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The Role of the Light Intermediate Chains in Cytoplasmic Dynein Function: a Dissertation

Sharon H. Tynan

University of Massachusetts Medical School

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THE ROLE OF THE LIGHT INTERMEDIATE CHAINS IN CYTOPLASMIC DYNEIN FUNCTION

A Dissertation Presented

By

Sharon Hughes Tynan

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
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In partial fulfillment of the requirements for the degree of:

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Approved as to style and content by:

____________________
Dr. Elizabeth Luna, Chair of Committee

____________________
Dr. Patricia Wadsworth, Member of Committee

____________________
Dr. Stephen Doxsey, Member of Committee

____________________
Dr. Stephen Lambert, Member of Committee

____________________
Dr. William Theurkauf, Member of Committee

____________________
Dr. Richard B. Vallee, Dissertation Mentor

____________________
Dr. Thomas B. Miller, Dean of the Graduate School of Biomedical Sciences

Department of Cell Biology
March 21, 2000
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"What a long, strange trip it's been ..."
-Grateful Dead
ABSTRACT

Cytoplasmic dynein is a multisubunit complex involved in retrograde transport of cellular components along microtubules. The heavy chains (HC) are very large catalytic subunits which possess microtubule binding ability. The intermediate chains (IC) are responsible for targeting dynein to its appropriate cargo by interacting with the dynactin complex. The light intermediate chains (LIC) are previously unexplored subunits that have been proposed to modulate dynein activity by regulating the motor or the IC-dynactin interaction. The light chains (LC) are a newly identified class of subunit which are also thought to have regulatory functions.

In the first part of this work, I analyzed the relationship between the four SDS-PAGE gel bands that comprise the light intermediate chains. 1- and 2-D electrophoresis before and after alkaline phosphatase treatment revealed that the four bands are derived from two different polypeptides, each of which is phosphorylated. Peptide microsequencing of these subunits yielded sequences that indicated similarity between them. cDNA cloning of the rat LICs revealed the presence of a conserved P-loop sequence and a very high degree of homology between the two different rat LICs and among LICs from different species.

The second series of experiments was designed to analyze the association of pericentrin with cytoplasmic dynein. First, various dynein and dynactin subunits were co-
associate with pericentrin in these experiments. Co-precipitation from $^{35}$S labeled cell extracts revealed a direct interaction between LIC and pericentrin. Comparison of pericentrin binding by LIC1 and LIC2 showed that only LIC1 was able to bind. Further investigation of the relationship between LIC1 and LIC2 demonstrated that each LIC will self-associate, but they will not form heterooligomers. Additionally, using co-overexpression and immunoprecipitation of LIC1, LIC2, and HC, I have shown that binding of the two LICs to HC is mutually exclusive.

Finally, I investigated the relationships between dynein HC, IC, and LIC by examining the interactions among the subunits. IC and LIC were both found to bind to the HC, but not to each other. Despite the lack of interaction between IC and LIC, they are, in fact, present in the same dynein complexes and they have partially overlapping binding sites within the N-terminal sequence of the HC. The HC dimerization site was determined to extend through a large portion of the N-terminus, and it includes both the IC and LIC binding sites, although these subunits are not required for dimerization.

Together these studies implicate the light intermediate chains in dynein targeting. Targeting of dynein to its cargo has been thought to be performed by the dynactin complex, and for one particular cargo, the kinetochore, there is considerable evidence to support this model. The results presented here suggest that the light intermediate chains appear to function in a separate, non-dynactin-based targeting mechanism.
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CHAPTER I

INTRODUCTION

Cells are able to transport themselves from one place to another, change shape, and move cellular components through their cytoplasm. Diffusion is too slow and non-specific to be solely responsible for these processes; specialized motor proteins have evolved which can efficiently perform motile functions. A variety of motors have been found which use cytoskeletal elements as tracks along which to move. These proteins include: myosin, an actin-based motor; axonemal dynein, a microtubule-based motor for ciliary and flagellar beating; and the microtubule-based cytoplasmic motors kinesin and cytoplasmic dynein.

Microtubule-based motors carry out many intracellular functions by moving cellular components along the extensive microtubule network. The kinesin family of motors supports motility towards the plus (rapidly growing) ends of microtubules (anterograde transport), with a few exceptions, while cytoplasmic dynein translocates its cargo towards the minus ends (retrograde transport). Proper functioning of these motor proteins is required for a wide range of functions, including: correct positioning of organelles, transport of vesicles, maintenance of the microtubules at the centrosome, axonal transport, aspects of mitosis, and nuclear migration (see reviews: Karki and Holzbaur, 1999; Vallee and Sheetz, 1996).

Microtubule motor proteins act as mechanochemical enzymes, hydrolyzing ATP to produce force. Kinesin and cytoplasmic dynein have large, globular head domains,
which are responsible for both the ATPase and microtubule binding activities. The ATPase of both proteins is activated by binding to microtubules, while ATP hydrolysis induces dissociation from microtubules (Paschal et al., 1987; Paschal and Vallee, 1987). Although the detailed mechanism of force production is not fully understood, the cyclic binding and release of microtubules in response to ATPase activity are likely to play a key role. Motor proteins also require a mechanism for attaching to their “cargo”. Kinesins and dynein are arranged so that the two head domains come together to form a base where accessory subunits may assist in binding to organelles and other structures (Vallee and Sheetz, 1996). Although the mechanism of cargo binding by the cytoplasmic motors is not fully understood, true kinesin has been proposed to attach to cargo by binding to a membrane-bound protein called kinectin. For cytoplasmic dynein, there is evidence that an accessory protein complex called dynactin is involved in attachment to cargo (see below).

**Cytoplasmic Dynein**

Cytoplasmic dynein was first identified as a microtubule associated protein (MAP1C) which has microtubule-activated ATPase activity and translocates microtubules when affixed to glass coverslips (Euteneuer et al., 1988; Lye et al., 1987; Paschal et al., 1987; Paschal and Vallee, 1987). Characterization of the ATPase activity of this motor, subunit composition, as assessed by SDS-PAGE of purified bovine brain MAP1C, and STEM analysis, revealed considerable similarity to axonemal dynein (Gibbons et al., 1978;
Paschal et al., 1987; Paschal and Vallee, 1987; Penningroth, 1986; Shpetner et al., 1988; Vallee et al., 1988), thus the name cytoplasmic dynein.

Cytoplasmic dynein is a large complex which contains four different classes of subunits: heavy chains (HCs), intermediate chains (ICs), light intermediate chains (LICs), and light chains (LCs). All of these classes, with the exception of the LICs, have homologues in axonomal dynein. The HCs are large (~530 kDa) polypeptides which contain four well-conserved ATPase (P-loop) consensus sequences (Koonce et al., 1992; Mikami et al., 1993; Zhang et al., 1993). The reason for the presence of multiple P-loops is not clear. The first is the most highly conserved and has long been suspected to be the primary site of ATPase activity (reviewed in Holzbaur and Vallee, 1994). Vanadate, a potent inhibitor of dynein ATPase activity (Gibbons et al., 1978; Paschal et al., 1987), mediates UV photocleavage at a site near the first P-loop sequence (Gee et al., 1997; Gibbons et al., 1987). The location of the cleavage site suggests that ATPase inhibition is due to vanadate blocking ATP from binding to the first P-loop. When full-length HC and HC containing a point mutation within the first P-loop sequence were overexpressed in cultured mammalian cells, the normal HC was found to be distributed throughout the cytoplasm, while the point mutant was localized to microtubules (Gee et al., 1997). This suggests that the mutant HC is incapable of ATP hydrolysis and, therefore, unable to release microtubules.
The HCs are also the site of microtubule binding activity. The actual microtubule binding site was elusive for many years, but it has now been found to lie within the HC primary sequence C-terminal to the P-loops, surrounded by sequence that is predicted to form an anti-parallel coiled-coil (Gee et al., 1997; Gee and Vallee, 1998). This coiled-coil appears to form an elongated stalk, which projects from the dynein head and can be seen in electron micrographs of dynein. The stalk presumably interacts with microtubules through a small globular region at the tip (Gee and Vallee, 1998). The stalk structure is intriguing for two reasons: first, it may keep some distance between the large dynein head and the microtubule, thus allowing more interactions with dynein or other MAPs per length of microtubule (Vallee and Gee, 1998). Second, ATP hydrolysis may produce a conformational change which would move the stalk relative to the rest of the head. Movement of the stalk could cause a change in the position of dynein (and the attached cargo) with respect to the microtubule.

The cytoplasmic dynein intermediate chains are subunits which bind to the base of the complex (Habura et al., 1999; King and Witman, 1990; Steffen et al., 1996; Tynan et al., 2000a) and interact with a protein complex called dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). The ICs, based on their location at the base of the molecule, have been thought to be involved in cargo binding, suggesting that the dynactin complex is also part of this process (see below). The light intermediate chains are a family of cytoplasmic dynein-specific subunits, which have also been referred to as light chains (Gill et al., 1994). They were designated light intermediate chains (Hughes et al., 1995)
to distinguish them from other cytoplasmic and axonemal dynein subunit classes, and to prevent confusion with axonemal light chains, homologues of which were subsequently found to be present in cytoplasmic dynein. The LICs are unique in that they contain a P-loop sequence, the function of which remains obscure (Gill et al., 1994; Hughes et al., 1995; Tynan et al., 2000a; Tynan et al., 2000b). Evidence obtained in the course of this thesis has shown that the LICs are involved in a cargo binding mechanism that is distinct from dynactin-mediated targeting (Purohit et al., 1999; Tynan et al., 2000b) (see below). The light chains were long thought to be absent from cytoplasmic dynein; however, several have recently been identified (Bowman et al., 1999; Harrison et al., 1998; King et al., 1996; King and Patel-King, 1995; Tai et al., 1998; Wang and Satir, 1998). The functions of the light chains within the dynein complex have not yet been determined, although they have been suggested to regulate cargo binding or motor activity (Tai et al., 1998).

The Dynactin Complex

Although purified cytoplasmic dynein is sufficient to cause gliding of microtubules on glass coverslips (Paschal and Vallee, 1987), it is not capable of supporting vesicle motility without the addition of an activating fraction (Schroer and Sheetz, 1991). Analysis of the activating fraction has revealed a 20S complex that is required for vesicle motility; this complex was named dynactin, for dynein activator. The dynactin complex consists of: a short, F-actin-like filament that is made up of the actin-related protein Arp1 and proteins that cap each end of the filament (Eckley et al., 1999; Garces et al., 1999;
Schafer et al., 1994); p150glued, the mammalian homologue of the Drosophila gene Glued, which is able to bind microtubules (Holzbaur et al., 1991; Gill et al., 1991); p50, also called dynamitin (Echeverri et al., 1996); and other LMW species that are not yet well characterized.

p150glued has structural similarity to a family of cytoskeletal linker proteins, or CLIPs. CLIP-170 (Pierre et al., 1992), which, unlike dynactin, has not been found to be part of a large complex. This protein binds to microtubules and has been implicated in docking endosomes onto microtubules, possibly to promote the subsequent recruitment of motors. This suggests a possible role for p150glued in dynein-dynactin based motility; it may tether both dynein and the vesicle to microtubules when dynein detaches during its ATPase cycle (Waterman-Storer et al., 1995). Recent evidence (King and Schroer, 2000) has shown that the processivity of dynein is slightly increased by dynactin, consistent with the tethering model of dynactin function.

The p150glued subunit of the dynactin complex has been shown to directly interact with the cytoplasmic dynein intermediate chain (Vaughan and Vallee, 1995; Karki and Holzbaur, 1995). The intermediate chains, based on their location in the base of the dynein complex, had been thought to be involved in targeting to cargo; therefore, finding that IC and p150glued directly interact suggested that dynactin is the dynein receptor on organelles. None of the dynactin subunits cloned thus far have been found to contain a transmembrane domain, indicating that it must attach to organelles in another way if it is
actually a receptor. It has been suggested that the Arp1 filament of dynactin could interact with a spectrin-like skeleton on organelles.

Evidence for dynactin involvement in cargo binding was provided using p50 (dynamitin) overexpression (Echeverri et al., 1996) in cultured mammalian cells. Overexpression of this subunit was found to dissociate the dynactin complex in cytoplasmic extracts and inhibit dynein functions in overexpressing cells (Burkhardt et al., 1997; Echeverri et al., 1996). Several vesicular organelles were found to be mislocalized, consistent with loss of dynein function. For example, endosomes and lysosomes, which are normally transported bidirectionally along microtubules by dynein and kinesin were located at the cell periphery, consistent with kinesin being the only functional motor. The Golgi complex, which dynein normally maintains in a perinuclear location in COS-7 cells was found to be scattered throughout the cytoplasm when p50 was overexpressed. There were some organelles not affected by p50 overexpression, particularly ER, mitochondria, and peroxisomes (Burkhardt et al., 1997; Presley et al., 1997), suggesting that these types of cargo may not bind to dynactin, or even use dynein, in retrograde transport.

p50 overexpression also provided direct evidence for cargo binding at kinetochores. Dynein (Pfarr et al., 1990; Steuer et al., 1990) and dynactin (Echeverri et al., 1996) have been localized to kinetochores during prometaphase, when they may function in microtubule capture or alignment of chromosomes at the metaphase plate (Echeverri et al., 1996). p50 overexpression causes dynein and dynactin both to be lost from
kinetochores, indicating that the dynactin complex is responsible for attaching dynein to this particular form of cargo, the kinetochore (Echeverri et al., 1996). Further evidence was provided by the finding that p50 itself binds to another kinetochore protein, ZW10 (Starr et al., 1998), mutations of which result in release of both dynein and dynactin.

The Light Intermediate Chains

The light intermediate chains are a cytoplasmic dynein-specific subunit family identified as 53/55 and 57/59 kDa doublets by SDS-PAGE. When this thesis work was initiated, nothing was known about this class of subunit, and they had been proposed to have regulatory functions. I set out to characterize this class of subunits, first by 2-D electrophoresis and phosphatase treatments to look for phosphorylation. While evidence for phosphorylation was obtained, the results of greater interest indicated that there are only two different LIC polypeptides (LIC1 and LIC2), and that they are related to each other (Chapter II). cDNA cloning of the two polypeptides (Chapter II and IV) demonstrated that they are actually highly homologous to each other at the primary sequence level and they have a well-conserved P-loop sequence.

