ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery

Noveera T. Ahmed, Chunlei Gao, Ben F. Lucker, Douglas G. Cole, and David R. Mitchell

1Department of Cell and Developmental Biology, State University of New York Upstate Medical University, Syracuse, NY 13210
2Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844

Introduction

Cilia and flagella are complex microtubule-based organelles composed of several hundred proteins (Li et al., 2004; Pazour et al., 2005). Failure to properly assemble just a single flagellar complex, such as outer arm dynein, results in primary ciliary dyskinesia in humans, which has been linked to chronic sinusopulmonary infections, reduced male fertility, and congenital organogenesis abnormalities due to defects in embryonic left–right asymmetry determination (Zariwala et al., 2007). Assembly of these organelles is a multistep process involving partial preassembly of complexes in the cytoplasm, transport of proteins and protein complexes into the flagellar compartment, assembly of a framework of outer doublet and central pair microtubules, and attachment of other components onto the microtubule framework. For example, outer dynein arms (Fowkes and Mitchell, 1998) and radial spokes (Qin et al., 2004) both undergo preassembly in the cytoplasm before entering the flagellar compartment. This process has been extensively studied in Chlamydomonas reinhardtii through the analysis of mutations that disrupt assembly of specific flagellar structures (Silflow and Lefebvre, 2001; Kamiya, 2002; Dutcher, 2003) and through studies of the intraflagellar transport (IFT) machinery essential to flagellar assembly and maintenance (Cole, 2003; Scholey, 2003).

Recent analysis of an ift46 mutant supports an IFT requirement for outer arm dynein assembly. IFT46 is an IFT complex B subunit whose absence in the ift46-1 strain results in very short flagella that lack many normal structures, including inner and outer row dyneins (Hou et al., 2007). A partial suppressor strain that expresses a truncated form of IFT46, sup ift46 1, assembles flagella of variable lengths that retain wild-type levels of inner arm dynein but fail to assemble outer arm dynein (Hou et al., 2007), which suggests that IFT46 plays an essential role in outer arm dynein assembly, and that ODAs require a unique interaction with IFT particles for their proper transport into flagella. Here, we provide evidence that the protein product of the ODA16 dynein assembly locus may regulate the link between outer arm dynein and IFT particles for their proper transport into flagella. ODA16 represents the first identified adaptor between an IFT cargo and an IFT subunit.

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or a docking complex that forms a dynein attachment site on the doublet surface (Fowkes and Mitchell, 1998; Kamiya, 2002). The ODA5 locus may encode a subunit of a third axonomal complex needed for dynein binding (Wirschell et al., 2004). However, some ODA loci do not apparently encode axonomal proteins and may therefore be directly involved in the assembly process. Here, we test the function of one such locus, ODA16, that does not appear to encode an axonomal protein, and which has other unique properties more consistent with a role in assembly or transport of the dynein motor (Ahmed and Mitchell, 2005). *Chlamydomonas* strains harboring mutations at ODA16 fail to assemble a full complement of outer arm dyneins onto axonemes, but show normal complementation in temporary diploids between *oda16* gametes and gametes with defects in cytoplasmic preassembly of the motor, docking, or accessory complexes needed for outer dynein arm assembly. This indicates that these complexes are likely unaffected by the *oda16* mutation. In addition, the few outer arm dyneins that do assemble on *oda16* axonemes appear functional (i.e., contribute to motility). Here, we eliminate several possible roles for the ODA16 protein during outer arm dynein assembly by showing that it does not act as a chaperone for doublet attachment, as a factor that modifies dynein to an assembly competent form, or as an axonomal docking site needed for outer arm dynein attachment. Instead, our results suggest that ODA16 assists in dynein transport from the cytoplasm into the flagellar compartment through an interaction with IFT46. Our data are consistent with a hypothesis that some axonomal components, including outer arm dynein, are released immediately upon transport into the flagellar compartment.

