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IC97 Is a Novel Intermediate Chain of I1 Dynein That Interacts with Tubulin and Regulates Interdoublet Sliding

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Our goal is to understand the assembly and regulation of flagellar dyneins, particularly the Chlamydomonas inner arm dynein called I1 dynein. Here, we focus on the uncharacterized I1-dynein IC IC97. The IC97 gene encodes a novel IC without notable structural domains. IC97 shares homology with the murine lung adenoma susceptibility 1 (Las1) protein—a candidate tumor suppressor gene implicated in lung tumorigenesis. Multiple, independent biochemical assays determined that IC97 interacts with both α- and β-tubulin subunits within the axoneme. I1-dynein assembly mutants suggest that IC97 interacts with both the IC138 and IC140 subunits within the I1-dynein motor complex and that IC97 is part of a regulatory complex that contains IC138. Microtubule sliding assays, using axonemes containing I1 dynein but devoid of IC97, show reduced microtubule sliding velocities that are not rescued by kinase inhibitors, revealing a critical role for IC97 in I1-dynein function and control of dynein-driven motility.

INTRODUCTION

Dyneins are minus–directed microtubule motors important for a variety of cellular functions, including membrane-bound organelle transport, assembly and orientation of the mitotic spindle, nuclear migration, assembly of the Golgi apparatus, and ciliary and flagellar motility. In the ciliary/flagellar axoneme, the outer and inner dynein arms convert the energy derived from ATP hydrolysis into microtubule sliding, which in turn drives flagellar beating and bending. Analysis using the model genetic organism Chlamydomonas reinhardtii has revealed that the inner arm dynein system is responsible for generation of the flagellar waveform—the size and shape of the flagellar bend (Brokaw and Kamiya, 1987; Kamiya, 2002; King and Kamiya, 2009). The inner dynein arms, of which there are at least seven isoforms (King and Kamiya, 2009), are heterogeneous in composition and structural arrangement on the axoneme, binding to the axoneme in precise locations to form part of a 96-nm repeating module along each doublet microtubule (Goodenough and Heuser, 1985; Piperno et al., 1990; Burgess et al., 1991; Mastronarde et al., 1992; Porter et al., 1992; Nicastro et al., 2006; Bui et al., 2008). The two-headed I1 dynein isoform, also called dynein f, is distributed uniformly along the length of the axoneme as a triad structure located proximal to each radial spoke S1 (Nicastro et al., 2006; Porter and Sale, 2000; Wirschell et al., 2007).

I1 dynein is among the best characterized inner arm isoforms, containing two heavy chains (α-HC and β-HC), three intermediate chains (IC138, IC140, and IC97—also termed IC110), and five known light chains (LC8, LC7a, LC7b, TcTex1, and TcTex2b) (Table 1; (Piperno et al., 1990; Myster et al., 1997; 1999; Harrison et al., 1998; Pazour et al., 1998; Perrone et al., 1998, 2000; Yang and Sale, 1998; DiBella et al., 2004a,b; Hendrickson et al., 2004). Recently, a new I1-dynein–associated protein, flagellar-associated protein (FAP)120, has been identified that interacts with IC138, LC7b, and IC97 (Ikeda et al., 2008; Bower et al., 2009). Multiple lines of evidence demonstrate that I1 dynein is an unusual dynein motor that plays a critical regulatory role in the axoneme (Smith and Sale, 1991; Kotani et al., 2007; Wirschell et al., 2007). The I1-dynein complex has been implicated as a target of the regulatory signals that control flagellar motility (Porter et al., 1992; Porter and Sale, 2000; Smith and Yang, 2004; Wirschell et al., 2007). Flagella that are lacking I1 dynein exhibit defective flagellar waveform and phototaxis, indicating that I1 dynein plays a role in these processes (Brokaw and Kamiya, 1987; Brokaw, 1994; King and Dutcher, 1997; Okita et al., 2005).

In Chlamydomonas, four independent loci, when defective, result in a specific failure to assemble the I1-dynein complex in the axoneme (DiBella and King, 2001). Three of these loci encode I1-dynein subunits; ida1, ida2, and ida7 mutants are defective in the α-HC, β-HC, and IC140, respectively (Myster et al., 1997; Perrone et al., 1998, 2000). Of particular use are novel
Within the axoneme, IC97 interacts with both IC138 and IC140 proteins that include IC138, LC7b, and FAP120 (Ikeda et al. 2009). This study revealed that both IC97 and IC138, although not necessary for I1-dynein assembly, are required for regulation of I1-dynein activity.

