner such that the globular heads containing the microtubule and ATP binding sites are at one end, and the carboxyl terminal tails are at the opposite end. These two ends are connected by an α-helical coiled-coil stalk that appears to be "kinked" approx 2/3 down its length. The light chain epitopes are at the carboxyl tail of the heavy chains, although the amount of interaction along the stalk is unclear. This figure is provided merely to show the approximate location of the different kinesin polypeptides and is not meant to reflect the intricate details of the subunit interactions. Although this illustration depicts a right-handed supercoil, the X-ray crystal structure of the GCN4 leucine zipper has revealed a left-handed supercoil (O'Shea et al., 1991). Thus, the directionality of the supercoil generated by the dimerization of the kinesin polypeptides may be left-handed. Illustration by Wendy Hiller-Gee (Hiller, 1989).

light chains (Fig. 1) (Hirokawa et al., 1989). Heavy chain epitopes recognized by monoconal antibodies are located to the globular head domains of the holoenzyme, thus identifying these regions as part of the heavy chains. The light chain epitopes are located to the fan-like tail portion at the opposite end of the holoenzyme. Further EM analyses indicate that the heavy chain globular head domains interact with microtubules, thus implicating the light chain-containing fan-shaped tail in organelle binding (Hirokawa et al., 1989). The elucidation of the heavy chain primary sequence verified this three-domain structure and was consistent with the hypothesis that a portion of the heavy chain polypeptide is present in each of the holoenzyme domains (Yang et al., 1989).

Molecular Genetics of Kinesin

Biochemical methodologies provided a substantial amount of information about the kinesin heterotetramer. However, a detailed analysis of each of the subunits was not readily accessible because of the inability to separate heavy chains and light chains in the absence of denaturing agents. The isolation of cDNA and genomic clones encoding for the individual kinesin subunits has provided a means to dissect each of these polypeptides. This approach has afforded further support for earlier biochemical data, initiated a detailed examination of kinesin structure and function, and provided a clearer understanding of organelle transport in the axon.

Kinesin Heavy Chains

The availability of monoclonal and polyclonal antibodies to the kinesin heavy chains facilitated isolation of cDNA and genomic clones initially from *Drosophila* (Yang et al., 1988,1989), and more recently from squid (Kosik et al., 1990), sea urchin (Wright et al., 1991), and rat brain (unpublished data, Cyr, Clendening, and Brady). The kinesin heavy chain is highly conserved evolutionarily. A comparison of heavy chain primary sequences across species reveals ~50–60% homology throughout the encoded polypeptide. The heavy chain primary sequence revealed several structural and functional features consistent with previous biochemical data. These features include a three-domain structure of the heavy chain consisting of an amino terminal globular head domain containing the consensus ATP and microtubule binding sites, a predicted α-helical coiled-coil stalk, and a globular carboxyl terminal region (Yang et al., 1989).

Amino Terminal Globular Head

The amino terminal 400 amino acids of the *Drosophila* heavy chain polypeptide are predicted to generate a globular head region of ~50 kDa (Yang et al., 1989). An analysis of the primary sequence of this region reveals the presence of a consensus ATP binding site (Yang et al., 1989). This observation is consistent with biochemical data demonstrating that the heavy chain subunit binds ATP (Gilbert and Sloboda, 1986; Penningroth et al., 1987; Bloom et al., 1988; Kuznetsov et al., 1989) and that this ATP binding site can be localized to a ~45-kDa chymotryptic fragment of the heavy chain (Kuznetsov et al., 1989). In vitro microtubule pelleting experiments utilizing truncated heavy chains demonstrated that in addition to the ATP binding site, the microtubule binding domain is also present within the globular head (Yang et al., 1989). These data substantiate rotary shadow EM data, which demonstrate kinesin holoenzyme interactions with microtubules via its globular heads (Hirokawa, et al., 1989). In vitro microtubule-gliding assays utilizing truncated recombinant *Drosophila* heavy chain polypeptides reveal that a segment containing the globular head region can promote microtubule binding and in vitro microtubule gliding (Yang et al., 1990). Thus, this region contains the minimal information necessary for force generation along microtubules.

