The LC7 Light Chains of Chlamydomonas Flagellar Dyneins Interact with Components Required for Both Motor Assembly and Regulation

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Members of the LC7/Roadblock family of light chains (LCs) have been found in both cytoplasmic and axonemal dyneins. LC7a was originally identified within Chlamydomonas outer arm dynein and associates with this motor’s cargo-binding region. We describe here a novel member of this protein family, termed LC7b that is also present in the Chlamydomonas flagellum. Levels of LC7b are reduced ~20% in axonemes isolated from strains lacking inner arm I1 and are ~80% lower in the absence of the outer arms. When both dyneins are missing, LC7b levels are diminished to <10%. In oda9 axonemal extracts that completely lack outer arms, LC7b copurifies with inner arm I1, whereas in ida1 extracts that are devoid of I1 inner arms it associates with outer arm dynein. We also have observed that some LC7a is present in both isolated axonemes and purified 18S dynein from oda1, suggesting that it is also a component of both the outer arm and inner arm I1. Intriguingly, in axonemal extracts from the LC7a null mutant, oda15, which assembles ~30% of its outer arms, LC7b fails to copurify with either dynein, suggesting that it interacts with LC7a. Furthermore, both the outer arm γ heavy chain and DC2 from the outer arm docking complex completely dissociate after salt extraction from oda15 axonemes. EDC cross-linking of purified dynein revealed that LC7b interacts with LC3, an outer arm dynein HC motor domain and also may part of the dynein complex. Several LC components so far found only in outer arm axonemal dynein possess putative regulatory regions (e.g., EF-hand and thioredoxin-like domains), associate directly with the HCs and likely influence motor function (Harrison et al., 2002; Sakato and King, 2003). In addition, a leucine-rich repeat LC interacts directly with the γ outer arm dynein HC motor domain and also may play a regulatory role (Benashski et al., 1999; Wu et al., 2000).

INTRODUCTION

Dyneins are large multisubunit complexes that function as microtubule-based molecular motors in eukaryotic cells (reviewed in (King, 2002; Vale, 2003). Ciliary and flagellar dyneins comprise the inner and outer arms and constitute a heterogeneous motor population that seems to have multiple force-generating roles. In these structures, the axonemal microtubules provide a scaffold for an extensive network of additional proteins that collectively regulate the waveform, beat frequency, and ultimately motility of these organelles. Therefore, defining the precise composition of these motor enzyme complexes and the mechanisms by which they are assembled and regulated is of major importance to understanding flagellar motility.

Overall, dynein molecular motors are built around two basic designs (for recent review, see Sakato and King, 2004). Cytoplasmic dynein and the outer arm and inner arm I1 axonemal dyneins contain two to three heavy chain motor units (~520 kDa each; HCs) that belong to the ancient AAA + family of ATPases (Neuwald et al., 1999; King, 2000). For example, Chlamydomonas outer arm dynein contains three distinct HCs (α, β, and γ) that exhibit different enzymatic and motor properties (for review, see DiBella and King, 2001). Dyneins also contain WD-repeat intermediate chains (ICs) that are associated with the N-terminal regions of the HCs and function in cargo and/or ATP-independent microtubule binding. Two general classes of light chains (LCs) are also part of the dynein complex. Several LC components so far found only in outer arm axonemal dynein possess putative regulatory regions (e.g., EF-hand and thioredoxin-like domains), associate directly with the HCs and likely influence motor function (Harrison et al., 2002; Sakato and King, 2003). In addition, a leucine-rich repeat LC interacts directly with the γ outer arm dynein HC motor domain and also may play a regulatory role (Benashski et al., 1999; Wu et al., 2000).

Members of the second LC class associate with the ICs and are located within the cargo-binding region. LCs from this latter group belong to three distinct protein families and in cytoplasm, incorporation of discrete isoforms is thought to define specific motor subtypes that may recognize particular intracellular cargoes (DiBella et al., 2001). Within flagella, the highly conserved LC8 protein associates with the outer dynein arms, inner arm I1, and the radial spokes, and also is involved in intraflagellar transport (King and Patel-King, 1995b; Harrison et al., 1998; Pazour et al., 1998; Yang et al., 2000).
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2001). LCs from the Tctex1/Tctex2 family were originally identified as putative distorters/sterility factors involved in the non-Mendelian transmission of murine h-aploptypes (Lader et al., 1989; Huw et al., 1995) and were subsequently found to be dynein LCs (King et al., 1996; Patel-King et al., 1997). In *Chlamydomonas*, LC2 (Tctex2a) is necessary for assembly of the outer arm motor unit onto the axoneme (Patel-King et al., 1997; Paozur et al., 1999), and Tctex2b is a component of inner arm I1 that contributes to the stability and motor function of that enzyme (DiBella et al., 2004). Tctex1 is an integral component of inner arm I1 (Harrison et al., 1998) and is also present in cytoplasmic dynein (King et al., 1996) where it is involved in attachment of specific cargoes (Tai et al., 1999).

