Light Chain 1 from the *Chlamydomonas* Outer Dynein Arm Is a Leucine-Rich Repeat Protein Associated with the Motor Domain of the γ Heavy Chain†

Sharon E. Benashski,‡ Ramila S. Patel-King,‡ and Stephen M. King*

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032-3305

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ABSTRACT: The LC1 light chain from *Chlamydomonas* outer arm dynein is tightly bound to the γ heavy chain. Molecular cloning revealed that LC1 is a member of the SDS22+ subclass of the leucine-rich repeat protein family and as such is likely involved in mediating interactions between dynein and the components of a signal transduction pathway. Through the combination of covalent cross-linking and vanadate-mediated photolysis, LC1 was found to associate with that portion of the γ HC that is C-terminal to the P1 loop. This region comprises most of the globular head domain of the heavy chain and includes the stalk-like structure that is involved in microtubule binding. Attachment of LC1 to this region represents the only known example of an accessory polypeptide directly associated with a dynein motor domain. Additional cross-linking experiments revealed that LC1 also interacts directly in situ with an ~45 kDa axonemal component; this interaction is disrupted by the standard high salt treatment used to remove the outer arm from the axoneme. These data suggest that LC1 acts to mediate the association between this 45 kDa axonemal polypeptide and the motor unit of the γ HC.

Dyneins are highly complex microtubule-based molecular motors that function to provide the motive force for ciliary and flagellar beating and which are involved in multiple motile events in the cytoplasm (e.g., vesicular transport, maintenance of the Golgi apparatus, mitotic spindle formation, etc.). In generic terms, these enzymes are constructed around one or more heavy chains (HCs,[1] >500 kDa), each of which forms a multilobed globular head structure (*1*) with an associated stem. It is these components that are responsible for ATP hydrolysis and for microtubule motor activity (see refs *2* and *3* for review). In dyneins with two or more HCs, an additional complex is located at the base of the stems. This structure contains several intermediate chains (ICs, 70−80 kDa) that are members of the WD-repeat protein family (*4*−*9*). These proteins are apparently involved in attachment of the motor enzyme to the appropriate cargo (*10*−*13*). Also associated with each dynein particle are a number of light chains (LCs, less than ~22 kDa) (*14*−*22*). These represent a very diverse group of molecules belonging to several distinct protein families (see below). Some dyneins contain additional polypeptides that do not readily fall into the classes described above. For example, cytoplasmic dynein has four light intermediate chains that are distantly related to ABC transporters (*23*, *24*).

One of the best studied dyneins is the outer arm from flagella of *Chlamydomonas*.[2] This ~2 MDa enzyme contains three HCs (α, β, and γ), two ICs, a trimeric docking complex, and eight LCs (*21*, *22*, *25*). The light chains can be divided into two classes based on their intradynein location (see Table 1). One class of light chains associates with the ICs at the base of the dynein particle, whereas the second set of light chains interact directly with the HCs. The former group includes the following.

(i) The highly conserved dimeric LC8 protein (~90% sequence identity between *Chlamydomonas* and humans) that is also associated with inner arm I1, cytoplasmic dynein, myosin V, neuronal nitric oxide synthase, and IκBα (*12*, *16*, *17*, *26*−*29*). In *Chlamydomonas*, LC8 is encoded at *fla14* and null mutants exhibit severe defects in flagellar assembly (*30*). In *Drosophila*, the lack of this protein leads to embryonic lethality (*31*).

(ii) LC6 is a closely related homologue of LC8 that likely is also dimeric (*26*, *32*).

(iii) LC2 is the *Chlamydomonas* homologue of the putative murine *t*-complex distorter Tctex2 (*33*); the related protein Tctex1 associates with the ICs of cytoplasmic dynein (*18*, *34*).

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* To whom correspondence should be addressed. Telephone: (860) 679-3347. Fax: (860) 679-3408. E-mail: king@panda.uchc.edu.

‡ These authors contributed equally to this work.

[1] Abbreviations: CD, circular dichroism; DMP, dimethyl pimelimidate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HC, heavy chain; IC, intermediate chain; LC, light chain; LRR, leucine-rich repeat; MBP, maltose binding protein; NMR, nuclear magnetic resonance; nNOS, neuronal nitric oxide synthase; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride.

[2] Previously, the non-HC components of the *Chlamydomonas* outer arm have been referred to by a variety of names based, in general, on molecular weight estimates. The designations used in this report recently have been adopted by several laboratories in an effort to promote an internally consistent nomenclature. Consequently, the intermediate chains are referred to here as IC1 (previously IC78 or IC80) and IC2 (previously IC69 or IC70). The light chains are now termed LC1−LC8 (see Table 1). The three outer arm heavy chains remain the α, β, and γ HCs. Components of the outer arm docking complex have been assigned a separate designation of DC.

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Table 1: Properties of *Chlamydomonas* Outer Arm Dynein Light Chains

designation	M_r	accession number	calcd mass (Da)	calcd p*I*	stoichiometry	intradynein association	properties	ref
LC1	22000	AF112476	22150	5.54	2	HC γ	leucine-rich repeat protein of the SDS22+ subclass	this study
LC2	20000	U89649[a]	15882	6.01	1	IC1/2	homologue of the putative *t* complex distorter Tctex2	*33*
LC3	19000	U43610[a]	17364	7.76	1	HC β	redox-active thioredoxin	*36*
LC4	18000	U34345	17787	4.22	1	HC γ	Ca^{2+} binding homologue of calmodulin	*38*
LC5	16000	U43609[a]	14179	8.34	1	HC α	redox-active thioredoxin	*36*
LC6	14000	U19484[a]	13856	6.80	2	IC1/2	homologue of LC8	*32*
LC7	11000	AF140239	11928	7.85	1	IC1/2	homologues present in cytoplasmic dynein	*35*
LC8	8000	U19490	10,321	7.51	4	IC1/2	dimeric, encoded at *fla14*, also a component of cytoplasmic dynein, inner arm I1, myosin V, nNOS, and IκBα	*17*, *27*−*30*, *32*

[a] The M_r assignments for the light chains encoded by sequences with these accession numbers have been revised.

(iv) LC7 has homologues in cytoplasmic dynein which affect axonal transport and mitosis (*35*).

Within the *Chlamydomonas* outer arm, each HC also is tightly associated with at least one light chain (reviewed in ref *3*). Both the α and β HCs interact with single light chains that are members of the thioredoxin superfamily; these proteins contain perfect copies of the redox active site and appear to be functional sulfhydryl oxidoreductases (*36*). Analysis of a *Chlamydomonas* mutant (*oda4-s7*) which expresses a truncated form of the β HC revealed that LC3 interacts with the N-terminal ~160 kDa of that HC (*37*). This region corresponds to the stem of the HC structure. Similarly, LC5 has recently been found to associate with that region of the α HC that is N-terminal to the ATP hydolytic site (H. Tedford and S. M. King, unpublished). These data suggest that both thioredoxin light chains likely bind to similar locations on these two closely related HCs. The third HC within the outer arm (γ) has two classes of light chains (LC1 and LC4) bound (*21*). Previously, we identified the LC4 protein as a novel member of the calmodulin superfamily (*38*). LC4 bound Ca^{2+} with an affinity of $\sim 10^{-5}$ M, suggesting a possible role in the photoshock response which is abnormal in the absence of the outer arms (*39*, *40*). During photoshock, the transition from a ciliary to a flagellar waveform and the consequent alteration in swimming direction are signaled by an increase in intraflagellar Ca^{2+} levels from $\sim 10^{-6}$ to 10^{-4} M (*41*). Thus, the γ HC may be a principle target for regulation in the control of outer arm activity.

Intriguingly, unlike the α and β chains, the γ HC contains two copies of a second tightly associated light chain of ~22 kDa (LC1). To determine the role LC1 plays in dynein activity, we have cloned this polypeptide and describe here its molecular structure and organization. LC1 is a novel member of the leucine-rich repeat protein family which represents a structural motif used to mediate protein−protein interactions; many proteins of this class are involved in binding to components of signal transduction pathways (*42*). Moreover, we find that LC1 is the only protein yet known to associate directly with the globular head domain (rather than with the stem) of a dynein HC. Further analysis of protein−protein interactions within the flagellum involving LC1 reveals that this polypeptide binds directly to an ~45 kDa axonemal component which is thereby targeted to the motor domain of the γ HC.