An Extended α-Helical Coiled-Coil Shaft

The heavy chain globular head domain is followed by a predicted α-helical coiled-coil rod that extends for ~56 nm in length (Yang et al., 1989). The primary sequence of this α-helical rod follows the heptad repeat pattern consistent with an α-helical coiled-coil structure. The heavy chain heptad repeats are interrupted by 24 amino acids, including a proline following the 147th amino acid (21st heptad) of the repeats (Yang et al., 1989). This interruption in the heptad repeat pattern is believed to account for the "kink" in the rod observed by EM analyses (Hirokawa et al., 1989; Scholey et al., 1989). The coiled-coil shaft region of the heavy chain polypeptide is thought to facilitate heavy chain dimerization, and a portion of this rod may play a role in heavy chain/light chain interactions (Cyr et al., 1991).

Small Carboxyl Terminal Globular Region

The *Drosophila* heavy chain polypeptide ends in a small ~10–15 kDa carboxyl terminal domain predicted to contain alternating types of conformation; thus, this area may also be globular in nature (Yang et al., 1989). This small domain is highly charged with ~24% basic and ~10% acidic amino acids. The heavy chain carboxyl terminal globular region presumably has some interactions with the kinesin light chains based on light chain localization to the holoenzyme tail region (Hirokawa et al., 1989), although the extent of these interactions is unclear.

Biochemical analyses of *Drosophila* heavy chains expressed in *E. coli* demonstrate that these subunits are sufficient for force generation in vitro (Yang et al., 1990). Such studies also suggest that posttranslational modifications, such as phosphorylation, are not necessary for in vitro microtu-

bule gliding. This finding is interesting in light of axoplasmic transport studies, which demonstrate that both kinesin subunits are phosphorylated during organelle motility (Elluru et al., 1991). Additional studies utilizing truncated versions of the recombinant heavy chain have begun to dissect further the functional areas of the heavy chain polypeptide.

The isolation of kinesin heavy chain cDNA clones has enabled specific, detailed questions to be addressed concerning heavy chain structure and function. A more complete understanding of the structure, and function of the kinesin holoenzyme has been initiated with the recent cloning, sequencing, and analysis of the kinesin light chains (Cyr, et al., 1991). Relatively little information was previously available concerning the light chains because of the inability to separate kinesin subunits biochemically from one another. Our current knowledge about these smaller subunits in combination with data concerning the heavy chains provides us with a clearer understanding of the role of this molecular motor in organelle transport.

Kinesin Light Chains

Biochemically purified light chains are a heterogenous class of ~64-kDa polypeptides that vary in isoelectric point and/or apparent mol wt (Wagner et al., 1989). The isolation of three light chain cDNAs indicates that some of these polypeptide isoforms are generated by differences in primary sequence (Cyr et al., 1991). Determination of the light chain primary sequence has provided a considerable amount of structural information about these smaller subunits. Several regions of the light chain primary sequence lend themselves to structural and functional predictions. These features include an amino terminal coiled-coil rod that may mediate heavy chain association and holoenzyme assembly, a possible organelle interaction site comprised of five 42 amino acid repeats, and a heterogeneous carboxyl terminus that may serve as an organelle targeting domain.

An α-Helical Amino Terminus Containing a Coiled-Coil Rod

The first 163 amino acids of the light chain polypeptide are predicted to be α-helical based on the high percentage of helix-forming residues and the absence of prolines. Within this larger α-helical region, one can identify 15 heptad repeats consistent with this region forming an α-helical coiled-coil structure.

The involvement of this α-helical coiled-coil domain in the interaction of two polypeptides is clear, but the identity of the subunit to which each light chain binds is less obvious. To date, the detailed interactions of the four individual kinesin subunits are unknown. Two possibilities present themselves: First, this region could mediate the formation of light chain dimers; second, this structural motif could be the site of light chain/heavy chain interactions. In the first case, the amino terminal α-helical coiled-coil region of the light chain polypeptides would serve to produce light chain dimers. These dimers might, in turn, interact with heavy chain dimers to generate the kinesin protein complex. Although plausible, no evidence supports this hypothesis. Electron micrographs of rotary shadowed kinesin holoenzyme show only a single coiled-coil rod domain (Hirokawa et al., 1989; Scholey et al., 1989). This rod corresponds to the "shaft" of kinesin. A second α-helical coiled-coil rod, ~16 nm in length, should be generated if light chain dimerization is mediated by such a structure. Although this length may be difficult to detect by EM, no such additional rod is observed. In addition, if this region is involved in light chain dimerization, some other domain must be involved in light chain/heavy chain association, but no candidate for alternative heavy chain interaction site is obvious. No other structural element in the light chain sequence suggests a site for interaction with the heavy chain.