In the second part of this work, I attempted to identify the subunit of dynein or dynactin that interacts with the centrosomal protein, pericentrin, which had been shown to be transported to the centrosome in a dynein-dependent manner (Young et al., 2000), and to associate with dynein (Purohit et al., 1999). I found that LIC was the only class of subunit which would bind specifically (Chapter III), suggesting that the LICs are
involved in cargo binding by dynein. Based on this first identified function for the LICs, I compared LIC1 and LIC2, and found that only LIC1 was able to bind to pericentrin. Furthermore, I found that the LICs bind to the dynein HC mutually exclusively, demonstrating that there are two functional subfractions of the dynein complex, which differ in their LIC content (Chapter IV).

In light of the LIC1-pericentrin interaction, I was interested in determining whether the LICs were located at the base of the dynein complex, where they must be for cargo binding. After locating the LIC binding site within the N-terminus of the HC sequence, I examined the interrelationships between IC, LIC, and HC. I found that both IC and LIC bind directly to the HC, but do not interact with each other. Although both subunit classes are thought to be involved in targeting, I found that both IC and LIC could bind to the HC at the same time. In the final phase of this thesis work, I mapped the binding sites of IC and LIC within the HC primary sequence, and determined the sequence responsible for HC dimerization (Chapter V).
Cytoplasmic dynein is a minus-end-directed microtubule associated motor protein (Paschal and Vallee, 1987). It has been implicated in retrograde axonal transport and movements of organelles, such as lysosomes and endosomes, towards the minus ends of microtubules (Lacey and Haimo, 1992; Lin and Collins, 1992; Paschal and Vallee, 1987; Schnapp and Reese, 1989; Schroer et al., 1989). The subcellular distribution of certain organelles, such as the Golgi apparatus, has also been attributed to cytoplasmic dynein (Corthesy-Theulaz et al., 1992). Immunological studies (Pfarr et al., 1990; Steuer et al., 1990) and *in vitro* reconstitution of chromosome-associated movements (Hyman and Mitchison, 1991) have also suggested that cytoplasmic dynein is associated with kinetochores, indicating that it may play a role in chromosome movement during mitosis.

Cytoplasmic dynein has been found to be structurally and biochemically related to axonemal dyneins (Lye et al., 1987; Paschal et al., 1987; Shpetner et al., 1988; Vallee et al., 1988), the ATPases responsible for flagellar and ciliary movements (Gibbons and Rowe, 1965). Despite the similarities between axonemal and cytoplasmic dyneins, there are also many differences, the most striking involving subunit composition. All dyneins contain high molecular weight heavy chains (HCs) which have multiple P-loop consensus sequences (Gibbons et al., 1991; Koonce et al., 1992; Mikami et al., 1993; Ogawa, 1991;
Zhang et al., 1993). Whether or not all of these domains are functional in ATP binding is not yet known. Cytoplasmic dynein also contains, along with the heavy chains, at least three electrophoretic species at 74 kDa, and a group of four species with apparent molecular weights of 53, 55, 57, and 59 kDa (Paschal et al., 1987). Axonemal dyneins show greater subunit complexity, with a variety of ICs ranging from 69 to 120 kDa, and numerous light chains of unknown function with M₉ of 10-20 kDa (Pfister et al., 1982; Piperno and Luck, 1979; Tang et al., 1982)(reviewed in Holzbaur and Vallee, 1994).

The cytoplasmic dynein 74 kDa subunits were cloned and shown to be homologous to a 70 kDa intermediate chain (IC70) of Chlamydomonas flagellar outer arm dynein (Paschal et al., 1992). The 70 kDa axonemal subunit has been shown by immunoelectron microscopy to be located at the base of the dynein molecule and is believed to be involved in binding the outer arm dynein to the axonemal A subfiber microtubule (King and Witman, 1990). By comparison, the 74 kDa cytoplasmic dynein IC has been postulated to be involved in attaching the cytoplasmic protein to organelles and kinetochores.

The function of the 53-59 kDa cytoplasmic dynein subunits, which we have termed light intermediate chains (LICs) (Hughes et al., 1993) is unknown. The present study was initiated to understand the basis for the electrophoretic complexity of these polypeptides and their relationship to other dynein subunits. We report here the primary structure of a 55 kDa cytoplasmic dynein LIC, along with evidence that the electrophoretic complexity of these polypeptides is due to the existence of multiple isoforms and to phosphorylation.
We have found that the 55 kDa LIC is unrelated to previously characterized axonemal and cytoplasmic dynein ICs and LCs. Furthermore, we find that the polypeptide contains a P-loop element, which has homology to a family of ATPases, the ABC transporter proteins.

**Materials and Methods**

*Protein Chemistry*

Cytoplasmic dynein was purified from calf brain white matter as previously described (Paschal et al., 1991), except that sucrose density gradients were prepared in 100 mM Tris-HCl pH 8.0, 4 mM MgCl₂. For peptide sequencing, the sucrose gradient fractions containing cytoplasmic dynein were electrophoresed on a 7% polyacrylamide gel as described (Laemmli, 1970). The protein was then transferred to PVDF (Millipore, Bedford, MA) in 10 mM CAPS, 10% MeOH for 45 minutes at 50 volts. The blot was stained with Coomassie Brilliant Blue R and the 53/55 kDa and 57/59 kDa doublets were excised for sequencing. The protein was subjected to *in situ* digestion with either trypsin or endoproteinase Glu-C. The peptides were eluted from the blot and separated by HPLC (Hewlett Packard 1090M) with a C8 microbore column (Aebersold, 1989; Fernandez et al., 1994). Automated peptide sequencing was performed on an Applied Biosystems 477A sequencer. Direct N-terminal sequencing was performed on undigested samples using an Applied Biosystems model 492 Procise sequencing system. All peptide sequence was determined in the Worcester Foundation for Experimental Biology Protein Chemistry Facility.
Alkaline phosphatase treatment of cytoplasmic dynein was carried out in 100 mM Tris-HCl pH 8.0, 4 mM MgCl₂, 0.1 mM ZnCl₂. Alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to a final concentration of 20-120 units/ml. Pyrophosphate was added to 50 mM in controls to inhibit dephosphorylation. All reactions were incubated at 37°C for 2 hours. The effects of alkaline phosphatase treatment were analyzed by SDS-PAGE and 2-D electrophoresis (O’Farrell, 1975) using the BioRad mini-PROTEAN II electrophoresis cell and mini tube module, followed by silver staining using the procedure of Oakley et al. (Oakley et al., 1980).

cDNA Cloning

A rat brain cDNA library in the Lambda ZAP II vector (Stratagene, LaJolla, CA) was screened by oligonucleotide hybridization. A partially degenerate oligonucleotide, 5'-AA(A/G)CCIGA(A/G)GA(T/C)GCITA(T/C)GA(A/G)GA(T/C)TT-3', was designed on the basis of amino acid sequence from peptide 4 (Table 1), with the exception that inosines were used at positions with four nucleotide choices. This oligonucleotide was ³²P-end labelled and used for hybridization in tetramethylammonium chloride (TMAC) hybridization solution (Jacobs et al., 1988) containing 3 M TMAC, 0.1 M NaPO₄, pH 6.8, 1mM EDTA, pH 8.0, 5X Denhardt’s solution, 0.6% SDS, 100 µg/ml denatured salmon sperm DNA at 39°C for 48 hours. The filters were washed in 3 M TMAC, 50 mM Tris-HCl pH 8.0, 0.2% SDS at room temperature for 15 minutes, followed by 36°C for 1 hour, 40°C for 15 minutes, and 44°C for 15 minutes. The TMAC was then removed from
the filter by three 10 minute washes in 2X SSC, 0.1% SDS at room temperature. Two clones, RAT1 and RAT2, were picked and isolated by two additional rounds of screening. Another clone, RP1, was subsequently isolated from the same library using probes random-primed from RAT1. Hybridization was performed overnight at 65°C in Rapid-hyb (Amersham, Arlington Heights, IL) hybridization buffer. The filters were washed in 2X SSPE, 0.1% SDS, 0.1% pyrophosphate, twice for 30 minutes at room temperature, then 1X SSPE, 0.1% SDS, 0.1% pyrophosphate, 30 minutes 65°C, and 0.7X SSPE, 0.1% SDS, 0.1% pyrophosphate twice for 30 minutes at 65°C.

In order to clone the 5' end of the cDNA, a restriction fragment of RP1 was used to rescreen the library. Screening was performed using Rapid-hyb as described above. The fifteen clones isolated were all "rescued" using helper phage according to the manufacturer (Stratagene). The plasmids were then probed with an oligonucleotide corresponding to the 5' end of LIC coding sequence from within RP1. Positive clones were sequenced (Sequenase, version 2.0, United States Biochemical, Cleveland, OH), and one was found to contain the N-terminal peptide sequence.

DNA sequencing was completed on both strands using nested deletions (Erase-A-Base: Promega, Madison, WI), convenient restriction sites, and oligonucleotides. All DNA and protein sequence was assembled and analyzed using the GCG DNA analysis programs, including MOTIFS and BESTFIT. The National Center for Biotechnology Information (NCBI) databases were scanned using BLAST (Altschul et al., 1990). The statistical
significance of the alignments was determined using RDF2 (Lipman and Pearson, 1985), and additional homology was examined using the BLOCKS Database Version 7.01 (Henikoff and Henikoff, 1994). The Protein Kinase Catalytic Domain Database (Hanks and Quinn, 1991) was also scanned for homology using FASTA (Pearson and Lipman, 1988).

Northern Blots
A multiple tissue northern (MTN) blot (Clontech Laboratories, Palo Alto, CA) was hybridized with probes random-primed from a Bgl II/Xho I fragment of RP1, which contains nearly the entire coding sequence of LIC53/55 and 664 bp of the 3’ untranslated sequence. The MTN was also hybridized with probes from the 3’ untranslated sequence alone. All hybridizations were performed in Rapid-hyb at 65°C overnight. The blots were washed with 2X SSPE, 0.1% SDS, 0.1% pyrophosphate for 15 minutes at room temperature, followed by 0.5X SSPE 0.1% SDS, 0.1% pyrophosphate at 65°C twice for 30 minutes each.

Results

Alkaline phosphatase reduces the complexity of cytoplasmic dynein light intermediate chains
By SDS-PAGE, four bands can be distinguished in the 50-60 kDa size range (Paschal et al., 1987). The four bands appear as two doublets and have been assigned molecular
weights of 53, 55, 57, and 59 kDa. In order to examine the complexity of the LICs further, cytoplasmic dynein was subjected to 2-D gel electrophoresis (Fig. 1B). Each of the four electrophoretic species seen by SDS-PAGE resolved into multiple spots.

To determine whether phosphorylation was responsible for the observed electrophoretic complexity, we treated cytoplasmic dynein with alkaline phosphatase. The effect of alkaline phosphatase was analyzed by SDS-PAGE (Fig. 1A) and 2-D gel electrophoresis (Fig. 1B). A clear reduction in the intensity of the 59 kDa band as well as the 55 kDa band was observed with increasing alkaline phosphatase concentration, while the 57 and 53 kDa bands increased in intensity. This change in pattern was also observed by 2-D gel electrophoresis (Fig. 1B, right panel), but relatively little effect on the number of spots corresponding to each remaining band was observed.

To learn more about the molecular basis for the complexity of the LICs, we set out to obtain primary sequence data by direct amino acid sequencing and by cDNA analysis. The LIC SDS-PAGE doublets were digested with either endoproteinase Glu-C or trypsin, and the resulting peptides were then purified and subjected to microsequencing. Multiple peptides were sequenced from each doublet (Table 1, all except sequence 1). Interestingly, two of the sequences generated from LIC57/59 peptides were related to sequences generated from LIC53/55 peptides (Table 1B). However, differences between sequences from the two doublets were also apparent. These data suggested that LIC53/55 and LIC57/59 represent related but distinct protein isoforms.
The full-length polypeptides were also subjected to N-terminal sequence analysis. The N-terminus of LIC57/59 appeared to be blocked, but a mixture of two overlapping sequences was obtained from the N-terminus of undigested LIC53/55 (Table 1A, sequence 1).

Using part of the amino acid sequence of peptide 4 of LIC53/55 (Table 1A), a 26-mer oligonucleotide was designed for screening a rat brain lambda ZAP II library. Two clones, RAT1 and RAT2, were isolated and found to encode multiple LIC53/55 peptide sequences. One of these clones was used to make random-primed probes to re-screen the library. One 4.4 kb clone, RP1 (Fig. 2), was isolated and sequenced completely. This clone was found to have part of a cDNA coding for malate dehydrogenase fused near the 5'-end of the LIC coding sequence. To obtain the remainder of the LIC sequence, oligonucleotides made from the 5'-most portion of the LIC sequence of RP1 were used to re-screen the library. Out of fifteen clones isolated at this stage, all of them were found to be identical to part of RP1, but only one contained enough 5' sequence to locate the initiator methionine, previously identified by N-terminal sequencing of the undigested LIC53/55 doublet (see Table 1, sequence 1). Clones contributing to the initial identification of LIC cDNAs and to the final cDNA sequence are presented in Fig. 2.

The 4.4 kb cDNA encodes a protein of 55 kDa (Fig. 3). Each of the peptides sequenced from LIC53/55 was found in the deduced amino acid sequence (Fig. 3). Only cDNAs
corresponding to the minor N-terminal sequence were found. All but one of the peptide sequences obtained from LIC57/59 could be aligned with the LIC53/55 deduced amino acid sequence (Table 1 and Fig. 3). The peptide data suggest that our cDNAs encode LIC53/55. This conclusion is based on the fact that peptide 11 (Table 1C), derived from LIC57/59, was absent from the deduced amino acid sequence, and peptide 7 (Table 1B) from LIC53/55 is closer to the deduced amino acid sequence than is the corresponding peptide from LIC57/59.

**Sequence analysis**

We compared the deduced amino acid sequence of LIC53/55 with the previously determined sequences of cytoplasmic and axonemal dynein intermediate and light chains. No clear relationship was observed with the five known rat cytoplasmic dynein intermediate chain isoforms (Paschal et al., 1992; Vaughan and Vallee, 1993), or IC70 (Mitchell and Kang, 1991) and IC78 (C. Wilkerson, personal communication) of *Chlamydomonas* flagellar outer arm dynein. Despite the size difference between the LICs and the *Chlamydomonas* flagellar outer arm dynein light chains (LCs; M_r 10-20 kDa), we compared our sequence with the one available light chain sequence (10 kDa) and found no significant relationship (S.M. King and R.S. Patel-King, unpublished results).

