IC138 Is a WD-Repeat Dynein Intermediate Chain Required for Light Chain Assembly and Regulation of Flagellar Bending

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Submitted August 12, 2004; Revised September 20, 2004; Accepted September 22, 2004

Monitoring Editor: Thomas Pollard

Increased phosphorylation of dynein IC IC138 correlates with decreases in flagellar microtubule sliding and phototaxis defects. To test the hypothesis that regulation of IC138 phosphorylation controls flagellar bending, we cloned the IC138 gene. IC138 encodes a novel protein with a calculated mass of 111 kDa and is predicted to form seven WD-repeats at the C terminus. IC138 maps near the BOP5 locus, and bop5-1 contains a point mutation resulting in a truncated IC138 lacking the C terminus, including the seventh WD-repeat. bop5-1 cells display wild-type flagellar beat frequency but swim slower than wild-type cells, suggesting that bop5-1 is altered in its ability to control flagellar waveform. Swimming speed is rescued in bop5-1 transformants containing the wild-type IC138, confirming that BOP5 encodes IC138. With the exception of the roadblock-related light chain, LC7b, all the other known components of the I1 complex, including the truncated IC138, are assembled in bop5-1 axonemes. Thus, the bop5-1 motility phenotype reveals a role for IC138 and LC7b in the control of flagellar bending. IC138 is hyperphosphorylated in paralyzed flagellar mutants lacking radial spoke and central pair components, further indicating a role for the radial spokes and central pair apparatus in control of IC138 phosphorylation and regulation of flagellar waveform.

INTRODUCTION

Our goal is to determine the mechanisms that regulate ciliary and eukaryotic flagellar bending. Based on informative mutations in Chlamydomonas, and effective in vitro functional studies, a surprisingly complex array of different dynein motors is required for generation and control of normal ciliary and flagellar bending (Mitchell, 1994; Gibbons, 1995; Porter, 1996; Porter and Sale, 2000; DiBella and King, 2001; Kamiya, 2002). For example, the outer arm dyneins are homogeneous structures responsible for control of beat frequency and power required for movement (Satir et al., 1993; Brokaw, 1994; DiBella and King, 2001; Kamiya, 2002). The inner arm dyneins, however, are more complex, composed of at least seven different dynein subspecies precisely organized in a 96-nm repeat pattern along each doublet microtubule of the axoneme (Porter, 1996; Porter and Sale, 2000). Diverse data indicate the inner arm dyneins control the size and shape of the flagellar bend (Brokaw and Kamiya, 1987; Brokaw, 1994; Kamiya, 2002). The mechanism for control of flagellar waveform involves additional structures (e.g., radial spokes, central pair apparatus, and the dynein regulatory complex) and control of dynein phosphorylation (Porter and Sale, 2000; DiBella and King, 2001; Kamiya, 2002; Smith and Yang, 2004).

The present study is focused on a single inner arm dynein, the I1 complex, also called the f-dynein (Goodenough et al., 1987; Piperno et al., 1990; Kagami and Kamiya, 1992; Porter et al., 1992). The I1 complex is a tripartite structure, or triad, located near the base of the S1 radial spoke, at the proximal end of the axonemal 96-nm repeat (Goodenough and Heuser, 1985; Piperno et al., 1990; Mastronarde et al., 1992; Smith and Sale, 1992b). This large 18S, two-headed complex (Figure 1A) is composed of two heavy chains, 1α and 1β; three intermediate chains, IC140, IC138, and IC97 (also referred to as IC110); and several light chains, including LC7a, LC7b, LC8, Tctex1, and Tctex2b (Goodenough and Heuser, 1985; Piperno et al., 1990; Smith and Sale, 1991, 1992b; Porter et al., 1992; King and Patel-King, 1995; Harrison et al., 1998; Perrone et al., 1998; Yang and Sale, 1998; Myster et al., 1999; Perrone et al., 2000; DiBella et al., 2004a,b).

Various data indicate that the I1 complex is an unusual dynein motor and plays a key regulatory role in the axoneme. Unlike other inner arm dyneins, the isolated I1 complex does not efficiently translocate microtubules in vitro motility assays (Smith and Sale, 1991; Kagami and Kamiya, 1992). Mutations in I1 result in failure of control of normal ciliary and flagellar waveform and phototaxis in Chlamydomonas, indicating a regulatory role in these processes (Brokaw and Kamiya, 1987; King and Dutcher, 1997). Additionally, mutations in I1 subunits suppress paralysis in a central pair mutant, indicating a functional link between I1 activity and the central pair apparatus mechanism for control of microtubule sliding (Porter et al., 1992). Consistent with this idea, in vitro functional assays, by using isolated axonemes or reconstituted axonemes, have revealed that the I1 com-
plex plays a key role in the regulation of microtubule sliding and that the mechanism involves changes in I1 phosphorylation (Smith and Sale, 1992a,b; Howard et al., 1994; Habermacher and Sale, 1996, 1997; Yang and Sale, 2000; Smith, 2002). In these in vitro experiments, IC138 was the only I1 subunit that was phosphorylated, and increased phosphorylation of IC138 correlated with decreased dynein-driven microtubule sliding (Habermacher and Sale, 1997; King and Dutcher, 1997; Yang and Sale, 2000). Furthermore, abnormal phosphorylation of IC138 also results in a failure in *Chlamydomonas* phototaxis (King and Dutcher, 1997). Together, the data indicate changes in IC138 phosphorylation regulate I1 activity and microtubule sliding.