**Results**

*Oda16 outer arm dyneins*  

*Chlamydomonas oda16* strains only assemble 10–20% of the wild-type amount of outer arm dynein into flagella, but this small remaining amount of dynein forms a strong attachment to axonomal microtubules and contributes to flagellar motility (Ahmed and Mitchell, 2005). Our previous electron microscopic analysis of *oda16* axonemes revealed variable numbers of outer row dynein arms per cross section but did not determine whether this represented a truly random variation or a proximal-distal gradient in dynein assembly. To see if the remaining outer arm dyneins in *oda16* flagella assemble preferentially near the base or tip of the axoneme, wild-type and *oda16* cells were compared using immunofluorescence with an antibody against an outer arm dynein intermediate chain (ODA-IC), ODA-IC2 (Fig. 1, A and B). As expected, dynein in wild-type cells is evenly distributed along both flagella and is also seen as a dispersed signal in the cell body. Fluorescence signal intensity was greatly decreased in *oda16* flagella, but its distribution was identical to that observed in wild-type flagella, which is consistent with an apparently random distribution of the remaining outer arms. The distribution of the dynein signal in *oda16* cell bodies remained nonlocalized as in wild-type cells, but its intensity was increased in *oda16* cells compared with wild-type cells.

We hypothesized that reduced flagellar outer arm assembly might result if *oda16* axonemes have reduced numbers of functional binding sites for dynein attachment or if the dynein complexes transported into *oda16* flagella are unable to bind efficiently. To test whether the remaining unoccupied outer dynein arm attachment sites in *oda16* flagella were capable of binding wild-type outer arm dynein in vitro, a high-salt extract of isolated, demembranated wild-type axonemes, which contains outer arm dynein but lacks ODA16 (Ahmed and Mitchell, 2005), was dialyzed to remove salt and incubated with *oda16* axonemes. After separation of axonemes from unbound dynein by centrifugation, the amount and specificity of dynein binding was assessed by Western blotting and electron microscopy of the pelleted axoneme fraction. As shown in Fig. 2 (A–C), outer arm dynein was restored to wild-type levels and appearance on these *oda16* axonemes. Therefore, ODA16 is not needed to modify axonemes for outer arm dynein attachment.

To test the alternative that ODA16 alters outer arm dynein into a form that binds with high affinity, and that few dyneins attach to *oda16* axonemes because of a dynein defect, outer arm dynein was extracted from *oda16* axonemes, concentrated to approximate the dynein levels of a wild-type extract, and then tested for its ability to bind to *oda16* axonemes. Blots show that outer arm dyneins were restored to near wild-type abundance in this experiment, and electron microscopy confirmed that a normal outer row dynein density was restored (Fig. 2, D–F). Thus, there are no apparent problems with either the axonomal binding sites or the dynein that can bind to those sites in *oda16* flagella.

**Dynein preassembly in *oda16* cytoplasm**  

We next examined the role of ODA16 in cytoplasmic preassembly of outer arm dynein. Although data from dikaryon rescue experiments are consistent with the presence of intact, preassembled dynein in *oda16* cytoplasm (Ahmed and Mitchell, 2005), we considered an alternative hypothesis. The ODA16 protein that is supplied to *oda16* mutant cytoplasm during dikaryon formation might be rapidly converting dynein from a non-preassembled form into one that is preassembled and able to be transported into flagella. We previously demonstrated that soluble extracts of wild-type cells, made by glass bead disruption in the absence of detergent, contain outer row dynein proteins preassembled into a complex that includes all three heavy proteins. In addition, ODA16 is reported to be a chaperone for doublet attachment, as a factor that modifies dynein arm attachment sites in *oda16* flagella. Therefore, ODA16 is not needed to modify axonemes for outer arm dynein attachment.

![Figure 1. Outer arm dynein distribution in the absence of ODA16.](image)

**Figure 1.** Outer arm dynein distribution in the absence of ODA16. Immunofluorescence images of wild-type (A) and *oda16* (B) cells stained with anti-ODA-IC2 show uniform distribution of outer arm dynein along the length of the flagella in both strains, although signal intensity is lower in the mutant flagella. Cytoplasmic distribution of ODA-IC2 is also similar in both strains, but abundance appears greater in mutant cytoplasm. Bar, 5 μm.