EXPERIMENTAL PROCEDURES

Axoneme Isolation, Dynein Purification, and Peptide Sequencing. Flagellar axonemes were prepared from *Chlamydomonas reinhardtii* strain 1132D (−), and the outer arm was extracted with 0.6 M NaCl as described in refs *43* and *44*. Outer arm dynein was subsequently purified by sucrose gradient centrifugation, and the peak fractions were concentrated using a Centricon 30 ultrafiltration unit (Amicon Corp., Danvers, MA) that had previously been treated with 5% Tween 20 to reduce the level of nonspecific protein binding. Axonemes lacking the outer arm were prepared from a strain bearing the *oda9* mutation (cc2245).

Dynein samples were separated in 5−15% polyacrylamide gels and blotted to PVDF membrane (Immobilon P^{sq}, Millipore, Woburn, MA). Following in situ digestion with trypsin, individual peptides were purified by reverse phase chromatography and sequenced at the Protein Chemistry-Facility, Worcester Foundation Campus, University of Massachusetts Medical School, Shrewsbury, MA.

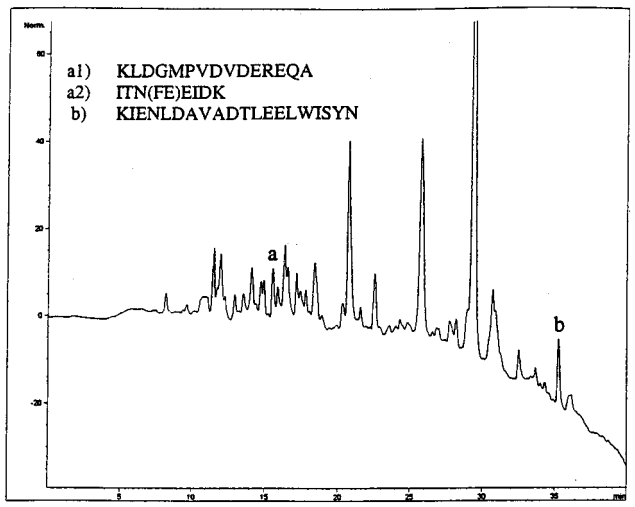

FIGURE 1: Analysis of tryptic peptides from LC1. Reverse phase chromatography of peptides derived from the tryptic digestion of electrophoretically purified LC1. Two peak fractions were subject to sequence analysis. The peak marked a yielded two peptide sequences that were distinguished on the basis of yield. Assignment of the initial residue (i.e., K or I) to peptides a1 and a2 was ambiguous, and two adjacent residues (FE) in the very low-level sequence a2 were misidentified (the sequence WG is encoded by the clone; see Figure 2). This analysis provided 40 unambiguous residue assignments.

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   1                    M  A  K  A  T  T  I  K  D  A  I  R  I  F     14
 181  AGCGAGCCGACCCCACAATGGCCAAGGCAACTACAATTAAGGATGCCATAAGGATTTTTG  240

  15  E  E  R  K  S  V  V  A  T  E  A  E  K  V  E  L  H  G  M  I     34
 241  AGGAGAGAAAGAGCGTTGTCGCAACAGAGGCGGAGAAGGTGGAGCTCCATGGCATGATTC  300

  35  P  P  I  E  K  M  D  A  T  L  S  T  L  K  A  C  K  H  L  A     54
 301  CGCCCATTGAGAAGATGGATGCTACCTTGTCGACCCTGAAAGCGTGCAAGCACCTGGCAC  360

  55  L  S  T  N  N  I  E  K  I  S  S  L  S  G  M  E  N  L  R  I     74
 361  TATCAACCAACAACATTGAGAAGATCAGCAGCTTGTCTGGCATGGAGAACCTCCGCATCC  420

  75  L  S  L  G  R  N  L  I  K  K  I  E  N  L  D  A  V  A  D  T     94
 421  TGTCGCTGGGGCGAAACCTCATCAAAAAGATTGAAAATTTGGACGCCGTGGCGGACACGC  480

  95  L  E  E  L  W  I  S  Y  N  Q  I  A  S  L  S  G  I  E  K  L    114
 481  TGGAGGAGCTGTGGATCTCCTACAACCAGATTGCCAGCTTGTCGGGCATCGAGAAGCTGG  540

 115  V  N  L  R  V  L  Y  M  S  N  N  K  I  T  N  W  G  E  I  D    134
 541  TGAACCTGCGGGTGCTGTACATGTCCAACAACAAGATCACCAACTGGGGTGAGATCGACA  600

 135  K  L  A  A  L  D  K  L  E  D  L  L  L  A  G  N  P  L  Y  N    154
 601  AGCTGGCGGCGCTGGATAAGCTGGAGGACCTGCTCCTGGCGGGCAACCCGCTGTACAACG  660

 155  D  Y  K  E  N  N  A  T  S  E  Y  R  I  E  V  V  K  R  L  P    174
 661  ACTACAAGGAGAACAACGCCACCAGCGAGTACCGGATAGAGGTGGTGAAGCGCCTTCCCA  720

 175  N  L  K  K  L  D  G  M  P  V  D  V  D  E  R  E  Q  A  N  V    194
 721  ACCTCAAAAAGCTGGACGGCATGCCCGTGGACGTGGACGAGCGGGAGCAGGCCAACGTGG  780

 195  A  R  G  G  *                                                 198
 781  CCCGCGGAGGATAGCACCGCTAGCTTTGGGCATTATGGGGGGAGACGTTCAAGGGTCGCG  840
 841  TAGGGTTTTGTAAGTCATCGAAGTGCAGAGGAATGCGCATGTTTTGCTTGCATGGCTGCG  900
 901  TCTCCTGGGAGTGTGGTGCGAGCACACACGGTGCAACGCAGGGCTGCACCAATGAGTGGA  960
 961  CGTCTCAAAGGTTGAGGTTCGGTTGCCATAGGTGGATTCCCAGGAAGGGCGATGAAGAGG 1020
1021  TTGCAGCTCCAGAGGGATTGGTTGCTCACCAGTGACCGATGCAAGGCGGGGCGCAATAGA 1080
1081  CAGTGGATGTAGGTGACCATTGGGAGACGGATCAGAGTGGCCGCGGTGGATGCAGACGGG 1140
1141  TAGAGGGGTGCACTTGCAGTCGGAGTTGTGCGTGTCACACGGACCACGCCACCTATAGGA 1200
1201  GCATGTCAAGTACAACAAGCACCAGCGGCGGCACAGTCACGGCTGTTGTAGACACGCACG 1260
1261  CTGGACGGCCTCCCAAGGGCAATTTGGAAGGCTGTTGGGGCCTTGGGGGCATTATTTCAG 1320
1321  GGTTTCTTGACCTGGGTGCGGGGCACCCTTGCTTGGTCGGGGTGTCCCGCCTTTGTAATG 1380
1381  TCGCAATCATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1440
1441  AAAAAAAAAAAAA                                                 1454
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FIGURE 2: Nucleotide and amino acid sequence of LC1. The nucleotide and corresponding amino acid sequences of LC1 are shown. Bold residues were identified directly by peptide sequencing. A perfect copy of the putative *Chlamydomonas* polyadenylation signal is underlined. This sequence is available under GenBank accession number AF112476.

Molecular Cloning and Analysis. A DNA segment encoding a portion of LC1 was initially obtained from first-strand cDNA derived from mRNA enriched for flagellar protein sequences using the PCR. The forward 4-fold degenerate primer 5′-GCGCGAATTCCTGGACGGYATGCCSGT-3′ was based on the peptide sequence LDGMPV using the *Chlamydomonas* codon bias and incorporated an *Eco*RI site and GC clamp at the 5′ end. The reverse primer was the standard oligo (dT) adaptor primer 5′-GCGCGTCGACTCG-AGT_{20}V-3′. The PCR product was used to obtain a full-length clone from a λZapII cDNA library (*9*). Multiple clones were obtained, and phagemids were rescued using helper phage. The longest clone was sequenced on both strands from double-stranded templates using Sequenase version 2.0 and a 7-deaza dGTP sequencing kit. Northern and Southern blots were prepared and probed as described previously (*32*).