Alternatively, this area may facilitate heavy chain/light chain interactions. Previous data indicate that the associations of light chains and heavy chains are relatively stable. These results

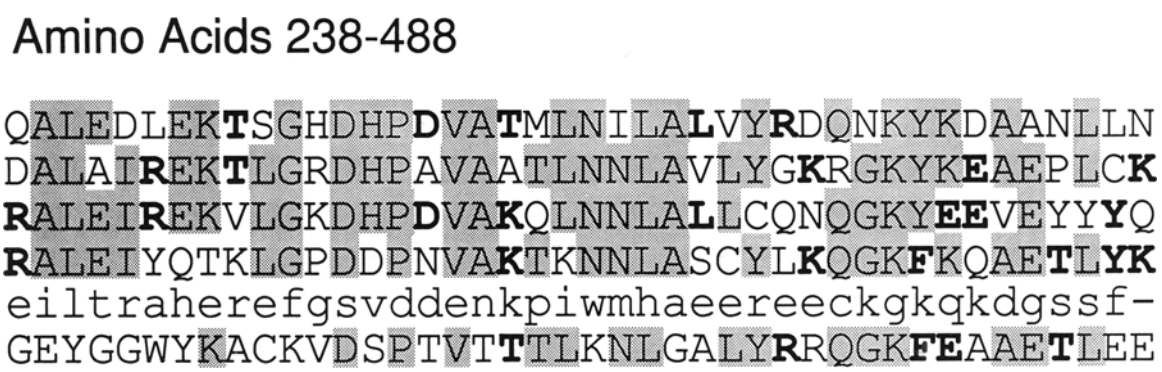

Fig. 2. The five imperfect amino acid repeats present in the light chain primary sequence. Five imperfect amino acid repeats of 42 amino acids each are present in the primary sequence of the kinesin light chains (Cyr et al., 1991). These repeats begin at Gln238 and extend through Glu488 with a 41 amino acid spacer (lowercase letters) located between the fourth and fifth repeats. Each repeat consists of 42 amino acids that center around several conserved amino acids. Sequences are aligned with identical amino acids present in at least three of the five repeats shaded, and amino acids present in two of the five repeats are in bold.

include the following observations: (1) Biochemical separation of light chains from heavy chains requires the use of denaturing agents (Bloom et al., 1988; Kuznetsov and Gelfand, 1986), and (2) immunoprecipitation of kinesin with either heavy chain or light chain monoclonal antibodies results in the coprecipitation of both kinesin subunits (unpublished data, Elluru, Stenioen, and Brady). This high-affinity association between the subunits has been demonstrated not to involve interchain disulfide bonds (Bloom et al., 1989). An interchain α-helical coiled-coil structure would account for the strong associations observed between the two constituents. No other light chain hydrophobic domain is present that could account for observed heavy chain/light chain stability. We believe that the possibility of this domain functioning as the interaction site is more consistent with available data.

Five Internal Repeats of 42 Amino Acids Each

In addition to the 15 heptad repeats located at the amino terminus, another repeat motif has also been identified within the light chain primary sequence. Five imperfect repeats extend within 251 amino acids (Fig. 2). These repeats consist of 42 amino acids, each containing five completely conserved amino acids and 15 residues that are found in four of the five repeats. The high degree of identity between the repeats and the fact that this area comprises 39% of the polypeptide length suggest that this specific motif is necessary for proper light chain function.

The precise structure of this region is unknown. These 42 amino acid repeats contain many stretches of α-helical-forming residues interrupted by helix-breaking amino acids. Although difficult to predict, this region appears to contain short helices separated by bends and turns. No hydrophobic core, indicative of a tightly packed globular domain, is present. Thus, this region is believed to be more diffuse in nature, consistent with the appearance of the holoenzyme fan-shaped tail region and the observed light chain susceptibility to proteolysis during biochemical purification. The hydrophobic periodicity observed in this region may be involved in interactions with organelle membrane surfaces or receptors present on organelles.