![Image](image)
chains and both intermediate chains (Fowkes and Mitchell, 1998). In extracts from some dynein assembly mutants, such as oda7, these subunits fail to preassemble, and some individual subunits show reduced cytoplasmic abundance, whereas in other mutants, such as docking complex mutant oda1, all subunits appear at normal abundance and in a stable complex. To assess dynein subunit stability in oda16 cytoplasm, blots of cytoplasmic extracts were probed with antibodies to the five larger outer arm dynein subunits. All five proteins show an increase in abundance in an oda16 extract compared with their levels in extracts of wild-type or oda4 (outer arm dynein heavy chain β [ODA-HCβ] mutant) cells (Fig. 3 A), and this increase in dynein subunit abundance is rescued back to wild-type levels by expression of an ODA16HA transgene. We previously showed that the HA-tagged ODA16 transgene phenotypically complements the oda16 assembly defect and that ODA16HA is expressed in ODA16-IR(HA) flagella at levels comparable to that of ODA16 in wild-type cells (Ahmed and Mitchell, 2005). These changes in heavy chain abundance can be observed directly in a Coomassie blue–stained gel (Fig. 3 A, top, HC) and by immunoblotting (Fig. 3 A, bottom). Probing this blot with anti-ODA16 shows that the ODA16 protein is expressed at wild-type levels in the oda4 cytoplasm, that no ODA16 is seen in the cytoplasm of oda16 cells, and that only the higher molecular weight HA-tagged ODA16 protein appears in the ODA16-IR(HA) strain. The increase in dynein abundance seen in oda16 cytoplasmic extracts correlates well with the increase seen by immunofluorescence in oda16 cell bodies (Fig. 1 B), and suggests that all of the dynein destined for flagellar assembly is synthesized in oda16 cells but accumulates in the cytoplasm because of its inefficient transport into the flagellar compartment.

To determine the assembly state of dynein subunits in these cytoplasmic extracts, ODA-HCβ was immunoprecipitated with an anti-HCβ monoclonal antibody. Western blots show that a complex containing all of the major outer arm dynein subunits was coprecipitated by this antibody from both wild-type and oda16 extracts (Fig. 3 B), which indicates that these subunits were successfully preassembled in the absence of the ODA16 protein. No subunits were precipitated from wild-type extracts by an unrelated anti-HA monoclonal antibody (Fig. 3 B, Ig lane) or from oda4 extracts by the anti-HCβ antibody (Fig. 3 B, oda4 lane). Immunoprecipitates of ODA16HA from ODA16-IR(HA) extracts failed to coprecipitate dynein proteins, and likewise, outer arm dynein immunoprecipitates from ODA16-IR(HA) cells contained no detectable ODA16HA protein (not depicted), which indicates that ODA16 is unlikely to be associated with dynein in this cytoplasmic pool.

To assess the ability of these preassembled dynein complexes to bind to axonemal docking sites, cytoplasmic extracts were mixed with axonemes from the outer row dynein assembly mutant pf28, an ODA-HCy mutant. In these experiments, a cytoplasm/flagella stoichiometric ratio of 2:1 was used, based on previous work showing that the apparent size of the cytoplasmic pool of flagellar precursors is sufficient for assembly of half-length flagella in the presence of protein synthesis inhibitors (Rosenbaum et al., 1969). As shown by the reappearance of normal levels of dynein heavy chains by a Coomassie stain (Fig. 3 C, top) and of all tested subunits by Western blots (Fig. 3 C, bottom),
the wild-type extract supported assembly of normal amounts of dynein onto pf28 axonemes. In contrast, an oda4 extract supported assembly of no HCα and only very small amounts of IC1, IC2, and HCγ, even though these subunits are present at approximately wild-type levels in the oda4 cytoplasm (Fig. 3 A). Dynein from the oda16 extract bound robustly to the pf28 axonemes. The ability of this dynein to bind when cytoplasm and axonemes are mixed in vitro, bypassing the need for a transport step, indicates that the ODA16 protein is not needed to make an assembly competent preassembled dynein complex, but only to facilitate the transport of this complex into the flagellar compartment. Because the ift46 mutant was also reported to be defective for outer dynein arm assembly (Hou et al., 2007), we included a cytoplasmic extract from this strain in our axoneme-binding experiment. As with oda16, the ift46 extract contained dynein that was able to bind with pf28 axonemes in vitro to restore all tested outer arm dynein subunits (see Fig. 3 C, last lane).