Fusion Protein Preparation and Antibody Production. Both the full-length LC1 protein and the C-terminal domain (residues 104−198) were obtained from the λ clone using PCR and subcloned across the *Xmn*I−*Xba*I sites in the pMAL-c2 vector (New England Biolabs, Beverly, MA). Following induction, this resulted in expressed proteins fused to the C-terminus of maltose binding protein via a hydrophilic linker that terminated in a factor Xa cleavage site. Both proteins were purified by amylose affinity chromatography. The MBP−LC1(104−198) fusion protein was used for polyclonal antibody production in rabbit R5932. Antiserum was blot purified, by the method described in ref *45* using the minor adaptations described in ref *17*, versus the full-length light chain obtained from the MBP−LC1(1−198) fusion protein by factor Xa digestion.

To prepare the recombinant LC1 protein for physical studies, the full-length LC1 protein was fused to an N-terminal His_{10} tag in the pET16b vector (Novagen, Madison, WI). The recombinant protein was purified by Ni^{2+} affinity chromatography and the tag removed by factor Xa digestion. This procedure resulted in a full-length LC1 protein containing a single additional His residue at the N-terminus.

Circular Dichroism Spectroscopy. The full-length recombinant LC1 protein was dialyzed extensively against 2 mM Tris-HCl (pH 8.0) and 12.5 mM NaCl to remove the cleaved His tag. Following dialysis, the CD spectrum of the protein was measured in the far-ultraviolet range between 190 and 280 nm using a Jasco J-715 spectropolarimeter.

Computational Methods. Searches of the Genbank and Expressed Sequence Tag databases were performed using both Gapped- and Ψ-BLAST as well as the original BLAST program. Sequence alignments were constructed using CLUSTALW. Secondary structure was predicted using PHD and the amphiphilic helix analyzed using HELICALWHEEL (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI). The molecular model of the repeat structure described in ref *46* was displayed using RASMOL.

Covalent Cross-Linking and Vanadate-Mediated Photolysis. Cross-linking of isolated axoneme and purified dynein samples with the amine-selective reagent dimethyl pimelimidate (9.2 Å linker length) and the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was carried out as described in refs *11* and *26*. Stock solutions of DMP and EDC were prepared in methanol and 30 mM Hepes (pH 7.5), 5 mM $MgSO_4$, 1 mM EDTA, and 25 mM KCl (HMEK buffer), respectively. For cross-linking with DMP, axoneme samples were exchanged into 100 mM triethanolamine, the cross-linker was added, and the reaction was allowed to proceed for 60 min at room temperature. The reaction was terminated by the addition of 250 mM Tris-HCl (pH 6.8), and the samples subsequently were prepared for gel electrophoresis. Zero-length cross-linking with EDC was performed on axoneme samples exchanged into HMEK buffer. The reaction was terminated by addition of gel sample buffer and heating at >95 °C.

To cleave the heavy chains by vanadate-mediated photolysis at the V1 site, 10 μM vanadate and 100 μM ATP were added to the purified dynein fractions. These samples were then placed on ice and irradiated with 365 nm light for 60 min before preparation for electrophoresis.

Electrophoresis and Immunoblotting. Following cross-linking and photolysis, axoneme and dynein samples were separated in either SDS-containing 5 to 15% acrylamide gradient gels or a 4% acrylamide/4 M urea system that contained no SDS in either the stacking or separating gels. Subsequently, samples were either stained with Coomassie blue or blotted to nitrocellulose in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.01% SDS, and 20% methanol. Immunoblotting procedures were as described previously (*33*) using the blot-purified R5932 antibody and the mouse monoclonal antibody 12γB (*47*) to detect LC1 and the γ HC, respectively.

RESULTS

Molecular Characterization of LC1. There are eight light chains within the *Chlamydomonas* outer arm that may be distinguished by one-dimensional electrophoresis (*21*). Following the standard high-salt extraction of the outer arm from the axoneme, LC1 (M_r = 22 000) copurifies with the γ HC and LC4 (a Ca^{2+}-binding homologue of calmodulin; *38*) in sucrose density gradients and following ion exchange and hydroxylapatite chromatography (*21*). Following transfer to PVDF membrane, samples of LC1 were digested with trypsin in situ and peptides eluting from the membrane were purified by reverse phase chromatography on a C_8 column (Figure 1). Two purified peptide samples were sequenced and provided a total of 40 unambiguous residue assignments. One sample (labeled a in Figure 1) contained two sequences that were distinguished on the basis of phenylthiohydantoin-amino acid yields.

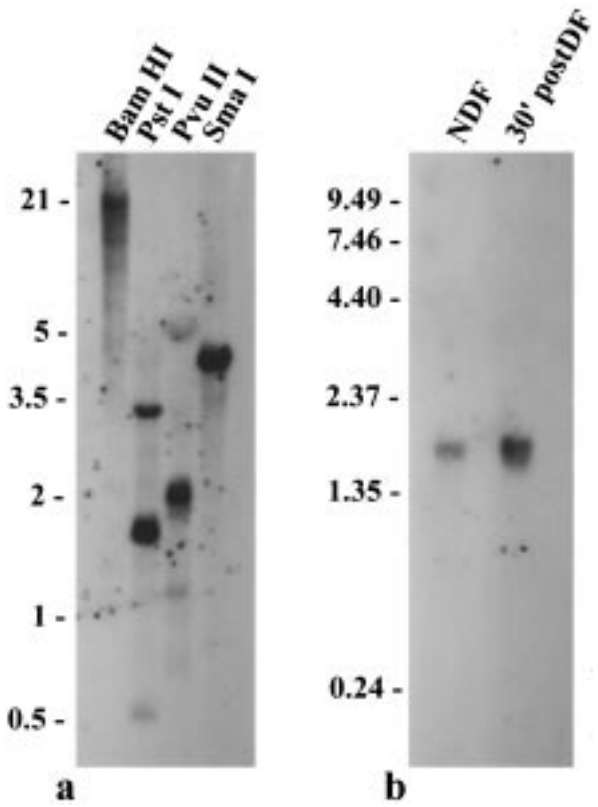

FIGURE 3: Southern and Northern blot analysis. (a) Southern blot of genomic DNA from *Chlamydomonas reinhardtii* strain S1D2 following digestion with *Bam*HI, *Pst*I, *Pvu*II, and *Sma*I. Single bands were found in *Bam*HI- and *Sma*I-digested samples. (b) Northern blot of total RNA obtained from nondeflagellated cells (NDF) and from cells that had been deflagellated and allowed to regenerate flagella for 30 min (30′ post DF). A single message that was upregulated following deflagellation was evident.

The peptide sequence LDGMPV was used for oligonucleotide design as it resulted in a primer with a low degeneracy once the *Chlamydomonas* codon bias had been incorporated. Using this gene-specific primer and an oligo(dT) adaptor primer, a PCR product of ~700 bp was obtained. Sequencing of the 5′ end of this product revealed that the predicted peptide sequence DVDEREQA followed directly from the sequence encoded by the primer. Thus, this product was considered likely to encode part of the LC1 protein and was therefore used to obtain a full-length clone from a *Chlamydomonas* λZapII cDNA library that is enriched for flagellar sequences (*9*).