A Heterogeneous Carboxyl Terminal Tail Region

The third distinctive region of the light chain is present at the carboxyl terminus of the polypeptide. As revealed by the isolation of multiple light

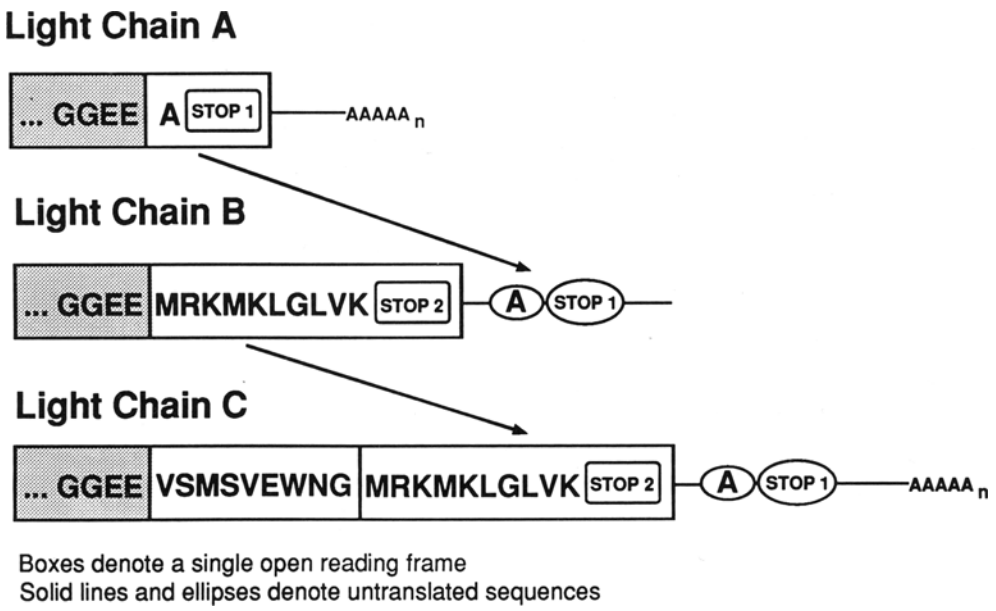

Fig. 3. Diagrammatic representation of kinesin light chain heterogeneity at the carboxyl terminus of the coding region. Open reading frames (denoted by boxes) for light chains A, B, and C are identical except for a 50-bp (light chain B) or 77-bp (light chain C) insertion at the carboxyl terminus. These insertions result in three light chain isoforms that are identical for the first 541 amino acids (shaded boxes), but differ in their carboxyl terminal amino acids. The open reading frame of light chain A terminated with an alanine. In light chain B, the alanine and subsequent stop codon (stop 1) seen in light chain A are contained within the 3' untranslated sequences, and an additional 50 bp have been alternatively spliced into the coding region. This insertion encodes for 10 amino acids, a new in-frame stop codon (stop 2), and 20 bp of additional 3' untranslated sequences. Light chain C is a light chain B variant, where an additional 9 amino acids have been inserted 5' to the light chain B addition, resulting in a 19 amino acid tail, 10 of which are found in light chain B. Untranslated sequences are denoted by the solid lines and ellipses. The cDNA clone isolated for light chain B is missing part of the 3' untranslated sequences and the poly(A) tail, and therefore, is not depicted in the figure. The portion of the 3' untranslated region that is present is identical to light chains A and C. Figure taken from Cyr et al., 1991.

chain cDNAs, this is a site of light chain diversity. Three isoforms are present in rat brain that differ in primary sequence only at the carboxyl termini (Fig. 3). Light chain A is the smallest of the isoforms containing only a single amino acid addition to the residues conserved in all three light chain classes. The light chain B polypeptide includes a 10 amino acid addition to the conserved residues. To generate light chain C, an additional 9 amino acids are inserted immediately 5' of the light chain B addition, resulting in a 19 amino acid tail (Fig. 3).

The genomic origin of this light chain heterogeneity has been examined by genomic Southern blot analyses and the isolation and partial sequencing of light chain genomic clones from rat. Genomic Southern analyses indicated that the three kinesin light chain mRNAs are generated from the alternative splicing of a single gene (Cyr et al., 1991). The mechanism of this alternative splicing has been examined by the isolation of rat genomic clones that have been partially sequenced in this region of mRNA diversity. These data enabled elucidation of the splicing mechanism utilized to generate the three kinesin light chain isoforms in rat brain. As depicted in Fig. 4, the mRNAs encoding kinesin light chains A, B, and C are generated by the use of an alternative splice donor site located within the 27-bp light chain C insertion and/or the exclusion of