The third LC family, LC7/Roadblock, was initially identified through molecular cloning of LC7 (henceforth termed LC7a) from the *Chlamydomonas* outer arm and isolation of *Drosophila* mutants defective in the roadblock gene (Bowman et al., 1998). In flies, roadblock mutant larvae suffer from posterior, sluggish motility that eventually leads to complete paralysis. These mutants also exhibit mitotic defects, along with severe axonal loss and nerve degeneration as a result of cargo accumulation, which is consistent with defects in retrograde axonal cargo transport. Recent studies have identified two mammalian roadblock (Robl) isoforms (Robl1 and Robl2) and indicate that both LCs can bind to cytoplasmic dynein ICs (Susalka et al., 2002) and form both homo- and heterodimers (Nikulina et al., 2004). In addition, the levels of Robl LCs may be modulated in response to specific cellular states. For example, one member of this LC class expressed in rat visual cortex is down-regulated in response to light stimuli (Ye et al., 2000). In *Chlamydomonas*, LC7a is an integral component of the outer arm and lack of this protein in the oda15 null mutant results in arm assembly defects and aberrant flagellar motility (Paozur and Witman, 2000).

To further define the role of LC7/Roadblock LCs in dynein function, we have identified and analyzed an additional member of this family in *Chlamydomonas*. This LC, termed LC7b, shares 48% and 47% identity with the *Drosophila* outer arm LCs in light chain (LC7a) and *Chlamydomonas* LC7a, respectively, and we demonstrate that LC7a and LC7b are both components of *Chlamydomonas* outer arm I1 and outer arm dynein. We find that association of LC7b is mediated, at least in part, by LC7a. Furthermore, zero-length cross-linking reveals that LC7b interacts directly with a component of the outer arm docking complex, the outer arm β heavy chain-associated thioredoxin, LC3, and with the IC138 IC that is required for phosphorylation-dependent control of inner arm I1 (Habermacher and Sale, 1996, 1997; Yang and Sale, 2000). These results provide the first evidence for how outer arm dynein is attached to the trimeric docking complex and suggest that members of this LC family play a role in both dynein-cargo attachment and motor regulation.

**MATERIALS AND METHODS**

**Strains and Media**

Wild-type (cc124) *Chlamydomonas reinhardtii* and the following mutant strains were used in this study: oda1, oda9, oda11, oda4, pf14, pf18 (obtained from *Chlamydomonas* Genetics Center, Duke University), oda15 (strain 31672; Paozur and Witman, 2000) and pf26/p50 (Piperno et al., 1990; WS4 isolate obtained from Dr. Winfield Sale, Emory University School of Medicine). All cells were grown in R (M medium plus 0.0075 M sodium acetate), or Tris-Acetate-Phosphate (TAP) media (Harris, 1989).

**Purification of Axonemes and Dynein**

Wild-type and mutant strains of *Chlamydomonas reinhardtii* were deflagellated with dibucaine by using standard methods (King et al., 1986) and demembranated with 1% IGEPLD (catalog no. 1L721; Sigma-Aldrich, St. Louis, MO) for 10 min to remove the plasma membrane. For purification, isolated axonemes were incubated with 0.6 M NaCl and then extracted with 0.6 M NaCl (King et al., 1986; Nakamura et al., 1997). NaCl-extracted proteins were precipitated using either 5-20% sucrose density gradients or by anion exchange chromatography (see below). Samples were routinely electrophoresed in 5-15% polyacrylamide gel and stained with Coomassie Brilliant Blue (CBB), or transferred to nitrocellulose for immunoblotting.

**Anion Exchange Chromatography**

To separate the different species of axonemal dynein, the 0.6 M NaCl axonemal extract was dialyzed against buffer A (20 mM Tris, pH 7.5, 60 mM KCl, 0.5 mM EDTA, 0.1 mM Tween 20, 1 mM dithiothreitol, 1 mM phenylmethyl-sulfonyl fluoride) and applied to an anion exchange column (Mono Q HR5/5; Pfizer, New York, NY) using a Biologics chromatography work station (Bio-Rad, Hercules, CA). Proteins were eluted at a flow rate of 0.5 ml/min with a linear salt gradient of 0-50% buffer B (buffer A with 1 M NaCl) and collected into 0.3-m1 fractions. To initially identify fractions pertinent to this study, samples were electrophoresed in 8% polyacrylamide gels, before staining for protein detection (Merrill et al., 1981).