The longest cDNA clone obtained was 1454 bp in length and contained a single open reading frame encoding a 198-residue protein with a mass of 22 150 Da and a calculated

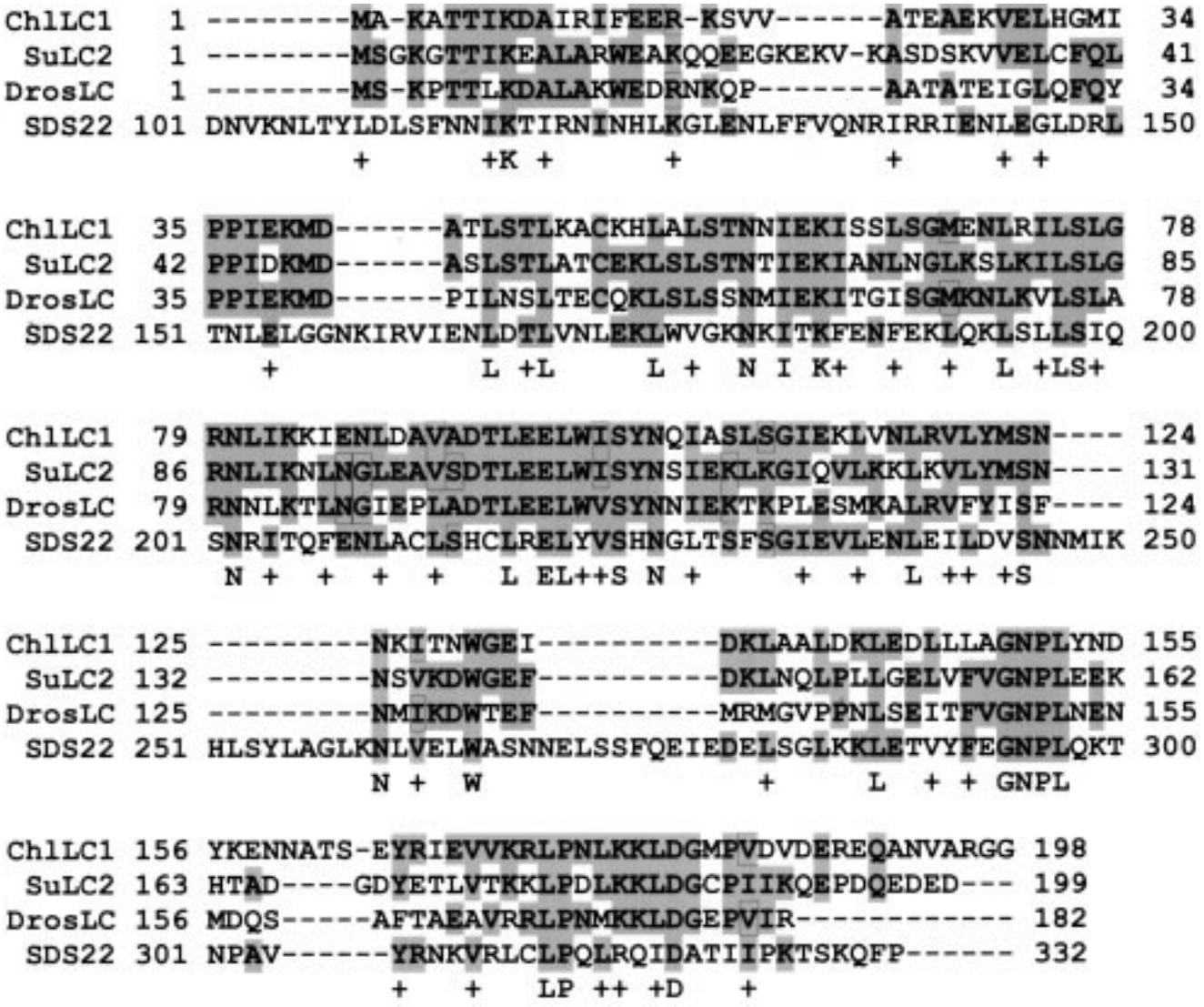

FIGURE 4: LC1 is related to the *S. pombe* SDS22(+) protein. Sequence comparison between *Chlamydomonas* LC1, sea urchin outer arm LC2 (GenBank accession number AB010054; K. Ogawa, published only in the database), *Drosophila* open reading frame from P1 clone DS07486 (GenBank accession number AC003925), and the SDS22+ protein from *S. pombe* (GenBank accession number A38439; *60*). The alignment was generated using CLUSTALW. Residues conserved in two or more sequences are shaded. A consensus sequence for invariant residues (and for conservative substitutions indicated by +) is shown below the alignment. The sequence of *Chlamydomonas* LC1 is 50, 46, and 33% identical with those of sea urchin LC2 and the *Drosophila* and fission yeast proteins, respectively.

p*I* of 5.54 (Figure 2). The three peptide sequences described in detail in Figure 1 were found within the encoded protein (42 of 44 residues correct; a Trp and a Gly were misidentified in the very low-level peptide sequence marked a2 in Figure 1), and the predicted basic residue was found to directly precede each tryptic fragment. There are four in-frame stop codons upstream of the initiator Met codon and a 597 bp 3′ untranslated region following the stop codon which includes a copy of the putative *Chlamydomonas* polyadenylation signal.

Southern blot analysis revealed a single band in both *Bam*HI- and *Sma*I-digested DNA, suggesting that there is only one *Chlamydomonas* gene for this light chain (Figure 3a). A single LC1 message of 1.51 kb was observed on Northern blots of total RNA prepared from cells that were actively regenerating flagella (Figure 3b). Message levels were significantly lower in cells that had not been deflagellated as observed previously for other integral axonemal components [e.g., the other γ HC-associated protein LC4 (*38*)]. RNA loadings were determined spectrophotometrically and verified by ethidium bromide staining. When the samples were probed for calmodulin, levels of induction similar to those reported previously were observed (*48*).

Analysis of the GenBank databases revealed that LC1 is related to several previously cloned proteins. It is most similar to the LC2 component of sea urchin outer arm dynein (AB010054; K. Ogawa, published only in the database) and is also closely related to a *Drosophila* homologue of the *Schizosaacharomyces pombe* SDS22+ protein (Figure 4). LC1 is 50% identical (59% similarity) with sea urchin LC2, 46% identical (58% similarity) with the *Drosophila* protein, and 33% identical (44% similarity) with *S. pombe* SDS22+. The probability of these matches occurring by chance [$P_{(n)}$] are 3.3×10^{-47}, 1.1×10^{-45}, and 2.7×10^{-10}, respectively. There is also a human version of SDS22+ in the Expressed Sequence Tag database (Z50749) which exhibits a similar degree of relatedness to *Chlamydomonas* LC1 as does the

