The Roadblock Light Chain Binds a Novel Region of the Cytoplasmic Dynein Intermediate Chain

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Running Title: Roadblock light chains bind the dynein intermediate chains
Summary

Cytoplasmic dynein is the major minus-end directed microtubule-based motor in eukaryotic cells. It is composed of a number of different subunits including three light chain families: Tctex1, LC8, and roadblock. The incorporation of the roadblock light chains into the cytoplasmic dynein complex had not been determined. There are two roadblock genes in mammals, roadblock-1 and roadblock-2. We find that both members of the roadblock family bind directly to all of the intermediate chain isoforms of mammalian cytoplasmic dynein. This was determined with three complementary approaches. A yeast two-hybrid assay demonstrated that both roadblock light chains interact with intermediate chain isoforms from the IC74-1 and IC74-2 genes in vivo. This was confirmed in vitro with both a solid phase blot overlay assay and a solution-binding assay. The roadblock binding domain on the intermediate chain was mapped to an approximately 74 residue region. The binding domain is downstream of each of the two alternative splice sites in the intermediate chains. This location is consistent with the finding that both roadblock-1 and roadblock-2 show no binding specificity for a single IC74-1 or IC74-2 intermediate chain isoform. In addition, this roadblock binding domain is significantly downstream from both the Tctex1 and LC8 binding sites supporting the hypothesis that multiple light chain family members can bind to the same intermediate chain.
Introduction

Cytoplasmic dynein is responsible for transporting a number of different cargoes to the minus-ends of microtubules (1), including kinetochores, endosomes, lysosomes and viruses (2-7). In addition, cytoplasmic dynein has been implicated in the positioning of the Golgi apparatus, the assembly and orientation of the mitotic spindle, and the transport of microtubules (8-13).

The cytoplasmic dynein complex is comprised of a motor domain and a cargo-binding domain, both of which are crucial for the proper functioning of the motor complex (14,15). Each domain is comprised of different cytoplasmic dynein subunits. The heavy chains of cytoplasmic dynein comprise the motor domain. This domain consists of both a microtubule binding site and an ATP hydrolysis site that is required to generate the power necessary to move the complex along the microtubule (16). The major form of cytoplasmic dynein contains two identical heavy chains per complex, each with a molecular weight of ~530 kDa. In contrast, the cargo-binding domain is comprised of multiple isoforms of the intermediate chains, light intermediate chains, and light chains.

There are two intermediate chains per complex and these subunits have a molecular weight of ~74 kDa. In mammals, there are two intermediate chain genes, IC74-1 and IC74-2. Alternative splicing and phosphorylation produce multiple intermediate chain isoforms (17-19). The intermediate chains bind to the p150Glued subunit of the accessory complex, dynactin (18,20). There are also multiple isoforms of the light intermediate
chains with molecular weights of 50-60 kDa which are the products of two light intermediate chain genes, LIC1 and LIC2 (21,22).

Three different mammalian light chain families were recently identified with molecular weights between 10 and 14 kDa: Tctex1, LC8, and roadblock. Unlike the two intermediate chain genes, which are closely related, each of the three light chain families has a distinct primary sequence. The Tctex1 light chain family is composed of two related members, Tctex1 and rp3 (23,24). Tctex1 and rp3 family members are differentially expressed in various tissues (24). Furthermore, Tctex1, but not rp3, binds to rhodopsin (25,26). In addition to Tctex1 and rp3, there are also several more distantly related members of this family (27). The LC8 light chain family is comprised of three different members, LC8a, LC8b, and LC8c (28). Yeast two-hybrid screens have found multiple binding partners to LC8 family members including neuronal nitric oxide synthase (29) and the BCL-2 family protein, BIM (30). The roadblock light chain family is comprised of two members, roadblock-1 and roadblock-2 (31). Mutations of the roadblock gene in *Drosophila* exhibited defects in both axonal transport and mitosis (31). Both Tctex1 and LC8 family members were shown to bind to the intermediate chains of cytoplasmic dynein (32-35). The binding sites for these two light chains were found to be just C-terminal of the second alternative splice region in the intermediate chain gene, indicating that Tctex1 and LC8 could bind any of the intermediate chain isoforms. In addition, the binding sites for both light chains on the intermediate chain were found to be distinct, implying that both Tctex1 and LC8 could bind to the same intermediate chain.
In this study, we sought to complete the analysis of the organization of the cytoplasmic dynein complex by determining how the roadblock light chains incorporated into the complex. We report here that both mammalian roadblock light chains, roadblock-1 and roadblock-2, bind directly to the intermediate chains of cytoplasmic dynein. The hypothesis that individual roadblock light chains specifically bound to different intermediate chain isoforms was also tested. It was found that both roadblock light chains bind all IC74-1 and IC74-2 intermediate chain isoforms. We have mapped the binding region to an approximately 73 amino acid domain of the intermediate chain, of which only a 53 amino acid portion is hypothesized to be capable of binding the roadblock light chain. This domain is located significantly downstream of the previously reported Tctex1 and LC8 binding sites, indicating that a single intermediate chain could bind each of the three light chain families.
Experimental Procedures