**Molecular Analysis of LC7b**

The entire coding region for *Chlamydomonas* LC7b was obtained by polymerase chain reaction (PCR) using wild-type *Chlamydomonas* first strand cDNA as the template. The forward primer 5’-CGGCTCTAGAGATGCAGATGAC (CA-630) and reverse primer 5’-CGCCGCAAGACCGCGCTTTTG-5’ were designed based on the entire coding sequence derived from the *Chlamydomonas* expressed sequence tag AV387124 and incorporate an XhoI site at the 5’ end and an EcoRI site at the 3’ end, respectively. After restriction digestion, the gel-purified product was subcloned into pBluescript SK- (Stratagene, La Jolla, CA) across XhoI/EcoRI restriction sites. The sequenced clone was used to probe a Southern blot of *Chlamydomonas* genomic DNA, and a northern blot of *Chlamydomonas* RNA from nondeflagellated cells and from cells 30-min postdeflagellation that were actively regenerating flagella. The full-length cDNA also was used to isolate an ~20-kb genomic fragment containing the full-length LC7b gene from a DASHlab (Stratagene) genomic DNA library previously generated from wild-type strain 1132D-5 (R. Patel-King and S. M.King, unpublished data).

**Preparation of Recombinant Protein and Antibody**

The full-length LC7b cDNA was subcloned into the pMAL-c2 vector (New England Biolabs, Beverly, MA) across the Xmal and EcoRI restriction sites. This resulted in the LC7b protein fused to the C terminus of maltose binding protein (MBP) via a 19-residue linker containing a factor Xa cleavage site. The overexpressed fusion protein was purified by amylose affinity chromatography. The full-length fusion protein was used as the immunogen to generate rabbit antiserum CT116. Nitrocellulose membrane containing recombinant LC7b was used to affinity-purify the antibody (Olmsed, 1986). A MBP-LC7a fusion protein and the LC7a-specific antibody R7175 were described previously (Bowman et al., 1999). Additional antibodies to LC7b include 1694A (IC2) and 1878A (IC1) (King et al., 1986, 1987; R5390 (LC5) (King and Patel-King, 1995b), R5391 (LC3) (Patel-King et al., 1996), R5391 (LC2) (Patel-King et al., 1997), R5391 (LC2) (Benashski et al., 1999), ODC2 (DC2 kindly provided by Drs. Ritsu Kamiya, University of Tokyo, Tokyo, Japan; and Ken-ichi Wakabayashi, University of Connecticut Health Center, Farmington, CT) (Wakabayashi et al., 2001, 2002; Y (IC1) (King et al., 1985), and oIC138 (Griff-2, IC138) and oIC140 (IC140) (Yang and Sale, 1998) (both kindly provided by Dr. Winfield Sale, Emory University School of Medicine, Atlanta, GA).

**Native Gel Electrophoresis of MBP-LC Fusion Proteins**

To examine the oligomeric state of LC7a and LC7b in solution, the MBP-LC fusion proteins were electrophoresed in gels of different acrylamide concentrations (4-20%) and pH 8.0. The retention coefficient (K_r) was determined from the negative slope of 100 [log (R_r × 100)]. The standard proteins used were jack bean urease (545-kDa hexamer; 272-kDa trimer), bovine serum albumin (132-kDa dimer; 66-kDa monomer), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and α-lactalbumin (14.2 kDa). A plot of log K_r vs. log M_r for these proteins was used as a standard curve from which the native molecular masses of the MBP fusion proteins could be determined.

**Chemical Cross-linking of Purified Dynein with the Zero-Length Cross-Linker 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC)**

To investigate protein–protein interactions involving LC7b, purified dynein was treated with 0–20 mM EDC dissolved in 100 mM HEPES, pH 7.4. Samples were incubated at room temperature for 60 min, and the reactions were terminated by addition of β-mercaptoethanol in HEMIS (30 mM HEPES, pH 8.0). The retention coefficient (K_r) was determined from the negative slope of 100 [log (R_r × 100)]. The standard proteins used were jack bean urease (545-kDa hexamer; 272-kDa trimer), bovine serum albumin (132-kDa dimer; 66-kDa monomer), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa), and α-lactalbumin (14.2 kDa). A plot of log K_r vs. log M_r for these proteins was used as a standard curve from which the native molecular masses of the MBP fusion proteins could be determined.
pH 7.5, 5 mM MgSO4, 0.5 mM EDTA, 25 mM KCl) to a final concentration of 0.1 M (Benashski and King, 2000).

**Densitometric Analysis of Wild-Type and Mutant Axonemes**

Various concentrations of wild-type and mutant axonemes were electrophoresed in 5–15% polyacrylamide gradient gels and either stained with Coomassie Blue or transferred to nitrocellulose. Blots were probed with CT116, 1878A (vs. IC1 of the outer arm; King et al., 1986) or /H9251 IC140 (vs. IC140 of inner arm I1; Yang and Sale, 1998). Signals were developed using a chemiluminescent substrate. Densitometry was performed on several different axonemal amounts for each sample to ensure that signals were within the linear response range, by using an Alpha Innotech Imaging System and AlphaEase Software (Athens, GA). Mutant axoneme amounts were normalized to wild-type by using the Coomassie Blue-stained /H9251 /H9252 tubulin dimer bands.