To further test the hypothesis that IC138 is a regulatory phosphoprotein, we cloned the gene and began characterization of mutant strains defective in IC138. We determined that like several other dynein intermediate chains, IC138 is a WD-repeat protein. The IC138 gene maps near the BOP5 locus (Dutcher et al., 1988), and the bop5-1 mutant displays a slow swimming phenotype that is rescued by the wild-type IC138 gene. The bop5-1 mutation results in the truncation of IC138 just before the last WD-repeat. Surprisingly, in bop5-1...
axonomes, the truncated IC138 assembles with all of the other known I1 subunits with the exception of LC7b. Consistent with a recent report (DiBella et al., 2004b), these results indicate that IC138 anchors LC7b in the I1 complex and that LC7b is required for normal control of bending. Furthermore, IC138 is hyperphosphorylated in axonomes from paralyzed flagella mutants lacking radial spokes and central pair apparatus. These data are consistent with the hypothesis that I1 is part of a regulatory network, including the radial spokes and central pair apparatus, which controls flagellar waveform.

**MATERIALS AND METHODS**

**Cell Strains and Growth Conditions**

The following *Chlamydomonas* strains were obtained from the *Chlamydomonas* Genetic Center (Dr. E. H. Harris, Department of Biology, Duke University, Durham, NC): CC-125 (wild-type, 137c mat+), CC-124 (wild-type, 137c mat−), CC-1036 (pD18 mat+), CC-1035 (pD17 mat+), CC-899 (pD10 mat+), CC-1877 (pD28 mat−), CC-2244 (ade1 mat+), and CC-3921 (ade1− [pS10]). The boI-1 strain was obtained from S. K. Dutcher (Washington University School of Medicine, St. Louis, MO) (Dutcher et al., 1988), and the 5A strain was described previously (Perrone et al., 1998). Double mutant strains boI5p217, boI5p218 were isolated. The radial spokes were purified from grown in HEPES Tris-ace- tophosphate medium or in modified Sager Granick minimal medium (Sager and Granick, 1953) with aeration on a 14:10-h light/dark cycle.

**Isolation of Axonomes, Dynein Purification, and Biochemical Analyses**

Flagella were isolated by the dibucaine method and demembranated using Nonidet (NP-40; Calbiochem, San Diego, CA) as described previously (Witman, 1986). Axonomes were resuspended in 10 mM HEPES, 5 mM MgSO4, 1 mM dithiobitol (DTT), 0.5 mM EDTA, 30 mM NaCl, 0.1 mM phenylmeth-ylsulfonyl fluoride, and 0.6 trypsin inhibitor units apotinin (HMDE-Na). Dystrophin immunoblotting was performed as described previously (Smith and Sale, 1991), and the concentration of 20× SDS-PAGE was performed as described previously (Smith and Sale, 1991), but by using Precision Plus Protein standards (Bio-Rad, Hercules, CA) to obtain clear bands. The protein band corresponding to IC138 was excised from the gel and microsequenced (performed by John Leszyk, University of Massachusetts, Worcester, MA). Peptides obtained are listed in Table 1. Amino acid sequences of peptides obtained by direct microsequencing of band purified IC138

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>AYRLYNVSHEDTLEEO . . . (PAS)</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>ANPDLLAVGYSAFCSSTPGAGAADPPL</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>GCGADTTTPNS</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>TPKPLLISNPTVLK</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>CSTSEYSELEYR . . . (PS)</td>
</tr>
<tr>
<td>Peptide 6</td>
<td>LEIWFALSTVKPVMHQ</td>
</tr>
<tr>
<td>Peptide 7</td>
<td>ATGVQATAWDISDTFR</td>
</tr>
<tr>
<td>Peptide 8</td>
<td>AAMUPEQDAPISR</td>
</tr>
<tr>
<td>Peptide 9</td>
<td>AGAVLPSISQLAGGVA</td>
</tr>
<tr>
<td>Peptide 10</td>
<td>PSAYHQGSMFAQGAPSYM</td>
</tr>
<tr>
<td>Peptide 11</td>
<td>DMFSS</td>
</tr>
</tbody>
</table>

The sequences used for design of primers PSS and P1AS are underlined, and the asterisk (*) indicates peptides identified by subsequent mass spectrometry analysis of purified IC138.