**Table 1. I1-dynein components and mutants**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Mol. wt.</th>
<th>Gene/mutant</th>
<th>Properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1aHC</td>
<td>523</td>
<td>ida1 (pf9, pf30)</td>
<td>Lacks inner arm II, slow swimming</td>
<td>Kamiya et al. (1991)</td>
</tr>
<tr>
<td>1βHC</td>
<td>511</td>
<td>ida2</td>
<td>Lacks inner arm II, slow swimming</td>
<td>Kamiya et al. (1991)</td>
</tr>
<tr>
<td>IC140</td>
<td>140</td>
<td>ida7</td>
<td>Lacks inner arm II, slow swimming</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>IC138</td>
<td>138</td>
<td>bop5</td>
<td>Partial I1 assembly, regulatory phosphoprotein part of IC138 subcomplex</td>
<td>King et al. (1997)</td>
</tr>
<tr>
<td>IC97 (IC110)</td>
<td>90 (110)</td>
<td></td>
<td>Non-WD repeat protein, homology to Las1/Casc1 proteins, part of IC138 subcomplex</td>
<td>Bower et al. (2009)</td>
</tr>
<tr>
<td>LC8</td>
<td>10</td>
<td>fla14</td>
<td>Required for flagellar assembly, part of I1 dynein, radial spokes and outer dynein arm</td>
<td>Yang et al. (2001)</td>
</tr>
<tr>
<td>LC7a</td>
<td>14</td>
<td>oda15</td>
<td>Required for outer arm assembly, slow swimming, associates with I1 dynein and may interact with LC7b</td>
<td>Pazour and Witman (2000)</td>
</tr>
<tr>
<td>LC7b</td>
<td>11</td>
<td></td>
<td>LC7/Robl family member, interacts with IC138, interacts with LC3 and DC2 of ODA</td>
<td>DiBella et al. (2004a)</td>
</tr>
<tr>
<td>Tctex1</td>
<td>13</td>
<td></td>
<td>Dimeric protein, potential cargo binding activity, also found in cytoplasmic dynein</td>
<td>Dibella et al. (2004a)</td>
</tr>
<tr>
<td>Tctex2b</td>
<td>13.7</td>
<td>pf16-D2</td>
<td>Not required for I1 assembly, stabilizes I1 dynein</td>
<td>DiBella et al. (2004a)</td>
</tr>
<tr>
<td>FAP120</td>
<td>42</td>
<td></td>
<td>Not required for I1 dynein assembly, ankryin repeat protein, part of IC138 subcomplex</td>
<td>Ikeda et al. (2009)</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Strains and Culture Conditions**

*Chlamydomonas* strains used in this study are summarized in Table 2. Cells were grown in Tris-acetate-phosphate medium or L-medium, with aeration on a 14:10-h light:dark cycle (Harris, 1989; Harris, 2009).

**Cloning and Sequencing of IC97 Sequences**

The cloning of IC97 was based on tandem mass spectrometry (MS/MS) identification of peptides derived from band-purified IC97 protein. Gene model C_850038 in the JGI *Chlamydomonas* genome database version 2.0 (http://shake.jgi-psd.org/cbure2/cbure2.home.html) encodes the IC97 gene (Supplemental Figure S1) and was used to design primers (Integrated DNA Technologies, Coralville, IA) for polymerase chain reaction (PCR) amplification of IC97 cDNA sequences (cDNA library 7 provided by Greg Pazour (University of Massachusetts, Amherst, MA). PCR products were cloned into pGEM-T-Easy (Promega, Madison, WI) or pcR2.1/GW8/TOPO cloning vectors (Invitrogen, Carlsbad, CA) and sequenced to verify intron–exon boundaries (DNA Sequencing Facility, Iowa State University, Ames, IA). The full-length cDNA is 2,973 kb.