Cloning of the human roadblock light chains. The human roadblock sequences (31) were used to design primers specific for roadblock-1 and roadblock-2. Roadblock-1 was amplified out of a human brain cDNA library by PCR using roadblock-1 specific primers and then inserted into pBluescript M13- vector (Stratagene) using the Sma1 site. Roadblock-2 was amplified from an EST (ATCC #1994230) using roadblock-2 specific primers and similarly introduced into the pBluescript M13- vector.

Yeast two-hybrid assay. Human roadblock-1 was subcloned into the pCR vector (Clontech) after EcoRI and XmaI restriction sites were introduced by PCR mutagenesis. Roadblock-1 was then digested out of the pCR vector and ligated into the pGADT7 activation domain vector of the Matchmaker system (Clontech). Rat intermediate chain constructs were similarly introduced into either the pGBKT7 or pGBT9 DNA-binding domain vector (Clontech). HF-7C yeast were co-transformed according to the Gietz LiAc Yeast Transformation protocol (36). Best results were obtained when the heat shock step was increased to two hours at 42° C. The yeast were resuspended in 100 µl of sterile water and 15 µl were dropped onto both minus (−) 2 and minus (−) 3 plates. The –2 plates, lacking both leucine (Leu) and tryptophan (Trp), showed co-transformation. The –3 plates, lacking Leu, Trp and histidine (His), demonstrate protein-protein interactions. To eliminate false positives arising from “leaky” HIS3 expression, 5 mM of a competitive inhibitor of the HIS3 pathway, 3-AT (3-amino-1,2,4-triazole), was added to the –3 plates. The plates were incubated at 30° C for 5-8 days. Positive interactions were
confirmed with a β-galactosidase assay that screened for the independent expression of the lacZ reporter gene.

**Yeast two-hybrid library screen.** Mouse roadblock-1 and roadblock-2 were cloned into a “bait” pAS2 DNA-binding domain vector (Clontech) and used to screen a mouse brain “prey” library. Sequencing of the individual “prey” plasmids and using the sequences to screen appropriate databases identified candidate roadblock light chain binding partners. The interaction of roadblock-1 and roadblock-2 and the candidate “prey” proteins was then verified by rescreening in a pair-wise yeast two-hybrid assay.

**In vitro synthesis of radiolabeled light chains.** A T7 promoter and Kozak sequence were introduced upstream of human roadblock-1, roadblock-2, and Tctex1 cDNA by PCR. The resulting cDNA was purified and used as the template in the Transcribe and Translate (TnT) Coupled Wheat Germ Extract System (Promega) according to manufacturer’s directions. The amino acid mixture used was lacking in methionine and 40 µCi of translational grade 35S methionine (Amersham Pharmacia) was added per 50 µl reaction. Best results were obtained when the reaction was incubated for two hours at 30°C.

**Blot Overlay assay.** The design and purification of full length IC74-1A, IC74-2C, and the truncations is described elsewhere (18,37). These constructs and purified bovine brain cytoplasmic dynein (38) were resolved on 4-16% or 4-20% gradient mini gels and transferred to 0.2 µm polyvinylidene difluoride (PVDF) membrane (BioRad) in a Hoeffer
blotting apparatus using 10 mM CAPS buffer pH 11 (Calbiochem). The blot overlay assay was as previously described (18) except in vitro synthesized radiolabeled light chain was used to probe the blot. The blot was then placed in a Phosphoimage cassette (Molecular Dynamics) and incubated at room temperature for approximately one week. The phosphoimage was analyzed with ImageQuant software. The location of the IC74-1A fragments was confirmed by screening the blot with the 74.1 intermediate chain antibody using enhanced chemical luminescence (ECL) (Pierce).

**Solution-binding assay using purified recombinant proteins.** 22.5 µl of in vitro synthesized radiolabeled light chain was incubated with 60 µg of either full length IC74-2C, a 1-125aa fragment of IC74-2C, or no intermediate chain. The mixture was next incubated on a shaker at 4° C for two hours. The mixture was then added to Protein A beads (Zymed) coupled with a pan intermediate chain antibody, 74.1, and incubated for three hours on a shaker at 4° C (38). The beads were washed twice with wash buffer (150 mM NaCl, 1 M Tris pH 8.1, 5 mM EDTA) and then once with de-ionized water. The beads were resuspended in 50 µl of 2X Lammelli sample buffer and 30 µl was resolved on a 4-20% gradient mini gel (ISC Bioexpress). The gel was then dried for 3 hours at 70° C (BioRad Model 583). The dried gel was then placed in a Phosphoimage cassette for approximately one week.
Results