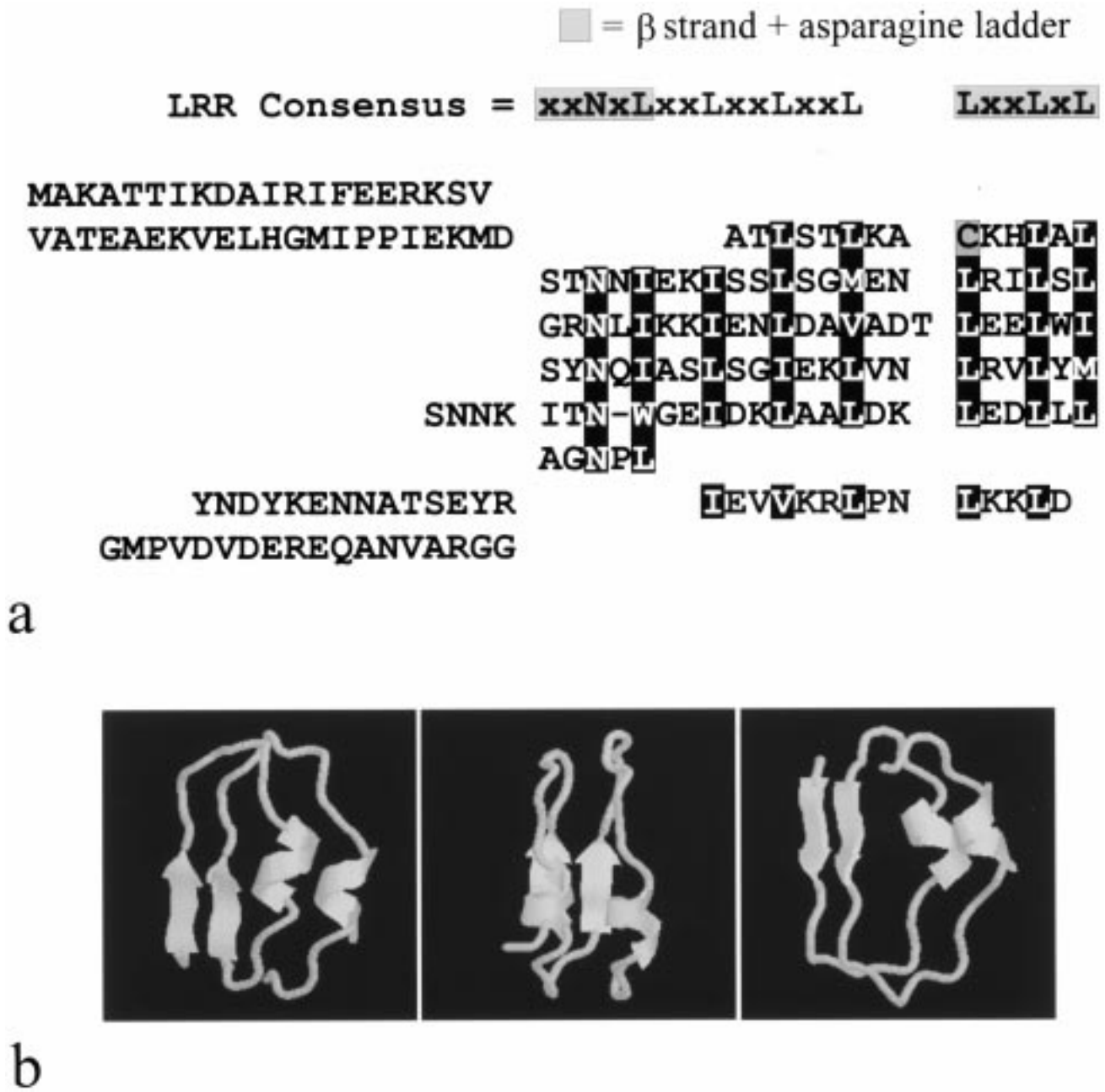

FIGURE 5: Organization of LRRs within LC1 and basic structure of the repeat. (a) The LC1 protein belongs to a class of polypeptides characterized by the presence of leucine-rich repeats. The basic SDS22+ subclass LRR consensus sequence is LxxLxxLxLxxNxIxxIxxLxx (where I and L are usually Ile and Leu, respectively, but can be any other bulky hydrophobic residue such as Met, Phe, or Val). LC1 contains four essentially complete and several partial motifs corresponding to this consensus. The N-terminal approximately 41 residues and the C-terminus do not conform to the LRR consensus, and one region (residues 7−23) is predicted to form an amphiphilic α helix. (b) A molecular model for two generalized LRRs is shown. Structurally, this motif forms an α helix, a β strand, and an "asparagine ladder" in the connecting loop. The ladder refers to the hydrogen bond network set up by the invariant Asn residue that is required to stabilize the tight turn between the structural elements. The coordinates were taken from ref *46* and were displayed using RASMOL.

fission yeast protein (not shown), and a partial coding sequence from a mixed organ (testis, lung, and B-cell) library exhibits significantly greater identity [AA923426; $P_{(n)} = 9.6 \times 10^{-26}$]. This latter clone may represent the human version of LC1.

Structural Properties of LC1. LC1 and SDS22+ belong to a subclass of proteins characterized by a leucine-rich repeat structure that has the general consensus LxxLxxLxLxxNx-IxxIxxLxx (*49*). In this consensus, I and L are usually Ile and Leu, respectively, but can be replaced by other bulky hydrophobic residues (e.g., Met, Phe, or Val). Analysis of the LC1 sequence revealed that it contains four essentially complete and several partial LRR motifs (Figure 5a). There are at least six distinguishable subclasses of LRR proteins; LC1 is a member of that group defined by SDS22+ (*49*). The structure of one LRR protein (porcine ribonuclease inhibitor which contains 15 LRRs) has been determined (*50*), and molecular models of the various subclasses have been generated (*46*, *49*). These analyses have revealed that the LRR motif has the general form of a β sheet, a tight turn stabilized by the invariant Asn residue, and an α helix (Figure 5b). Thus, the LC1 protein should contain both α helical and β sheet structures. This prediction has been confirmed by CD spectroscopy of the recombinant protein which shows

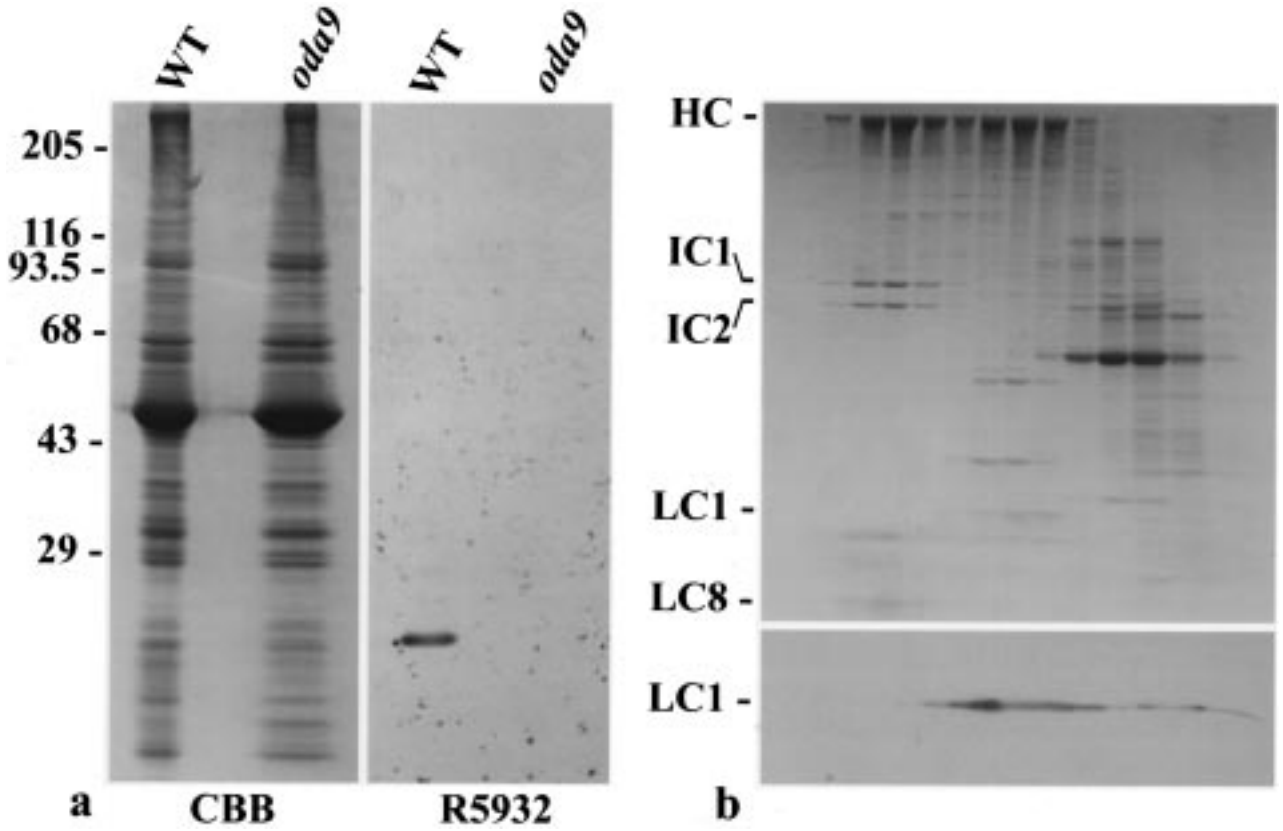

FIGURE 6: Immunological analysis of LC1 from *Chlamydomonas* axonemes. (a) Axonemes were prepared from wild-type cells and a strain lacking outer arms (*oda9*). Following electrophoresis in 5 to 15% acrylamide gels, the samples were either stained with Coomassie blue (CBB) or blotted to nitrocellulose and probed with the R5932 antibody raised against the C-terminal domain of LC1. The antibody is highly specific and recognizes a single band in wild-type axonemes; this immunoreactive band is missing in *oda9* axonemes, confirming that LC1 is specifically an outer arm component. (b) A high-salt extract of wild-type axonemes was sedimented in a 5 to 20% sucrose gradient, and equal volumes of each fraction were electrophoresed in 5 to 15% acrylamide gels. The upper panel shows the Coomassie blue-stained gel and the lower strip a nitrocellulose blot probed with the R5932 antibody to detect LC1. This protein comigrates with the γ HC at ~12 S. There is also some LC1 present in fractions extending from the 12 S peak to the top of the gradient.

evidence for both types of secondary structural elements (data not shown).