Restriction fragment length polymorphism (RFLP) mapping of the IC138 gene was performed as described previously (Porter et al., 1996; Perrone et al., 2000). The IC138 gene was used as a probe on genomic Southern blots to identify EcoRI/XhoI RFLP between two polymorphic *Chlamydomonas* strains. The IC138 gene was then hybridized to mapping filters containing DNA isolated from tetrat pregyny between multiply marked *Chlamydomonas* strains and cosegregation of the RFLP was analyzed with respect to >50 genetic and molecular markers. Linkage to the genetic mutation p97 (28 cM) and the two molecular markers CK1 (30 cM) and CNC63 (45 cM) placed the IC138 gene on the left arm of linkage group XII/XIII. The position of the IC138 gene on linkage group XII/XIII was more precisely determined using sequence from the 3′ untranslated region and single nucleotide polymorphisms (Kathir et al., 2003). The ability of the IC138 gene to rescue the boI-1 motility defect was tested by cotransformation as described previously (Perrone et al., 1998). Briefly, boI-1 was crossed into an arginine requiring background (p77) then boI- 1arg7 cells were cotransformed with a selectable marker, pARG7.8, and a full-length genomic clone of IC138, pBx. Arg-positive transformants were picked into liquid medium and scored under a dissecting scope for possible rescue of the boI-1 swimming phenotype. Cells with apparent wild-type motility were further analyzed by phase contrast microscopy to measure swimming speed, as described below.

**Antibody Production and Western Analyses**

An insert including nucleotides 11–167 of IC138 (cDNA clone B4) was subcloned into the petE-28(a) expression vector (Novagen, Madison, WI). The His-tagged B4 peptide was expressed in inclusion bodies of *Escherichia coli* (BL21). The inclusion bodies were solubilized in 8 M urea, and the His-tagged fusion protein was purified on a Ni+ column following the manufacturer’s instructions (Novagen). The fusion protein remained soluble following dialysis in phosphate-buffered saline (PBS) and was used to generate polyclonal antibodies in rabbits (Spring Valley Laboratories, Woodbine, MD). Western blotting was performed on polyvinylidene difluoride. The IC138 antibody was used at a dilution of 1:250. The following rabbit polyclonal antibodies were used at the indicated dilutions: α-IC140 (1:10,000), α-1Eh (1:10), α-Lc8 (1:100), α-Tetex1c (1:50), α-Tetex2b (1:50), α-Lc7a (1:50) (King and Patel-King, 1995; King et al., 1996; Mysteer et al., 1997, Yang and Sale, 1996; Bowman et al., 1999; DiBella et al., 2004a,b). The horse-radish peroxidase-conjugated goat anti-rabbit secondary antibody was used and immunoreactivity was detected by enhanced chemiluminescence (Amersham Biosciences).

**Analysis of Motility and Microtubule Sliding Assay**

The forward swimming velocity of freely swimming cells was measured as described previously using phase contrast microscopy and video tape analysis (Porter et al., 1992; Mysteer et al., 1997, 1999). The average swimming speed for each strain was calculated from a minimum of 20 individual cells. Beat frequency was measured stroboscopically using dark field microscopy and synchronization of strobe frequency with the flagellar beat (Mitchell and Kang, 1991).

Microtubule sliding velocity was measured as described previously (Howard et al., 1994; Habermacher and Sale, 1996, 1997). Briefly, isolated flagella were first resuspended in a buffer containing 10 mM HEPES, 5 mM MgSO4, 1 mM EDTA, 0.5 mM MgCl2, and 50 mM potassium acetate (HMDE), with no protease inhibitors. An aliquot of flagella was demembranated with 0.5% NF-40 in HMDE, and 8–10 μl of axonomes was added to a glass perfusion chamber constructed with a coverslip mounted on a slide by using
double stick tape. The axonemes were then washed with 50 μl of HMDEK containing 1 mM ATP. Microtubule sliding was initiated by the addition of HMDEK + ATP supplemented with 1–2 μg/ml protease (Nagarase; Sigma-Aldrich, St. Louis, MO). Microtubule sliding was visualized using dark field microscopy through a Zeiss Axiovert 35 and recorded by a silicon intensified camera (VE-1000; Dage-MTI, Michigan City, IN) and a VCR equipped with a jog/shuttle device as described previously (Habermacher and Sale, 1996). The average microtubule sliding velocity was calculated from a minimum of six experiments with a sample size of at least 60 independent axonemes.

