Discrete PIH proteins function in the cytoplasmic preassembly of different subsets of axonemal dyneins

Ryosuke Yamamoto, Masafumi Hirono, and Ritsu Kamiya

Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan

Axonemal dyneins are preassembled in the cytoplasm before being transported into cilia and flagella. Recently, PF13/KTU, a conserved protein containing a PIH (protein interacting with HSP90) domain, was identified as a protein responsible for dynein preassembly in humans and *Chlamydomonas reinhardtii*. This protein is involved in the preassembly of outer arm dynein and some inner arm dyneins, possibly as a cofactor of molecular chaperones. However, it is not known which factors function in the preassembly of other inner arm dyneins. Here, we analyzed a novel *C. reinhardtii* mutant, *ida10*, and found that another conserved PIH family protein, MOT48, is responsible for the formation of another subset of inner arm dyneins. A variety of organisms with motile cilia and flagella typically have three to four PIH proteins, including potential homologues of MOT48 and PF13/KTU, whereas organisms without them have no, or only one, such protein. These findings raise the possibility that multiple PIH proteins are commonly involved in the preassembly of different subsets of axonemal dyneins.

Introduction

The motility of cilia and flagella depends on dynein molecules that constitute the inner and outer arms on the outer doublet microtubules of the axoneme. Loss of axonemal dyneins in humans causes various serious disorders, collectively called primary ciliary dyskinesia, that involve sinusitis, bronchitis, pneumonia, and situs inversus (Marshall, 2008). *Chlamydomonas reinhardtii* has a single complex of outer arm dynein and seven major subspecies of inner arm dyneins termed "a" to "g" (King and Kamiya, 2009). Outer arm dynein is necessary for flagellar beating at high frequency, whereas inner arm dyneins are important for proper flagellar waveforms (Brokaw and Kamiya, 1987). Each species of dynein is composed of multiple subunits, which are thought to be preassembled in the cytoplasm before the dynein is transported into cilia and flagella. The necessity of cytoplasmic preassembly has been demonstrated for outer arm dynein (Fowkes and Mitchell, 1998; Omran et al., 2008). A conserved PIH (protein interacting with Hsp90) family protein (Zhao et al., 2008), PF13/KTU is necessary for the cytoplasmic preassembly of outer arm dynein and a subset of inner arm dyneins (Omran et al., 2008). This protein is thought to work as a cofactor of heat shock proteins. Its defect causes primary ciliary dyskinesia in humans and a nonmotile phenotype in *C. reinhardtii*.

In this study, through the analysis of a novel *C. reinhardtii* mutant deficient in inner arm dyneins, we identified another PIH protein that most likely functions in dynein preassembly. This mutant, *ida10*, was found to have a mutation in the gene encoding another PIH protein, MOT48. Together with the previous study (Omran et al., 2008), our findings suggest that *C. reinhardtii* uses PF13/KTU and MOT48 separately for the preassembly of outer arm dynein and for that of some inner arm dyneins, although there is some redundancy. In addition, we found that *C. reinhardtii* has a third conserved PIH protein, TWI1, which is a putative homologue of a zebrafish protein, TWISTER. This protein is involved in a motile cilia-dependent phenomenon in fish (Sun et al., 2004). Thus, all three PIH proteins of *C. reinhardtii* seem to be closely related to cilia and flagella motility, possibly all functioning in the preassembly of axonemal dyneins.

Correspondence to Ritsu Kamiya: kamiyar@biol.s.u-tokyo.ac.jp

Abbreviations used in this paper: BLAST, Basic Local Alignment Search Tool; CBB, Coomassie brilliant blue; PIH, protein interacting with HSP90.

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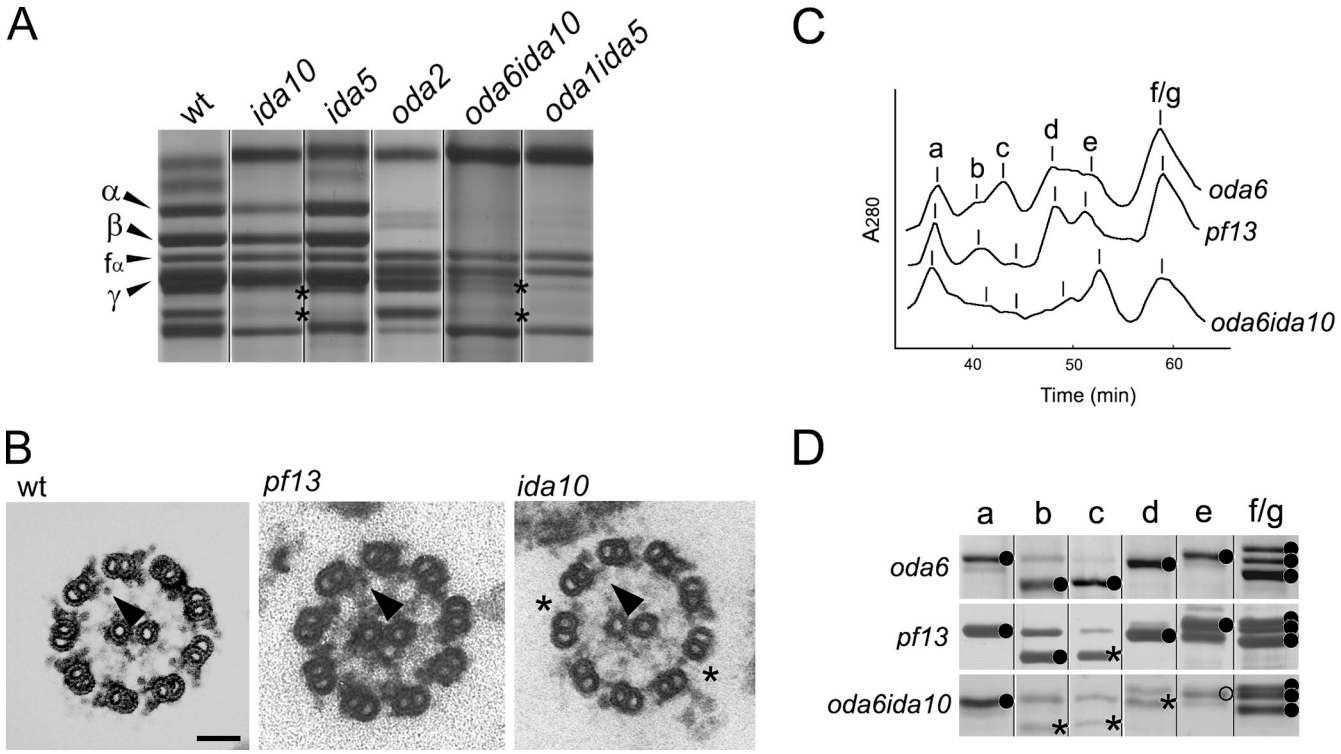