The deduced amino acid sequence of LIC53/55 was also analyzed for secondary structure and for the presence of structural motifs (see Materials and Methods). No substantial
blocks of alpha-helix, coiled-coil alpha-helix, or beta-sheet were observed. However, the MOTIFS program revealed a P-loop sequence near the N-terminus, from amino acid 60 to 68 suggesting a possible nucleotide-binding site.

While a comparison to the Protein Kinase Catalytic Domain Database failed to reveal similarity to known kinases, a search of the protein sequence databases using the BLAST program revealed short segments of homology with a variety of proteins, including several nucleotidases. Prominent among these were members of the ABC transporter family of ATPases. Screening the databases with a 29 amino acid sequence from the P-loop region of LIC53/55 (amino acids 47-76) revealed the highest degree of homology with the ABC transporters (Table 2). Analysis of the LIC53/55 sequence using the BLOCKS program, which searches for multiple blocks of homology among members of multi-gene families, again revealed a relationship with the ABC transporters. However, two additional downstream sequence elements shared by ABC transporters were not detected in LIC53/55.

Gill et al. (1994) reported the cloning of cDNAs encoding a 56 kDa chicken brain cytoplasmic dynein subunit referred to as DLC-A. Comparison of that sequence with LIC53/55 revealed 64% amino acid identity and 80% similarity including conservative amino acid substitutions (Fig. 4). We note that amino acid sequences derived from the bovine LIC57/59 peptides are closer to the chicken DLC-A than to the amino acid sequence deduced from the LIC53/55 cDNAs. Of particular interest, peptide 11 (Table
1C), derived from LIC57/59, is clearly related to amino acids 402-417 of the DLC-A sequence (Fig. 4), but is absent from the LIC53/55 sequence. Furthermore, the N-terminal sequence obtained from bovine LIC53/55 is completely absent from DLC-A. These results suggest that DLC-A is the chicken homologue of LIC57/59.

Northern blot analysis

To gain further insight into the relationship between LIC isoforms, Northern blot analysis was performed (Fig. 5). LIC transcripts of 4.4, 3.5, and 2.0 kb were observed. The 4.4 kb species was found in all tissues examined and was the most prominent species in most tissues. The 2.0 and 3.5 kb transcripts were particularly abundant in testis, although they were detectable in other tissues. The same blot was hybridized with a probe to the 3'-untranslated sequence. The 4.4 kb and 3.5 kb bands were observed in all tissues, while hybridization with the 2.0 kb species was eliminated (data not shown).

Discussion

The LICs represent the least well-characterized components of cytoplasmic dynein. The present study indicates that these polypeptides are related to each other and constitute a new dynein subunit class which is distinct from the heavy, intermediate, and light chains.
We have found that the electrophoretic complexity of the LICs is greater than previously recognized (Paschal et al., 1987). However, our results also indicate that the complexity may derive from extensive modification of as few as two polypeptide isoforms. Alkaline phosphatase treatment substantially simplified the one-dimensional LIC electrophoretic pattern (Fig. 1A), suggesting that phosphorylation is responsible for some of the observed complexity. High levels of alkaline phosphatase failed to eliminate all of the 2-D electrophoretic complexity (Fig. 1B). This observation may reflect alkaline phosphatase-insensitive phosphorylation, other post-translational modifications, or even greater isoform diversity than that revealed by our peptide and cDNA sequence analysis.

We obtained two distinct N-terminal sequences from Edman degradation of the p53/55 electrophoretic doublet (Table 1). It is unlikely that this heterogeneity contributed to the observed electrophoretic complexity because the two amino acids included in the longer sequence are uncharged. Despite the fact that the shorter sequence was more abundant than the longer sequence, the latter corresponded to the amino acid sequence deduced from cDNA analysis. The shorter sequence could reflect alternative splicing of LIC transcripts; however, the observed heterogeneity seems equally likely to reflect limited proteolysis at the N-terminus of the polypeptide.

The results of the present analysis and that of Gill et al. (1994) are most readily understood in terms of two LIC genes. In the present study, peptide sequences derived from LIC53/55 were all found in the deduced amino acid sequence. Amino acid
sequences from two LIC57/59 peptides were found to be related to sequences from LIC53/55 peptides, but the sequence of peptide 7 (Table 1B) is actually much closer to sequences in chicken DLC-A. In addition, sequence from LIC57/59 peptide 11 (Table 1C) was only found in DLC-A, and the amino terminal sequence of LIC53/55 was unique to the LIC53/55 deduced amino acid sequence. Together these results suggest that each of the two LIC isoforms have been conserved throughout vertebrate evolution. Furthermore, because sequence differences are distributed across the length of the isoforms, it seems more likely that they represent products of distinct genes, rather than being the results of alternative splicing.

We identified three LIC transcripts in rat at 4.4, 3.5 and 2.0 kb at high stringency (Fig. 5), one of which (2.0 kb) was not recognized by a 3'-untranslated probe. In view of its size, it is likely that the 4.4 kb species corresponds to LIC53/55. However, the origin of the 3.5 kb species, which also hybridized with the 3'-untranslated probe, is uncertain. We note that the 3'-untranslated sequence for LIC53/55 contains repetitive elements (Fig. 3). Thus, the relationship between the 3.5 kb and 4.4 kb transcripts is uncertain. Gill, et al. (1994) described DLC-A cDNAs of 2.4 and 1.6 kb in chicken which were identical except for unusual alternative polyadenylation site usage. They suggested that these sequences correspond to the 2.4 and 1.6 kb transcripts observed in their study, but no evidence is provided to confirm this prediction. Thus, it remains uncertain whether the transcripts observed in our study encode two or three LIC isoforms, and the correspondence between rat and chicken transcripts remains to be resolved.
Relationship to other dynein polypeptides and ATPases

The present study, together with that of Gill, et al. (1994), appears to complete the molecular cloning of the known cytoplasmic dynein subunits. The complex array of cytoplasmic dynein subunits (Paschal et al., 1987) can now be seen to represent only three polypeptide classes. Whether axonemal dyneins will prove to have components related to the LICs, and whether cytoplasmic dynein will prove to have components related to the axonemal LCs, remains to be determined. However, it is clear that molecular analysis of the numerous dynein subunits which have been identified biochemically (reviewed in Holzbaur and Vallee, 1994) will likely be required to determine their structural and functional interrelationships.

We observed LIC53/55 to contain a P-loop element near the N-terminus of the polypeptide. While such elements are found in many nucleotidases, it is difficult to assess whether or not they are functionally significant. This issue is particularly acute for the dyneins, which contain from four to five P-loop sequences per heavy chain. It is not known if more than one of these is functionally important (reviewed in Holzbaur and Vallee, 1994).

However, in the case of the LICs we have detected additional homology beyond the P-loop sequence with a subset of ATPases, the ABC transporters (also called traffic ATPases). Most of these proteins are involved in regulation of cell surface channels.
The LICs contain only a portion of the conserved ABC transporter sequence; thus, we feel it is unlikely that they are closely related to the ABC transporters in function. However, the extended homology between the LICs and the ABC transporters around the P-loop region strongly suggests some common feature in their mechanisms of action, presumably ATP binding and hydrolysis. Future work in our laboratory will be directed at testing the LICs for enzymatic activity.

Potential roles for the LICs in cytoplasmic dynein function

An intriguing question is the possible role for an additional ATPase in the dynein complex. One interesting possibility is in the regulation of organelle and kinetochore binding. Recent work has suggested that the link between cytoplasmic dynein and other subcellular structures may be quite complex and subject to an elaborate regulatory mechanism involving an interaction with an additional polyprotein complex, the dynactin (or Glued) complex (Gill et al., 1994; Holzbaur et al., 1991; Paschal et al., 1993; Schroer and Sheetz, 1991). A direct interaction between cytoplasmic dynein and dynactin has not yet been observed. Nonetheless, in a search for subcellular receptors for cytoplasmic dynein, we have found evidence for a direct interaction between the cytoplasmic dynein ICs and p150\textsuperscript{Glued} (K.T. Vaughan, E.L.F. Holzbaur and R.B. Vallee, unpublished observations). Curiously, p150\textsuperscript{Glued} has also been found to have a microtubule-binding site (Waterman-Storer et al., 1993), which would be predicted to interfere with dynein-mediated motility rather than to stimulate it. Together these data suggest that the dynein-p150\textsuperscript{Glued} interaction must be regulated.
We suggest that a possible role for the LICs is in regulating these interactions. Perhaps the LICs serve to order a series of interactions between the two complexes and the cellular substrates for dynein-mediated motility (such as organelles). In this case, the LICs may behave as low turnover, kinetic "switches", ordering the steps in a complex pathway.
Figures

Figure 1. The effect of alkaline phosphatase treatment on cytoplasmic dynein light intermediate chains

A) 20S dynein was treated with 20-120 units/ml of alkaline phosphatase for 2 hours, then run on a 9% SDS-polyacrylamide gel and silver stained. The top of the gel including the cytoplasmic dynein heavy chain is not shown due to intense silver staining. For untreated dynein (lane 1), the predominant bands are 55 and 59 kDa. With increasing alkaline phosphatase concentration, the 55 and 59 kDa bands shift to 53 and 57 kDa, respectively (see lane 5 for maximum effect). The far right lane shows alkaline phosphatase inhibition by 50 mM pyrophosphate. B) 2-D gel of untreated LICs (left), and LICs treated with 80 units/ml alkaline phosphatase (right).
Figure 2. Line diagram of LIC35/55 cDNAs

Only the clones contributing to the initial identification of the LIC cDNA (RAT1) and to the final cDNA sequence (RP1 and FP2) are presented. The dotted line at the 5' end of RP1 indicates the region which was found to encode malate dehydrogenase rather than LIC35/55. Numbering corresponds to the final nucleotide sequence in Figure 3.
Figure 3. Primary sequence of LIC53/55

The nucleotide and deduced amino acid sequences of LIC53/55 are shown. cDNA and protein sequence numbering is presented in the left margin. Peptide sequences are underlined and the P-loop consensus sequence is double underlined. A consensus polyadenylation signal was identified at nucleotides 4296-4301 (AATAAA). This sequence can be retrieved from GenBank using Accession number U15138.
Figure 4. Comparison of the primary structure of rat LIC53/55 and chicken DLC-A

LIC53/55 and DLC-A were aligned using BESTFIT. The double underlined region represents peptide 11 (Table 1), the only peptide sequence not found in LIC53/55. | represents identity, : represents strong conservative substitutions, and . represents weak conservative substitutions. The two sequences are 64% identical overall plus 16% conservative substitutions.
| LIC 53/55 | MAFVEVEKL, LAGPSGPAAVMGLD... TEEDRAQQGFLMSILLIRVSTRASRKLPSGMNLIVFGDGSQATLMTHLGA 77 |
| DLC-A | MAAVRAGSFQGSAASAAGWASAGELRASQEREEDCQNLMSCLSEVSTKSLPSGKVLGQEGDQGATSLIQGSI 80 |
| LIC 53/55 | EQGKKGKLPSVLRTSSC0VWILDGLYKKGKLVFAVASILKSTLVLIFVADMSRPTINLESKOIAVSLR 157 |
| DLC-A | EYLLGGYVLPSVHEMDDQDCNMLDGDLGLYKKGKLVKGAENGSLKDLTVLVDMSRPTANSLEGSKASVVR 160 |
| LIC 53/55 | HHHWKIPPAEMDLERHRMDPQYIPEEMCQGSQRQGLTSQSSDEENVALPLGDNYLTHFLIPVVTCZKDAV 237 |
| DLC-A | HHNKLIPPEAMDLERHRMDPQYIPEEMCQGSQRQGLTSQSSDEENVALPLGDNYLTHFLIPVVTCZKDAV 240 |
| LIC 53/55 | SIIEKRDYDHEHDFQIAMLQPLQALYTVSVESEQNLDLVIDYKTVHATQFHTIPALVQEDVIFIPAGMDNE 317 |
| DLC-A | SIIEKRDYDHEHDFQIAMLQPLQALYTVSVESEQNLDLVIDYKTVHATQFHTIPALVQEDVIFIPAGMDNE 320 |
| LIC 53/55 | KIALLHEMPTTVKPDAYEFTFIPVRLHVDEKELAADMEQVPILEKQ 382 |
| DLC-A | KIALLHEMPTTVKPDAYEFTFIPVRLHVDEKELAADMEQVPILEKQ 382 |
| LIC 53/55 | GQGAPSVPAMSP. GTSVIIKDPDHNKRMABAESKGLASFFEMSSLLKKTGPSPASAGGVQSGKAXSGTSEGQPRSLTEQC 461 |
| DLC-A | GSVTIESVSTIPAGSKQIKIPNKGATSEKVLAPFFSSLKKTGPSPASAGGVQSGKAXSGTSEGQPRSLTEQC 461 |
| LIC 53/55 | COTQGATVESLSEQIEMRTIKPO... GNVEKSTENAA 497 |
| DLC-A | KSSQKVPVTEVQAELDRIKRPEMVSPSPTSEPBEKGA 514 |
**Figure 5. Multiple tissue northern blot**

A rat multiple tissue northern blot was hybridized with random-primed probes from the coding sequence of LIC53/55 including 664 bp of 3' untranslated sequence. The positions of hybridizing bands at 4.4 kb, 3.5 kb and 2.0 kb are indicated.
Table 1. Comparison of rat LIC 53/55 deduced amino acid sequence to bovine LIC peptides

<table>
<thead>
<tr>
<th></th>
<th>cDNA</th>
<th>LIC 53/55 (major)</th>
<th>LIC 53/55 (minor)</th>
</tr>
</thead>
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<td>APVGVEKKL</td>
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<td></td>
<td>LIC 53/55</td>
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<td>LIC 53/55 (minor)</td>
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<td>GGPASVPSAPGTSV</td>
<td>GGPASVPSAPGTSV</td>
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<tr>
<td>B 7</td>
<td>cDNA</td>
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<td>DFQDYIEPEEQGSPQR</td>
</tr>
<tr>
<td></td>
<td>LIC 53/55</td>
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<td>DFQDYIEPEEQGSPQR</td>
</tr>
<tr>
<td></td>
<td>LIC 57/59</td>
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<td>DFQDYIEPEEQGSPQR</td>
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<td>11*</td>
<td>LIC 57/59</td>
<td>DVSSNVAAYVAPAGSK</td>
<td>DVSSNVAAYVAPAGSK</td>
</tr>
</tbody>
</table>

* sequence 1 is the N-terminal sequence of LIC 53/55. Two different sequences were obtained. Protein with the major sequence at the N-terminus was approximately 2.5 times more abundant than protein with the minor sequence.