An IC97 cDNA probe containing base pairs 1343–2685 (Supplemental Figure S1B) was used to screen a BAC library (Clemson University Genomics Institute, Clemson, SC) and a 9.791-kb genomic EcoRI-HindIII fragment containing the IC97 gene was identified and subcloned into pBlueScript SK-vector (plasmid IC97-H). IC97 is found in the flagellar proteome as FAP94 (Pazour et al. 2005) and is published under accession FJ156240 and FJ156241 for the gene and cDNA sequences, respectively.
Table 2. Chlamydomonas strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Defect</th>
<th>Motility</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC124</td>
<td>Radial spokes, I1 dynein, retrograde IFT</td>
<td>WT</td>
<td>Harris (1989, 2009)</td>
</tr>
<tr>
<td>CC1331 (fla14-3)</td>
<td>Radial spokes, I1 dynein, retrograde IFT</td>
<td>Paralyzed</td>
<td>Yang et al., unpublished</td>
</tr>
<tr>
<td>CC-4077 (ida7-1::IC140 5A&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>Truncated IC140; lacks outer dynein arm</td>
<td>Slow-jerky swimming</td>
<td>Perrone et al. (1998)</td>
</tr>
<tr>
<td>ida7-1::IC140 5A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC-4080 (bop5-1)</td>
<td>Partial I1 dynein assembly; IC138 point mutant</td>
<td>Slow-smooth swimming</td>
<td>DUTcher et al. (1988); Hendrickson et al. (2004)</td>
</tr>
<tr>
<td>bop5-2</td>
<td>Partial I1 dynein assembly; null IC138 allele</td>
<td>Very slow swimming, spinning cells</td>
<td>Bower et al. (2009); DUTcher et al. (1988)</td>
</tr>
<tr>
<td>CC-2664 (ida1)</td>
<td>Lacks I1 dynein; 1αHC mutant</td>
<td>Slow-smooth swimming</td>
<td>Kamiya et al. (1991); Myster et al. (1997)</td>
</tr>
<tr>
<td>CC-2666 (ida2)</td>
<td>Lacks I1 dynein; 1βHC mutant</td>
<td>Slow-smooth swimming</td>
<td>Kamiya et al. (1991); Perrone et al. (2000)</td>
</tr>
<tr>
<td>CC-2668 (ida3)</td>
<td>Lacks I1 dynein; gene product unknown</td>
<td>Slow-smooth swimming</td>
<td>Kamiya et al. (1991)</td>
</tr>
<tr>
<td>CC-2670 (ida4)</td>
<td>Lacks inner arm isoforms α, c, and d</td>
<td>Slow-smooth swimming</td>
<td>Perrone et al. (1998); Yang and Sale (1998)</td>
</tr>
<tr>
<td>CC-3921 (ida7)</td>
<td>Lacks I1 dynein; IC140 mutant</td>
<td>Slow-smooth swimming</td>
<td>Kamiya et al. (1991); Perrone et al. (1998); Yang and Sale (1998)</td>
</tr>
<tr>
<td>CC-262 (p17 mt-), 5c12B, pf28 pf30 ssh1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Lacks radial spoke head; RSP1 mutant; HA-tagged α&lt;sub&gt;1&lt;/sub&gt;-tubulin; Lacks outer dynein arm; Lacks I1 dynein; suppressor of short mutation</td>
<td>Paralyzed</td>
<td>McVittie (1972); Huang et al. (1981); Kozminski et al. (1993)</td>
</tr>
<tr>
<td>pf28 pf30 ssh1&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>Paralyzed</td>
<td>Ledizet and Piperno (1986); Freshour et al. (2007)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Yang, Yang, Wirschell, and Davis (unpublished) determined that the pf5 mutation in strain CC1331 is an allele at the FLA14 locus (fla14-3) that encodes the LC8 protein. Other strains denoted as pf5 in the Chlamydomonas Stock Center contain a mutation that is tightly linked to the FLA14 locus and thus are not pf5-fla14-3 alleles.

<sup>b</sup> Assembly defects in fla14-3 include an effect on the retrograde IFT motor as evidenced by assembly of half to full-length flagella; no defects in assembly of the outer dynein arm are observed; II-dynein assembly defects are restricted to loss of IC97 and FAP120 specifically; and the radial spokes are reduced resulting in the paralyzed flagellar phenotype.

<sup>c</sup> The ida7-1::IC140 5A strain was first described in Perrone et al. (1998).

<sup>d</sup> Strain pf28 pf30 ssh1 is a triple mutant lacking both the outer dynein arm and I1 dynein; the ssh1 mutation allows for wild-type length flagella in the double dynein mutant background (Ledizet and Piperno, 1995; Freshour et al., 2006).

Computational Analyses

Sequence alignments were performed using LaserGene SeqMan (DNAStar, Madison, WI). The LaserGene EditSeq module was used for translations and to estimate the predicted mass of the IC97 protein. Primers were designed to amplify homologous sequences. The COILS program (www.ch.embnet.org/software/COILS_form.html) was used to predict coiled-coil regions in IC97 and its orthologues. ClustalW, version 1.82, was used for alignment and comparison of the murne Las1 protein (AACQ3498.1) and IC97 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Isolation of Axonemes, Dynein Purification, and Biochemical Analyses

Flagella were isolated as described previously (Wittman, 1986) and demembranated using NP-40 (Calbiochem, San Diego, CA). Axonemes were resuspended in HMDEKP (30 mM HEPES, pH 7.4, 5 mM MgSO<sub>4</sub>, 1 mM dithiothreitol [DTT], 0.5 mM EDTA, 25 mM KCl, and 1 mM phenylmethylsulfonyl fluoride) plus the protease inhibitors aprotinin and leupeptin (Sigma-Aldrich, St. Louis MO). Dyneins were extracted in HMDEKP plus 0.6 M KCl and fractionated by fast-performance liquid chromatography (FPLC) on a MonoQ column (GE Healthcare, Piscataway, NJ) as described previously (Kagami and Kamiya, 1995).

Chemical cross-linking was carried out using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) by treating microtubule-bound I1-dynein fractions or treating isolated axonemes with EDC for 30–60 min at room temperature, respectively (Benashski and King, 2000; Wirschell et al., 2008). All cross-linking reactions were performed in HMDEKP buffer (30 mM HEPES, pH 7.4, 5 mM MgO<sub>4</sub>, 0.5 mM EDTA, and 25 mM KCl) supplemented with protease inhibitors. Reactions were neutralized with a 20-fold molar excess of β-mercaptoethanol. Cross-linked axonemes were collected by centrifugation and resuspended to 2 mg/ml with sample buffer. SDS-PAGE was performed by standard techniques using Precision Plus dual-color protein standards to estimate relative mobility (M<sub>r</sub>) (Bio-Rad Laboratories, Hercules, CA).