ODA16 localization

Biochemical fractionation of flagella has shown that ODA16 resides primarily in the flagellar matrix and does not copurify with axonemal outer arm dynein (Ahmed and Mitchell, 2005). To better understand the mechanism by which this protein affects outer arm dynein assembly, we visualized its distribution in cells by immunofluorescence and Western blotting. When wild-type C. reinhardtii were double labeled with anti-ODA16 and anti-acetylated tubulin antibodies, ODA16 was seen to localize predominantly to the peribasal body region, with weaker staining seen throughout the entire flagellar length (Fig. 4 A and B). This distribution is strikingly different from outer arm dynein distribution, which is mostly in the flagella starting above the transition zone and dispersed in the cytoplasm (Fig. 1), but is similar to that seen for several IFT components including the IFT kinesin subunit FLA10 (Vashishtha et al., 1996). To directly compare ODA16 and FLA10 localization, oda16 cells expressing HA-tagged ODA16 were double labeled with anti-FLA10 and anti-HA antibodies. By immunofluorescence, the distribution of ODA16HA is unchanged by the addition of the tag, and ODA16HA and FLA10 approximately colocalize in both the flagella and the peribasal body region (Fig. 4 C).

To compare the relative abundance of ODA16 in flagella and cytoplasm, blots of whole cell protein were compared with protein from equal numbers of deflagellated cell bodies and with flagella isolated from an equal number of cells (Fig. 4 D). Blots of 10-fold and 50-fold higher amounts of flagella samples were included to provide a semiquantitative comparison. ODA16 appears to be at least 50-fold more abundant in the cytoplasm than in flagella, somewhat similar to the distribution of an IFT complex B protein (IFT46), which is between 10-fold and 50-fold more abundant in cytoplasm, whereas axonemal ODA-IC2 appears about equally abundant in cell body and flagellar fractions.
Immediately after cell fusion (15 min after mixing cells), both outer row dynein (IC2) and ODA16 were present in flagella from the ODA16-1R(HA) cell, but neither protein was present in flagella from the oda2 cell (unpublished data). After 40 min, ODA16HA was present around both pairs of basal bodies and within both pairs of flagella, whereas outer arm dynein was still only detectable in one pair of flagella (Fig. 5, A–C). By 140 min, ODA16HA and outer arm dynein were both detectable in all four flagella (Fig. 5, D–F). The outer arm dynein signal intensity in the oda2 flagella increased gradually between 40 and 90 min after mating, and was uniformly distributed along the flagellar length at all time points examined. In summary, we conclude that ODA16 is present in both cytoplasmic and flagellar compartments, that its abundance is much greater in the cytoplasm than in flagella, and that its distribution is independent of dynein distribution but similar to, and dependent on, IFT complex distribution.

**Oda16 yeast two-hybrid screen**

To identify proteins that interact with ODA16, we conducted a yeast two-hybrid screen, and used mammalian resources to take advantage of the high overall level of sequence similarity (>70%) between algal and mammalian ODA16 homologues. A human ODA16 cDNA was subcloned into a yeast two-hybrid vector and used as bait to screen a mouse testis–derived cDNA library. The 168 clones initially selected for growth dependence on a temperature-sensitive flagellar assembly strain fla10−, IFT subunit abundance in flagella is reduced even at the permissive temperature because of a reduction in FLA10 kinesin levels, but enough IFT is maintained to support assembly of full-length flagella whose axonemal structure and motility remain wild type (Pedersen et al., 2006). ODA16 levels in fla10− flagella were compared with levels in wild-type flagella to determine if ODA16 localization to the flagellum is dependent on FLA10 kinesin activity. As illustrated in Fig. 4 E, ODA16 levels were reduced in fla10− flagella, as were levels of IFT46 and FLA10, whereas levels of axonemal ODA-IC2 remained normal. These results show that ODA16 levels in flagella depend on transport into the flagellar compartment by IFT, and suggest that ODA16 maintains its flagellar abundance through association with the FLA10 kinesin or another IFT complex protein.