Roadblock-1 interacts with multiple intermediate chains in an *in vivo* yeast two-hybrid assay. We recently identified roadblock-1 as a component of purified bovine brain cytoplasmic dynein (28). However, how the roadblock-1 light chain was incorporated into the cytoplasmic dynein complex was unknown. Three pieces of evidence led us to hypothesize that roadblock-1 binds to the intermediate chains of cytoplasmic dynein. First, previous experiments demonstrated that the intermediate chain base of the cytoplasmic dynein complex could be separated biochemically from the heavy chain motor domain (21). Analysis of the fractionated cytoplasmic dynein subunits revealed that the Tctex1 and LC8 light chains copurify with the intermediate chains on a sucrose gradient (24). Similarly, the roadblock related protein, LC7, is part of the IC-LC complex in flagellar dynein (39,40). Thirdly, both the Tctex1 and LC8 light chains have been recently demonstrated to bind directly to the intermediate chains (32-35). To determine whether the roadblock-1 light chain also binds to the intermediate chains of cytoplasmic dynein, a yeast two-hybrid assay was used. A roadblock-1/pGADT7 construct and three intermediate chain yeast two-hybrid constructs (either pGBT9 or pGBKT7) were made. There are two different cytoplasmic dynein intermediate chain genes (18). The intermediate chain genes are alternatively spliced to produce a number of different isoforms. We screened the binding of roadblock-1 to one IC74-1 product, IC74-1B, and two IC74-2 products, IC74-2B and IC74-2C. The results indicate that roadblock-1 interacts with each of these intermediate chains (Fig. 1). The interactions
were confirmed with a β-galactosidase assay (not shown). This experiment confirmed our hypothesis that the roadblock-1 light chain interacts with the intermediate chains.

**Both roadblock-1 and roadblock-2 bind to intermediate chain in an *in vitro* blot overlay assay.** To confirm the *in vivo* results that roadblock-1 binds to the intermediate chains of cytoplasmic dynein, we utilized an *in vitro* solid phase blot overlay assay. Purified brain cytoplasmic dynein, which contains all IC74-1 and IC74-2 intermediate chain isoforms, was resolved with SDS-PAGE and blotted to PVDF. The blot was incubated with $^{35}$S labeled roadblock-1 synthesized *in vitro* (Fig. 2A). Roadblock-1 specifically interacted with a band of the approximate weight of the intermediate chains of cytoplasmic dynein (Fig. 2B). This radiolabeled band was identified as the cytoplasmic dynein intermediate chain by immunochemistry. The other roadblock family member, roadblock-2, had not previously been shown to be a component of cytoplasmic dynein. Using the blot overlay assay, we determined that roadblock-2 also binds specifically to the intermediate chains of cytoplasmic dynein. As a positive control, we used the Tctex1 light chain, which has been shown to directly bind to the intermediate chains (32). Some low level non-specific binding in the 45-60 kDa region of the blot was observed for the three light chains. These blot overlay results confirm that roadblock-1 interacts with the intermediate chain and also demonstrates that the related family member, roadblock-2, interacts with cytoplasmic dynein intermediate chain.

**Roadblock-1 and roadblock-2 bind multiple intermediate chains in a yeast two-hybrid assay.** To further confirm the interaction between the roadblock light chains and
the intermediate chains, we used both roadblock-1 and roadblock-2 to screen a brain 

yeast two-hybrid library (Fig. 3). This approach also allowed us to determine if 

roadblock-1 and roadblock-2 interacted with any other subunits of cytoplasmic dynein. 

This library has previously been shown to contain the Tctex1 and LC8 light chains (27). 

Interactions between both roadblock light chains and the intermediate chains IC74-2A, 

IC74-2B, and an IC74-1 fragment were identified in the screen. The IC74-1 fragment 

was missing the first N-terminal 187 amino acids, a segment that includes the regions that 

are alternatively spliced to produce either IC74-1A or IC74-1B. This indicates that both 

roadblock light chains are capable of binding either IC74-1A or IC74-1B. A second 

construct was also identified that was only missing the first 43 amino acids and was 

determined to be IC74-1A (not shown). This indicated that the region of roadblock 

binding was found between amino acids 187 and 643 (the end) of the IC74-1 intermediate 

chain. In this screen, no colonies encoding cytoplasmic dynein heavy chain, light 

intermediate chains, or members of the Tctex1 and LC8 family were identified. 

However, roadblock light chain was identified as a binding partner, demonstrating that 

these light chains self-associate (Nikulina and King, manuscript in preparation).