RESULTS

IC138 Sequence Analysis

The initial strategy for cloning IC138 was to obtain partial peptide sequence (Table 1), design oligonucleotide primers for RT-PCR, and use the PCR product for screening libraries, and additional PCR (Figure 1B). Sense and antisense degenerate primers P1A51 and F551, listed in Materials and Methods, were designed based on the underlined amino acids indicated in Table 1. We obtained the cDNA sequences as outlined in the legend of Figure 1. The Chlamydomonas IC138 is found in six BAC clones (17C8, 23K17, 4P7, 9D3, 29I21, and 32D15) and is located in scaffold 16, contig 37 (Chlamydomonas reinhardtii genome version 2; http://genome.jgi-psf.org/chlre2/chlre2.home.html). IC138 also found in two expressed sequence tags (BQ188669.1 and AV643468.1). The full-length genomic IC138 clone (GenBank accession no. AY743342) is novel and contains 11 exons and 10 introns (Figure 1B). The predicted protein is novel and composed of 1057 amino acids with a predicted mass of 111.1 kDa, a calculated pI of 5.73, and contains the peptides listed in Table 1 and underlined in Figure 2. Consistent with the other members of the dynein IC family (Wilkinson et al., 1995; Yang and Sale, 1998), the sequence predicted a series of seven WD-repeats in the C-terminal portion of the protein (bold text, Figure 2). Alignment of IC138 with known Chlamydomonas dynein IC proteins indicates that IC138 contains a similar arrangement of seven WD-repeats (Figure 3 and that IC138 is more similar to the outer dynein arm IC78 compared with IC69 (Figure 3B).

Database searches for sequences similar to Chlamydomonas IC138 revealed a human IC138 homologue (GenBank accession no. NM_024763) and mouse IC138 homologue (GenBank accession no. XM_143950). Following the standard nomenclature, we propose that the human IC138 be referred to as DNA13 and the mouse IC138 as Dna13. DNA13 has been mapped to position 1p32.1 in the human genome, whereas Dna13 has been mapped to chromosome 4 in the mouse genome. IC138 and DNA13 share 34% identity and 49% similarity, whereas there is 30% identity and 56% similarity between IC138 and Dna13 (Figure 3C). There is 58% identity and 68% similarity between DNA13 and Dna13. Motif prediction programs indicate both DNA13 and Dna13 are WD-repeat proteins, similar in organization to other dynein IC proteins. DNA13 has five canonical WD-repeats, whereas Dna13 is predicted to have six WD-repeats, all of which are clustered in the C-terminal region of both proteins. The existence of human and mouse homologues of IC138 suggests that these proteins also may have evolutionarily conserved roles. These genes become candidates for loci involved in primary cilia dyskinesia (El Zein et al., 2003).

The IC138 Gene Maps Near the BOP5 Locus and IC138 Is Truncated in bop5-1 Axonemes

Molecular mapping procedures placed the IC138 gene on the left arm of linkage group XII/XIII, between two outer arm dynein loci, ODA6 and ODA9 (Figure 4A; Kathir et al., 2003). This map position places the IC138 gene in proximity to a novel motility mutation, bop5-1, whose mutant gene product had not previously been identified. bop5-1 was first isolated as a suppressor of the Chlamydomonas mutant pf10, which exhibits abnormal swimming speed and ineffective flagellar beat (Dutcher et al., 1988). The bop5-1 mutation partially suppresses the pf10 motility defect; however, neither the bop5-1/pf10 double mutant nor the bop5-1 suppressor alone is wild-type (Dutcher et al., 1988). For example, the average swimming speed of bop5-1 cells is significantly slower than wild-type cells (see below). To determine whether the IC138 gene is defective in bop5-1, we sequenced the entire IC138 gene in bop5-1 and found that it contained a single point mutation at nucleotide 4725 that changes a guanine to a thymine. The conceptual translation suggests that the mutation converts codon 974 from glutamic acid to a premature stop codon (Figure 4B) that would result in the premature stop codon.
Figure 3. IC138 structural domains and homologues. (A) Schematic illustration of the dynein intermediate chains comparing the positions of the seven WD/β-sheet repeats among several axonemal dynein intermediate chains. Alignment of these sequences indicates that the WD/β-sheet repeats are all clustered within the C-terminal portion of the proteins. (B) The MegAlign Program (DNASTAR) was used to generate a phylogenetic tree of dynein intermediate chains. (C) Alignment of the Chlamydomonas IC138 (C.r. IC138) and its homologues human IC138 (DNAI3), mouse IC138 (Dna13).