**Electron Microscopy of oda15 Axonemes**

Samples were prepared for electron microscopy by using the method of Schroeter et al. (1994). Axoneme samples were fixed with 2.5% glutaraldehyde, 1% tannic acid (#1764; Mallinckrodt, St. Louis, MO) in 100 mM sodium cacodylate, pH 7.4. Intact pellets were then transferred into a postfix solution (100 mM cacodylate, pH 7.4, 1% osmium tetroxide, 0.8% potassium ferricyanide) and subsequently washed in cacodylate buffer. Fixed samples were incubated in 1% tannic acid, 100 mM cacodylate, pH 7.4; stained en bloc with 0.5% aqueous uranyl acetate; and dehydrated in an ethanolic series in propylene oxide. Samples were transitioned into plastic using increasing concentrations of Polybed (Polysciences, Warrington, PA) in propylene oxide. Blocks were polymerized at 60°C for 2–3 d, sectioned, and poststained with 2% aqueous uranyl acetate for 30 min and lead citrate (Sato, 1967). Sections were examined in a Phillips CM10 transmission electron microscope operating at an accelerating voltage of 60 kV.
RESULTS

Identification and Molecular Analysis of a Novel LC7/ Roadblock Dynein Light Chain

To identify additional dynein components in Chlamydomonas flagella, we searched the Chlamydomonas expressed sequence tag (EST) and nonredundant databases by using LC7a from outer arm dynein (accession no. AF140239) as the initial query sequence. The search identified Chlamydomonas EST AV387124, which is 1362 base pairs in length and contains a single open reading frame encoding a 100-residue protein with a calculated molecular weight of 11,147 Da and a pI of 5.56. This protein (here termed LC7b) exhibits 47% sequence identity (63% similarity) with LC7a. A phylogenetic tree illustrating the relationship between LC7b and other members of this LC family is shown in Figure 1a. A secondary structure prediction made using Predictprotein (Rost and Sander, 1993) suggests that LC7b contains α-helices in the N-terminal and central sections of the polypeptide, with two short β strands between the helices and three additional strands in the C-terminal regions (Figure 1b). This predicted arrangement of secondary structural elements is consistent with similar analyses for other LC7/Roadblock family members (Koonin and Aravind, 2000; Nikulina et al., 2004) and is clearly distinct from the LC8 (Liang et al., 1999) and Tctex1/Tctex2 (Wu et al., 2001) LC families.

A Southern blot of Chlamydomonas wild-type genomic DNA digested with SmaI and BamHI produced single bands when probed with the full-length LC7b cDNA, suggesting that this protein is encoded by a single gene (Figure 1c). A single band of ~1.36 kb was readily detected in a northern blot of RNA isolated from cells that were actively regener-
ating flagella (Figure 1d); this band was barely detectable in RNA from nonflagellated cells, suggesting that LC7b is a flagellar protein. The LC7b gene was cloned using the cDNA construct as the initial probe and found to contain six exons (Figure 1e). Restriction fragment length polymorphism (RFLP) analysis revealed that the LC7B gene is located on linkage group XII/XIII, between TUB1 and TUB2.

**LC7b Is Present in Flagellar Axonemes**

The entire LC7b coding region was subcloned into the pMAL-c2 vector and expressed as an N-terminal fusion with MBP. The full-length fusion protein was used as the antigen to generate rabbit polyclonal antiserum CT116. After separation from MBP, immobilized LC7b protein was used to affinity purify the LC7b antibody. Western blotting against recombinant outer dynein arm LCs revealed that CT116 reacted only with its target antigen, and specifically did not recognize LC7a (Figure 2a) or any other axonemal dynein components (Figure 2b).

To determine whether LC7b is indeed an integral dynein LC, axoneme samples isolated from wild-type and mutant strains lacking various flagellar structures were probed with the CT116 antibody (Figure 2c). LC7b was detected in wild-type axonemes and similar levels were observed in mutants lacking a subset of inner arms I2/3 (ida4), radial spokes (pf14), and central pair components (pf18). The levels of LC7b were reduced by ~20% in axonemes from mutants lacking inner arm II (ida1), and by ~80% in the absence of the outer arms and the outer arm docking complex (oda1) or outer arms alone (oda9) (Table 1). The LC7a-null mutant, oda15, also showed a significant decrease. The most dramatic reduction (of >90%) was seen in a mutant (pf28pf30) lacking both the outer arms and inner arm I1. The decreased amounts of LC7b in mutants lacking individual dyneins and the severely diminished levels observed in the pf28pf30 double mutant imply that this protein associates with both the outer arms and inner arm I1. This is consistent with the arrangement of four outer arms and one copy of inner arm I1 in the 96-nm axonemal repeat.