We previously found that ODA16 enters flagella in the absence of outer arm dynein (Ahmed and Mitchell, 2005), thus ODA16 is likely to interact directly with the IFT machinery for its transport rather than indirectly through an association with dynein, and its abundance and distribution in the flagellar compartment should not be directly linked to that of assembled outer row dyneins. To test these assumptions, dikaryons were formed by mating ODA16-1R(HA) (tagged ODA16, wild type for outer row dynein assembly) with oda2 (untagged ODA16, defective for outer row dynein assembly), and the distribution of outer row dynein and ODA16HA were visualized by immunofluorescence 40 min and 140 min after mixing cells (Fig. 5). Immediately after cell fusion (15 min after mixing cells), both outer row dynein (IC2) and ODA16 were present in flagella from the ODA16-1R(HA) cell, but neither protein was present in flagella from the oda2 cell (unpublished data). After 40 min, ODA16HA was present around both pairs of basal bodies and within both pairs of flagella, whereas outer arm dynein was still only detectable in one pair of flagella (Fig. 5, A–C). By 140 min, ODA16HA and outer arm dynein were both detectable in all four flagella (Fig. 5, D–F). The outer arm dynein signal intensity in the oda2 flagella increased gradually between ~40 and 90 min after mating, and was uniformly distributed along the flagellar length at all time points examined. In summary, we conclude that ODA16 is present in both cytoplasmic and flagellar compartments, that its abundance is much greater in the cytoplasm than in flagella, and that its distribution is independent of dynein distribution but similar to, and dependent on, IFT complex distribution.
Gal4 promoter driven by the two-hybrid interaction were re-tested for expression of a Gal4-dependent β-galactosidase gene. DNA was recovered from 13 colonies positive for expression of both two-hybrid reporter genes, and sequencing revealed four independent clones, which encoded mouse homologues of a cytoplasmic protein (pellino2), a mitochondrial protein (creatine kinase), and two flagellar proteins (inner arm dynein light chain p28 and IFT complex B subunit IFT46). One representative of each clone was reintroduced into yeast and crossed with either a control strain carrying the empty Gal4 DNA-binding domain vector or a strain carrying a vector expressing the ODA16-Gal4 DNA-binding domain fusion protein. Only the IFT46 clone retained a positive signal on retesting (Fig. 6 B), whereas p28 showed a false positive signal when combined with the empty vector (Fig. 6 A). All three selected two-hybrid MmlIFT46 cDNA sequences contained full-length coding sequences in frame with the Gal4 activation domain, and also contained sequences from the 5' untranslated region of MmlIFT46 that added linkers of up to 125 amino acids between the Gal4 activation domain and IFT46.

Although mouse and human ODA16 sequences are highly conserved, MmlIFT46 and CrIFT46 share only 36% identity and 50% similarity (Fig. 6 C). To confirm that the C. reinhardtii homologues of ODA16 and IFT46 interact, pull-down assays were conducted with bacterially expressed proteins. ODA16 with an amino-terminal GST tag and IFT46 with an amino-terminal HIS tag were coexpressed, and IFT46HIS was purified from bacterial extracts with nickel-coated magnetic beads. Blots show that IFT46HIS could pull down ODA16GST but not GST alone (Fig. 6 D), which supports the conclusion that these two proteins interact directly in vitro. In a reciprocal experiment, IFT46HIS copurified with ODA16GST but not with GST alone (unpublished data). As a further test of the ability of ODA16 to interact with native IFT46, ODA16GST was used to pull down interacting proteins from an NP-40-generated flagellar extract containing flagellar matrix proteins. As illustrated in Fig. 5 E, IFT46 was precipitated with ODA16GST but not with GST alone.

**In vivo interaction between ODA16 and IFT particles**

To confirm that these *Chlamydomonas* proteins interact in vivo, detergent-generated flagellar matrix extracts from the HA-tagged ODA16-1R(HA) strain were immunoprecipitated with anti-HA antibodies. Blots of the resulting pellets show that IFT complex B (IFT46 and IFT81) and the FLA10 IFT motor are specifically coprecipitated with ODA16HA (Fig. 6 F). In contrast, outer arm dynein subunits were not detectable in these precipitates (unpublished data). Collectively, these results support a model in which an association between ODA16 and the IFT complex, mediated at least in part by a direct interaction between ODA16 and IFT46, is essential for efficient transport of outer arm dynein into the flagellar compartment, but dynein itself, once it reaches the matrix, does not remain associated with this IFT–ODA16 complex.