IC138 hyperphosphorylation was determined by comparing untreated and phosphate-treated axonemes (calf intestinal phosphatase [CIP]) by SDS-PAGE. Briefly, axonemes were treated with buffer or CIP for 30 min at room temperature and then directly fixed for SDS-PAGE analysis.

IC138 phosphorylation status was determined by in vitro phosphorylation of isolated axonemes using radioactive [γ-<sup>32</sup>P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) (Yang and Sale, 2000). IC138 hyperphosphorylation was determined by incubating IC138 hyperphosphorylated axonemes with radioactive [γ-<sup>32</sup>P]ATP to a final concentration of 40 μM, and axonemes were incubated at 30°C for another 5 min. Samples were immediately fixed with sample buffer for analysis on 5% SDS-PAGE, transferred to nitrocellulose, and subsequently detected by autoradiography. The migration of IC138 was confirmed by Western blot of unlabeled axonemes run on the same gel.

MS/MS analyses of band-purified IC97 and the IC97 cross-linked product were performed at University of Massachusetts Proteomic Mass Spectrometry Laboratory by Dr. John Leszyk. MS/MS spectra were used to search the JGI Chlamydomonas Genome database, version 2.0.

Reconstitution of I1-Dynein Complexes onto Isolated Axonemes

FPLC-purified I1 dynein and IC97 fractions derived from the double mutant ida7-1::IC140 5A ssh1 were dialyzed and then combined with isolated pf28 pf30 ssh1 axonemes. The reactions were incubated for 15 min at room temperature in HMDEKP buffer. Axonemes and supernatant fractions were collected by centrifugation and reconstitution of I1-dynein components analyzed by Western blot.

Antibody Production and Western Blot Analyses

A restriction fragment containing nucleotides 1737-2492 of the IC97 cDNA (Supplemental Figure S1B) was digested from clone pMW173.1 with BamHI and EcoRI and subcloned into the pet28b expression vector (Novagen, San Diego, CA) to create plasmid pMW185.1 (Supplemental Figure S1B).
Cloning IC97 Sequences
To verify the exact sequence of the protein encoded by gene model C_850038, we designed primers based on predicted exons and the peptides identified by MS/MS. Sequences from a wild-type Chlamydomonas cDNA library were amplified by PCR and sequenced to confirm the complete open reading frame (ORF) plus 412-base pairs of 5' and 282-base pairs of 3' UTRs. The ORF predicts a 759-amino acid protein with a mass of 81.5 kDa (Supplemental Figure S1A). Using the antisense strand of the cDNA as a probe, we performed a Northern blot on RNA derived from cells either before or during flagellar regeneration. An ~2.8-kb transcript is detected that is up-regulated in response to deflagellation and flagellar regrowth (data not shown), a hallmark of mRNAs encoding flagellar proteins (Silflow et al., 1982).

IC97 Is a Non-WD Repeat IC That Has Orthologues in Ciliated Organisms
The predicted protein was analyzed by BLAST and found to have homology to several proteins, including a Ciona intestinalis axonemal protein (BA688834.1) and a murine protein, Las1 (Zhang et al., 2003; Manenti et al., 2004), that is part of the multigenic Pasi locus associated with an inherited predisposition to pulmonary carcinoma in mice. Alignment of the Chlamydomonas and murine proteins shows the homology spans the entire sequence (Figure 1). Other than a small region in the N terminus that is predicted to form a coiled-coil, the IC97 protein does not have any notable motifs including WD-repeat domains that are found in other dynein intermediate chains.

IC97 Antiserum Detects the IC97 Component of I1 Dynein
To determine whether the gene we identified by MS/MS does indeed encode the IC97 subunit of I1 dynein, we made a specific polyclonal immune sera to an epitope-tagged fusion protein containing amino acids 443-693 of the predicted IC97 protein (Supplemental Figure S1B). In Western blots of isolated axonemes, the antibody detects a band having a mass of ~90,000 on SDS-PAGE (Figure 2A), well in agreement with the predicted mass of 81.5 kDa (The M_r of 90,000 is estimated from our SDS-PAGE gels. The mass of IC97 has been previously estimated as 97 and 110 kDa; Porter et al., 1992; Habermacher and Sale, 1997. This discrepancy is most likely due to differences in the molecular weight standards used). This band is specifically missing in I1-dynein–deficient axonemes (ida1, ida3, and ida7), indicating that the antisera detects an I1-dynein component. To further verify this, we purified I1 dynein by FPLC fractionation. The antisera detects the same size band in purified I1-dynein (dynein-f peak) fractions (Figure 2B). These results demonstrate that our antibody specifically detects the IC97 component of I1 dynein.