**LC7a and LC7b are Both Components of the Outer Dynein Arm and Inner Arm I1**

To confirm the location of LC7b, axonemes were treated with 0.6 M NaCl, which removes dynein components, and the soluble extracts were fractionated in 5–20% sucrose den-

<table>
<thead>
<tr>
<th>Strain</th>
<th>LC7b</th>
<th>IC1</th>
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<td>cc124</td>
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<td>oda15</td>
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<tr>
<td>ida1</td>
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<tr>
<td>oda1</td>
<td>13</td>
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<td>n.d.</td>
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<tr>
<td>oda9</td>
<td>17</td>
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n.d., not determined.

a Values were determined by quantitative densitometry of CBB-stained gels and normalized based on the α and β tubulin band signal.

b not present.
In ida1 extracts, which lack inner arm subtype II, the majority of LC7b copurified with the trimeric outer dynein arm (Figure 3a); minor pools of LC7b also were observed sedimenting higher in the gradient and likely represent dissociation products. In addition, when the same extract was fractionated under conditions that cause the outer arm to partially dissociate (i.e., the absence of Mg$^{2+}$ and an increase in hydrostatic pressure; Nakamura et al., 1997), LC7b comigrated with the αβ HC dimer (Figure 3b).

The location of DC2 from the outer arm docking complex also is shown in Figure 3, a and b, and illustrates that significant levels of this protein copurify with LC7b only in the presence of Mg$^{2+}$. Furthermore, in ida9 axonemal extracts, the majority of LC7b cofractionated with components of inner arm II (Figure 4a), whereas in wild-type, it copurified with both sets of arms (Figure 4b). To ensure that LC7b associated with each dynein, wild-type and ida9 high salt extracts also were fractionated by anion exchange chromatography. A linear KCl gradient was used to elute the various dynein subspecies. After purification, fractions were electrophoresed in 5–15% acrylamide gels and either stained with Coomassie Blue (top) or blotted to detect LC7b. Molecular weight markers are indicated to the left and the positions of the outer arm and/or inner arm II ICs are indicated to the right. In ida9 extracts, inner arm II eluted at 380–400 mM salt (fractions 94–99). LC7b also eluted within these same fractions. In wild-type extracts, LC7b copurified with both sets of arms (fractions 90–99).

Figure 4. LC7b copurifies with both the outer arms and inner arm II. In both a and b, 0.6 M NaCl axonemal extracts were loaded onto 5–20% sucrose gradients. After sedimentation, fractions were electrophoresed in 5–15% acrylamide gels and either stained with Coomassie Blue (top) or blotted to detect LC7b. Molecular weight markers are indicated at left. (a) In ida9 extracts, the majority of LC7b comigrated with inner arm II (fractions 5–8). (b) In wild-type extracts, LC7b copurified with the outer arms and inner arm II (fractions 2–8), suggesting that it associates with both classes of arms. In both c and d, dialyzed 0.6 M NaCl axonemal extracts were subjected to anion exchange chromatography. A linear KCl gradient was used to elute the various dynein subspecies. After purification, fractions were electrophoresed in 5–15% acrylamide gels and either stained with Coomassie Blue (top) or blotted to detect LC7b. Molecular weight markers are indicated to the left and the positions of the outer arm and/or inner arm II ICs are indicated to the right. (c) In ida9 extracts, inner arm II eluted at 380–400 mM salt (fractions 94–99). LC7b also eluted within these same fractions. (d) In wild-type extracts, LC7b copurified with both sets of arms (fractions 90–99).
LC7b Also Associates with IC138 from Inner Arm I1

In a similar manner to that described above, inner arm I1 purified from the oda1 mutant was treated with EDC (Figure 7a) and probed with CT116. A major, reactive band of $M_r$ 133,000 was detected, suggesting that LC7b had formed a complex with an inner arm IC (Figure 7b). An immunoblot of EDC-treated oda1 dynein using an antibody against IC140 revealed no cross-linked products of the appropriate $M_r$ (our unpublished data). However, when a similar sample was probed for IC138, the same cross-linked product was detected (Figure 7, c and d). This product was not present in untreated dynein, indicating that it resulted from the zero-length cross-linking reaction and that LC7b and IC138 interact directly (Figure 7, b and c). An additional minor band of $M_r$ 17,900 containing LC7b also was present in the cross-linked samples. Immunoblots using antibodies specific for Tctex1, Tctex2b, LC7a, and LC8 did not identify any similar sized cross-linked products, indicating that the $M_r$ 17,900 band did not contain any of these LCs (our unpublished data). The simplest interpretation is that this band represents an LC7b dimer as also was observed in cross-linked outer arm dynein.