**Discussion**

Outer arm dynein assembly is a multistep process that involves preassembly of subunits in the cytoplasm, movement of complexes into flagella, and assembly of these complexes into a functional dynein arm. Most mutations that disrupt this process have been traced to genes encoding subunits of the dynein motor itself or subunits of complexes that form binding sites for motor attachment, but *oda16* appears to be an exception. Based on this and our previous studies, ODA16 should not be considered a new dynein subunit or docking site protein, as it is not needed to form a functional motor complex or docking complex (Ahmed and Mitchell, 2005), does not partition biochemically as an axonemal protein, and has a distribution resembling that of IFT proteins, not axonemal proteins (Fig. 4). We demonstrate here that ODA16 is also unlikely to function as a chaperone at the outer doublet attachment step, as it is not needed to make an assembly competent binding site or to make an assembly competent dynein (Fig. 2). ODA16 is also not required to chaperone the preassembly of dynein subunits in the cytoplasm because complexes are preassembled in *oda16* cytoplasm and are competent to bind to axonemes (Fig. 3). Instead, ODA16 appears to be needed only for efficient transport of outer arm dynein motor complexes into the flagellar compartment. Because low levels of outer arm dynein manage to assemble in the absence of ODA16 (Ahmed and Mitchell, 2005), we conceive the role of ODA16 to be a cofactor or adaptor that enhances the ability of the IFT machinery to transport outer arm dynein.

Several other axonemal components, including the inner arm dyneins and radial spokes, have been shown to enter the
flagellar compartment as partially assembled complexes and to require IFT for incorporation into flagella. In contrast, evidence of a role for IFT in outer dynein arm assembly has been questioned. Piperno et al. (1996), using cytoplasmic complementation in dikaryons between strains that carried the temperature-sensitive IFT kinesin mutation, fla10ts, concluded that outer row dynein could assemble after IFT had been blocked by a temperature shift, whereas inner row dynein could not. In that study, mating was used to fuse a gamete carrying a dynein assembly defect with a gamete that was wild type for dynein assembly, and the recovery of dynein into the mutant flagella was analyzed by immunofluorescence. However, recent experiments with fla10ts strains have shown that IFT is required for downstream signaling steps essential to mating-based cell fusion in Chlamydomonas (Wang et al., 2006), and therefore, the mated cells analyzed by Piperno et al. (1996), and therefore, the mated cells analyzed by Piperno et al. (1996), likely retained at least a small residual IFT activity that could have supported outer dynein arm assembly. Thus, assembly of inner and outer row dyneins may both require IFT yet differ in some essential aspect of the transport mechanism such as the need to reach the flagellar tip for cargo release.

Axonemal protein assembly is thought to depend on transport from a peribasal body loading zone to the flagellar tip (Qin et al., 2004; Pedersen et al., 2006), but outer arm dyneins could be an exception. Concentration at a peribasal body location has been described for inner row dynein light chain p28 (Piperno et al., 1996), intermediate chain IC138 (Hou et al., 2007), and radial spoke protein 3 (Qin et al., 2004), but outer row dynein displays no such concentrated peribasal body localization (Fig. 1;
functions as an IFT-associated adaptor for a specific peripher-
alsemblage and maintenance of flagella as organelles but instead
subunit essential for any common role of IFT particles in the as-
mitchell, 2005). Thus, if ODA16 is an IFT subunit, it is not a
with motile axonemes that retain outer row dynein (ahmed and
fl age lar length or on the flagellar abundance of IFT subunits.
primarily affect outer row dynein assembly, and have no affect on
assembly loci; for some of these loci, gene products have not been
 assaulting such cargoes with IFT particles around basal bodies and their subsequent release upon entry into
flagellar compartment. Similar to the adaptors for other
intracellular transport processes, ODA16 could mediate changes in
affinity with the transport machinery as the cargo moves from
one compartment to another. Screens for assembly mutations that
affect axonemal components such as outer arm dyneins have not
been saturated, as only single alleles exist at several dynein as-
sembly loci; for some of these loci, gene products have not been
identified. It is therefore likely that other axonemal structures are
transported into the flagellar compartment through interactions
with IFT adaptors that are functionally similar to ODA16.

Materials and methods

C. reinhardtii strains

Strain 137c served as genetic background and wild-type control for all
experiments. Additional strains used include oda1 pf28 [mitchell and
baum, 1985], fA10° (walther et al., 1994), oda16-1 and oda16-18 (HA)
(ahmed and mitchell, 2005), oda4 (okagaki and kamiya, 1986), and
h46 [hou et al., 2007].

Protein extraction and fractionation

Flagella and flagellar fractions were prepared as described previously
(ahmed and mitchell, 2005). High-salt extracts prepared from 137c or
oda16 axonemes were dialyzed against HMDEK (30 mM Hepes, 5 mM
MgSO₄, 1 mM DTT, 0.5 mM EGTA, and 25 mM potassium acetate, pH 7.4) for 18 h at 4°C. For outer arm dynein rebinding experiments, the dialyzed extract was incubated with oda16 axonemes in HMDEK for 2 h on ice and sedimented in a microcentrifuge for 15 min.