Secondary structure prediction (made using PHD; not shown) of the N-terminal region of LC1 preceding the LRR segment revealed that residues 7−23 have a high probability of exhibiting helical structure. Further analysis suggested that this predicted helical segment is amphiphilic with a very significant clustering of polar and nonpolar residues to opposite sides of the helix (not shown). The C-terminal region of LC1 also does not conform well to the LRR consensus.[3]

LC1 Is Specifically Associated with the γ Heavy Chain of the Outer Dynein Arm. To further investigate the role of LC1 in dynein function, it was necessary to obtain a specific antibody as a probe. Accordingly, the C-terminal region of LC1 (residues 104−198) was fused to maltose binding protein and the recombinant molecule used to raise antisera in rabbit R5932. This antiserum was then blot purified versus the full-length LC1 protein prior to use. Immunoblot analysis of axonemes from wild-type cells revealed a single immunoreactive band with an M_r of 22 000. As predicted, this band was completely absent in axonemes prepared from the mutant *oda9* which lacks outer dynein arms (Figure 6a). Examination of dyneins extracted from wild-type axonemes with 0.6 M NaCl and sedimented through a 5 to 20% sucrose density gradient revealed that LC1 was present at ~12 S as is the γ HC (Figure 6b). Thus, the γ HC and LC1 cofractionate and therefore interact directly with each other. A second light chain (LC4) is also known to associate with this subunit of the outer arm (*38*).

Intriguingly, some LC1 protein was also evident in fractions extending from the 12 S peak toward the top of the gradient. This suggests that LC1 has a tendency to dissociate from the γ HC during extensive ultracentrifugation. A similar hydrodynamic pressure-induced dissociation of the γ HC from the other components of the outer arm has been recently reported (*51*). This raises the possibility that the actual stoichiometry of LC1 within the outer arm may be higher than the 2 copies per particle previously reported.

LC1 Is Bound to the Motor Domain of the γ HC. The α and β HCs within the *Chlamydomonas* outer dynein arm are both tightly associated with thioredoxin-like light chains (LC5 and LC3, respectively). These components interact with the N-terminal ~160 kDa of the HCs [i.e., with the stems rather than with the globular head domains (*37*); H. Tedford and S. M. King, unpublished]. To determine whether LC1 is attached to the analogous region of the third (γ) HC within the outer arm, we first employed DMP (an amine-selective reagent with a 9.2 Å linker length) to covalently attach LC1

[3] Essentially complete ^{1}H, ^{15}N, and ^{13}C backbone resonance assignments for the LC1 protein have been made which support the predicted secondary structure (*62*).

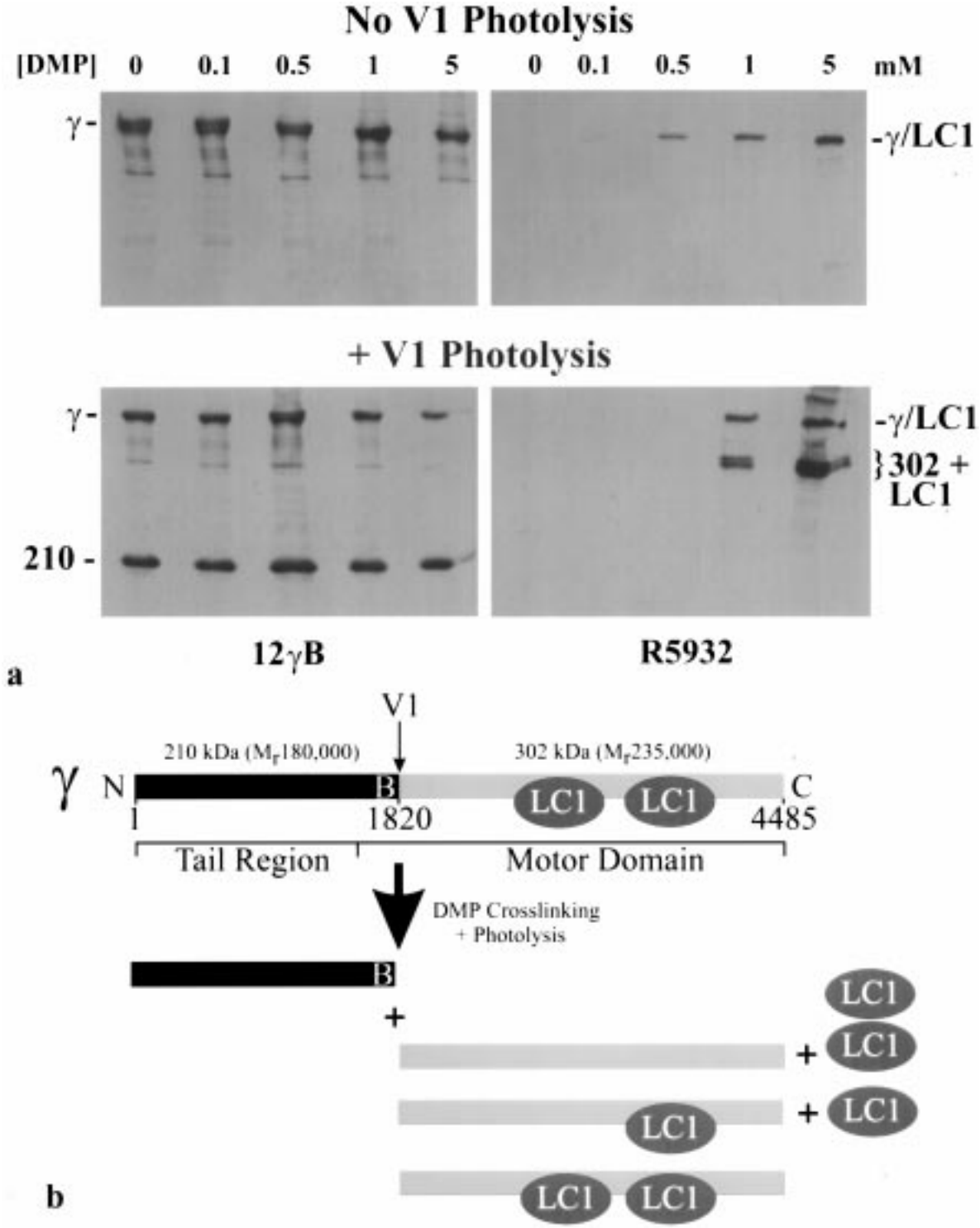

FIGURE 7: LC1 associates with the motor domain of the γ HC. (a) The purified γ subunit (containing the γ HC, LC1, and LC4) from the outer dynein arm was treated with 0−5 mM DMP. Subsequently, the cross-linked samples were electrophoresed in 4% acrylamide/4 M urea gels, blotted to nitrocellulose, and probed either with the 12γB monoclonal antibody or with R5932 to detect the N-terminal region of the γ HC and the LC1 protein, respectively (upper panels). Note that proteins of ~150 kDa or less migrate at the dye front in this gel system, and therefore, the LC1 protein is not visible in un-cross-linked samples. The samples shown in the lower panels were subject to vanadate-mediated photolysis following cross-linking. This reaction cleaves the γ HC at the V1 site within the ATP hydrolytic domain and yields an N-terminal fragment corresponding to the stem of the heavy chain and a larger C-terminal region containing the motor unit. As is evident in the upper right panel, DMP treatment results in the formation of a large conjugate containing LC1 that migrates with the upper portion of the wider γ HC band detected by 12γB. Following photocleavage, the 12γB antibody detects the smaller N-terminal V1 fragment (of 210 kDa), whereas the R5932 antibody reacts specifically with a larger doublet band. The additional band above the γ HC−LC1 product in the lower panel probed with antibody R5932 represents the interface between the 2.5% acrylamide stacking gel and the 4% acrylamide separating gel that became distorted during blotting. (b) Structural map of the γ HC indicating the products generated by DMP cross-linking and vanadate-mediated photolysis. Photocleavage of the γ HC−LC1 conjugate yielded the N-terminal fragment containing the 12γB epitope and a larger doublet band derived from the C-terminal region of the HC cross-linked to either one or both copies of LC1.

to the γ HC (Figure 7). Treatment of sucrose gradient-purified dynein with this reagent resulted in formation of a single new band containing LC1 that comigrated with the upper region of the wider γ HC band as detected by the 12γB monoclonal antibody (Figure 7, upper panels). This result indicates that the LC1 protein has been covalently bound to the HC by the cross-linker.