**Both roadblock-1 and roadblock-2 bind to the cytoplasmic dynein intermediate 

chain in a solution-binding assay.** The fact that both roadblock light chains bound a C-

terminal IC-1 fragment in the yeast two-hybrid assay suggested that the binding domain 

for roadblock-1 and roadblock-2 was downstream of both the Tctex1 and LC8 binding 

sites. To further define the interaction of the roadblock light chains with the intermediate 

chain, an *in vitro* solution-binding assay was used. For these experiments, we used the
ubiquitously expressed IC74-2C intermediate chain (41). The IC74-2C isoform has the two alternative splicing regions removed. IC74-2C is found in all mammalian cell types, and in many cultured cells it is the only intermediate chain detected. Bacterially expressed IC74-2C intermediate chain and a 1-125aa fragment of IC74-2C were purified from bacterial lysates (18). Note that the 1-125aa fragment of IC74-2C is equivalent to a 1-159aa fragment of IC74-1A, which contains both splicing regions. $^{35}$S labeled light chains, roadblock-1, roadblock-2 and Tctex1, were synthesized *in vitro* and separately incubated with the intermediate chain or intermediate chain fragment. The pan intermediate chain antibody, 74.1, was used to immunoprecipitate the intermediate chain and any bound light chain. The epitope for the 74.1 antibody is located in the first 60 amino acids of the intermediate chain (not shown). The immunoprecipitates were resolved by SDS-PAGE, and the dried gel was analyzed by autoradiography. Note that the antibody binding does not block the binding of either the roadblock light chains or Tctex1 to the intermediate chain. Both roadblock-1 and roadblock-2 co-immunoprecipitated only with the full length intermediate chain. This demonstrates that they directly bind to IC74-2C and it confirms the yeast two-hybrid assay results (Fig. 4). Since neither roadblock-1 nor roadblock-2 bound to the 1-125aa fragment of IC74-2C, the roadblock binding domain is located between amino acid 125 of IC74-2C and the C-terminus (amino acid 612) of the intermediate chain. In contrast, Tctex1 co-immunoprecipitated with full length IC74-2C and the 1-125 fragment of IC74-2C. This is consistent with published results that indicate the Tctex1 binding site on IC74-2C is found between amino acids 106-115 (32). It had previously been reported that *in vitro* synthesized Tctex1 was immunoprecipitated with endogenous intermediate chain present
in an *in vitro* translation mixture (26). However, when the wheat germ extract is used for the *in vitro* synthesis reaction we found no immunoprecipitation of either Tctex1 or the two roadblock light chains without the addition of exogenous intermediate chain (Fig. 4). This is consistent with findings that the cytoplasmic dynein intermediate chain, and other dynein subunits, cannot be identified in plant databases (42). The results from this experiment indicate that both roadblock light chains bind IC74-2C *in vitro* and that the roadblock binding site is C-terminal to the Tctex1 site.

**Roadblock binding to IC74-1A truncation constructs.** To further define the roadblock binding domain on the cytoplasmic dynein intermediate chain, we utilized an IC74-1A truncation series in the blot overlay assay. All constructs contained the N-terminal, which is recognized by the 74.1 intermediate chain antibody (18). The IC74-1A constructs were resolved by SDS-PAGE and transferred to PVDF. To confirm the location of the intermediate chain fragments, the blot was probed with the 74.1 intermediate chain antibody (Fig. 5A). Each of the six intermediate chain constructs was identified as well as smaller fragments that are the products of premature translation termination (18). The blots were then incubated with either $^{35}$S labeled roadblock-1 or Tctex1 and the binding of the light chain to the fragments was determined by autoradiography (Fig. 5). Roadblock-1 strongly bound to full length IC74-1A, the 1-404aa fragment, and the 1-314aa fragment. The intensity of each of these three bands appears to be equivalent. Roadblock-1 did not bind to the 1-242aa, 1-228aa, 1-150aa, or the 1-123aa fragments. This indicates that the roadblock binding domain on the intermediate chain is between amino acids 242 and 314. In contrast, Tctex1 bound to full
length IC74-1A, the 1-404aa fragment, the 1-242aa fragment, and the 1-228aa fragment. Tctex1 did not bind to either the 1-150aa or the 1-123aa fragment. The results from this blot overlay assay indicate that the Tctex1 binding site is between 123aa and 228aa on the intermediate chain. This is consistent with recent results that indicate that the Tctex1 binding site on IC74-1A is between amino acids 139-157 (32). Surprisingly, Tctex1 consistently did not interact with the 1-314aa IC741A fragment. This could be due to the 1-314aa fragment not renaturing properly for Tctex1 binding, since Tctex1 bound both the smaller 1-242aa fragment and the 1-228aa fragment. The results of this experiment identify a roadblock binding domain on the intermediate chain that is significantly downstream of both the Tctex1 and LC8 binding sites.
Discussion