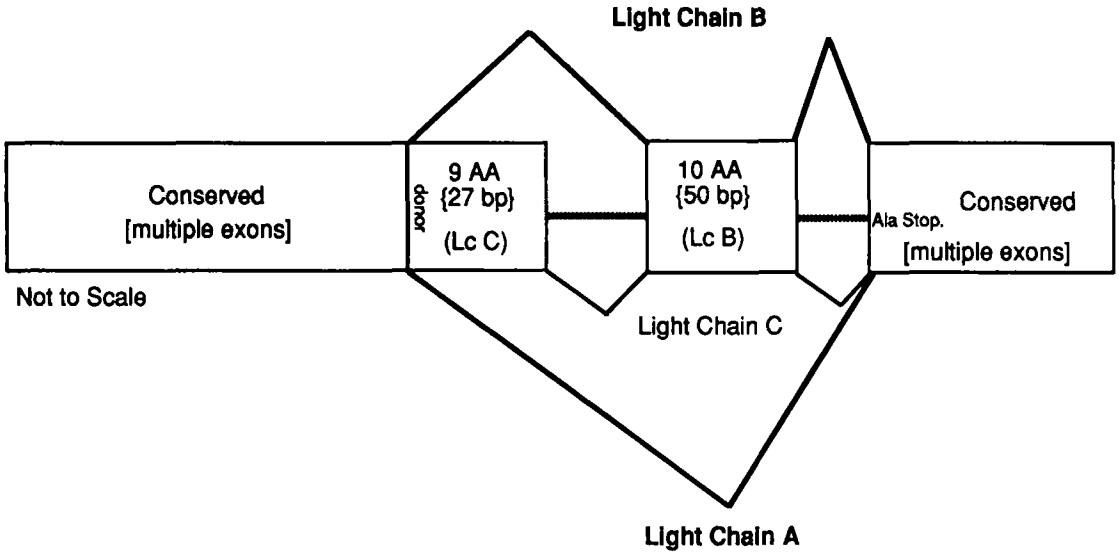

Fig. 4. Intron/exon boundaries at the alternatively spliced region of the kinesin light chain gene. The isolation and partial sequencing of kinesin light chain genomic clones from rat have enabled a determination of the splicing mechanism utilized to generate the three light chain mRNAs. As depicted in the diagram, the light chain C insertion is contiguous with the previous exon, thus representing the last 27-bp of this exon. mRNAs for light chains A and B utilize an alternative splice donor site located at the first 6-bp of the light chain C insertion to remove these 27-bp as part of the intron. The light chain B 50-bp insertion is a single exon that can be included or excluded from the mRNAs. Light chain A mRNA removes the 27-bp of the light chain C insertion and also excludes the light chain B 50 bp exon. Light chain B results from the utilization of the light chain C alternative splice donor site, but does not remove the light chain B exon. Light chain C does not employ the alternative donor site, but rather utilizes an additional donor site located immediately 3' to the 27-bp insertion. This mRNA also includes the light chain B exon, resulting in a 77-bp insertion. As indicated, several exons are expected to generate the 5' and 3' conserved regions of the light chain mRNAs. Exon locations are depicted by boxes. Introns are denoted by dashed lines. The splicing schemes necessary to generate each of the light chain mRNA species are shown.

the 50-bp exon that encodes the 10 amino acids and the 20-bp of 3' untranslated sequences of the light chain B insertion.

The functional implications of having three kinesin light chains has yet to be determined. However, the proposed role of the light chains in organelle binding suggests that the light chain polypeptide isoforms may serve to target and bind to differing specific organelle classes. This hypothesis is particularily attractive in light of the predicted amphipathic helix structure of the light chain B addition (also present in light chain C). Amphipathic helices are known to target many mitochondrial proteins to the mitochondria (Douglas et al., 1986). Since the kinesin heterotetramer is presumably assembled in the cytoplasm, a targeting mechanism must be present to enable binding to organelles transported by this molecular motor. A targeting domain at the light chain carboxyl terminus may serve this function.

Reevaluating Axonal Transport

The availability of cellular and molecular probes for numerous motor proteins has initiated a reevaluation of our knowledge of axonal transport and the mechanisms of force generation in the axon. Recent molecular genetic data have provided a clearer understanding of some of the motors believed to be involved in these processes and also have enabled a clarification of the roles played by motors that had originally been thought to participate in axonal transport. Additional data

are still necessary to dissect the intricate details of kinesin-based organelle motility, and the generation of force by cytoplasmic dynein and other putative axonal mechanochemical enzymes. Current advances in both molecular and cell biological techniques should enable this information to be attained within the next several years. These data should provide a basis for determining the underlying molecular mechanisms of the processes involved in axonal transport and intracellular motility in general.

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