Irradiation of dynein HCs with ultraviolet light in the presence of ADP and vanadate results in a photocleavage reaction that specifically cleaves the peptide backbone at the P-loop motif within the ATPase active site (also referred to as the V1 site). Vanadate-mediated photolysis of the γ HC yields two fragments with M_rs of 180 000 and 235 000 (*52*). As the large dynein HCs migrate anomalously during gel electrophoresis, the actual masses of these fragments as calculated from the sequence (*53*) are considerably higher: 210 and 302 kDa, respectively. The smaller V1 fragment contains the 12γB epitope (*52*) and represents the N-terminal region of the molecule. It is this domain which forms the stem of the dynein particle. The larger C-terminal V1 fragment corresponds to the motor domain of the HC; no antibody probe specific for this region of the γ HC exists at present.

As expected, photolysis of un-cross-linked dynein samples yielded a single V1 photocleavage product (210 kDa) containing the 12γB epitope (Figure 7, lower left panel). No additional bands recognized by 12γB were observed following photolysis of DMP-treated samples. This suggests that the light chain was not attached to the N-terminal region of the γ HC. Examination of the DMP-treated samples following vanadate-mediated photolysis with the R5932 antibody identified a new doublet band with an M_r of ~250 000 (predicted actual mass of ~325 kDa) that represents the larger C-terminal photocleavage product from the γ HC conjugated to the LC1 protein (Figure 7, lower right panel). Thus, LC1 is cross-linked to that region of the γ HC that is located C-terminal to the site of ATP hydrolysis. The appearance of the $M_r \sim 250\,000$ band as a doublet most likely represents formation of a cross-linked product containing the larger C-terminal photocleavage fragment of the γ HC attached to either one or both copies of LC1 (see Figure 7b). These results strongly suggest that the LC1 protein is closely associated with the dynein motor unit.

Intra-Axonemal Interactions Involving LC1. As a class, LRR proteins are involved in protein−protein associations and in many cases mediate attachment to components of signal transduction pathways. However, within the isolated dynein particle, there is no evidence that LC1 interacts with any component except the γ HC. Therefore, to further investigate the role of LC1 in dynein function, we used zero-length cross-linking to stabilize the weak and/or transient interactions within the axoneme that are disrupted by the high-salt treatment employed to extract the outer arm for purification. EDC covalently attaches interacting carboxyl and amino groups via an isopeptide bond. Importantly, this cross-linking reagent provides no components to the final cross-linked product, and therefore, only amino acyl residues in direct contact can become covalently linked (see ref *11* for a further discussion of carbodiimide chemistry).

Wild-type axonemes were treated with various concentrations of EDC and the products separated by gel electrophoresis and blotted to nitrocellulose. Cross-linked species containing the LC1 protein were detected using the R5932 antibody (Figure 8). In samples treated with 0.5−50 mM EDC, a single major product with an M_r of ~66 000 containing LC1 was observed. This apparent mass suggests that the 22 kDa light chain has become cross-linked to an ~45 kDa protein. However, as isolated, the outer dynein arm does not contain a protein component with this approximate mass. Consistent with this, the $M_r \sim 66\,000$ product was not observed when sucrose gradient-purified dynein samples were treated with EDC. This indicates that the $M_r \sim 66\,000$ product derives from cross-linking between LC1 of the outer arm and some other non-dynein axonemal component. The $M_r \sim 66\,000$ product does not appear to derive from cross-linking between LC1 and either tubulin or actin (a component of the inner arms), as immunostaining of EDC-treated axoneme samples with specific monoclonal antibodies did not reveal a cross-linked product with the appropriate M_r.

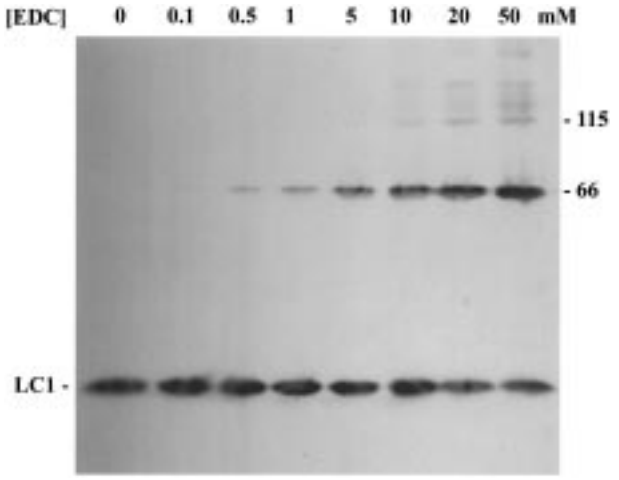

FIGURE 8: LC1 interacts directly with an ~45 kDa axonemal protein. Wild-type axonemes were subject to zero-length cross-linking by treatment with 0−50 mM EDC for 60 min. Cross-linked products were separated in a 5 to 15% acrylamide gradient gel and transferred to nitrocellulose. This figure shows the nitrocellulose blot probed with the R5932 antibody to detect cross-linked products containing LC1. A single major conjugate with an M_r of ~66 000 was observed in axoneme samples treated with 0.5−50 mM EDC. This suggests that the 22 kDa LC1 protein interacts directly with an ~45 kDa axonemal component. At the higher EDC concentrations, several additional minor products with an M_r of ≥115 000 containing LC1 were observed.

At cross-linker concentrations at or above 10 mM, several additional minor bands containing LC1 were observed. The smallest of these migrated at an M_r of 115 000, and the larger bands exhibited sequential mass increases of ~50 kDa. The most likely interpretation of this result is that the LC1−45 kDa protein conjugate has become cross-linked to one or more copies of tubulin within the doublet microtubules. However, EDC treatment of axonemes resulted in many tubulin-containing cross-linked products with masses of more than ~110 kDa (i.e., the tubulin dimer). Therefore, it was not possible to assess this hypothesis directly using the specific antibodies.

DISCUSSION

A Dynein Motor Domain-Associated Light Chain. LC1 is the largest of the eight light chains associated with the outer dynein arm from *Chlamydomonas* flagella (see Table 1). Using a combination of chemical cross-linking and vanadate-