IC97 Interacts with α- and β-Tubulin Components
The IC97 protein sequence does not predict any notable domains or motifs that would lend insight into the function of IC97. Thus, to better understand the role of IC97 in I1 dynein, we took advantage of EDC chemical cross-linking to identify IC97-binding proteins. EDC is a proven reagent for identifying axonemal interactions, particularly for the axonemal dyneins (King et al., 1991; Yang and Sale, 1998; Benashski and King, 2000; DiBella et al., 2004a; Hendrickson et al., 2004). Western blots of EDC-treated axonemes were probed with the IC97 antibody, which detected an EDC cross-linked product, with an M_r of ~140,000, suggesting

**RESULTS**

Identification of the IC97 Subunit of I1 Dynein
To identify the IC97 subunit, I1-dynein, purified by FPLC was resolved on SDS-PAGE, and proteins were stained with Coomassie blue (data not shown). The band corresponding to the IC97 subunit was excised, digested with trypsin, and peptides were separated by liquid chromatography. MS/MS analyses of the resulting peptides were used to search the Chlamydomonas genome database and identified a candidate gene model C_850038 (Supplemental Figure S1A). The gene model prediction is influenced by the presence of gaps in the genomic sequence. As a result, three peptides seem to reside in predicted introns and one peptide resides in the predicted 5' untranslated region (UTR). Gene model C_850038 (FAP94) was identified in the KCl fraction of the flagellar proteome (Pazour et al., 2005), a result consistent with a dynein protein.

Microtubule Binding Assays and Tubulin Blot Overlays
Purified tubulin (Cytoskeleton, Denver, CO) was polymerized at 37°C in the presence of 10 mM Taxol (Cytoskeleton), and microtubules were collected by centrifugation. The I1-dynein–bound microtubule pellets were cross-linked by SDS-PAGE and analyzed by Western blot and protein stain.

**Immunoprecipitation of IC97-interacting Proteins**
Immunoprecipitation was performed as described previously (King et al., 1991). Briefly, EDC-treated wild-type axonemes were solubilized with SDS-sample buffer and diluted 20-fold with Tris-buffered saline (TBS) buffer (10 mM Tris, pH 7.5, and 150 mM NaCl). The diluted axonemes were precleared with protein A beads (Sigma-Aldrich) for 2-3 h, and then incubated with protein A beads bound with the IC97 antiserum. The immunocomplexes were collected, washed with TBS buffer plus 1% NP-40 and 0.05% Tween 20 and fixed with SDS-sample buffer. The immunoprecipitated were separated by SDS-PAGE and analyzed by Western blot and protein stain.

**RESULTS**

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IC97 (Mr of ~90,000) is in direct contact with an axonemal protein with an estimated mass of 50 kDa (Figure 3A). To determine the identity of the IC97-interacting protein, we used the IC97 antiserum to immunoprecipitate the IC97 cross-linked product in sufficient yield for identification (Figure 3B). MS/MS analysis of the IC97 cross-linked product identified peptides corresponding to both α- and β-tubulin in addition to IC97. The identification of IC97 peptides demonstrates that the band isolated for MS/MS is the IC97 cross-linked product. The Mr of the cross-linked product is ~140,000 on SDS-PAGE, suggesting that IC97 does not cross-link to a tubulin dimer, but rather IC97 independently cross-links to α- and β-tubulin. Hence, the cross-linked product is a mixture of IC97-α-tubulin and IC97-β-tubulin species. In support of this model, the ratio of IC97:α-tubulin:β-tubulin peptides recovered is 1.5:1:1, indicating that both α- and β-tubulin are present in equivalent amounts compared with a nearly twofold amount of IC97.

To further validate the results obtained by MS/MS, we used several approaches. First, we immunoprecipitated the IC97 cross-linked product (Figure 3C, IC97) from SDS-solubilized EDC-treated axonemes containing an HA-tagged α1-tubulin (Kozminski et al., 1993). The IC97 cross-linked product is detected using an antibody to the HA tag, indicating that it contains the epitope-tagged tubulin (Figure 3C, HA). Second, we bound purified I1 dynein to Taxol-stabilized microtubules and then performed EDC cross-linking. The IC97 cross-linked product is formed indicating that IC97 interacts with tubulin components in vitro (Figure 3D). Third, we took advantage of a blot overlay assay that uses biotinylated tubulin as a probe to identify tubulin-interacting proteins (Yanagisawa and Kamiya, 2004). Blot overlays of purified dynein fractions detect a tubulin-interacting band at the expected size for IC97 in purified-I1 dynein (dynein-f peak) fractions (asterisk in Figure 3E).