# peptide 11 was sequenced from LIC 57/59. The deduced amino acid sequence of LIC 53/55 does not contain any sequences with significant homology.

* Peptides 2 and 5 were generated by endoprotease Glu-C digestion. All other peptides were generated by digestion with trypsin.
Table 2. LICs show homology to the extended P-loop sequence of ABC transporter proteins

<table>
<thead>
<tr>
<th>LIC53/55</th>
<th>LPSGKNILVFGEDGSGKTTLMTKL</th>
<th>LPSGKSVLGLGEDGAGKTSLIGKI</th>
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<tbody>
<tr>
<td>DLC-A1</td>
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<td></td>
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<tr>
<td>VirB11&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>C. elegans Multidrug Resistance Protein&lt;sup&gt;4&lt;/sup&gt;</td>
<td>IPSGKSVAFIGRNAAGKSTLLRMI</td>
<td></td>
</tr>
<tr>
<td>Sulfate Permease&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>klbA gene product&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbomycin Resistance Protein&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The underlined amino acids are conserved with LIC53/55.

1<sup>gp</sup> X79088; 2<sup>pir</sup> F47301; 3<sup>sp</sup> P24586; 4<sup>sp</sup> P34712; 5<sup>sp</sup> P16676; 6<sup>gp</sup> L05600; 7<sup>gp</sup> M80346
CHAPTER III
PERICENTRIN DIRECTLY INTERACTS WITH CYTOPLASMIC DYNEIN LIGHT INTERMEDIATE CHAIN

The centrosome is the major microtubule nucleating organelle in animal cells (Kellogg et al., 1994; Zimmerman et al., 1999). It is usually composed of a pair of centrioles surrounded by a protein matrix from which microtubules are nucleated (Gould and Borisy, 1977; Szollosi et al., 1972). The centrosome proteins pericentrin and γ-tubulin are localized to the matrix material where they form a unique lattice-like network (Dictenberg et al., 1998). The lattice appears to represent the higher order organization of γ-tubulin rings, structures comprised of γ-tubulin and several other proteins that appear to provide the templates for nucleation of microtubules at the centrosome (Moritz et al., 1995; Schnackenberg et al., 1998; Zheng et al., 1995). γ-Tubulin and pericentrin are also part of a large cytoplasmic protein complex that may represent the fundamental subunit of microtubule nucleation before its assembly at the centrosome (Dictenberg et al., 1998).

The assembly and molecular organization of the centrosome is important for bipolar spindle assembly during mitosis (for review see Waters and Salmon, 1997). Functional abrogation or depletion of pericentrin or γ-tubulin disrupts centrosome assembly and organization, and creates structural defects in microtubule asters and spindles (Doxsey et al., 1994; Felix et al., 1994; Stearns and Kirschner, 1994). The molecular motor cytoplasmic dynein and the nuclear mitotic apparatus protein (NuMA) also play key roles...
in the organization of spindle poles (Karki and Holzbaur, 1999; Merdes and Cleveland, 1997).

The precise role of pericentrin in spindle function is currently unknown. The protein has been shown to contribute to the organization of microtubule arrays in both interphase and mitosis. Pericentrin antibodies introduced into mouse oocytes and *Xenopus laevis* embryos disrupt the organization of centrosomes and meiotic and mitotic spindles (Doxsey et al., 1994). Moreover, when added to *Xenopus* extracts, the antibodies inhibit the assembly of microtubule asters.

To further examine the role of pericentrin in spindle organization, the protein was overexpressed in somatic cells (Purohit et al., 1999). Pericentrin overexpression did not have any detectable effects on interphase centrosomes, although spindle organization was disrupted in mitotic cells. Mitotic defects observed include: multipolar, monopolar, and distorted spindles; mispositioned spindles; and spindles with misaligned chromosomes at prometaphase. The spindle defects caused by excess pericentrin are similar to those seen upon overexpression of the p50 (dynamitin) subunit of dynactin (Echeverri et al., 1996). Since the basis of the defects caused by dynamitin overexpression was found to be inactivation of cytoplasmic dynein (Echeverri et al., 1996), the localization of dynein and dynactin to spindle poles and kinetochores was assessed (Purohit et al., 1999) in pericentrin overexpressing cells. Dynein staining was seen to be diminished at kinetochores and spindle poles, while dynactin was unaffected. Dynactin overexpression
has also been found to disrupt the normal localization of many organelles (Burkhardt, 1998; Echeverri et al., 1996). One of the most dramatic effects being on the Golgi apparatus, which was found to be scattered throughout the cytoplasm of COS-7 cells, rather than at its normal perinuclear location. Pericentrin overexpression was also seen to cause this type of disruption (Purohit et al., 1999). These results suggested an interaction between dynein and pericentrin. Immunoprecipitations from extracts of cells overexpressing pericentrin were found to contain cytoplasmic dynein intermediate chain, and conversely, immunoprecipitations with anti-dynein antibodies contained overexpressed pericentrin. Immunoprecipitations with anti-dynactin antibodies, however, did not precipitate overexpressed pericentrin.

To further investigate the relationship between dynein and pericentrin, we tested a number of dynein and dynactin subunits in a co-overexpression and immunoprecipitation assay with pericentrin. Of all the subunits tested, only cytoplasmic dynein LIC was found to interact with pericentrin. Co-precipitation from extracts of $^{35}$S labelled, overexpressing cells revealed that a LIC fragment and pericentrin were the only proteins specifically immunoprecipitated, demonstrating a direct interaction between pericentrin and cytoplasmic dynein light intermediate chain.
Materials and Methods

cDNA Constructs

The preparation of cDNAs encoding full-length rat p150glued (Vaughan et al., 1999), human dynamitin (Echeverri et al., 1996), rat myc-tagged cytoplasmic dynein intermediate chain 2C (IC2C; Vaughan and Vallee, 1995), rat FLAG-tagged cytoplasmic dynein heavy chain (Mazumdar et al., 1996), full-length pericentrin-HA (Purohit et al., 1999), and full-length and truncated rat LIC (Tynan et al., 2000b) have been described elsewhere.

Antibodies

Affinity-purified rabbit IgG was prepared from sera raised against the COOH terminus of pericentrin (Doxsey et al., 1994). Anti-HC polyclonal was a gift from Dr. Atsushi Mikami (unpublished results). Anti-p150glued (Vaughan et al., 1999; Vaughan and Vallee, 1995), anti-p50 (Echeverri et al., 1996), and anti-myc (Gee et al., 1997) have been described elsewhere. Anti-HA was purchased from BAbCO (Richmond, CA).

Immunoprecipitation

COS-7 cells were cultured in DMEM (GibcoBRL, Grand Island, NY) with 10% fetal calf serum and 100 μg/ml penicillin and streptomycin (GibcoBRL). 50-75% confluent cells were transfected with Lipofectamine (GibcoBRL) for 6-14 hrs. in serum-free DMEM, then the medium was removed and replaced with complete DMEM. 24-40 hours after
transfection, the cell monolayers were rinsed 2X in D-PBS and scraped into modified RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 1 mM EGTA, 1 μg/ml leupeptin and aprotinin and 1mM AEBSF). The cells were lysed in the RIPA buffer on ice for 20 minutes, then debris was sedimented in a microfuge at full speed for 10 minutes. The resulting extracts were used for immunoprecipitation with either anti-HA and protein G agarose beads (Amersham) or affinity purified anti-pericentrin with protein A agarose beads (Amersham). Immunoprecipitations were incubated at 4°C overnight with gentle agitation. Beads were washed 5X in modified RIPA buffer and eluted with 2X SDS-PAGE sample buffer at 100°C for 5 minutes. Eluates were immunoblotted and probed with indicated antibodies.

\[35S\text{-labelled Immunoprecipitations}\]

COS-7 cells were labelled with 100 μCi \[35S\text{-methionine}\] per plate for 4 hours in methionine-deficient DMEM, after which they were harvested and lysed as described above. Protein G beads were pre-blocked for 3 hours in unlabelled, untransfected COS-7 cell extract, after which they were washed 3X and used in the immunoprecipitation with anti-HA monoclonal antibody.

\[\text{Results}\]

\[\text{Endogenous pericentrin and cytoplasmic dynein interact with each other}\]

In order to verify an \textit{in vivo} interaction between cytoplasmic dynein and pericentrin, we
immunoprecipitated endogenous pericentrin from untransfected COS-7 cells and probed immunoblots with anti-HC. Despite the very low levels of endogenous pericentrin in non-transfected cells (Doxsey et al., 1994), we were able to specifically detect HC when large numbers of cells were used for the immunoprecipitation (fig. 6).

*Cytoplasmic dynein light intermediate chain interacts with pericentrin*

In order to determine which subunit of dynein or dynactin is responsible for the apparent dynein-pericentrin association, we compared the ability of several dynein and dynactin subunits to bind to pericentrin in a co-overexpression and immunoprecipitation assay (fig. 7). Of the subunits tested for the ability to co-precipitate with pericentrin (dynein subunits HC, IC, LIC, and dynactin subunits p150\textsuperscript{glued} and p50 (dynamitin)), only LIC was found to be able to interact. A fragment of LIC (N174-myc, fig. 7) was also seen to co-immunoprecipitate, suggesting specificity in the interaction.

*Pericentrin binds directly to cytoplasmic dynein light intermediate chain*

HA-pericentrin and LIC-N174-myc were co-immunoprecipitated from \textsuperscript{35}S-labelled overexpressing cultures (fig. 8). On SDS-PAGE gels the full-length LIC is distorted by the antibody heavy chain band, so the LIC-N174-myc fragment was used because it is well-separated from the antibody. Anti-HA was used to precipitate the HA-pericentrin, and the resulting pellet was run on an SDS-PAGE gel and blotted onto PVDF. The PVDF blot was thoroughly dried and exposed to film. HA-pericentrin and N174-myc were the only specific bands seen in the anti-HA precipitation. There is also a band at
approximately 80 kDa, which is seen in the beads alone sample, also, indicating that it is non-specific. These results demonstrate that LIC-N174-myc and HA-pericentrin interact with each other directly; no endogenous COS-7 cell proteins are required to mediate the association.

**Discussion**

Pericentrin overexpression has profound effects on the organization, positioning, and function of mitotic spindles, and on the organization of the Golgi complex (Purohit et al., 1999). Several studies show that cytoplasmic dynein is involved in processes affected by pericentrin overexpression (for reviews see Holzbaur and Vallee, 1994; Karki and Holzbaur, 1999; Vallee and Sheetz, 1996). Consistent with a role for cytoplasmic dynein in mediating the pericentrin overexpression phenotype has been the observation that dynein staining intensity at prometaphase kinetochores and spindle poles is reduced (Purohit et al., 1999). Our data indicate that the interaction is direct and specifically mediated by the light intermediate chains of the motor protein complex. Thus, this study provides additional evidence for a dynein-pericentrin interaction and identifies the first functional role for LICs.

The function of the light intermediate chains has been obscure. They have only been identified in cytoplasmic forms of dynein and contain well-conserved P-loop elements of unknown function near their NH$_2$ termini (Gill et al., 1994; Hughes et al., 1995; Paschal et al., 1987). Previous studies have implicated a different class of dynein subunit, the
intermediate chains, in subcellular targeting. The intermediate chains reside at the base of the dynein complex and interact with the p150^{glued} subunit of the dynactin complex (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Dissociation of the dynactin complex by dynamitin overexpression was found to release dynein from prometaphase kinetochores. Together, these data supported a role for dynactin in anchoring dynein to at least one form of subcellular cargo through the intermediate chains (Echeverri et al., 1996). This mechanism has received further support from evidence that mutations in ZW10, a dynactin-anchoring kinetochore component, also release dynein from the kinetochore (Starr et al., 1998).

The current study identifies an additional and previously unsuspected mechanism for linking dynein to its cargo. The presence of cytoplasmic dynein, but not dynactin, in pericentrin immunoprecipitates strongly suggests that dynactin is not necessary for the pericentrin/dynein interaction. Co-expression of recombinant dynein and dynactin subunits with pericentrin reveals a direct interaction with the light intermediate chains as an additional class of cargo binding subunit. Whether these polypeptides serve in a subset of dynein-mediated processes, such as interactions with soluble protein complexes versus membranous organelles or kinetochores, remains to be determined.

Whether light intermediate chain-mediated dynein interactions are completely independent of dynactin also remains to be resolved. Examination of the behavior of GFP-pericentrin in living cells has revealed clear centripetal transport of pericentrin-
containing particles to the centrosome (Young et al., 2000). This behavior is correlated with a cell cycle-dependent accumulation of pericentrin and γ-tubulin at the centrosome, which is strongly inhibited by nocodazole, antibody to cytoplasmic dynein intermediate chain, or overexpressed dynamitin. These data, together with the identification of a pericentrin-dynein interaction demonstrates that recruitment of pericentrin and γ-tubulin to centrosomes involves dynein-mediated transport. Since pericentrin previously has been shown to interact with the γ-tubulin complex (Dictenberg et al., 1998), we currently believe that pericentrin functions as a molecular scaffold that transports important activities to the centrosome and anchors them at this site.

The ability of dynamitin overexpression to inhibit centrosome protein recruitment suggests a role for dynactin in pericentrin-mediated transport, despite the lack of evidence in the current study for a role for dynactin in the dynein-pericentrin interaction. It is conceivable that dynactin disruption affects pericentrin accumulation via a mechanism unrelated to direct pericentrin transport, such as the disruption of the microtubule cytoskeleton. Alternatively, dynactin could regulate dynein-mediated pericentrin motility independent of a role in linking pericentrin to dynein. Such a model contrasts with an obligatory role for dynactin in the attachment of dynein to kinetochores (Echeverri et al., 1996), but is consistent with our current evidence for an involvement of alternative dynein targeting mechanisms in different cellular processes. Finally, it is possible that pericentrin interacts with dynein by a bivalent mechanism involving both
the light intermediate chains and dynactin, but that the latter interaction is poorly preserved in vitro.