Prior to this work, the roadblock light chain family was the only subunit whose location in the cytoplasmic dynein complex was unknown. The data in this report establish that both roadblock-1 and roadblock-2 interact with all five known intermediate chain subunits, IC74-1A, IC74-1B, IC74-2A, IC74-2B, and IC74-2C. Three complementary methods were utilized to demonstrate that the two mammalian roadblock light chains bind the cytoplasmic dynein intermediate chain. Both roadblock-1 and roadblock-2 interact with the intermediate chain isoforms from the IC74-1 and IC74-2 genes in vivo in a yeast two-hybrid assay. Two in vitro methods were used to confirm these findings. First, a solid phase blot overlay assay demonstrated that both roadblock-1 and roadblock-2 interact specifically with the intermediate chains of purified cytoplasmic dynein from bovine brain. Secondly, roadblock-1 and roadblock-2 bound specifically to the ubiquitous intermediate chain, IC74-2C, in a solution-binding assay. We have further identified a 74 amino acid domain of the intermediate chain necessary for roadblock light chain binding between amino acids 242 and 314. This domain is C-terminal to the region of alternative splicing, consistent with the finding that the roadblock light chains bind all intermediate chain isoforms. It is possible that the interaction identified in the yeast two-hybrid assay is not a direct interaction of the two co-transformed proteins, but rather one mediated by their mutual binding to an endogenous yeast protein. However, this possibility is unlikely since no proteins homologous to roadblock can be identified in the Saccharomyces cerevisiae database (31). In addition there is only 24% identity, primarily in the WD repeat region, between the 74 kDa mammalian intermediate chain
and the 58 kDa *Saccharomyces cerevisiae* pac11p intermediate chain (43). With this identification of the roadblock binding domain on the intermediate chain, the location of each of the subunits in the cytoplasmic dynein complex is now known. Members of all three of the light chain families directly bind to the intermediate chain at distinct, non-overlapping sites.

Our results emphasize the importance of the intermediate chain for the assembly and organization of the dynein complex. While the dynein heavy chains are responsible for generating the power to move along microtubules, it is the base of the complex that is important for binding cargo. The largest component of the cargo-binding base, the intermediate chain, has a number of important functional domains (Fig. 6). The coiled-coil domain at the N-terminus is part of a 123 amino acid region necessary for the binding to a region of 611 amino acids of p150, a subunit of the accessory protein dynactin (18,20,44). Two alternative splicing regions, separated by a serine rich area, follow the coiled-coil domain. Phosphorylation of one of these serines is utilized for the regulation of dynein binding to p150, and thus dynactin and vesicular cargo (37). The Tctex1 and LC8 light chain binding sites immediately follow the second region of alternative splicing (32,33,45).

The C-terminal region of the intermediate chain is composed of 7 WD repeat motifs (46,47). Domains containing WD repeats have been demonstrated to form a propeller structure (48). The entire structure must be properly folded to form a platform to which other proteins bind. A WD repeat domain is important for the interaction of the
intermediate chain with the N-terminus of dynein heavy chain and mapping studies suggest that a complete WD repeat domain is crucial for intermediate chain-heavy chain binding (49). The intermediate chain binding site on the heavy chain has been mapped to a 150 residue region in *Dictyostelium* and a 250 residue region in mammalian cytoplasmic dynein (50,51). In addition to binding to the intermediate chains, the heavy chains also bind to the light intermediate chains. In mammalian cytoplasmic dynein, the light intermediate chains bind to a ~250 residue region near the N-terminal of the heavy chain (51). It is interesting to note that the binding sites for the intermediate chains and the light intermediate chains on the heavy chain overlap by approximately 50 amino acids. However, the two subunits bind in a non-exclusive manner allowing both intermediate chains and light intermediate chains to incorporate into the same complex (51).

The intermediate chains also serve as a scaffold for light chain binding in the dynein complex. Our results indicate that the roadblock light chain binds to the intermediate chain significantly downstream of the Tctex1 and LC8 binding sites. The C-terminal portion of the roadblock binding domain overlaps the initial twenty-one amino acids of the first WD repeat. Since the entire WD domain structure must be properly folded in order for proteins to bind, it is unlikely that the roadblock light chain is interacting with just a portion of the first WD repeat (48). This suggests that the roadblock binding domain is restricted to approximately 242-294aa of IC74-1A. Since roadblock-1 binds to both IC74-1 and IC74-2 isoforms, we compared the sequences of IC74-1A and IC74-2C to look for areas of similarity in this region. We found that in this 53aa region there are
only five non-conservative substitutions. Despite this conservation, the region between the end of the LC8 site and the beginning of the WD repeat domain had not previously been shown to have any functional significance. Furthermore, the Tctex1 and LC8 light chain intermediate chain binding regions are separated by only eight amino acids, yet the roadblock binding site is separated from the other light chain binding regions by at least 70 amino acids. The wide separation between the roadblock binding site and the other light chain binding sites supports the hypothesis that all three light chain families bind to the same intermediate chain.