The LC7b Subunit of I1 Fails to Assemble in \textit{bop5-1}

We postulated that truncation of IC138 in \textit{bop5-1} would result in a failure to assemble a complete I1 complex. We used Western blot analysis and protein stains to determine whether the I1 complex is accurately assembled in \textit{bop5-1}. Wild-type and \textit{bop5-1} axonemes contain similar amounts of I1 proteins, including the 1α Dhc, IC140, IC138, LC8, Tctex1, and Tctex2b (Figure 6A). These results were verified in three independent experiments. Furthermore, based on silver stained gels, \textit{bop5-1} axonemes also contain 1β Dhc (our unpublished data) and IC97 (Figure 6B).

The dynein light chains LC7a and LC7b are located in the outer dynein arm as well as the inner dynein arm I1 complex (Piperno and Luck, 1979; Pfister et al., 1982; Harrison et al., 1998; DiBella et al., 2004b). Therefore, to determine whether LC7a and LC7b are present in the I1 complex it was necessary to perform the Western blot on axonemes from the double mutant \textit{bop5-1oda7}, which contains the truncated IC138 and is missing the outer arm dynein. LC7a is reduced in \textit{oda7} axonemes, as expected, as well as in axonemes from the \textit{bop5-1oda7} double mutant. However, although it is present in \textit{oda7} axonemes, LC7b is missing in axonemes isolated from the \textit{bop5-1oda7} (Figure 6C), consistent with its failure to assemble in the I1 complex in \textit{bop5-1} axonemes. This result was repeated in three independent experiments and indicates that the C terminus, including the last WD-repeat, is required for LC7b assembly into I1. We were unable to analyze LC8 in a similar manner because LC8 is also found in the radial spoke, in addition to the inner arm dynein I1 and the outer arm (Piperno and Luck, 1979; Pfister et al., 1982; Harrison et al., 1998; Yang et al., 2001).

We also examined whether the truncation in IC138 in \textit{bop5-1} alters the stability of the I1 complex after salt extraction and purification by MonoQ chromatography or zonal centrifugation on sucrose gradients. As described before for the I1 complex in wild-type axonemes, IC138 and IC140 from \textit{bop5-1} copurify in the f-fraction (our unpublished data; Kagami and Kamiya, 1992), as well as in the 18S I1 complex peak fractions on sucrose gradients (our unpublished data; Smith and Sale, 1991; Porter et al., 1992). Thus, despite the C-terminal truncation in IC138 and the failure to assemble
LC7b, the II dynein still forms a discrete 18S complex in bop5-1 axonemes.

**EDC Cross-Linking of IC138 to a ~97-kDa Axonemal Protein**

Previous studies have shown that IC140 forms a ~250-kDa cross-linked product when using a zero length cross-linker EDC (Yang and Sale, 1998). To determine whether the ~250-kDa cross-linked product is a result of a direct interaction between IC138 and IC140, we performed EDC cross-linking on isolated axonemes and analyzed the results by Western blots by using antibodies to IC138 and IC140. In wild-type axonemes, an ~250-kDa cross-linked product is observed using both IC138 and IC140 antibodies (Figure 7). This result could be explained by three possibilities: 1) IC138 and IC140 cross-linked to each other, 2) both proteins independently cross-linked to a third protein, or 3) both IC138 and IC140 are cross-linked to different proteins of similar sizes.

To distinguish these possibilities, we took advantage of both the bop5-1 mutant and an ida7 allele that contains a truncated IC140, “5A” (Perrone et al., 1998). The prediction is that if IC138 and IC140 are cross-linking to each other, then in the presence of either a truncated IC138, as in the case of bop5-1, or a truncated IC140, as in the case of 5A, the sizes of the IC138 and IC140 cross-linked product should be identical on Western blots. However, in the bop5-1 axonemes the IC138 cross-linked product is smaller than the IC140 cross-linked product and, in 5A axonemes, the IC140 cross-linked product is smaller than the IC138 cross-linked product (Figure 7). Moreover, in every case the cross-linked product is...
~97 kDa larger than the parent band. These results indicate that IC138 and IC140 are not cross-linked to each other using EDC. Instead, both proteins independently interact with another protein, possibly the third IC protein in I1, IC97. Further testing of this model will require antibodies to IC97.

IC138 Is Hyperphosphorylated in Paralyzed Flagellar Mutants

As reviewed in the Introduction, the basis for our focus on IC138 is that changes in IC138 phosphorylation correlate with changes in the control of dynein-driven microtubule sliding and the regulation of flagellar motility (Habermacher and Sale, 1997; King and Dutcher, 1997; Yang and Sale, 2000). For example, inhibition of microtubule sliding, which is characteristic of paralyzed flagella mutants, correlates with increased phosphorylation of IC138. Furthermore, mutations that result in hyperphosphorylation of IC138 also result in decreased axonemal microtubule sliding velocity and failure to phototax (King and Dutcher, 1997). To further test the idea that IC138 is abnormally phosphorylated in paralyzed flagellar mutants, we developed a Western blot based assay of IC138 phosphorylation. The strategy was to perform Western blots on control axonemes, or axonemes first treated with alkaline phosphatase (CIP), before preparation for SDS-PAGE, and then examine changes in IC138 mobility in acrylamide gels (King and Dutcher, 1997).