The identification of a direct interaction between pericentrin and cytoplasmic dynein light intermediate chain supports the previous model (Purohit et al., 1999) that pericentrin overexpression disrupts spindles by sequestering dynein. In this model, the excess pericentrin would bind to light intermediate chain, interfering with normal dynein cargo-binding, possibly by competing with other light intermediate chain interactions in the cell. Alternatively, cargo binding could be disrupted by steric interference between overexpressed pericentrin and the intermediate chain-dynactin interaction. Mapping studies have, in fact, shown the binding sites for the intermediate and light intermediate chains to be in close proximity within the dynein heavy chain (Tynan et al., 2000a). Further work will be required to identify the full range of light intermediate chain functions.
Figures

Figure 6. *Endogenous pericentrin and cytoplasmic dynein co-immunoprecipitate*

Lysates from nontransfected cells were used for immunoprecipitation with affinity purified anti-pericentrin antiserum (lane 1) or no antibody (beads, lane 2), and proteins were immunoblotted with anti-DHC antibody.
Figure 7. HA-pericentrin interacts with the light intermediate chain of dynein

Dynein and dynactin components were expressed alone or together with HA-pericentrin in COS-7 cells (+ or -). Cells were lysed, HA-pericentrin was immunoprecipitated, and blots were probed with myc, FLAG, or dynactin antibodies, as indicated on the right. LIC (LIC-myc) and a COOH-terminal fragment of LIC (N174-myc) co-precipitated with HA-pericentrin, whereas other dynein components, dynein heavy chain (HC-FLAG), dynein intermediate chain (IC-myc), and dynactin components (p50 and p150glued) did not co-precipitate. Supernatants (Sups) from immunoprecipitations are shown on the right. HA-pericentrin precipitation was similar in all samples, as confirmed by coomassie blue staining (data not shown).
**Figure 8.** The cytoplasmic dynein light intermediate chain interacts directly with HA-pericentrin

$^{35}$S-methionine-labelled COS-7 cells co-expressing HA-pericentrin and N174-myc were used for immunoprecipitation with anti-HA antibodies or no antibody (beads). HA-pericentrin and N174-myc co-precipitated specifically with HA antibodies. The identity of N174-myc was confirmed by immunoblotting (data not shown). The ~80kDa band represents non-specific binding to the protein G beads.
Cytoplasmic dynein is a large, multi-subunit complex (Paschal and Vallee, 1987) which functions as a molecular motor that moves cellular components towards the minus ends of microtubules and determines the distribution of many vesicular organelles (Vallee and Sheetz, 1996). Cytoplasmic dynein has also been found to be involved in many aspects of mitosis, where it is found at kinetochores, spindle poles, and the cell cortex (Busson et al., 1998; Echeverri et al., 1996; Gonczy et al., 1999; Pfarr et al., 1990; Steuer et al., 1990).

The cytoplasmic dynein complex is composed of four subunit classes: the heavy (HCs), intermediate (ICs), light intermediate (LICs), and light chains (LCs). The dynein heavy chains are large (532 kDa) polypeptides which contain four ATPase domains and are responsible for microtubule binding and catalytic activity (Vallee and Sheetz, 1996). The intermediate chains are a diverse set of subunits derived by alternative splicing from two different genes (Vaughan and Vallee, 1995). The ICs have been found to be responsible for the interaction of dynein with a second complex called dynactin, which is required for dynein-based motility, by directly binding to the p150glued dynactin subunit (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Dynactin is thought to be involved in linking dynein to various organelles in the cell; thus the intermediate chains have been
proposed to have an important function in dynein targeting (Echeverri et al., 1996). The LCs are a diverse family of low- and very low molecular weight subunits (Bowman et al., 1999; Harrison et al., 1998; King and Patel-King, 1995); a role in subcellular targeting has been proposed (Tai et al., 1999).

The HCs, ICs, and LCs all have homologous counterparts in flagellar and ciliary forms of dynein. The LICs, however, are unique to cytoplasmic dynein. They contain a P-loop consensus sequence of unknown function (Gill et al., 1994; Hughes et al., 1995). 2-D electrophoresis of both rat and chicken LICs reveals numerous LIC species, at least some of which result from phosphorylation of the LIC polypeptides (Gill et al., 1994; Hughes et al., 1995). Based on molecular cloning of one of the rat LICs and peptide microsequencing, we proposed that there are at least two different LIC genes per organism (Hughes et al., 1995). Comparison of the chicken sequence, DLC-A, to the sequence of our rat LIC2 clones and bovine LIC peptide sequences suggests that DLC-A is the chicken isoform of LIC1, and that LIC1 and LIC2 are different gene products (Hughes et al., 1995). Northern blotting suggests that both LICs have a wide tissue distribution (Gill et al., 1994; Hughes et al., 1995).

Recently, we demonstrated that recombinant full-length and truncated LIC polypeptides bind to pericentrin, a structural component of the centrosome that is thought to be involved in organizing microtubule nucleating material. Pericentrin has been observed to move in a linear fashion along microtubules towards the centrosome (Young et al.,
2000). Quantitative analysis of centrosomal components during the cell cycle indicated that pericentrin, along with γ-tubulin, accumulates from G1 through metaphase, at which time the centrosomal level drops dramatically (Dictenberg et al., 1998), although somewhat different results have been reported for γ-tubulin in a different system (Khodjakov and Rieder, 1999). Using a co-overexpression and immunoprecipitation assay, we tested a number of dynein and dynactin subunits for the ability to bind to pericentrin; of all of the expression constructs used, only full-length and truncated LIC were found to co-immunoprecipitate with overexpressed pericentrin. Furthermore, when we repeated this experiment in 35S-labelled cells, pericentrin and a LIC fragment were the only specific immunoprecipitated species detected, demonstrating a direct interaction. These results suggest that the LICs, like the ICs, are responsible for linking dynein to its cargo.

In this study, we report the cDNA cloning and sequencing of rat LIC1, establishing the existence of two LIC genes, and we compare the ability of both LIC1 and LIC2 to bind to pericentrin. We find that LIC1, but not LIC2, is capable of this interaction, and we have identified the central portions of both LIC1 and pericentrin as the interacting regions. To determine whether distinct pericentrin-binding forms of the dynein complex exist, we examined the LIC content of dynein and have found that the LICs form homooligomers, but not heterooligomers, and that in triple-overexpression studies, HC can bind to either LIC1 or LIC2, but not both. These results indicate that the LICs specify different subtypes of dynein, only one of which interacts with pericentrin.
Materials and Methods

cDNA cloning and sequencing

A rat brain cDNA library in the Lambda ZAP II vector (Stratagene, La Jolla, CA) was screened by plaque hybridization as previously described (Hughes et al., 1995) with probes random-primed (DECAprime II kit, Ambion, Inc., Austin, TX) from rat LIC2 cDNA (Hughes et al., 1995). Isolated cDNA clones were sequenced (Sequenase, version 2.0, Amersham, Arlington Heights, IL) and compared to LIC peptide and LIC2 cDNA sequence (Hughes et al., 1995). We identified one LIC1 cDNA and used probes random primed from it to isolate additional LIC1 cDNAs from the library. The 5' end of the cDNA was obtained using the Marathon cDNA Amplification Kit with rat brain mRNA (Clontech, Palo Alto, CA). The entire LIC1 cDNA sequence was assembled and analyzed using the GCG DNA analysis programs, including BESTFIT and PILEUP. The National Center for Biotechnology Information (NCBI) databases were scanned using BLAST (Altschul et al., 1990). Alignments of sequences were made using GCG programs and BOXSHADE.

Mammalian Expression Constructs

LIC1 and LIC2 mammalian expression constructs (See fig. 12) were made by removing the β-gal sequence from pCMVβ (Clontech) with NotI (NEB, Beverly, MA) digestion and inserting LIC1 and LIC2 full-length or partial cDNAs. The LIC cDNAs had 3' end NotI sites and coding sequence for epitope tags: myc (MEQKLISEEDLN), HA (YPYPVPDYA), or FLAG (DYKDDDDK) added by PCR. NotI restriction sites were
added to the 5' ends by either PCR or first cloning into pARK (gift from Dr. Melissa Gee), which has NotI sites flanking the MCS. LIC1 P-loop point mutations were made using the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene). To produce an amino-terminal fragment of pericentrin (pHAI/pericentrin 1-575), we cut the full length HA-pericentrin construct (Purohit et al., 1999) with PaeR7I creating a clone extending from the start codon (nucleotide 295) to nucleotide 2019. A truncated central domain of pericentrin containing nucleotides 2587-4317 of the full-length pericentrin was constructed (pHAI/pericentrin 764-1341) by PCR amplification using clone lambda Pc1.2 (Doxsey et al., 1994), a 5' primer with a 5' EcoRI restriction enzyme site (5'GCGAATTCTGAAACGCCAACAT3'), and a 3' primer with a 3' PaeR7I site (5'CGATTTCTCTGCTTTATCC3'). The PCR product was digested with EcoRI and PaeR7I and ligated into the pHAI plasmid digested with EcoRI/PaeR7I. A third truncated form of pericentrin coding for the carboxy terminus (pHAI/pericentrin 1341-1920) was constructed by digesting lambda pc1.2 with XbaI. The restriction fragment was cut by PaeR7I and inserted into pHAI to yield a clone containing nucleotides 4317-6309, with a stop codon at nucleotide 6054.

**Antibodies**

The LIC1 and LIC2 cDNAs were put into the NdeI site of pET 15b (Novagen, Inc., Madison, WI) expression vector and expressed in *E. coli* strain BL21DE3. The bacteria were lysed by French press and the cell debris was pelleted. The bacterial cytoplasm was passed over a nickel affinity column (Novagen), washed and eluted with imidazole
Anti-myc polyclonal antibody used was previously described (Gee et al., 1997); anti-HA polyclonal and monoclonal antibodies were purchased from BAbCO (Richmond, CA); and anti-FLAG M2 monoclonal antibody and affinity resin were purchased from Sigma (St. Louis, MO). Secondary HRP-conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch Labs, Inc. (West Grove, PA).

**Co-immunoprecipitation assays**

COS-7 cells were grown in DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco BRL). The cells were transfected (Lipofectamine, Gibco BRL) for 6-12 hours with 1-4 ug of DNA. Transfections were individually optimized for each construct and combinations of constructs. 30-48 hours after transfection, the cells were harvested by washing the monolayer twice with D-PBS and scraping into a modified RIPA buffer (100mM NaCl, 1mM EGTA, 50mM Tris, pH 8.0, 1mM AEBSF, 2 ug/ml leupeptin and pepstatin). The
cells were placed in a microfuge tube on ice for 20 minutes and then spun in a microfuge at maximum speed for 10 minutes. The resulting extract was used for all co-immunoprecipitation experiments with Protein G (Amersham) or Protein A (Pierce) sepharose beads and the appropriate antibody. Immunoprecipitations were incubated overnight at 4°C with gentle agitation. The beads were then washed with modified RIPA buffer five times at room temperature and eluted into 2X SDS-PAGE sample buffer at 100°C for 5 minutes. The entire eluate and a sample of the supernatant were used for immunoblotting.

Results

LIC1 cDNA cloning and sequencing

Bovine LIC peptide sequences generated from the 57/59 kDa electrophoretic doublet in our earlier study (Hughes et al., 1995) included some which showed similarity to, but were clearly distinct from, LIC 53/55 peptide sequences. Other peptide sequences were homologous to LIC2 sequence deduced from cDNA clones. One LIC 57/59 peptide sequence was unique, but corresponded to part of the deduced sequence from chicken DLC-A, suggesting the existence of a second LIC gene in rat. To test this possibility, we isolated additional LIC cDNAs from a rat brain cDNA library using probes that were random primed from our LIC2 cDNA (Hughes et al., 1995). Sequencing of cDNAs and comparison to LIC2 and peptide sequences (Hughes et al., 1995) allowed us to identify a novel cDNA clone, which we termed LIC1. This clone
was used to re-probe the library to obtain additional LIC1 clones. The 5' end of LIC1 was completed using 5'-RACE with rat brain mRNA. Two different mRNAs were represented among LIC1 clones obtained; they differed only in their polyadenylation site, consistent with what was reported for chicken DLC-A (Gill et al., 1994). The complete cDNA sequence of rat LIC1 has been deposited in GenBank with accession number AF181992. The deduced amino acid sequence is shown in fig. 9 (Lic1). Using BESTFIT, we find rat LIC1 to be 65% identical (71% similar) to rat LIC2 and 81% identical (90.8% similar) to DLC-A. The rat LIC1 sequence contains regions corresponding to all of the peptide sequences we previously obtained from the LIC57/59 doublet, including the peptide that was found only in DLC-A, thus confirming our hypothesis that DLC-A is the chicken isoform of LIC1. We note that a LIC1 antiserum cross-reacted with LIC2, consistent with the high degree of sequence conservation; surprisingly, an anti-LIC2 serum was monospecific (fig.10).

Figure 9 shows a BOXSHADE comparison of rat LIC1, LIC2, and DLC-A, with a human LIC2 and a LIC from *C. elegans* found by scanning GenBank with the rat LIC1 deduced amino acid sequence. All of these sequences contain a P-loop consensus (fig. 9, asterisks) sequence. We searched the *S. cerevisiae* genome with LIC protein sequence and found no matches. Short (approx. 15 amino acids) regions, which show the highest homology among all LICs (fig. 9) also did not find any matches.
We note that during the course of these experiments we also obtained cDNA clones that indicated that there are four alternative LIC2 isoforms in rat; the human LIC2 isoforms have all appeared in GenBank (Maeda et al., 1998). We have obtained no comparable pattern in our LIC1 cDNAs.

**LIC1 specifically binds to pericentrin**

Since rat LIC1 and LIC2 are highly homologous to each other, we were interested in potential functional similarities or differences between them. Our initial analysis of pericentrin binding made use of LIC1, but the relative ability of different LICs to interact with pericentrin was not explored (Purohit et al., 1999). Fig. 11 shows co-immunoprecipitation assays in which HA-pericentrin was overexpressed with LIC1-myc or LIC2-myc. Anti-HA immunoprecipitates of pericentrin were probed with anti-myc to detect the LICs. Only LIC1, and not LIC2, was found to co-immunoprecipitate with pericentrin.