Structural prediction algorithms suggest that the roadblock light chains are members of a superfamily of proteins implicated in NTPase regulation (52). The roadblock light chain binds next to the WD repeat domain, the region that binds the heavy chain. If the roadblock light chain has such a function, its location is consistent with the hypothesis that it regulates either the interactions between the dynein subunits or the functional properties of the heavy chain.

Cytoplasmic dynein has many different functions in a cell. Unlike the kinesin superfamily, which has multiple heavy chains that have been implicated in specific functions, the major cytoplasmic dynein has only one heavy chain (53,54). It is thought that the cargo-binding subunits of cytoplasmic dynein, the intermediate, light intermediate, and light chains, are responsible for the transport of specific cargoes. Consistent with this hypothesis, each of these subunits has multiple isoforms and many appear to be responsible for particular functions. Both Tctex1 and the LC8 light chains
have been identified as binding partners in many yeast two-hybrid assays (29,55-57). Recent work has also shown that the light intermediate chain 1 (LIC1) containing cytoplasmic dynein binds to the centrosome component, pericentrin, while dynein with LIC2 does not (58).

The roadblock light chain is crucial for the proper functioning of the cytoplasmic dynein complex. Mutations of the roadblock gene in Drosophila result in defects in mitosis and axonal degeneration. The degeneration is caused by vesicle accumulations in axons that are similar to those produced by mutations of other motor protein subunits (59,60). In particular, there were specific accumulations of small, clear vesicles that resembled the distinct TrkA/NGF vesicle (31). Since there are six other roadblock-like genes in Drosophila, this suggested that this roadblock light chain specifically binds to this TrkA/NGF vesicle for retrograde transport. However, it remains to be determined if there are specific binding partners for mammalian roadblock-1 and roadblock-2.

We demonstrate that both roadblock light chains bind to all the intermediate chain isoforms. Previously, it has only been hypothesized that the Tctex1 and LC8 cytoplasmic dynein light chains bind both IC74-1 and IC74-2 intermediate chain isoforms (32,33). In this study, we confirm part of this hypothesis by determining that Tctex1 directly binds to both IC74-1A and IC74-2C. It is thought that cytoplasmic dyneins with the different intermediate chain isoforms are responsible for specific cellular functions. For example, the IC74-2C isoform is ubiquitous and in many cells is sufficient for constitutive dynein function (17,19). The distribution of other intermediate chains is more restricted, for
example, IC74-1A is only found in neurons, suggesting it has a neuron specific function. This hypothesis is supported by the observation that the expression of IC74-1A and the other intermediate chain isoforms are developmentally regulated in brain, and that the changes in expression levels occur just prior to axon extension (17,19,61). Changes in the relative expression levels of the roadblock light chains have also been observed. In hepatocellular carcinoma tissue, the expression levels of roadblock-1 and roadblock-2 are altered relative to control tissue samples (62). In addition, the roadblock-1 light chain is highly expressed in the visual cortex and upon sensory stimulation, its mRNA and protein levels are rapidly down regulated (63). It is thus possible that independent regulation of the expression levels of the different interacting cytoplasmic dynein subunit isoforms creates different domains for specific cargo binding or dynein regulation.

It has also been suggested that the light chains may serve as an intramolecular glue responsible for keeping the cytoplasmic dynein complex intact. Since there are both two members of each light chain family, and two intermediate chains per complex, it is possible that light chain dimers reinforce the integrity of the cargo-binding base (27,28,34). Interestingly, intermediate chain binding to the roadblock light chain family differs from Tctex1 light chain binding. During our experiments, we repeatedly observed that the binding of the Tctex1, roadblock-1, and roadblock-2 to the intermediate chain had different sensitivities to the concentration of salt. In the solution-binding assay, it was observed that washing the immunoprecipitates in 150 mM NaCl did not appear to affect the interaction of either light chain with the intermediate chain. However, in the presence of 1 M NaCl, it was observed that while Tctex1 binding to the intermediate
chain was unaltered, roadblock-1, and roadblock-2 no longer bound the intermediate chain. This suggests a significant difference in the molecular basis for the binding of these light chains to the intermediate chain.