LC7a Is Required for the Stable Association of LC7b, the $\gamma$ Heavy Chain, and DC2 with the Outer Arm

To further investigate the putative interaction between LC7b and DC2 detected by EDC cross-linking, we took advantage of the mutant oda15, which is defective for LC7a and exhibits a slow, jerky swimming (Pazour and Witman, 2000). Immunoblot analysis using the R7178 antibody confirmed that LC7a is completely missing in this strain, although considerable amounts of other outer arm dynein polypeptides (e.g., the $\gamma$ HC-associated LC1 protein) are present (Figure 8a). This observation suggested that oda15 still retains significant numbers of outer dynein arms. To assess this, we examined oda15 axonemal cross-sections and found widely varying numbers of outer arms, ranging from a full comple-

Figure 5. Outer dynein arm LC7a is also a component of inner arm I1. (a) Approximately 150 $\mu$g of wild-type (cc124) and oda1 axonemes and (b) oda1 purified 18S dynein were electrophoresed in 5–15% gradient gels then either stained with CBB (top) or transferred to nitrocellulose (bottom) and blotted to detect LC7a using the R7178 antibody. LC7a is present in oda1 axonemes and is also a component of purified inner arm I1.
ment to none (mean 3.2), associated with the doublet microtubules (Figure 8b). This observation is consistent with the quantitative densitometry analysis (Table 1), which indicates that 20% of outer arm IC1 is retained in oda15 axonemes. Longitudinal sections revealed that, in regions containing outer arms, runs of motors were assembled with a 24-nm repeat as in wild-type. However, gaps occurred in the arrangement at apparently random intervals (Figure 8c), and many microtubule segments seemed completely devoid of attached dyneins. IC140 levels also were reduced to approximately one-third that seen in wild-type strains (Table 1), suggesting that inner arm II assembly is also compromised in the oda15 mutant.

Dyneins were extracted from oda15 axonemes and sedimented in a sucrose density gradient under conditions that normally preserve association of the outer arm with the trimeric docking complex (Takada et al., 1992; Figure 3). Electrophoretic and immunoblot analysis revealed that LC2, LC8, and both ICs from the outer arm cosedimented at ~18S in fractions 6–9 (Figure 9). However LC1 and the γ HC cosedimented at ~12S, as also occurs when Mg"+ is lacking in the gradient. As in wild-type samples, the peak of IC140

Figure 6. LC7b cross-links to DC2 from the outer arm docking complex and the LC3 thioredoxin. Purified outer arm dynein from ida1 axonemes was cross-linked with 0 or 20 mM EDC. Samples were electrophoresed in 5–15% gradient gels and then either stained with CBB or transferred to nitrocellulose and blotted to detect various outer arm components. Molecular weight markers are indicated on the left. (a) CBB stained gel of 0 and 20 mM EDC-cross-linked dynein. (b) Blots of 0 and 20 mM EDC-cross-linked dynein probed with CT116 to detect LC7b-reactive products. Three cross-linked species of M, ~19,200, 27,400, and 94,700 were identified. Two additional, nonspecific bands indicated by arrows were occasionally observed with this antibody preparation. (c) Identical samples to those shown in b were probed to detect DC2-containing species. (d) Samples similar to those shown in b and c were probed to detect LC3-containing species. (e) A single lane containing the 20 mM EDC-cross-linked sample was cut lengthwise and then probed with either αDC2 (left) or CT116 (right). Realignment of both halves revealed a common reactive band detected by both antibodies. (f and g) Similar procedure as in e, however the left sides of the blots were probed with monoclonal antibodies 1878A against IC1 (f), or 1869A against IC2 (g). The M, 94,700 band contains both LC7b and DC2 but does not include IC1 or IC2. (h) A similar blot of EDC-treated dynein was probed with antibodies against LC7b and LC3. The M, 27,400 band contains both proteins.
from inner arm I1 occurred slightly higher in the *oda15* sucrose gradient than the outer arm, suggesting that at least some of this dynein also assembled in the mutant. Interestingly however, LC7b did not comigrate with either the outer arms or inner arm I1, but rather completely dissociated and was found at the very top of the gradient. Furthermore, we observed that the docking complex protein DC2 also was released and sedimented at ~7S, rather than remaining associated with the outer arm (Figure 3). Consequently, we conclude that LC7a is required for the stable association of LC7b, the docking complex, and the γ HC subcomplex with the remainder of the outer arm dynein.