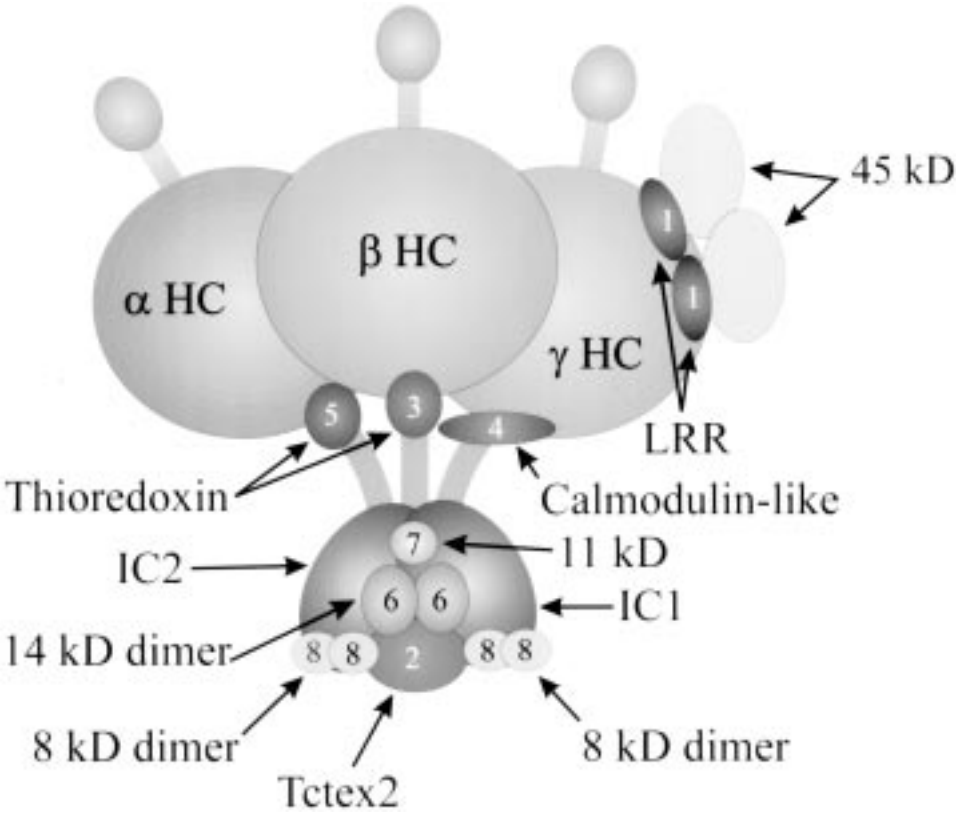

FIGURE 9: Architecture of the outer dynein arm. A model showing the locations and protein−protein associations within the *Chlamydomonas* outer dynein arm. The three HCs contribute the stems and globular head domains. These heads each include a small stalk-like structure at the top which is thought to contain the microtubule binding site (*54*, *55*). The precise location of LC1 relative to the stalk on the γ HC head is not known. The two WD repeat-containing ICs are located in the basal region. The general location of each light chain is also shown. Note that little is yet known about the precise protein−protein interactions within the intermediate chain−light chain complex. Furthermore, this diagram does not include the trimeric docking complex that is essential for the correct placement of the outer arm within the axoneme as it is not yet clear how that structure interacts with dynein polypeptides. A synopsis of the properties of outer dynein arm light chains can be found at http://www2.uchc.edu/~king/.

mediated photolysis, we have found that the LC1 protein is bound to that region of the γ HC that is C-terminal to the first P-loop. This region of the γ HC comprises most of the globular head (see ref *3* for a review) and includes the stalk-like structure that has recently been suggested to contain the microtubule binding domain (*54*, *55*). The determination that LC1 binds the C-terminal region of the γ HC represents the first known example of a dynein accessory component associated with the motor domain of a HC. Thus, LC1 is in the appropriate location to bind and thereby target specific modulators to the γ HC.

Several lines of evidence support the hypothesis that the C-terminal region of dynein HCs are involved in signal transduction controlling motor function. For example, small deletions in this area from the β HC of the *Chlamydomonas* outer arm suppress flagellar paralysis imposed by mutations in radial spoke and central pair complexes (*56*, *57*). Importantly, several mutations in the γ HC also lead to suppression of paralysis phenotypes, suggesting that signal transduction from the central pair−radial spoke complex occurs through the γ HC to impinge on outer arm activity (*58*). Within the α HC, proteolytic mapping indicates that at least one (and probably two) region within the globular head is subject to rapid phosphorylation and/or dephosphorylation in vivo (*59*). Together, these findings strongly support a role for the C-terminal portion of the HC in regulatory control of dynein motor function in addition to mediating the ATP-sensitive HC−microtubule interaction.

LC1 Is a Leucine-Rich Repeat Protein. LC1 is a member of the leucine-rich repeat protein family. Many members of this diverse class of polypeptide are involved in binding to components of signal transduction pathways. For example, SDS22+ (which is the archetype for the subclass to which LC1 belongs) acts as a positive modulator of protein type 1 phosphatase at the metaphase−anaphase transition in fission yeast (*60*). Other LRR proteins include RAD1 and RAD7 involved in DNA damage repair, the TrkC receptor protein kinase, and *Drosophila* flightless-1 (see ref *42* for a more comprehensive listing).

LC1 Interacts Directly with an ~45 kDa Axonemal Component. In an attempt to understand the protein−protein interactions in which LC1 is involved, we employed covalent cross-linking using EDC. This reagent yields an isopeptide bond between adjacent carboxyl and amino groups. Importantly, EDC does not contribute any components to the final product (the carbodiimide is hydrolyzed to a urea during the reaction) and thereby yields a zero-length cross-link. Therefore, only proteins that are in direct contact can be covalently linked using this reagent. When purified dynein was treated with EDC, we observed no products containing LC1. However, treatment of intact axonemes yielded a single major ~66 kDa band, suggesting that LC1 was cross-linked to an ~45 kDa protein. This component must therefore be in direct contact with LC1 in situ and associate via an interaction that is disrupted during high-salt extraction of the outer arm. Furthermore, this observation necessarily implies that the

~45 kDa protein is thus in close proximity to the γ HC motor.

Molecular Architecture of the Outer Dynein Arm. Our current understanding of protein−protein associations within the outer arm dynein particle and the general location of the various components is illustrated in Figure 9. Each HC has at least one tightly associated light chain. However, only in the case of the γ HC is a light chain bound to the head domain. Both redox-active thioredoxins (LC3 and LC5), which bind to the β and α HCs, interact with the N-terminal portions of their respective HCs (*37*; H. Tedford and S. M. King, unpublished). The fourth HC-associated light chain is the Ca^{2+}-binding LC4 protein. No information is currently available concerning its location, and therefore, its diagrammed position on the γ HC stem is speculative. As both copies of LC1 are located on the head domain, they have been illustrated as interacting with two copies of the ~45 kDa component. It remains unclear what further protein−protein associations this novel polypeptide might be involved in. However, it is interesting to note that at high cross-linker concentrations, additional minor bands were obtained that showed mass increases in units of ~50 kDa. One possibility is that these additional products represent sequential cross-linking of the LC1−45 kDa protein conjugate to multiple copies of tubulin in the microtubule lattice. However, as there are many cross-linked products containing tubulin generated during these experiments, it was not possible to assess this hypothesis directly using specific antisera.

At the base of the dynein particle is an additional structure referred to as the intermediate chain−light chain complex (*61*). This contains the ICs which are both essential for assembly of the entire particle. IC1 interacts directly with microtubules and consequently may be involved in structural attachment of the arm to the axoneme (*11*, *12*). However, this IC is not sufficient for binding as an additional trimeric docking complex is necessary for the attachment of extracted dyneins to their correct location within the axoneme (*25*). This docking complex is not shown in Figure 9 as it is not yet clear how that structure interacts with the remainder of the outer arm.

Also located in the basal domain are four distinct light chains (several of which are present in multiple copies). At least one of these components (LC8) plays an essential structural role as null mutants fail to assemble outer arms (*30*).

Structural Features of LC1. There are at least six subclasses of LRR proteins (*49*). LC1 is clearly a member of that subclass defined by SDS22+. The three-dimensional structures of two LRR proteins have been determined [porcine ribonuclease inhibitor (*50*) and a nitrogen fixation ORF from *Azotobacter* which is a LRR variant (*50*)], and several others have been modeled (*49*). Generally, this repeat structure consists of a β strand and an α helix interconnected via a tight turn. The turn is stabilized by a hydrogen bonding network set up by the invariant Asn residue. Sequence alignments for LC1 suggest the presence of four or five LRR repeats, and CD spectroscopy confirms that LC1 contains both α helix and β sheet structures. Intriguingly, the N- and C-termini of LC1 are predicted to be α helical, and we have now demonstrated that point directly by assigning the backbone resonances from the ^{1}H, ^{15}N, and ^{13}C multidimensional NMR spectra (*62*). Thus, the LC1 protein appears to contain a novel structural fold. At least within the ribonuclease inhibitor, the LRRs (of which there are 15) are oriented in a horseshoe shape and present an inner concave surface (formed from the β strands from each repeat) that binds ribonuclease. It is possible that a similar orientation occurs in LC1 with the β sheet face interacting with the ~45 kDa axonemal component, while other regions (such as the N- and/or C-terminal domains or the opposite face of the LRR region) are involved in permanent attachment to the γ HC. The β strands of LC1 are mainly hydrophilic and indeed contain at least 10 residues that could be involved in EDC-mediated cross-linking reactions.

In conclusion, we document here the molecular structure of the LC1 component from *Chlamydomonas* outer arm dynein and its function in targeting a novel axonemal polypeptide to the motor domain of the γ HC.

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