This assay revealed that the phosphorylation state of IC138 is altered in paralyzed flagellar mutants (Figure 8). In wild-type axonemes, IC138 runs as a relatively discrete band on 5% gels irrespective of CIP treatment (Figure 8A, compare lanes 1 and 2). In mia2-1 cells, which are known to contain hyperphosphorylated IC138 (King and Dutcher, 1997), IC138 migrates as a smeared band. After alkaline phosphatase treatment, the mia2-1 IC138 migrates as a discrete band (Figure 8A, lane 8), similar to IC138 from wild-type axonemes. As predicted from previous work (Habermacher and Sale, 1997), IC138 in axonemes from paralyzed flagellar mutants pf17 (lacking radial spoke head) or pf18 (lacking the central pair apparatus) is not a discrete band (Figure 8A, lanes 3 and 5). Rather, IC138 seems to run as a more slowly migrating and smeared band, consistent with a more highly phosphorylated IC138. Consistent with this interpretation, alkaline phosphatase treatment resulted in a discrete IC138 band, similar to the IC138 migration pattern in wild-type axonemes (Figure 8A, lanes 4 and 6). Similar results were obtained for axonemes isolated from pf14 cells (our unpublished data).

To determine whether the bop5-1 mutation might affect the phosphorylation state of IC138, we also analyzed bop5-1 in a radial spoke or central pair mutant background. Interestingly, bop5-1 does not suppress paralysis in the radial spoke mutant pf17, or in the central pair mutant pf18 (our unpublished data). In addition, the truncated IC138 in axonemes from bop5-1 migrates as a discrete band, indicating the truncation does not result in any obvious changes in the phosphorylation of IC138 (Figure 8A, lane 9). However, IC138 in the double paralyzed mutant, bop5-1pf17, slowly migrates as a smeared band, characteristic of pf mutants (Figure 8A, lane 11). A similar migration pattern was observed for IC138 in axonemes isolated from a bop5-1pf18 double mutant (our unpublished data). Thus, the bop5-1 IC138 subunit seems similar to the wild-type IC138 subunit with respect to its pattern of phosphorylation.

To further assess how the bop5-1 IC138 might alter motility, we also measured microtubule sliding velocity in axonemes by using a videomicroscopy assay. The microtubule sliding velocities of wild-type and bop5-1 axonemes were essentially identical (Figure 8B). The velocity of microtubule sliding in the double mutant bop5-1pf17 was identical to that of pf17 axonemes (Figure 8B). Moreover, addition of kinase inhibitors (Habermacher and Sale, 1997; Yang and Sale, 2000) restored microtubule sliding to near wild-type velocity for both pf17 and bop5-1pf17. The truncation of IC138 in bop5-1 therefore does not interfere with the rescue of microtubule sliding induced by protein kinase inhibitors.
DISCUSSION

Here, we further analyze flagellar dynein IC IC138, a regulatory subunit of inner arm dynein II. Key conclusions from this study include that IC138 is a WD-repeat protein and that in vivo the WD-repeat structure is important for assembly of the roadblock-like light chain, LC7b, a newly identified component of the I1 complex (DiBella et al., 2004b). The basis for these conclusions is that the IC138 gene rescues the bop5-1 motility defects; that the bop5-1 allele contains a mutation that results in the deletion of the seventh WD-repeat of IC138; and although the truncated IC138 and the other I1 proteins assemble in bop5-1 axonemes, LC7b fails to assemble in the complex. Significantly, whereas bop5-1 cells display wild-type beat frequency, the cells swim slowly. Therefore, a relatively modest truncation in IC138 structure reveals the importance of the WD-repeat structure for roadblock light chain assembly, inner arm I1 complex function and control of flagellar waveform.