**DISCUSSION**

*Chlamydomonas* Contains Two Members of the LC7/Roadblock Family

Previously, we identified LC7 (here termed LC7a) within the outer dynein arm as a founding member of the LC7/Roadblock class of dynein light chains (Bowman et al., 1999). Here, we describe a second member of this LC family, termed LC7b, found in *Chlamydomonas* that is ~50% identical to both LC7a and the *Drosophila* cytoplasmic dynein LC, roadblock. The LC7/Roadblock dynein LCs belong to an ancient superfamily involved in the regulation of NTPases.
One member of this family, MglB from *Myxococcus xanthus*, seems to modulate the GTPase MglA, which is involved in the regulation of gliding motility (Hartzell and Kaiser, 1991a; 1991b). Mammals also contain two members of this family that are differentially expressed (e.g., Robl1 but not Robl2 has been detected in brain). Furthermore, levels of Robl are dramatically altered in response to light stimuli in the rat visual cortex (Ye et al., 2000), and the balance between Robl1 and Robl2 in human hepatocellular carcinoma tissue is shifted compared with healthy liver tissue (Jiang et al., 2001).

**LC7a and LC7b Associate with Both the Outer Dynein Arm and Inner Arm I1**

Four outer dynein arms and one inner arm I1 normally assemble within a 96-nm repeat along the axonemal microtubules. Consistent with this arrangement, we found that LC7b levels are ∼80% below wild type in mutants completely lacking the outer arms or outer arms and docking complex (oda1, oda9) and ∼20% reduced in the absence of inner arm I1 (ida1) (Table 1). LC7a is almost completely absent from the axoneme when both classes of motor are missing (pf28pf30), and furthermore, this protein copurifies with both dyneins in sucrose density gradients and following anion exchange chromatography. These data indicate that this LC associates with both classes of dynein arm and further implies that it cannot assemble in the absence of other dynein components. This latter property is a common feature of many dynein proteins and is consistent with their preassembly into subcomplexes within the *Chlamydomonas* cytoplasm (Fowkes and Mitchell, 1998).

Previously, we identified LC7a within *Chlamydomonas* outer arm dynein, and found that this protein was greatly reduced, but not absent, in mutant axonemes prepared from strains lacking that dynein (Bowman et al., 1999). In this report, we demonstrate that LC7a is also an integral component of inner arm I1. Consequently, these two dyneins contain the same two members of this LC class. In cytoplasmic dynein, Robl1 and Robl2 are capable of forming both homo- and heterodimers (Susalka et al., 2002; Nikulina et al., 2004), suggesting that they assemble in pairs within the dynein motor unit. We observed that both LC7a and LC7b fusion

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**Figure 8.** Axonemes from oda15 contain randomly placed outer dynein arms. (a) Approximately 50 μg of wild-type (cc124) and oda15 (LC7a null mutant) axonemes were electrophoresed in 5–15% gradient mini gels then either stained with Coomassie Blue or transferred to nitrocellulose and blotted to detect the outer arm proteins, LC1 (using antibody R5932; Benashski et al., 1999) and LC7a (R7178). Reduced levels of LC1 were found in oda15, signifying the presence of outer arms; however, the LC7a protein was completely absent. (b) A longitudinal section of an oda15 axoneme prepared by thin section electron microscopy. The section outlined is shown at higher magnification, below, and shows stretches of outer arms (arrowheads) and randomly spaced gaps (brackets) where arms are missing. (c) Random cross sections of oda15 axonemes reveal a widely varying complement of outer arms.

(Koonin and Aravind, 2000). One member of this family, MglB from *Myxococcus xanthus*, seems to modulate the GTPase MglA, which is involved in the regulation of gliding motility (Hartzell and Kaiser, 1991a; 1991b). Mammals also contain two members of this family that are differentially expressed (e.g., Robl1 but not Robl2 has been detected in brain). Furthermore, levels of Robl are dramatically altered in response to light stimuli in the rat visual cortex (Ye et al., 2000), and the balance between Robl1 and Robl2 in human hepatocellular carcinoma tissue is shifted compared with healthy liver tissue (Jiang et al., 2001).
proteins also form dimers in solution, and our cross-linking
data further suggests that at least LC7b forms homodimers
within both axonemal dyneins. Thus, the LC7/Roadblock
LCs follow the general characteristic that LC components
within the dynein IC/LC complex exist as dimers, e.g., the
inner arm proteins Tctex1 (Harrison et al., 1998; Wu et al.,
2001) and Tctex2b (DiBella et al., 2004); outer arm LC6 (Pfis-
ter et al., 1982; King and Patel-King, 1995b), and LC8, which
is present in both dyneins (Pfister et al., 1982; King and
Patel-King, 1995b; Liang et al., 1999) (Figure 10). The one
apparent exception to this rule is the LC2 (Tctex2a) compo-
nent of the outer arm, which is present at a stoichiometry of
one per dynein (Pfister et al., 1982; Patel-King et al., 1997).
However, we have recently identified an additional outer
arm component related to Tctex1 that may act as its partner
(L.M.DiBella and S. M.King, unpublished data).