All LICs sequenced to date contain a P-loop consensus sequence, indicating potential ATPase activity. Since this P-loop is well conserved from human to *C. elegans*, it is likely that it plays a functional role in LIC activities. In order to test for any effect of ATPase activity on the LIC1/pericentrin interaction, we constructed two different full-length LIC1 constructs with point mutations in the P-loop sequence. Because it is difficult to predict the effects on binding and/or nucleotide hydrolysis in response to point mutations (Saraste et al., 1990), we tested a mutation of the invariant lysine (K80) to
glutamic acid (E) and a mutation of the threonine (T81) to alanine. Both of these point mutants were used in the co-immunoprecipitation assay with pericentrin. We were unable to detect any binding differences between these mutants and wild type LIC1 (See fig. 12, and data not shown).

To determine whether specific regions within LIC1 and pericentrin were important in their interaction, we evaluated a series of truncation mutants of both LIC1 and pericentrin in our co-immunoprecipitation assay. Fig. 12 shows LIC1 truncations in a summary of the results of the co-immunoprecipitation assays using full-length HA-pericentrin (data for several of these truncations are shown in figs. 11 and 13B). This series of truncations shows that the region of LIC1 between amino acids 140 and 236 is important in the interaction. Mutants that contain any part of this region were found to interact with HA-pericentrin in the assay, while mutants that do not contain any of this region did not interact.

Figure 13A shows three fragments of pericentrin, the N-terminal, central, and C-terminal portions. The full-length LIC1 would not co-express with all of the pericentrin fragments, so N- and C-terminal truncated LIC1 fragments were used (fig. 13B). We found that both bind to the central portion of pericentrin, amino acids 764-1341.
The LICs specify different subtypes of dynein

In light of the finding that pericentrin binds to LIC1, but not LIC2, we tested the ability of LICs to homooligomerize or heterooligomerize. We overexpressed LIC1-HA with LIC1-myc or LIC2-myc (fig. 14A). When LIC1-HA was immunoprecipitated with anti-HA, only LIC1-myc was found to co-precipitate, indicating that LIC1 forms homooligomers, but not heterooligomers. Fig. 14B shows that oligomers will also form from LIC2-myc and LIC2-Flag. Homooligomers also occurred when the myc-tagged constructs had point mutations in the P-loop sequences (data not shown), indicating that a functional P-loop is not required for LIC self-association.

Finally, we used triple overexpression to determine whether LIC1 and LIC2 homooligomers were present in the same or different dynein complexes (fig. 15). LIC1-HA and LIC2-myc were overexpressed along with C1140-myc, a dynein heavy chain construct that binds to the LICs (Tynan et al., 2000b). Figure 15A shows the triple overexpression extract immunoprecipitated with both anti-HA and anti-LIC2. When probed with anti-myc, C1140-myc was found in both immunoprecipitates, but LIC2-myc was only found in anti-LIC2, and not in anti-HA precipitations. Figure 15B shows the same blots re-probed with anti-HA to localize LIC1-HA. LIC1-HA is found only in anti-HA, and not in anti-LIC2 immunoprecipitations. This experiment demonstrates that each LIC independently associates with HC; LIC1 and LIC2 do not bind to HC simultaneously.
Discussion

We have identified a second LIC gene in rat, confirming our earlier hypothesis (Hughes et al., 1995) and further accounting for the considerable diversity in LIC forms observed in purified cytoplasmic dynein complexes. We have also demonstrated that LIC1 is specific for pericentrin binding and that the LICs form homooligomers, but not heterooligomers. Through triple-overexpression studies, we have shown that the two LIC isoforms cannot bind to HC simultaneously. These results together provide evidence for the existence of functionally distinct cytoplasmic dynein complexes which differ by LIC content.

LIC sequences

From comparisons of rat LIC2 and chicken DLC-A sequences we previously hypothesized the existence of two different but related LIC genes in rat (Hughes et al., 1995). The amino acid sequences of LIC1 and LIC2 differ throughout the entire length, and the DNA sequences are only 68% identical, with no large (>30 nt) stretches of complete identity, supporting the idea that they are derived from different genes. LIC1 and LIC2 amino acid sequences account for all eleven peptide sequences that we obtained from bovine cytoplasmic dynein LICs (Hughes et al., 1995), suggesting that there are no additional LIC genes.
BLAST searches of the *C. elegans* genome, which is nearly complete, with either LIC sequence yield only one match. The gene product is a LIC that is equally homologous to both LIC1 and LIC2. These results suggest that *C. elegans* has only one LIC gene. Since the full range of LIC functions is not known in any species, it remains to be seen whether a single LIC in *C. elegans* can carry out all LIC functions, or if the diversity provided by two separate vertebrate LICs allows for additional roles for this class of subunit. No sequences homologous to LIC have been found in the *S. cerevisiae* genome, apparently indicating that LIC is not involved in the limited functions attributed to yeast dynein. It is also interesting to note that the LICs have not appeared among nuclear migration mutants of *A. nidulans* (the nud mutants) (Morris, 1976; Xiang et al., 1994) and *N. crassa* (the ropy mutants) (Plamann et al., 1994). All other dynein subunit classes and some dynactin subunits have been identified in the screening of these mutants, suggesting that the LICs may not be involved in nuclear migration.

*The LIC1/Pericentrin Interaction*

The isoform specificity of pericentrin binding that we describe here adds strong support for the specificity of the LIC-pericentrin interaction. The interaction site (amino acids 140-236 of LIC1) is underlined in fig. 9. This region shows a high degree of identity between all LICs, except for amino acids 201 to 219, which appears to be isoform-specific. This region may account for the binding specificity of LIC1.
The P-loop sequence of LIC1 is located N-terminal to the interaction region. Mutations in the P-loop do not have any obvious effect on the LIC1/pericentrin interaction. Some fragments of LIC1 bind more efficiently to pericentrin than full-length LIC1 does; for example, LIC1N174 and LIC1C173 each contain part of the interacting region, and each co-immunoprecipitate with pericentrin more efficiently than full-length LIC1. The most likely explanation for this observation is that when LIC1 is truncated, regulatory elements are removed, allowing for maximum binding between LIC1 and pericentrin. Conversely, the pericentrin fragment that binds to LIC1 does not bind any better than full-length pericentrin, suggesting that any regulation of the interaction occurs with LIC1, rather than pericentrin. We would expect the LIC1/pericentrin interaction to be highly regulated to allow for the slow accumulation and rapid dissociation of pericentrin at the centrosome.

γ-tubulin has been found to co-immunoprecipitate with and accumulate at the centrosome with pericentrin (Dictenberg et al., 1998). γ-tubulin does not associate with the central portion of pericentrin (W. Zimmerman and S. Doxsey, unpublished results), in contrast to LIC1 (fig.13). This suggests that pericentrin may be capable of associating with LIC1 (and thus dynein) and the γ-tubulin containing γ-TURC complex at the same time. This would allow for the γ-TURC complex to be transported to the centrosome at the same time as pericentrin, without any components of the complex directly binding to dynein.
Significance of dynein subtypes

Our findings that only LIC1 binds to pericentrin and that LIC1 and LIC2 binding to HC is mutually exclusive suggests that a substantial subfraction of dynein is incapable of binding to pericentrin. LIC1-containing dynein can bind to pericentrin, while LIC2 dynein cannot, and there may be LIC-less dynein that also cannot. This adds diversity to the pool of dynein in the cell. The subcellular targeting of dynein may be simplified by the existence of dynein subtypes, each of which is responsible for a given set of dynein functions. Pericentrin binding is likely to be one of several functions of LIC1, since there appears to be considerably more LIC1 than pericentrin in cells. This suggests that other mechanisms are still required for the specific binding of dynein to various cellular components, but alternative LIC subunit content provides some amount of specificity.

We have speculated that the LICs may be responsible for a non-dynactin based targeting mechanism for dynein, perhaps specific for soluble proteins rather than membrane-bound organelles (Purohit et al., 1999). In this view, the LICs would function independently of dynactin, with each having different targeting specificities. Dynactin-mediated targeting of dynein would, presumably, involve either a subfraction of dynein which lacks LICs or in which the LICs have been inactivated. However, given that p50 overexpression has been observed to disrupt the accumulation of pericentrin at centrosomes (Young et al., 2000), it is also quite possible that dynactin and the LICs function in concert in certain situations to support both binding and motility. Further work is necessary to determine the actual relationship between LIC- and dynactin-mediated functions.
Figures

Figure 9. BOXSHADE comparison of cytoplasmic dynein LIC sequences

The amino acid sequence of rat LIC1 (Lic1) compared to other LIC sequences: rat LIC2 (Lic2) (Hughes et al., 1995); human LIC2 (humlic2) (GenBank accession number O43237); chicken LIC1 (Dlca) (Gill et al., 1994); and C. elegans LIC (celegans) (Derived from a cosmid sequence with GenBank accession number Z73422). Asterisks indicate the amino acids that comprise the P-loop consensus sequence (GXXGXGKT/S). Underlining shows the pericentrin binding site.
Figure 10. Polyclonal antisera raised against bacterially expressed cytoplasmic dynein LICs
COS-7 cell extract was immunoblotted with antiserum raised against rat LIC1 (lane 1) and LIC2 (lane 2).
Figure 11. Comparison of cytoplasmic dynein LIC1 and LIC2 binding to pericentrin

Constructs listed on the right were overexpressed with (+) and without (-) overexpression of HA-pericentrin. Anti-HA immunoprecipitations were immunoblotted and probed with anti-myc to detect the LICs. Supernatants are shown at the right to demonstrate overexpression of LIC.
Figure 12. Cytoplasmic dynein LIC1 fragments bind to pericentrin

Various truncation constructs of LIC1 were co-expressed with HA-pericentrin. The gray box represents the P-loop sequence; X within the box (LIC1TA and LIC1KE) represents a point mutation within the P-loop sequence. On the right, + indicates positive co-immunoprecipitation with HA-pericentrin; - indicates negative co-immunoprecipitation. Black bar summarizes the deduced pericentrin binding region. Data for several immunoprecipitations are shown in fig. 11 and fig. 13.
Pericentrin binding:
(Summary)

LIC1
LIC1TA
LIC1KE
LIC1C173
LIC1C160
LIC1C140
LIC1C110
LIC1N110
LIC1N174
LIC1N236
LIC1N349
LIC2

Amino Acid #

Pericentrin-HA Binding
**Figure 13.** Cytoplasmic dynein LIC1 co-immunoprecipitations with pericentrin fragments

A) Three pericentrin fragments were constructed for co-immunoprecipitation. The amino acids included in each fragment are listed at the right; all fragments had N-terminal HA tags. B) LIC constructs listed at right were co-overexpressed with the pericentrin constructs listed at the top. Pericentrin fragments were immunoprecipitated with anti-HA. Anti-myc was used to probe immunoblots of the precipitations for LIC1-myc fragments.
A

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B

- LIC1C173-myc
- LIC1N174-myc
**Figure 14. Cytoplasmic dynein LICs form homooligomers but not heterooligomers**

Supernatants from immunoprecipitations are shown at the right to verify expression of LIC fragments. A) LIC1-HA overexpressed in combination with LIC1-myc and LIC2-myc (indicated at the top) was immunoprecipitated with anti-HA and immunoblots were probed with anti-myc. Only LIC1-myc co-precipitated with LIC1-HA. B) LIC2-myc, which did not co-precipitate with LIC1-HA, does co-precipitate with LIC2-FLAG.
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Anti-HA IPs | Sups

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Anti-FLAG IPs | Sups

LIC2-myc-
**Figure 15. Cytoplasmic dynein HC binds to either LIC1 or LIC2, but not both**

Overexpressed constructs are indicated above the lanes. A) Triple overexpression of a HC construct which binds LICs and ICs (HC-C1140-myc), LIC2-myc, and LIC1-HA were immunoprecipitated with anti-HA (left side) and anti-LIC2 (right side). Samples of the extracts used for both sets of immunoprecipitations are shown in the center. Immunoblots were probed with anti-myc to detect both HC-C1140-myc and LIC2-myc. B) The blots from panel A were reprobed with anti-HA to detect LIC1-HA. In the right hand section, there is a non-specific smudge, which appeared upon overexposure of the blot; no specific LIC bands were seen in that panel.
Cytoplasmic dynein is a very large complex that functions as a minus-end directed microtubule-based motor protein (Paschal and Vallee, 1987). Dynein has been implicated in many cellular functions, including several aspects of mitosis, as well as a variety of interphase processes, including vesicle transport and organelle positioning (Vallee and Sheetz, 1996). The dynein complex has been found to be made up of a number of subunit classes: heavy chains (HC), intermediate chains (IC), light intermediate chains (LIC) and light chains (LC). All cytoplasmic dynein subunits except for the LICs have homology with subunits of axonemal dynein, the motor complex that powers flagellar and ciliary beating. The organization of the subunits within the complex is not fully known.

The cytoplasmic dynein complex is two-headed, with each head being attached to a stem; the stems come together to form a base, which is involved in attachment to cargo. The head structure contains four ATPase consensus sequences, which are thought to power the motor (Holzbaur and Vallee, 1994). Additional insight into the structure of the heavy chain has recently been gained with the identification of a specific microtubule binding site which lies at the tip of a small stalk protruding from the dynein head (Gee et al.,
1997). The stalk seems to be responsible for force transmission between the globular portion of the motor domain and the microtubule surface.

The ICs have been localized by immunoelectron microscopy to the base of the dynein complex (Steffen et al., 1996). In this position, they are perfectly situated for binding to dynactin and other targeting or cargo-binding polypeptides. Recently, the IC interaction site was mapped to the N-terminal region of the heavy chain sequence, the region thought to form the base, using *Dictyostelium* cytoplasmic dynein subunits (Habura et al., 1999). The HC dimerization site was deduced to be immediately C-terminal to the IC binding site.

The location of the light intermediate chains within the dynein complex is unknown. We have recently found that LIC1 binds specifically to pericentrin (Purohit et al., 1999; Tynan et al., 2000b), a centrosomal protein which is known to be transported to the centrosome in a dynein-dependent fashion (Young et al., 2000). These results have revealed a targeting function for the LICs.

This study was initiated to gain further insight into the apparent function of the LICs in targeting. To this end, we have mapped its binding site within the dynein complex and compared it to the sites for IC binding and HC dimerization. We have found that the LICs, as well as the ICs, bind to the base of the complex, putting them both in position to be involved in targeting. Additionally, we have shown that the LICs and ICs bind to the
heavy chain on overlapping binding sites, but have no contact with each other. This suggests a model of dynein in which the LICs and ICs are on opposite sides of the complex and may interact simultaneously with other large structures.