Sequence and secondary structural predictions have shown that most dynein intermediate chains studied are members of the WD-repeat protein family (Ogawa et al., 1995; Wilkerson et al., 1995; Yang and Sale, 1998). The exceptions to date include dynein IC1 from sea urchin sperm tail axonemes and dynein IC3 from Chlamydomonas sperm tail axonemes, neither of which contains WD-repeats (Ogawa et al., 1996; Padma et al., 2001). Using the same criteria established in Yang and Sale (1998), including both a classical definition of a WD-repeat (Neer et al., 1994; Smith et al., 1999) and predicted β-sheet “propeller” structure, not strictly conforming to the traditional WD definitions (Faber et al., 1995; Neer and Smith, 1996), IC138 is another member of the dynein IC WD/β-sheet family, predicted to contain seven repeats located near the C terminus. As proposed previously, one simple model is that each WD-repeat dynein IC contains seven WD/β-sheet repeats (Yang and Sale, 1998) and that the repeats are likely organized into a β-sheet propeller structure (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996) to mediate protein–protein interactions required for dynein assembly and anchoring to cargo. Given this model, it may be surprising that IC138 lacking the seventh WD/β-sheet repeat can still assemble and support much of the assembly of the I1 complex. Thus, this observation is one of the most informative features of bop5-1. An additional informative feature of the bop5-1 phenotype, as discussed below, is that the WD/β-sheet repeat may mediate dynein light chain anchoring in the I1 complex.

The WD/β-sheet repeat class of dynein intermediate chains is thought to be located at the base of the dynein structure (Figure 1A), in a position to mediate assembly and anchoring to cargo (King and Witman, 1990; Paschal et al., 1992; Ogawa et al., 1995; Perrone et al., 1998; Yang and Sale, 1998). Evidence includes direct electron microscopic (EM) immuno-localization of IC78 in outer arm dynein (King and Witman, 1990), and IC74 in cytoplasmic dynein (Steffan et al., 1996), and EM analysis of inner arm dynein II mutants lacking either of the motor domains (Myster et al., 1999; Perrone et al., 2000; Porter and Sale, 2000). In Chlamydomonas and humans, mutant alleles of IC genes result in failure of outer dynein assembly, demonstrating their essential role (Mitchell and Kang, 1991; Wilkerson et al., 1995; Pennarun et al., 1999; DiBella and King, 2001; Kamiya, 2002). Furthermore, cross-linking studies revealed that IC78 and IC69 physically interact, indicating they are both located at the base of the outer arm dynein (King et al., 1991). Sequence comparison between intermediate chains of the outer and the inner arm dyneins suggest that IC138 is most similar to IC78 (Ogawa et al., 1995; Wilkerson et al., 1995). This conclusion is consistent with the previous proposal that IC140 in the inner arm dynein II is most similar to IC69 in outer arm dynein (Yang and Sale, 1998). Thus, based on EM analysis of II mutants and analogy to the outer arm dynein intermediate chains, both IC140 and IC138 are likely located at the base of the I1 complex mediating assembly and function of inner arm dynein as illustrated (Figure 1A).

The bop5-1 mutant, originally recovered as a suppressor of the pf10 motility phenotype (Dutcher et al., 1988), has proven to be informative with respect to IC138 structure and function and the role of I1 in control of flagellar bending. The phenotype of the bop5-1 mutant is distinct from other mutants that fail to assemble the complete I1 complex. I1 mu-
Figure 5. IC138 rescues motility in bop5-1. bop5-1 cells that had been transformed with full-length IC138 and stable diploids containing both the wild-type IC138 gene and the bop5-1 IC138 gene were analyzed. (A) Swimming speeds of freely swimming cells were measured. The decreased swimming speed seen in bop5-1 is rescued by wild-type IC138. (B) By Western analysis, only the full-length IC138 is found in the axonemes of the stable diploids (#1, #2, and #4) and the bop5-1 cells transformed with the wild-type IC138 gene (2A, E6).
with control of dynein-driven microtubule sliding and regulation of flagellar bending (Habermacher and Sale, 1997; King and Dutcher, 1997; Yang and Sale, 2000). These studies further indicated that increased phosphorylation of IC138—“hyperphosphorylation”—correlated with inhibition of dynein activity and altered flagellar waveform (King and Dutcher, 1997). In the current studies, we postulated that IC138 in paralyzed flagellar mutants also would be hyperphosphorylated. Consistent with this hypothesis, our Western blot assay revealed that IC138 is hyperphosphorylated in axonemes from paralyzed flagellar axonemes defective in radial spoke or central pair assembly. This result supports a model in which a network of structures including the central pair apparatus, radial spokes, inner arm dynein I1, and IC138, and possibly the dynein regulatory complex, participate in control of flagellar motility (Porter and Sale, 2000; Smith and Yang, 2004).