The intermediate chains are located at the base of the cytoplasmic dynein complex and are important for dynein binding to cargo (18,20,64,65). We report here that both roadblock light chains bind to all the isoforms of the cytoplasmic dynein intermediate chain. The roadblock binding region is located significantly downstream of the other two light chain binding sites, in a novel conserved region of the intermediate chain. Thus all three light chain families are components of the base of the cytoplasmic dynein complex. This data supports the hypothesis that all three light chain families bind to the same intermediate chain.
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Figure Legends

Figure 1. Roadblock-1 interacts with both IC74-1 and IC74-2 cytoplasmic dynein intermediate chain isoforms in a yeast two-hybrid assay. A. Minus (–) 2 plate lacking leucine and tryptophan (Leu and Trp). Colony growth on this plate indicates co-transformation of the DNA-binding domain (either pGBT9 or pGBKT7) and activation domain (pGADT7) vectors. B. Minus (–) 3 plate lacking Leu, Trp and Histidine (His) (HIS3 is the reporter gene). Colony growth indicates an interaction between the two proteins. C. Schematic diagram of the plates. Transformation with pGADT7 [1] and pGBKT7 [2] alone was used as negative controls. Co-transformation with PGADT7 and pGBKT7 [3] was used as a positive control for the –2 co-transformation plate and a negative control for the –3 interaction plate. Roadblock-1 interacted with IC74-1B, 2B, and 2C [4-6]. There was no interaction when the light chain or intermediate chains were co-transformed with their respective empty partner plasmids [7-10].

Figure 2. Roadblock-1 and roadblock-2 bind directly to the intermediate chains in vitro in a blot overlay assay. A. Autoradiograph of 35S labeled light chains synthesized in vitro and resolved on a 4-20% gradient SDS-polyacrylamide gel. The only proteins synthesized with radiolabel are the respective cytoplasmic dynein light chains. Tctex1 has a molecular weight of ~14 kDa and migrates slower than the ~12 kDa roadblock-1 and roadblock-2. The position of the molecular weight standards is indicated on the right side of the gel. B. Interaction of cytoplasmic dynein intermediate chains and light chains. Purified bovine brain cytoplasmic dynein was resolved on a 4-16% gradient SDS-
polyacrylamide gel and transferred to PVDF. The blots were incubated with \(^{35}\)S labeled light chain, either Tctex1, roadblock-1 (RB-1) or roadblock-2 (RB-2), and exposed to a phosphor screen. The autoradiograph of each blot is shown. To confirm that it was the intermediate chain (IC) that was specifically recognized, a blot was screened with the 74.1 intermediate chain antibody (74.1). The position of the molecular weight standards is indicated on the left side of the blot.

Figure 3. Roadblock-1 and roadblock-2 interact with both IC74-1 and IC74-2 cytoplasmic dynein intermediate chain isoforms from a brain library. A. Minus (−) 2 plate lacking both Leu and Trp. Colony growth on the −2 plate indicated co-transformation of the activation domain (pACT2) vector and the DNA-binding domain (pAS2) vector. B. Minus (−) 3 plate lacking Leu, Trp, and His (HIS3 is the reporter gene). Colony growth on the −3 plate indicates an interaction between the two proteins. C. Schematic diagram of the −2 and −3 plates. Co-transformation with pACT2 and pAS2 [1] was used as a positive control for the −2 co-transformation plate and as a negative control for the −3 interaction plate. A positive control for both the −2 and −3 plates was the direct interaction of pVA3-1 + pTD1-1 [2]. Roadblock-1 and roadblock-2 were cloned into the pAS2 vector and used to screen a brain library. Roadblock-1 [3-5] and roadblock-2 [7-9] were found to bind to IC74-2A, IC74-2B, and an IC74-1 fragment. The IC74-1 fragment was sequenced and found to be missing the first 187aa. This indicates that this N-terminal 187aa region does not contain the roadblock binding site. There was no interaction when the light chain or intermediate chains were co-transformed with their respective empty partner plasmids [6, 10-13]. This screen extends our previous
results that roadblock-1 and roadblock-2 interact with both IC74-1 and IC74-2 intermediate chain isoforms.

**Figure 4. Both roadblock-1 and roadblock-2 bind directly to full length IC74-2C, but not the first 125 aa of IC74-2C, in a solution-binding assay.** *In vitro* synthesized $^{35}$S labeled light chain, (A) roadblock-1, (B) roadblock-2, and (C) Tctex1, was incubated with either full length IC74-2C intermediate chain (**FL**), the first 125 amino acids of IC74-2C (**125**), or no intermediate chain (-). After a two-hour incubation, the reaction solutions were immunoprecipitated with the 74.1 antibody. The immunoprecipitate was resolved on a 4-20% gradient SDS-polyacrylamide gel, dried, and exposed to a phosphor screen. Only the light chain region is shown. The autoradiographs demonstrate that both roadblock-1 and roadblock-2 bind to full length IC74-2C, but not the first 125 amino acid fragment. This indicates that the roadblock binding domain on the intermediate chain is between approximately 125aa and the end (amino acid 612) of IC74-2C. In contrast, Tctex1 binds to both full length IC74-2C and the first 125 amino acids. This is consistent with published results that the Tctex1 binding site is between 106-123aa of IC74-2C. The results of this experiment indicate that the roadblock binding domain on the intermediate chain is downstream of the Tctex-1 binding site.