**Materials and Methods**

*Mammalian Expression Constructs*

LIC1-myc, LIC2-myc, LIC1-myc and LIC2-myc point mutants, IC-myc, HC-FLAG, HC-C1140-myc, HC-C260-myc, and HC-N1137-FLAG constructs are described elsewhere (Gee et al., 1997; Tynan et al., 2000b; Vaughan et al., 1995). Additional HC 5' end constructs were made by adding a myc tag at the appropriate location using PCR, then cloning into HC-C1140-myc in the pARK vector at the PmlI site of HC. The entire clone was then inserted into pCMVβ (Clontech) from which the β-galactosidase sequence had been removed by NotI (NEB, Beverly, MA) digestion. Other HC fragments were made by adding NotI sites, myc tag, and Kozak sequence by PCR using VENT DNA polymerase (NEB), followed by NotI digestion and direct cloning into NotI digested pCMVβ.

*Antibodies*

Anti-myc and anti-LIC polyclonal antibodies used have been described elsewhere (Gee et al., 1997; Tynan et al., 2000b); anti-HA monoclonal and polyclonal antibodies were purchased from BAbCO (Richmond, CA); anti-FLAG M2 monoclonal antibody and
affinity resin were purchased from Sigma (St. Louis, MO); anti-IC monoclonal antibody 74.1 was purchased from Chemicon (Temecula, CA); and anti-HC polyclonal antibody was a gift from Dr. Atsushi Mikami. Secondary HRP-conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch Labs, Inc. (West Grove, PA).

Co-immunoprecipitation assays

All co-immunoprecipitations were performed as previously described (Tynan et al., 2000b). Briefly, COS-7 cells were grown in DMEM+10%FCS+Pen/Strep (Gibco BRL, Grand Island, NY) to 80% confluence, then transfected for 12 hours with appropriate DNA using Lipofectamine transfection reagent (Gibco BRL). 30-48 hours after transfection, cells were scraped into modified RIPA buffer (100mM NaCl, 1mM EGTA, 50 mM Tris, pH 8.0, 1% NP-40, 1mM AEBSF, 2μg/ml leupeptin and pepstatin), lysed on ice for 20 minutes and spun in a microfuge for 10 minutes. Immunoprecipitations were performed using protein G (Amersham) beads or M2 affinity resin (Sigma) overnight at 4°C with gentle agitation. Beads were washed 5 times with modified RIPA buffer and eluted with 2X SDS PAGE sample buffer at 100°C for 3 minutes. The entire eluate and a sample of the supernatant were used for immunoblotting.

Results

LICs and ICs bind to the HC at the base of the complex

To begin to develop a clear picture of how the cytoplasmic dynein subunits are associated
with each other, we used co-overexpression and immunoprecipitation of various combinations of dynein subunits to test whether one subunit would co-precipitate with the other. In these experiments, the high levels of protein being used minimize the contribution of indirect interactions that are mediated by endogenous protein, as demonstrated by negative results (fig. 16, panel C) and triple overexpression (fig. 20).

Figure 16 (A and B) shows co-immunoprecipitation of LIC1, LIC2, and IC with full-length heavy chain, demonstrating that each of these subunits bind independently to the HC. The IC and LIC do not co-precipitate (fig. 16C), suggesting that they bind independently to the HC. In previous work (Tynan et al., 2000b) we found that pericentrin binding by LIC1 and LIC multimerization was not affected by point mutations in the P-loop sequences (LIC1: K80 to E; T81 to A; LIC2: K67 to E). Figure 17 shows that these same point mutations do not appreciably affect HC binding.

The IC has been reported to interact with the HC near the N-terminus (Habura et al., 1999). This region is likely to be at the base of the complex, where the ICs have been observed by EM to be located (King and Witman, 1990; Steffen et al., 1996). We used HC fragments to identify the general location of LIC binding. In this experiment, we overexpressed HC fragments in COS-7 cells, immunoprecipitated, and then looked for co-precipitating endogenous subunits (fig. 18). Full-length HC and the N-terminal fragment (C1140-myc) co-immunoprecipitated IC, LIC1, and LIC2, while the C-terminal
three-quarters of the HC (N1137-FLAG) did not precipitate any endogenous dynein subunits.

**LICs and ICs are present in the same dynein complexes**

Previously, we demonstrated that LIC1 and LIC2 binding to the dynein complex is mutually exclusive, thus specifying different subtypes of dynein, and that LICs can function as targeting subunits of dynein (Tynan et al., 2000b). The ICs also function in targeting, so it is possible that the LICs may be alternative to the ICs, and that only one of the subunits would be present in a dynein molecule.

In order to determine conclusively whether LICs and ICs are present in the same complex, we took two approaches: first, we immunoprecipitated endogenous dynein complex from COS-7 cells using anti-IC antibody 74.1, and probed the immunoprecipitate with anti-HC, IC, and LIC polyclonal antibodies (fig. 19). The results show that both LIC1 and LIC2 are co-immunoprecipitated with IC, and are both capable of binding to IC-containing dynein.

As an alternative approach to this question, and as a control to the other co-immunoprecipitation experiments, we performed a triple over-expression and immunoprecipitation experiment. In this case, we overexpressed the myc-tagged N-terminus of HC with myc-tagged IC and LIC, immunoprecipitated LIC, and probed for all of the subunits with anti-myc antibody. Fig. 20 shows that IC co-immunoprecipitates
in the triple expression, confirming the previous result that LICs and ICs can co-exist in dynein complexes.

**LIC and IC binding sites**

To further our understanding of dynein subunit interactions, we created numerous rat HC expression constructs for mapping LIC and IC binding sites within the rat cytoplasmic dynein HC. The summary of mapping co-immunoprecipitations is shown in fig. 21 (data from several of the fragments is shown in fig. 22), with the deduced LIC and IC binding sites shown at the bottom of each panel. Comparison of LIC1 and LIC2 indicated that the binding sites are identical, as predicted. The LIC binding site was found to lie between amino acids 649 and 800 on the rat HC, and the IC binding site between amino acids 446 and 701.

**HC dimerization site**

Figure 23 shows a summary of co-immunoprecipitations using the HC fragments with full-length HC (several examples of the data are shown in fig. 24). These results indicate that HC dimerization is mediated by an extended region of the HC sequence, which includes the LIC and IC binding regions, and extends more toward the motor (head) domain than the subunit binding sites. Interestingly, there is a region at the extreme N-terminus that does not show binding to any of the subunits tested, including HC itself. We note that in the HC-FLAG/HC-myc co-immunoprecipitation (see fig. 24, top panel) overexposure of the Western blot revealed numerous myc-reactive degradation products
that are absent from the immunoprecipitate. Since the myc tag is at the C-terminus, this result provides independent evidence that C-terminal fragments do not bind to the full-length HC.

**Discussion**

In this study, we have found that the cytoplasmic dynein ICs and LICs bind directly to the HC, and we have mapped the LIC and IC binding sites within the rat cytoplasmic dynein HC sequence. Both subunits bind to the N-terminus of the HC, with their binding sites overlapping within the primary sequence. Despite the close proximity of their binding sites, we have detected no interaction between the ICs and LICs. However, we have demonstrated that they co-immunoprecipitate in the presence of HC, indicating that they interact indirectly through this polypeptide. Additionally, we have found that the LIC1 and LIC2 binding sites are identical. Finally, we have found that a large portion of the N-terminal region of the HC, including, but substantially greater than, the IC and LIC binding regions, is responsible for HC dimerization.

In previous work (Purohit et al., 1999; Tynan et al., 2000b) we tested numerous dynein and dynactin subunits for the ability to bind to pericentrin, and found that only LIC1 interacts. Since pericentrin has been found to be transported to the centrosome in a dynein-dependent fashion (Young et al., 2000), our data supported a role for LIC1 in mediating the binding of dynein to this particular form of cargo. The localization of the LIC binding site to the N-terminus of the heavy chain, which presumably places the LICs
at the base of the dynein complex, further supports a targeting role. The LICs bind to a region of the rat heavy chain located between amino acids 649 and 800. Our previous work (Tynan et al., 2000b) showed that LIC1 and LIC2 exhibit a high degree of sequence homology yet are present in distinct dynein complexes. Our finding that they share a common HC binding site, along with their inability to heterooligomerize (Tynan et al., 2000b), suggests that they compete with each other for HC binding.

The LIC and IC binding sites are distinct but overlapping within the HC primary sequence, as demonstrated by our co-immunoprecipitation results (figs. 21 and 22). The LICs co-immunoprecipitate with the HC constructs N649C907 and C674, but not with N547C649. These results demonstrate that the part of the C674 HC fragment that contains LIC binding elements is between amino acids 649 and 674. This region is within the boundaries of the IC binding site, amino acids 446 to 701. Despite this clear overlap of the binding regions, we have found that LIC and IC can bind to a common dynein HC (fig. 19 and 20), although they do not interact with each other directly when free in solution (fig. 16). Presumably, the surfaces within the folded HC for LIC and IC binding must be topologically distinct to allow for independent binding (see below).

The IC binding site within the Dictyostelium HC has been reported to lie between amino acids 629 and 730 (Habura et al., 1999). This region of the Dictyostelium HC is homologous to amino acids 589-690 of the rat heavy chain, and is similar to the IC binding site deduced from our current data (446-701). However, we have found the N-
terminal boundary of the binding site to lie between amino acids 446 and 549, upstream of the N-terminal boundary in the *Dictyostelium* HC. This difference may reflect the phylogenetic distance between rat and *Dictyostelium* or differences in the sensitivities of the methods used for assaying polypeptide interactions (see below). Comparing the rat HC sequence to that of the *Dictyostelium* HC, there is a very high degree of homology within the IC binding region; the deduced *Dictyostelium* binding site shows 64% identity (72% similarity) with the equivalent region of rat HC. Extending the binding site from rat HC amino acid 589 to 516 still gives 61% identity (69% similarity), but the degree of homology is considerably lower beyond this point. Based on these considerations, it seems reasonable to deduce that the IC binding site begins at or around amino acid 516 on the rat HC (amino acid 559 in *Dictyostelium*). Beyond the C-terminal end of the deduced IC binding site, a high degree of homology persists. This region corresponds to the remainder of the LIC binding site identified here (amino acids 649 to 800 of rat HC), which shows 56% identity (62% similarity) with the equivalent *Dictyostelium* sequence.

We have found that HC dimerization occurs across a large portion of the N-terminal region of the HC (amino acids 300-1140). Our data differ significantly from those of Habura, et al. (Habura et al., 1999) in this regard, who found the *Dictyostelium* HC dimerization site to include amino acids 627 to 780, plus additional, unidentified sequence C-terminal to this region. A major difference in these two reports may involve the sensitivity of methods being used. Habura, et al. (Habura et al., 1999) used HC fragments translated in reticulocyte lysates and assayed for protein-protein interactions by
cross-linking the fragments or by binding them to bacterially-expressed HC. Cross-linking yielded fewer dimerizing fragments than did binding of \textit{in vitro} translated HC fragments to bacterially expressed HC, suggesting (Habura et al., 1999) that there are significant differences in sensitivities even between these methods. These approaches involve the use of low concentrations of fragment, in contrast to the co-overexpression method used here. Because of the higher levels of protein involved in our co-expression assay, it may be better suited for detecting partial binding sites (fig. 21-24; Purohit et al., 1999; Tynan et al., 2000b). Conceivably, some of the interactions detected in our study could be nonspecific, but this possibility is disputed by several observations. First, several HC fragments that tested positive in the HC-HC interaction assay (for example, N907C1100 and N261C440, fig. 21-24) were clearly negative when tested against ICs and LICs. In addition, other fragments clearly interacted differentially with the LICs and ICs, with N547C649 interacting with IC but not LIC, and N758C907 interacting with LICs but not ICs (fig. 21 and 22); only N649C907 showed interaction with all of the subunits tested (fig. 21-24). Finally, the co-immunoprecipitation assay has proven to be very selective in other contexts (Purohit et al., 1999; Tynan et al., 2000b).

The HC/HC interaction site identified in the current study is much more extensive than those found for the ICs and LICs. We have shown that several HC fragments that do not bind to IC or LIC bind to HC, adding further support to the contention (Habura et al., 1999; Mazumdar et al., 1996) that the HCs do not need accessory subunits for dimerization. This important conclusion is further substantiated by the observation that
LIC or IC co-overexpression was not required for HC-myc/HC-FLAG dimerization (see fig. 23 and 24). Whether binding of LIC and IC to the HC requires HC dimerization is not clear. LIC and IC binding cannot be tested in the absence of HC dimerization using the fragments produced in the current study, and it remains to be seen whether such a test will be possible.

We also note that the C-terminal boundary of the HC self-association domain determined in the current study ends between amino acids 900 and 1100. Previous work has suggested that the N-terminus of the motor domain lies between amino acid 1137 and 1455 (Gee et al., 1997; Samso et al., 1998). Our finding that the N1137 fragment of the HC does not bind to full-length HC is consistent with this model. Finally, there is a relatively short sequence at the N-terminus extending to amino acid 300 that does not seem to be involved in binding to any of the subunits examined here. This region may play a role in binding to some of the LCs or other as yet unidentified proteins.