IC97 Interacts with IC138 and IC140 within I1 Dynein

To further characterize how IC97 assembles within I1-dynein, we took advantage of unique I1-dynein mutants that assemble partial I1-dynein complexes. Figure 4A shows Western blots of isolated axonemes from three mutant Chlamydomonas IC97 is homologous to murine Las1. Alignment of the Chlamydomonas IC97 sequence and its murine orthologue, Las1, shows a high degree of conservation throughout the two proteins. Chlamydomonas IC97 is listed under accession FJ156241 and Las1 under accession AAQ934981. The alignment was performed using the ClustalW server at http://www.ebi.ac.uk/Tools/clustalw2/index.html. IC97 and Las1 are 26% identical and 41% similar overall (expect value e−19).
strains: bop5-1—expressing and assembling a truncated IC138 but missing the LC7b and FAP120 subunits of the IC138 subcomplex (Hendrickson et al., 2004; Ikeda et al., 2008), ida7-1::IC140 5A—expressing and assembling a truncated IC140 lacking the N terminus (see table 1 in Perrone et al., 1998), and bop5-2—an IC138-null allele that assembles I1 dynein lacking IC138, LC7b, and FAP120 (Ikeda et al., 2009; Bower et al., 2009). These observations are consistent with other reports, indicating that IC97 is a component of the IC138 subcomplex and dependent upon IC138 for assembly into I1-dynein (Ikeda et al., 2008; Bower et al., 2009). This interaction is not mediated by LC7b or FAP120 because IC97 assembles in bop5-1, where LC7b and FAP120 are missing (Hendrickson et al., 2004; Ikeda et al., 2008). Thus, we conclude that IC97 is interacting with IC138 directly. Furthermore, in the bop5-1 mutant, IC97 copurifies with I1-dynein by FPLC fractionation, indicating that it is stably associated with I1 dynein (Hendrickson et al., 2004).
unpublished data). In contrast, although IC97 assembles in ida7-1::IC140 5A, it does not cofractionate with I1 dynein (derived from the ida7-1::IC140 5A oda9 double mutant and represented by IC138 in Figure 4B), indicating that IC97 requires the N terminus of IC140 for stable association with the I1-dynein complex and suggesting that IC97 and IC140 also interact. Together, these results suggest that IC97 interacts with both IC138 and IC140 within I1-dynein, further defining interactions of I1-dynein subunits in the higher order dynein motor complex.

IC97 Is Not Required for I1-Dynein Assembly or for Anchoring in the Axoneme

The results from the bop5-2 mutant demonstrate that IC97 is not required for I1-dynein assembly or anchoring in the axoneme (Bower et al., 2009). To verify this, we tested whether I1-dynein complexes that lack IC97 (derived by salt extraction from strain ida7-1::IC140 5A oda9) can rebind I1-dynein–deficient axonemes in an in vitro reconstitution assay. Supplemental Figure S2 shows that I1-dynein complexes can rebind to I1-deficient axonemes in the absence of IC97, indicating that IC97 is not required for I1-dynein anchoring to the axoneme.

Furthermore, analysis of a newly identified LC8 mutation fla14-3 (identified as the defective gene in the motility mutant pf5), demonstrates that I1 dynein (identified by IC140) assembles without IC97 and FAP120 (Figure 4C). The fla14-3 mutant expresses a larger LC8 protein due to a read-through mutation in the LCS stop codon (Figure 4C; Yang, Yang, Wirschell, and Davis, unpublished data). LC8 is a component of several flagellar complexes, including the retrograde intraflagellar transport (IFT) motor, the outer dynein arm, I1 dynein, and the radial spokes. This particular fla14 allele affects assembly of the I1-dynein components listed above and the radial spokes but does not affect assembly of the outer dynein arm. There also may be an effect on the retrograde IFT motor as evidenced by assembly of half- to full-length flagella. A detailed characterization of the assembly defects in fla14-3 is described in Yang, Yang, Wirschell, and Davis (unpublished data).

IC97 Is Required for Control of Microtubule Sliding

Because IC97 is not required for assembly of I1 dynein, we predicted that IC97 may function with IC138 in the regulation of I1-dynein activity. Based on analysis of bop5-1 axonemes, FAP120 and LC7b do not seem to function in the regulatory pathway that controls I1 dynein (Hendrickson et al., 2004; Ikeda et al., 2008). Therefore, we reasoned that we could use the fla14-3 mutant to test the role of IC97 in regulation of I1-dynein activity using the microtubule sliding assay. We did not need to combine the fla14-3 mutation with a mutation such as pf17 that disrupts radial spoke assembly because fla14-3 itself is inherently defective in assembly of the radial spokes (Huang et al., 1981; Yang, Yang, Wirschell, and Davis, unpublished data). Defects in radial spoke assembly lead to disruption of the signaling pathway that regulates I1 dynein, resulting in hyperphosphorylated IC138 and inhibition of microtubule sliding. Given the radial spoke defect in fla14-3, we predict that IC138 is hyperphosphorylated in fla14-3 axonemes. As expected, IC138 is hyperphosphorylated, similar to the radial spoke mutant pf17, indicating that there is a defect in the regulatory pathway in fla14-3 axonemes, a result that is consistent with the radial spoke defect present in this mutant (Figure 5A; compare CIP-treated to untreated axonemes; also see Hendrickson et al., 2004).