Additionally, this result provides further support for the idea that, in the absence of the radial spoke or central pair components, dyneins are “globally” inactivated (Huang et al., 1982). The phosphorylation of IC138 is apparently diagnostic of inhibition of dynein. Furthermore, bop5-1 does not suppress paralysis in the radial spoke and central pair mutants examined, and IC138 remains hyperphosphorylated. However, the global phosphorylation of IC138 is evidently not sufficient to cause paralysis. For example, mutant cells totally lacking the I1 complex (pf9, pf30, ida1, ida2, or ida7) or defective in IC138 phosphorylation (min2-1) are motile (Browkaw and Kamiya, 1987; Kamiya et al., 1991; Porter et al., 1992; King and Dutcher, 1997; Perrone et al., 1998). However, it is important to note that each of these mutants is defective in flagellar waveform and phototaxis (King and Dutcher, 1997), consistent with a role for the I1 complex in control of flagellar bending.

The bop5-1 motility phenotype further indicates that IC138 plays a key role in control of inner arm dynein I1 and regulation of flagellar waveform. Here, we also show that the bop5-1 mutant has a slow swimming phenotype but displays wild-type beat frequency. The simplest interpretation is that the bop5-1 mutation, truncation of IC138, alters flagellar waveform. In accordance with this interpretation, transformation of bop5-1 with the wild-type IC138 gene restores swimming speed. The transformed bop5-1 cells also assemble the full-length, wild-type IC138 in the axoneme, indicating that rescue involves replacement of the truncated IC138. The bop5-1 motility defect is therefore likely caused by the IC138 C-terminal truncation and is likely to involve the failure of LC7b assembly in the I1 complex. Because LC7b is also closely associated with several outer arm dynein subunits (DiBella et al., 2004b), one hypothesis is that both LC7b and IC138 may be important for the efficient coordination of inner arm and outer arm dynein activity. Further studies will be needed to address this interesting possibility.

Figure 6. LC7b fails to assemble in I1 from bop5-1. (A) Western analyses were performed on axonemes isolated from wild-type and bop5-1 cells by using antibodies to the 1α dynein heavy chain (1α Dhc), two intermediate chains (IC138 and IC140), and the light chains (LC8, Tctex1, and Tctex2b). (B) Silver-stained SDS-PAGE of I1 fractions indicating that IC97 (arrowhead) is assembled in axonemes from bop5-1 cells in an amount comparable with the wild-type positive control derived from pf28 axonemes and lacking in the negative control derived from pf28ty30 axonemes. IC140, IC138, and truncated IC138 are indicated with asterisks. (C) Further analysis of bop5-1 and the double mutant bop5-1oda7 reveals that LC7b, but not LC7a fails to assemble when IC138 is truncated. The tubulin region of the gel was excised and Coomassie stained to demonstrate protein loads.

Figure 7. EDC cross-links IC138 to an ~97-kDa axonemal protein. Axonemal proteins were isolated from wild-type (WT), bop5-1 and 5A (truncated IC140) cells and cross-linked using the zero-length cross-linker EDC. The samples were then separated on a 3–6% gradient gel and transferred to nitrocellulose. The blots were probed with α-IC140 or α-IC138. The IC138 and IC140 EDC cross-linked products (indicated by the asterisk) are coincidentally at the same position for WT axonemes (~250 kDa) but has shifted in the bop5-1 IC138 blot as well as the 5A IC140 blot by ~97 kDa in each case, indicating that IC138 and IC140 both interact with an axonemal protein of ~97 kDa.
Figure 8. IC138 is hyperphosphorylated in radial spoke and central pair mutants. To determine whether the phosphorylation state of IC138 is altered in paralyzed flagellar mutants, axonemes isolated from wild-type, pf17, pf18, mia2-1, bop5-1, and bop5-1pf17 were treated with CIP. (A) Based on a comparison of CIP-treated (−) versus control, untreated (+) axonemes, IC138 seems to be hyperphosphorylated in mia2-1 and the paralyzed mutants pf17, pf18, and bop5-1pf17. In contrast, IC138 from wild type and bop5-1 is not hyperphosphorylated. (B) Flagella were isolated and demembranated immediately preceding the addition of ATP and protease. Microtubule sliding velocity is expressed as micrometers per second.

ACKNOWLEDGMENTS

We are grateful to Drs. Steve King and Linda DiBella (University of Connecticut, Storrs, CT) for antibodies to the dynein light chains and sharing data on roadblock light chains LC7a and LC7b before publication, Dr. Carolyn Silflow (University of Minnesota, St. Paul, MN) for fine resolution mapping of IC138, and Drs. Pete Lefebvre (University of Minnesota, Elizabeth Smith (Dartmouth College), and Greg Pazour (University of Massachusetts) for DNA libraries. We thank Dr. Maureen Wirschell and Laura A. Fox for support and comments on the manuscript. T.W.H. was supported by a National Institutes of Health postdoctoral training award GM-00680, and this work was supported by grants from the National Institutes of Health (GM-68101 to P.Y., GM-55667 to M.E.P, and GM-51173 to W.S.S.).

REFERENCES