To compare relative protein abundance in whole cells, cell bodies, and flagella, protein was prepared from equal numbers of cells before or immediately after pH shock deflagellation (Witman, 1986) by resuspending all cell volumes of water containing 3 volumes of acetone. Flagella were prepared from a known number of cells by dibucaine deflagellation (Witman, 1986) and appropriately diluted to provide SDS-PAGE samples of known stoichiometry relative to whole cell and cell body samples.

Cytoplasmic extracts were made from autolysin-decelled worms by washing cells in HMDEK50 (HMDEK prepared with 50 mM potassium acetate), suspending ~2×10⁶ cells with 1.5 ml of HMDEK50, and vortexing with 1.5 ml of 0.5-mm glass beads in a 15 ml conical tube (three times for 1 min). Broken cells were spun in a microcentrifuge (Hermle) for 30 min, and supernates were frozen in liquid N₂ and stored at −70°C for later use. For binding of cytoplasmic proteins to p28 axonemes, extract from 6×10⁶ cells was diluted to 0.5 ml with HMDEK50, mixed with axonemes prepared from 3×10⁶ cells, and incubated on ice for 1 h. Axonemes were pelleted, washed with 1 ml HMDEK50, and prepared for SDS-PAGE.

Antibodies and immunodetection

Antibodies used include anti-acetylated tubulin (clone 6-11B-1; Sigma-Aldrich), affinity-purified rabbit anti-ODA16 (Ahmed and Mitchell, 2005), mouse monoclonal anti-ODA-HCp (C11.6), anti-ODA1C1 (C1.1), anti-ODA-IC2 (C11.4; Mitchell and Rosenbaum, 1986), mouse monoclonal anti-ODA-HCv (12Y8; a gift of S.M. King, University of Connecticut Health Center, Farmington, CT; King et al., 1983), rabbit polyclonal anti-ODA-HCv (B3B; Fowkes and Mitchell, 1998), mouse monoclonal anti-HA epitope 12CAS (Roche), rat monoclonal anti-HA epitope 3F10 (Roche), rabbit anti-GST (Sigma-Aldrich), rabbit anti-FLA10 (a gift of J.L. Rosenbaum, Yale University, New Haven, CT), rabbit anti-IFT81 and -IFT139 (Cole et al., 1998), and rabbit anti-IFT46 (a gift of S.M. King, University of Connecticut Health Center, Farmington, CT; King et al., 1983), rabbit polyclonal anti-ODA-HCv (12Y8; a gift of S.M. King, University of Connecticut Health Center, Farmington, CT; King et al., 1983), rabbit anti-IFT81 and -IFT139 (Cole et al., 1998), and rabbit anti-IFT46 (a gift of H. Qin, Texas A&M University, College Station, TX; Hou et al., 2007).

For blots, proteins separated by SDS-PAGE were transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore), and peroxidase-labeled secondary antibodies were detected on Biomax Light film (Kodak) using Signal West Dura Extend Duration Substrate (Thermo Fisher Scientific). Molecular mass was estimated based on migration of Benchmark protein ladder (Invitrogen) standards. Scanned images of stained gels and films were generated in Photoshop 6.0 (Adobe). Indirect immunofluorescence followed previously described methods (Cole et al., 1998). Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse secondary fluorescent antibodies were used at a 1:1,000 dilution (Invitrogen). Images were visualized using a microscope (microphot-FX; Nikon) using a 60× 1.4 NA plan Apo objective (Nikon), and captured using Spot Advance 4.0.0 software (Diagnostic Instruments, Inc.). Images were cropped and adjusted using Photoshop 6.0.

Electron microscopy

Specimens for thin section electron microscopy were prepared as previously described (Mitchell and Sale, 1999). Images were taken using a microscope (100CXII; JEOL Ltd.) operated at 80 kV. Negatives were scanned and imported in Photoshop 6.0, inverted, and adjusted for contrast and median density.