Fig. 25 shows a representation of our current understanding of the structural organization of the dynein complex. Although the IC and LIC binding sites overlap within the HC primary sequence, both subunits are present in the same dynein pool as demonstrated by immunoprecipitation of endogenous LIC-containing dynein in COS-7 cells using anti-IC antibodies, as well as by triple overexpression and co-immunoprecipitation (fig. 19 and 20). This suggests that the LICs and ICs must be associated with different surfaces of the complex. HC dimerization must occur on yet another surface of the HC based on similar
considerations. This three-dimensional picture of the dynein complex would allow for the ICs to bind to the dynactin complex on one side of dynein, while LICs could bind cargo proteins, such as pericentrin, on a different dynein surface. Pericentrin may also be bound to other binding partners, such as the γ-TURC complex. It remains to be seen whether dynein can use dynactin-mediated and LIC-mediated interactions simultaneously, and how such interactions would be orchestrated to maintain proper localization of various cellular components.
Figures

**Figure 16.** Cytoplasmic dynein LICs and ICs both bind to the HC but not to each other

COS-7 cells were co-transfected with different dynein subunits. Cell lysates were used for immunoprecipitations with (A, B) anti-FLAG to immunoprecipitate the HC-FLAG, and (C) anti-IC. Immunoprecipitations were immunoblotted and probed with anti-myc to detect myc-tagged IC and LIC. Expressed proteins are indicated above each lane. In all panels, immunoprecipitation pellets (IPs) are on the left and supernatants (sups) are on the right. Note that no myc-reactive species are detected in the LIC and IC single expression controls.
Figure 17. Cytoplasmic dynein LIC P-loop point mutations do not affect HC binding

COS-7 cells transfected with HC-FLAG plus normal or point mutant LICs were used for immunoprecipitations with anti-FLAG. Immunoprecipitations were immunoblotted and probed with anti-myc to detect myc-tagged LIC species.
Figure 18. Endogenous cytoplasmic dynein ICs and LICs bind to the N-terminal region of HC

COS-7 cells overexpressing HC constructs (listed above lane) were used for immunoprecipitations with anti-FLAG (left lanes) and anti-myc (right lane). Immunoblotted precipitates were probed with anti-IC and anti-LIC to detect endogenous COS-7 dynein subunits co-precipitating with overexpressed HC and HC fragments.
Figure 19. LICs bind to IC-containing dynein

Untransfected COS-7 cells were used for anti-IC immunoprecipitation. The precipitates were immunoblotted and then blots were probed with a mixture of anti-IC, anti-LIC, and anti-HC antibodies. Despite the lack of interaction between LICs and ICs (fig. 16), the LICs co-immunoprecipitated with the ICs.
Figure 20. ICs bind to LIC-containing dynein

COS-7 cells double- and triple-transfected with LIC-myc, IC-myc, and HC-C1140-myc were used for anti-LIC immunoprecipitation. Resulting pellets were immunoblotted and probed with anti-myc to localize all overexpressed proteins. Immunoprecipitation pellets are shown at the left and extracts at the right.
**Figure 21. Mapping of LIC and IC binding sites within the cytoplasmic dynein HC**

Various HC constructs are listed at the left and depicted graphically. Data for the most informative fragments are shown in fig. 22. Positive co-immunoprecipitation is indicated by blue; negative co-precipitation is indicated by red. The deduced binding sites are indicated at the bottom in dark blue. A) co-immunoprecipitations with full-length IC; B) co-immunoprecipitations with full-length LICs. LIC1 was used unless noted otherwise. † indicates HC fragments that were also tested with LIC2. Only the most important HC fragments were tested with both LIC1 and LIC2. †† (fragment N400C602) indicates that only LIC2 was tested because the antibody light chain was obscuring the result in LIC1 (anti-HA) immunoprecipitations; the antibody light chain was not detected in LIC2 (anti-FLAG) immunoprecipitates.
IC binding summary:
(a.a. 446-701)

Amino Acid #

A

B
**Figure 22. Co-immunoprecipitation of IC and LIC with dynein HC fragments**

HC fragment names are listed to the left. Top) myc-tagged HC fragments were co-expressed with (+) or without (-) IC-myc, immunoprecipitated with anti-IC, and immunoblotted with anti-myc. Note smudges in N446C549, N649C907, and N655C907 single-expression lanes resulting from weak reactivity of the secondary antibody with the immunoprecipitating IgG light chain. Bottom) myc-tagged HC fragments were co-expressed with LIC2-FLAG and immunoprecipitated with anti-FLAG (except for N758C907, which was co-expressed with LIC1-HA and immunoprecipitated with anti-HA), and immunoblotted with anti-myc. Immunoprecipiation pellets are in left lanes, supernatants in the right.
Figure 23. Characterization of the cytoplasmic dynein HC dimerization domain

Various myc-tagged HC fragments were co-expressed with full-length HC-FLAG for co-immunoprecipitations. All immunoprecipitations were performed with anti-FLAG and immunoblots were probed with anti-myc to detect the HC fragments, with the exception of N1137-FLAG, which was co-expressed with myc-tagged full-length HC, immunoprecipitated with anti-FLAG and the immunoblot was probed with anti-HC (see fig. 24). As in figure 21, blue is positive, red is negative, and dark blue shows the deduced dimerization domain. The data of the most informative constructs is shown in fig. 24.
HCFLAG
C1140myc
C907myc
C800myc
C689myc
C260myc
N199C300myc
N261C440myc
N446C549myc
N400C502myc
N547C649myc
N649C907myc
N748C907myc
N907C1100myc
N1137FLAG

HC binding summary:
(a.a. 300-1137)

Amino Acid #
Figure 24. Mapping of the cytoplasmic dynein HC dimerization domain

See figure 23 for description of antibodies used. The HC fragment names are listed at the left. The HC-myc and HC-FLAG co-immunoprecipitation, upon overexposure, revealed numerous myc-reactive degradation products. Since the myc-tag is on the C-terminus, these are C-terminal fragments, and are not detected in the precipitation, consistent with the HC dimerization domain being at the N-terminus. Immunoprecipitation pellets are shown in the left lanes, supernatants in the right.
Figure 25. Schematic diagram of cytoplasmic dynein subunit interaction sites

The location of the motor domain and the microtubule binding stalk (Gee et al., 1997) are shown within the primary sequence of the HC. The subunit binding domains are those deduced from analysis of overlapping fragments (Fig. 21 and 23; IC, LIC, HC “binding summaries”). The N-terminal boundary of the motor domain is not yet fully defined (indicated by a dotted line).
CHAPTER VI
DISCUSSION

The work presented in this dissertation has significantly contributed to cytoplasmic dynein research by describing a previously unexplored class of subunits, the light intermediate chains. These studies have demonstrated the existence of two highly homologous LICs, one of which has been shown to be involved in the targeting of cytoplasmic dynein to a specific cargo, pericentrin. Additionally, the interactions among the intermediate, light intermediate, and heavy chains have been explored to provide a foundation for future studies into the interrelationships of subunit function.

The light intermediate chain family

The light intermediate chain class of dynein subunits is made up of LIC1 and four spliceoforms of LIC2 (Gill et al., 1994; Hughes et al., 1995; Maeda et al., 1998; Tynan et al., 2000b); no evidence has been found for additional LIC polypeptides. The LICs are highly homologous to each other and are conserved between rat, human, chicken, and C. elegans. There is evidence that some organisms (Xenopus (Niclas et al., 1996) and C. elegans (Tynan et al., 2000b)) may have only one LIC. This supports a model for gene duplication and subsequent divergent evolution. It will be interesting to compare the contribution of a single LIC to dynein activity versus the full range of functions of LIC1 and LIC2 in higher organisms.
Searches of the *S. cerevisiae* genome sequence have not revealed any LIC homologues (Tynan et al., 2000b). In budding yeast, dynein and dynactin have been shown to be important for nuclear migration, which is vital to proper segregation of daughter nuclei (Eshel et al., 1993; Saunders et al., 1995). All dynein and several dynactin subunit homologues, with the notable exception of LICs, have been found to be mutated in strains of yeast that are defective for nuclear migration. Similar results have been found in other genetic systems which are defective in nuclear migration: *A. nidulans* (the nud mutants) (Morris, 1976; Xiang et al., 1994) and *N. crassa* (the ropy mutants) (Plamann et al., 1994). Together these results suggest that the LICs do not contribute to the role of dynein in nuclear migration.

All of the LICs identified to date (see fig. 9) contain a P-loop consensus sequence of unknown function. None of the binding interactions found for the LICs (self-association, HC binding, and pericentrin binding) have been altered detectably by point mutations in the P-loop sequence. The P-loop region is homologous to ATP binding consensus and a small (24 amino acids) region of surrounding sequence of a family of ATPases known as the ABC transporters. The LICs are clearly not related to these proteins, as they lack approximately 200 amino acids of highly conserved sequence which defines the family. The 24 amino acid long extended P-loop region of homology with *bona fide* ATPases suggests that the LIC P-loop is likely to be functional (Hughes et al., 1995). An ATPase domain could, conceivably, affect the affinity of LICs for one or more of their binding partners, which would not be detectable in co-overexpression and immunoprecipitation
assays. Alternatively, ATP hydrolysis may be activated by one or more regulatory signals, such as phosphorylation. This also might not be detectable in overexpressing cells, since kinases or other modifying enzymes might not be able to modify all of the overexpressed protein. Finally, the P-loop may be involved in an unknown LIC function, or it may be inactive. More work is required to determine whether the P-loop is, in fact, functional, and if so, what the function may be.

**The pericentrin/light intermediate chain interaction**

Pericentrin is a unique form of dynein cargo; most other cargoes that have been identified are membrane-bound organelles and vesicles, and large structures, such as kinetochores. Pericentrin, in contrast, is likely to be a soluble protein or complex during transport. The LICs may be part of a specific binding mechanism for dynein to associate with soluble proteins; this would imply that there are a number of soluble proteins to be transported, some of which may bind to LIC2, and some to LIC1. Pericentrin is not likely to be the only LIC1-binding cargo, as there is much more LIC1 in the cell than pericentrin. NuMA (*nuclear mitotic apparatus protein*) has also been shown to form a complex with dynein and dynactin (Merdes et al., 1996). The subunit of dynein or dynactin which mediates this interaction is unknown; since NuMA is a soluble protein, the LICs are good candidates for binding to this cargo.

In COS-7 cells, the Golgi apparatus is a constitutive minus-end organelle. During mitosis, the stacks are dispersed throughout the cell, and are rapidly transported to their
normal perinuclear localization upon re-formation of the interphase microtubule network. In contrast, pericentrin accumulation at the centrosome occurs very slowly throughout the cell cycle, until metaphase, when the level at spindle poles drops dramatically (Dictenberg et al., 1998). This distinctive pattern of pericentrin accumulation may be due to regulation of pericentrin binding at the centrosome or by regulation of transport. The LICs may be involved in a mechanism to regulate the transport to result in slow accumulation.

Regulation of transport may occur via regulation of cargo binding. This is consistent with results seen in *Xenopus* melanophores (Reese and Haimo, 1999), where kinesin II was found on both aggregated and dispersed membranes, but dynein and dynactin were only found on aggregated membranes. These results suggest that kinesin II is present constitutively, but dynein is only present when the membranes are being transported in a dynein-driven direction. The phosphorylation, potential ATPase activity, and other as yet undescribed posttranslational modifications of the LICs could all be regulatory signals to enhance or inhibit binding of dynein to specific cargo.

*Dynactin vs. light intermediate chains*

Dynamitin (p50) overexpression has been found to inhibit the accumulation of pericentrin at the centrosome (Young et al., 2000), although in biochemical assays pericentrin did not interact with the dynactin complex (Purohit et al., 1999). Despite this apparent paradox, there are several models which could explain these observations. First, dynactin has been
proposed to have dual functions, targeting dynein to membranous vesicles and kinetochores, and tethering dynein and the cargo to the microtubule during the ATPase cycle to increase the processivity of dynein (King and Schroer, 2000; Waterman-Storer et al., 1995). Therefore, in pericentrin transport, dynactin may be playing only a single role, that of tethering dynein to the microtubule, while LIC attaches to the cargo. Alternatively, dynamitin overexpression could be interfering in an indirect manner by sequestering dynein with an inactive dynactin fragment. Another model is that pericentrin could bind to both dynein and dynactin in vivo, but its interaction with dynactin is poorly preserved in vitro. Finally, p50 overexpression may produce secondary effects, such as disruption of the microtubule array (Burkhardt et al., 1997; Echeverri et al., 1996), which would prevent proper pericentrin transport, even if using a mechanism unrelated to dynactin.

**Model for dynein targeting**

Bidirectional movements have been proposed to be regulated by coordination of dynein and kinesins (reviewed in Vallee and Sheetz, 1996). As noted above, recent work has suggested that kinesin II may be constitutively bound to such organelles, while dynein and dynactin bind only when actively transporting (Reese and Haimo, 1999). Directionality and net rate of vesicle transport may depend on the ratio of active kinesin to dynein on the organelle surface. This mechanism would require only regulation of dynactin binding to the cargo for appropriate transport. One model that has been proposed is that the short F-actin-like filament of dynactin could interact with a spectrin
network on the surface of organelles (Vallee and Sheetz, 1996). In this model, the binding mechanism would be similar for many membrane-bound vesicles and organelles, although the binding of dynactin to the kinetochore via dynamitin and ZW10 would be a special case. Alternatively, ZW10 or related proteins may be found on the surface of organelles for dynactin to bind to in targeting dynein.

Another example of a cargo with unique regulatory requirements is mitochondria. The transport of mitochondria on microtubules is unusual in that, although there is true bidirectional movement, much of their movement is saltatory, and they can remain stationary for significant periods of time. Additionally, there is evidence that mitochondria may be transported to regions of the cell which have greater immediate need for ATP (reviewed in Hollenbeck, 1996). Dynamitin overexpression is not seen to disrupt motility or localization of mitochondria (S. Tynan, unpublished results; Burkhardt, et al., 1997), suggesting that dynactin is not required for their minus-end motility. To date, no other interphase minus-end cytoplasmic motors other than dynein have been discovered. The minus-end kinesins appear to function only in mitosis. These observations all support a novel mechanism for regulation of dynein-driven transport in mitochondrial motility. Regulation of dynein association via one or both LICs could be responsible for appropriate retrograde transport of mitochondria. Consistent with this possibility, overexpressed fragments of LIC2 have been found to localize to the outer surface of mitochondria in COS-7 cells (S. Tynan, unpublished observations).
Future directions

This thesis project has added greatly to the dynein field, and, at the same time, it has generated even more new questions. In future work, it will be intriguing to determine the full range of LIC binding partners and assess what they have in common. Additionally, it is important to elucidate the regulatory mechanisms that are involved in LIC1/pericentrin binding. This may give additional insight into the function of both LICs and pericentrin, as well as overall regulation of the dynein complex. As discussed above, dynactin seems to have dual roles in dynein function, i.e., in cargo binding and in tethering dynein to the microtubule. The actual relationship between dynactin function and LIC-based cargo binding must be studied in more detail. Analysis of the subunit arrangement within the dynein complex predicts that both dynactin and pericentrin are able to bind at the same time, suggesting that multiple cargos could be simultaneously transported by dynein. Alternatively, dynactin may be required for transport, even when it is not involved in targeting, as appears to be the case for pericentrin.
REFERENCES