To test whether IC97 plays a regulatory role, we used the in vitro microtubule sliding assay to compare I1-dynein function in axonemes that contain IC97 (wild-type and pf17) with fla14-3 axonemes that lack IC97. Consistent with previous reports, relative to wild-type axonemes, microtubule sliding is greatly reduced in pf17 axonemes (Figure 5B). Like other radial spoke mutants, microtubule sliding velocities in fla14-3 axonemes are greatly reduced (Figure 5B). However, unlike other radial spoke mutants, the slow microtubule sliding in fla14-3 axonemes is not rescued by kinase inhibitors (Figure 5B). These results are consistent with the hypothesis that IC97 plays a critical role, along with IC138, in the regulation of I1-dynein activity.

The central pair/radial spoke signaling mechanism has been shown to impinge upon the IC138 subunit of I1 dynein and rescue of microtubule sliding velocities correlates with dephosphorylation of IC138 (Habermacher and Sale, 1997). To further understand the failure to rescue microtubule sliding velocities with kinase inhibitors, we used an in vitro phosphorylation assay to test whether there is a defect in dephosphorylation of IC138 in fla14-3 axonemes. Isolated
Figure 5. IC97 is required for regulation of microtubule sliding. (A) Western blots of isolated axonemes from pf17 and fla14-3 were probed with an antibody to the IC138 I1-dynein subunit. Axonemes were untreated or treated with CIP. IC138 is extensively phosphorylated (hyperphosphorylated) in radial spoke mutants such as pf17 and in the fla14-3 mutant (compare the untreated vs. treated bands; Hendrickson et al., 2004). (B) Microtubule sliding velocities were measured in wild-type, pf17, and fla14-3 axonemes (bars indicate mean ± SD). Sliding rates were reduced in fla14-3 axonemes relative to wild type—a result consistent with other radial spoke mutant axonemes (pf17). In contrast to pf17 axonemes, the sliding rates of fla14-3 axonemes do not increase upon addition of the kinase inhibitors protein kinase inhibitor (PKI) or DRB indicating that IC97 is required for PKI/DRB-mediated rescue of microtubule sliding. (C) In vitro phosphorylation of pf17 or fla14-3 axonemes by using [γ-32P]ATP was performed in the presence or absence of CKI-specific inhibitors. In pf17 and fla14-3 axonemes, when axonemes are pretreated with kinase inhibitors (CKI-7 or DRB), radioactive phosphate incorporation into IC138 is reduced, indicating that IC138 is largely dephosphorylated.

axonemes from pf17 and fla14-3 were incubated with radioactive [γ-32P]ATP after treatment with the protein kinase inhibitors CKI-7 or 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole (DRB) (shown to block IC138 phosphorylation as well as rescue microtubule sliding in isolated axonemes; Yang and Sale, 2000). Consistent with previous reports (Yang and Sale, 2000), incorporation of radioactive phosphate is reduced when pf17 axonemes are treated with the kinase inhibitors (Figure 5B, pf17, bottom). In fla14-3 axonemes, kinase inhibitor treatment also reduces radioactive phosphate incorporation (Figure 5B, fla14-3, bottom). Although this assay does not detect dephosphorylation at specific residues (see Discussion), the reduction of phosphate incorporation indicates that IC138 is dephosphorylated and that the kinases and phosphatases that regulate IC138 are intact. Thus, although IC138 phosphorylation in axonemes from fla14-3 is inhibited with CKI-7 or DRB, microtubule sliding was not restored to wild-type velocity, further indicating an essential role for IC97 in regulation of I1 dynein.

DISCUSSION

Here, we describe the IC97 subunit of the conserved I1 dynein motor. IC97 interacts directly with tubulin, and along with IC138, plays a role in regulation of I1 dynein and control of microtubule sliding.

IC97 Interacts with Tubulin Subunits in the Axoneme

Based on multiple lines of evidence, we determined that IC97 interacts with both α- and β-tubulin subunits (Figures 3 and 6A and Supplementary Figure S2). Our data suggest that IC97 is in direct contact with the A-tubule of the outer doublet, near the I1-dynein docking site. This is consistent with a close association of the I1-dynein IC/LC domain with the wall of the A-tubule (Nicastro et al., 2006; Bui et al., 2008).

Given that IC97 is not required for I1-dynein assembly, an IC97–tubulin interaction cannot explain the precise localization of I1 dynein within the 96-nm repeat. The specific localization of I1 dynein in the axoneme may still require additional factors (King and Kamiya, 2009).

The precise function of the IC97–tubulin interaction is unclear. One possibility is that it is part of a mechanism that detects curvature of the axoneme for control of dynein activity and axonemal bending. Experimental and theoretical evidence indicate that mechanical feedback from bending of the axoneme is involved in regulation of dynein activity (Hayashibe et al., 1997; Brokaw, 2002; Lindemann, 2004; Murata and Shingyoji, 2004; Brokaw, 2008; Hayashi and Shingyoji, 2008). It is possible that these mechanically based control mechanisms involve multiple interactions of the dynein motors with the axoneme that include direct interactions with tubulin. Of particular interest is whether these tubulin interactions with dynein intermediate chains are altered during axonemal bending.

IC97 Interactions with I1-Dynein Subunits

In Figure 6A, we propose a model for how IC97 interacts with the axoneme and I1-dynein components. Shown in red are interactions between IC97-tubulin, IC97-IC140, and the IC97-IC138 subcomplex, including FAP120.