**LC7a Is Not Absolutely Required for Outer Dynein Arm
Assembly**

Many components (including some LCs) of both the outer
dynein arm and inner arm II are essential for assembly of
the motor unit onto the axoneme, e.g., the 1α and 1β HCs
from inner arm II (Kamiya et al., 1991; Porter et al., 1992;
Myster et al., 1997; Perrone et al., 2000); IC140 from the II
dynein (Perrone et al., 1998; Yang and Sale, 1998); the outer
arm β and γ HCs (Mitchell and Rosenbaum, 1985; Kamiya,
1988; Sakakibara et al., 1993); IC1 and IC2 from the outer arm
(Kamiya, 1988; King et al., 1995; Wilkerson et al., 1995); LC8,
a LC required for IFT that also is found in the outer arm,
inner arm II, and radial spokes (King and Patel-King, 1995b;
Pazour et al., 1998); and the outer arm LC, LC2 (Pazour et al.,
1999). However, LC7a is apparently not absolutely required
for this process, because the LC7a-null mutant *oda15* can
assemble ~20% of its outer arms and ~35% of the II class of
inner arm. Analysis of purified dyneins from this strain
indicated that the outer arms were unstable, because LC7b,
the γ HC, and DC2 dissociated from the motor unit after
extraction even when purified in the presence of Mg2+
under conditions that maintains their integrity within wild-
type dynein. Furthermore, LC7b also completely dissoci-
ated, suggesting that it requires LC7a to form stable
interactions with both dyneins.

**Does LC7b Mediate Attachment of the Outer Arm to the
Docking Complex?**

The trimeric docking complex (DC1-DC3) is essential for
correct placement of the outer dynein arm onto the axoneme
(Koutoulis et al., 1997; Wakabayashi et al., 2001; Takada et al.,
2002), although a fraction of dyneins can assemble in the
absence of DC3 (Casey et al., 2003). Our cross-linking data
reveal that DC2 and LC7b are in direct contact in purified
outer arm dynein. This finding, together with the observa-

![Figure 9](image-url)
tion that both proteins dissociate from dyneins extracted from *oda15* axonemes suggest that these proteins interact in the flagellum. This is the first evidence that links a component of the docking complex to the outer arm. Although the interaction of LC7b and DC2 represents the only identified connection between these two structures (Figure 10), this does not rule out the possibility of additional interactions between other dynein and docking complex components. The instability of the γ HC in this mutant raises the possibility that it also interacts with LC7b. However, in *ida1* sucrose gradients purified under conditions that cause the γ HC and DC2 to dissociate from the remaining components of the outer arm, LC7b copurifies with the αβ HC docking complex. EDC cross-linking revealed that LC7b is also in direct contact with the β HC-associated thioredoxin LC3. This observation raises the intriguing possibility that LC3 and the DC3 docking complex protein that binds Ca\(^{2+}\) in a redox-sensitive manner (Casey et al., 2003), are in close proximity.

A protein interaction map for the outer dynein arm is shown in Figure 10 (top) and illustrates the complexity of associations that exist within this dynein motor. In addition to direct associations with LC3 and DC2, additional potential linkages involving LC7a and other subunits within the cargo-binding region are indicated. For example, as the Robl1 and Robl2 cytoplasmic dynein LCs both bind to the IC74 IC in a region distinct from the LC8 and Tctex1 binding sites (Susalka et al., 2002), IC1 and IC2 from the axonemal outer arm are also potential LC7a and LC7b interacting partners.

**LC7b Interacts with a Regulatory Subunit of Inner Arm I1**

Within inner arm I1, we found that LC7b cross-links to IC138; however, because LC7b also detaches from inner arm I1 in the absence of LC7a, it must again form additional associations within this dynein (Figure 10). Both cAMP-dependent protein kinase (Hasegawa et al., 1987; Howard et al., 1997) and casein kinase 1 (Yang and Sale, 2000) have been implicated in the regulation of microtubule sliding through inner arm I1 (Smith and Sale, 1992). Moreover, IC138 is the only known phosphorylated subunit within this dynein; implicating it as a major regulatory component involved in this process (Habermacher and Sale, 1997). Interaction of LC7b with IC138 places it in an intriguing position within this dynein that may transduce regulatory signals.

In conclusion, we have identified a novel member of the LC7/Roadblock family in *Chlamydomonas*. We show that both LC7a and LC7b associate with outer arms and I1 inner arms and that LC7a mediates a stable association between several dynein components, including LC7b within both dyneins. These LCs participate in multiple interactions within the outer arm and their relationship with the docking complex seems to play a major role in the assembly and stability of this dynein motor. In addition, within inner arm I1, LC7b associates with a major regulatory subunit in a manner that is stabilized by LC7a.

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