**Figure 5. The roadblock-1 binding site is between approximately 228aa and 314aa on the intermediate chain.** Blots resolving the IC74-1A truncation series were probed with either (A) the intermediate chain antibody 74.1, (B) $^{35}$S-labeled roadblock-1, or (C) $^{35}$S-labeled Tctex1. The truncation protein resolved in each lane is indicated on the top of
the panels. The location of each of the truncation series is shown on the left.  **A.** The 74.1 intermediate chain antibody recognized each of the truncated constructs. Smaller fragments were detected that were premature termination products (18).  **B.** Roadblock-1 bound to full length IC74-1A, the 1-403aa fragment and the 1-314aa fragment. Roadblock-1 did not bind to the 1-242aa, the 1-228aa, the 1-150aa, or the 1-123aa fragments.  **C.** Tctex1 bound to full length IC74-1A, the 1-403aa fragment, the 1-242 fragment and the 1-228aa fragment. This indicates that the roadblock binding domain on the intermediate chain is between amino acids 242-314.

**Figure 6. Schematic diagram of the cytoplasmic dynein intermediate chain. (Top)**
Diagram of the intermediate chain gene of cytoplasmic dynein. The coiled-coil domain makes up the first sixty amino acids of the intermediate chain. The two alternative splice sites are indicated in red (A1 and A2). The serine rich region (S) is located between the two alternative splice sites. The seven WD repeat motifs are indicated in purple. The binding sites for the p150 subunit of dynactin, Tctex1, LC8, and the Heavy Chain are shown as blue bars.  **(Middle)** Summary of roadblock binding data. Roadblock-1 did not bind the 1-123aa, 1-150aa, 1-228aa, or 1-242aa IC74-1A truncations, shown as red bars. Roadblock-1 strongly bound to both the 1-314aa and 1-404aa fragments, shown as green bars. The IC74-1 fragment that interacted with both roadblock-1 and roadblock-2 in a yeast two-hybrid assay is also shown in green. The roadblock binding domain is shown as a light blue bar.  **(Bottom)** Final intermediate chain diagram complete with the roadblock binding domain. Note that the last twenty-one amino acids of the roadblock binding domain overlaps with the initial part of the first WD repeat and thus are not likely
to be part of the binding domain. The final roadblock binding domain is between
approximately amino acids 242-294 of IC74-1A. Data was compiled from the following
papers to create this figure: (18,20,32,33,37,46,47,49)
**Abbreviations:** IC, intermediate chain; LIC, light intermediate chain; LC, light chain; PVDF, polyvinylidene difluoride; aa, amino acids.

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Figure 1

A. -2 Plate

B. -3 Plate

C. Schematic Diagram

1. pGADT7 alone
2. pGBKT7 alone
3. pGADT7 + pGBKT7
4. Rmy2480d > pGADT7 + IC74-kB/pGBKT7
5. Rmy2480d > pGADT7 + IC74-kB/pGAL19
6. Rmy2480d > pGADT7 + IC74-kC/pGAL19
7. Rmy2480d > pGADT7 + pGBKT7
8. pGADT7 + IC74-kB/pGBKT7
9. pGADT7 + IC74-kB/pGAL19
10. pGADT7 + IC74-kC/pGAL19

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Figure 3

A. -2 Plate

B. -3 Plate

C. Schematic Diagram

1. pAS2-I + pACT2
2. pAA-I + pTDB-I
3. orn/Blodo+1pAS2-I + 1Cy64dpACT2
4. orn/Blodo+1pAS2-I + 1Cy642dpACT2
5. orn/Blodo+1pAS2-I + 1Cy662dpACT2
6. orn/Blodo+1pAA-I + pACT2
7. orn/Blodo+2pAS2-I + 1Cy64dpACT2
8. orn/Blodo+2pAS2-I + 1Cy642dpACT2
9. orn/Blodo+2pAS2-I + 1Cy662dpACT2
10. orn/Blodo+2pAA-I + pACT2
11. pAS2-I + 1Cy64dpACT2
12. pAS2-I + 1Cy642dpACT2
13. pAS2-I + 1Cy662dpACT2
|   | Roadblock-1 | Roadblock-2 | Tctex1 |
|---|-------------|-------------|--------|
| FL | 125         | 125         | 125    |
|    |             |             |        |
A. 74.1 Screen of IC74-1A Fragments

B. Roadblock-1 Blot Overlay

C. Tctex-1 Blot Overlay

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Figure 5
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Figure 6
The roadblock light chain binds a novel region of the cytoplasmic dynein intermediate chain

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