Based on the analysis of the bop5-2 mutant (Bower et al., 2009), we conclude that IC97 assembles into I1 dynein in an IC138-dependent manner, suggesting that these two I1-dynein subunits interact. This interaction is not mediated by the N terminus of IC138, IC7b, or FAP120 because IC97 is present in bop5-1, where these proteins are missing (Figure 4C) (Hendrickson et al., 2004; Ikeda et al., 2009; Bower et al., 2009). This result suggests that IC97 and IC138 interact directly.

The IC97-IC140 interaction is supported by analysis of the ida7-1::IC140 5A strain (Perrone et al., 1998). Although an intact I1 dynein assemblies in this mutant, IC97 is specifically lost upon extraction of I1 dynein from the axoneme (Figure 4B), indicating that IC97 requires the N terminus of IC140 for a stable association with I1 dynein in vitro. No other I1-dynein subunit seems to dissociate in the ida7-1::IC140 5A mutant, suggesting that the IC97-IC140 interaction is also direct.

The model also depicts a possible interaction between IC97 and LC8. Analysis of the fla14-3 mutant indicates that assembly of IC97 and FAP120 are specifically disrupted. However, we predict that the loss of IC97/FAP120 from I1 dynein in fla14-3 is indirect (see below).

IC97 Is Required for I1-Dynein–mediated Control of Microtubule Sliding In Vitro

I1 dynein is an essential part of a regulatory pathway that controls microtubule sliding and regulates axonemal bend-
ing. Figure 6B is founded in part on analysis of microtubule sliding in axonemes isolated from paralyzed flagellar mutants defective in assembly of the radial spokes or central pair structures (reviewed in Porter and Sale 2000; Smith and Yang 2004; Wirschell et al., 2007).

Here, we provide evidence that assembly of IC97 is necessary for regulation of I1 dynein and control of microtubule sliding. fla14-3 axonemes assemble an intact I1-dynein that appears to lack only the IC97 and FAP120 subunits (Figure 4C). This mutant allele of LC8 manifests assembly defects in I1 as well as the radial spokes (Huang et al., 1981; Yang, Yang, Wirschell, and Davis, unpublished data). The defect in radial spoke assembly intrinsic to this mutant allele allowed us to use the microtubule sliding assay to directly test the effects of the loss of IC97 on the regulatory pathway that controls I1 dynein. Similar to radial spoke mutants like pf17; IC138 is hyperphosphorylated in fla14-3 (Figure 5A). Furthermore, despite inhibition of IC138 phosphorylation, fla14-3 axonemes do not increase microtubule sliding rates upon treatment with kinase inhibitors, indicating that IC97 is required, along with IC138, for control of I1 dynein. Evidently, dephosphorylation of IC138 is not sufficient for control of microtubule sliding; the assembly of IC97 also is required (Figure 6B).

We cannot rule out the possibility that the microtubule sliding defect in fla14-3 is a result of the mutation in LC8 and a novel defect in radial spoke assembly that allows fla14-3 to behave differently than other radial spoke mutants. However, we propose that the failure to rescue microtubule sliding velocities in fla14-3 is due to the specific loss of IC97 for the following reasons. 1) Recent models propose that the LC8 dimer functions in a structural capacity; establishing and driving the formation of large macromolecular complexes like the dynein motors (Williams et al., 2007; Barbar, 2008; King, 2008). For example, the IC of cytoplasmic dynein is partially disordered and gains structure upon binding to LC8 (Nyarko et al., 2004). Thus, loss of IC97/FAP120 from fla14-3 I1-dynein may be the result of a defective I1-dynein structure that is not competent to recruit these subunits. Such a model would indicate that IC97/FAP120 and LC8 do not interact directly. 2) More importantly, IC97 requires IC138 for assembly into I1 dynein and together they are localized within a subcomplex at the base of I1-dynein (Bower et al., 2009). IC97 can assemble in the absence of LC7b and FAP120...
IC97 Is Related to Proteins Implicated in Pulmonary Carcinoma

IC97 has orthologues in many species that contain motile cilia and flagella. Of interest is the observed homology between IC97 and Las1—a protein implicated as a lung tumor susceptibility gene (Zhang et al., 2003). Las1 is highly expressed in lung and testis— tissues that contain motile cilia and in the kidney—a tissue containing immotile, primary cilia (Zhang et al., 2003). Moreover, in dividing cells, Las1 is expressed in G2 and its degradation during mitosis is required for cell cycle progression (Liu et al., 2007). The finding that Las1 is a microtubule-binding protein (Liu et al., 2007) is consistent with the IC97–tubulin interaction described here, suggesting that an interaction with tubulin may be a conserved feature of IC97/Las1 function. The significance of the homology between *Chlamydomonas* IC97 and the Las1 protein involved in lung tumorigenesis is unclear, but raises the questions of whether Las1 localizes to motile cilia, as well as primary cilia, and whether Las1 plays functional roles both in cilia and outside of the axone.}

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