Yeast two-hybrid screen

The coding region of a full-length cDNA clone of the human ODA16 homologue (available from GenBank/EMBL/DDB under accession no. BC036377; clone ID 4824989; Thermo Fisher Scientific) was amplified from genomic DNA (available from GenBank/EMBL/DDB) to create pAC1-CYH2-HsODA16 with homology to human ODA16 (5′-ATGCTAAGGGATCCCTCAACAGGCAACGCAAG-3′), which adds an NdeI site before the start codon, and GenBam1r (5′-CTAGCAAGGATCCTCACTGACC-3′), which adds a BamHI site after the stop codon. The PCR product was cloned between Ndel and BamHI in the yeast Gal4-binding domain vector pAS1-CYH2 to create pAC1-CYH2-HsODA16. An amplified sample of a mouse testis-derived cDNA library cloned into Gal4 activation domain vector pACTII was provided by S. Dutcher (Washington University in St. Louis, St. Louis, MO). Vector plasmid pAS1-CYH2, control plasmids pSE111 (pACTII containing SNF4) and pSE112 (pAS1-CYH2 containing SNF4), and Saccharomyces cerevisiae strains Y187 containing 25 mM, 50 mM, or 100 mM 3,5-aminotriazole, HsODA16-Gal4BD localization to the nucleus was also confirmed by immunofluorescence.

The yeast two-hybrid screen was conducted as described previously (Clark et al., 2006) with the following alterations. 3.5 × 10⁹ Y190 cells containing pAS1-CYH2-HsODA16 were transformed with the mouse cDNA library (3.5 × 10⁶ clones) and plated onto SD + 25 mM 3,5-aminotriazole + 10 μg/ml adenine. Of 101 survivors, 18 rescored as positive for a lacZ gene driven by a Gal4-dependent promoter. DNA was recovered from 13 lacZ-positive clones strains and sequenced, and we identified four independent clones encoding an uncharacterized protein (available from GenBank/EMBL/DDB) with homology to Cff146 (three clones), an axonemal dynein light chain (accession no. BC118619) with homology to Cpo28 (one clone), pellino 2 (accession no. NM_033602), which lacks algal homologues (one clone), and mitochondrial creatine kinase 2 (accession no. NM_198415; eight clones). Y190 cells containing pAS1-CYH2 or pAS1-CYH2-HsODA16 were mated with Y187 cells containing one representative of each positive clone from the screen. As a positive control, pSE112 and pSE111-containing strains (SNF4Δ/IFFT4Δ) interaction were included. After growth in Sc-Leu1Δ medium for 6 d, a dilution series was spotted onto selection plates (25 mM 3,5-aminotriazole + 10 μg/ml adenine) and scored after 6 d.

Pull-down assays and immunoprecipitation

For pull-downs with bacterially expressed proteins, either pGEX-ODA16 (GST-tagged ODA16; Ahmed and Mitchell, 2005) or pGEX-4T2 (GST alone) were co-transformed into B21/1ΔDe3 Escherichia coli with pSF-Duet1-IFT46F (His-tagged IFT46). The full coding sequence of Chlamydomonas IFT46 was subcloned into pRSF-Duet using the EcoRI and SalI sites of MCS-1. His tag–based pull-down assays were performed using the MategHist Protein Purification System (Promega) according to the manufacturer’s instructions, except that 500 mM NaCl was added to all buffers used for the Ni6His pull-downs. Protein was separated and transferred as described for immunodetection. For pull-downs and immunoprecipitations of proteins expressed in Chlamydomonas, flagellar matrix was prepared by freeze-thaw as described previously (Ahmed and Mitchell, 2005). GST and GST-ODA16p fusion proteins were incubated with wild-type flagellar matrix, and precipitated proteins were blotted with polyclonal anti-IFT46. For anti-HA immunoprecipitation, 400 μg of Flagellar matrix from oda16-1R(HA) was precloned with protein A/G agarose (Santa Cruz Biotechnology, Inc.) followed by incubation with either normal mouse IgG (Santa Cruz Biotechnology, Inc.) or monoclonal anti-HA antibody 12CAS (Roche) for 2 h, and precipitation with 20 μl of protein A/G agarose for 1 h on ice. Blots of immunoprecipitates were probed with polyclonal anti-IFT46, monoclonal anti-IFT81, polyclonal anti-FLA10, or monoclonal anti-HA antibodies. For immunoprecipitation of cytoplasmic proteins, preclared cytoplasmic extracts from ~10⁶ cells were incubated with 2 μg of mouse monoclonal anti-HA (as a control Ig) or mouse monoclonal anti-HCip1 C11.6 to precipitated proteins coassembled with HClip.

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