Figure 1. **Dynein defects in the *ida10* axoneme.** (A) Equal amounts of axonemes from wild type, *ida10*, *ida5*, *oda2*, *oda6ida10*, and *oda1ida5* were run on a 3–5% urea gel and stained with silver. Only the dynein heavy chain region is shown. The pattern of *ida10* is similar to that of *ida5*, which indicates that single-headed inner arm dyneins (asterisks) are reduced. Note that outer arm dynein heavy chains are also slightly reduced compared with wild type or *ida5*. The thick bands seen in the uppermost region are those with contaminating membrane proteins. All lanes are from the same gel. Black lines indicate that intervening lanes have been spliced out. (B) Cross-sectional images of typical axonemes from wild type, *pf13*, and *ida10*. In *ida10*, the inner lobe of the inner arm dynein image, corresponding to the density of single-headed dyneins (Kamiya et al., 1991), is significantly reduced in intensity compared with wild type or *pf13* (arrowhead). The outer arm dynein is also missing on some outer doublet microtubules (asterisks). Bar, 50 nm. (C) Elution patterns of extracts from the *oda6*, *pf13*, and *oda6ida10* axonemes. Desalted samples were subjected to chromatography on a Mono Q column. Dynein b, c, and d are greatly reduced in *oda6ida10* compared with *oda6*. Dynein c is reduced in the *pf13* axoneme. (D) Gel patterns showing the dynein heavy chains in the peak fractions (circles and asterisks) from the same urea gels. Dynein b, c, and d are greatly diminished in *ida10* (asterisks). Dynein e is also apparently reduced (open circle), although its reduction is not clear in C.

Results and discussion

Isolation of a novel *C. reinhardtii* dynein-deficient mutant, *ida10*

A novel *C. reinhardtii* mutant, *ida10*, was isolated from a library of UV-mutagenized cells that displayed motility defects. Its flagellar motility was somewhat variable from one cell to another and from culture to culture; it tended to show better motility when cultured for a long time or when differentiated into gametes. In vegetative cells during logarithmic growth, a majority of cells swam more slowly (~80 μm/s) than wild-type cells (~140 μm/s). The flagellar beat frequency (~40 Hz) was also lower than wild-type frequency (~65 Hz). The rest of cells were completely non-motile or showed only sporadic twitching of flagella.

Urea gel electrophoresis detected abnormal dynein composition in the axoneme. *C. reinhardtii* has a single species of outer arm dynein and seven major species of inner arm dyneins (King and Kamiya, 2009). Inner arm dyneins are classified into a double-headed type containing two heavy chains (subspecies f, also called I1) and a single-headed type containing one heavy chain (subspecies a, b, c, d, e, and g; Kagami and Kamiya, 1992). In *ida10* axonemes, bands representing single-headed inner arm dynein species were substantially reduced compared with those of wild type (Fig. 1 A). This pattern is similar to that of *ida5*, an inner arm dynein–deficient mutant with a mutation in the conventional actin gene and lacking inner arm dynein subspecies a, c, d, and e (Kato-Minoura et al., 1997). The bands representing outer arm dynein heavy chains were also slightly reduced in *ida10*, which suggests that it has a slight deficiency in outer arm dynein also. In contrast, two heavy chains of dynein f/I1 appeared normal (Fig. 1 A).

Inner arm dynein subspecies b, c, and d are greatly reduced in *ida10*

Dynein composition in the *ida10* axonemal high-salt extract was analyzed by ion-exchange column chromatography on a Mono-Q column (Kagami and Kamiya, 1992). To facilitate analysis, we used a double mutant of *ida10* and *oda6*, a mutant lacking outer arm dynein. The elution pattern of the *oda6ida10* axonemal extract indicated a great reduction in the amounts of single-headed inner arm dyneins b, c, and d (Fig. 1, C and D), and possibly a modest reduction in dynein e (Fig. 1 D). The amount of the inner arm dynein c in *ida10* was <10% of that in the wild-type axoneme, as estimated from the band density in Western blot patterns (Fig. 2 A). In contrast, the amounts of inner arm dynein a, f/I1, and g were almost normal or only slightly reduced

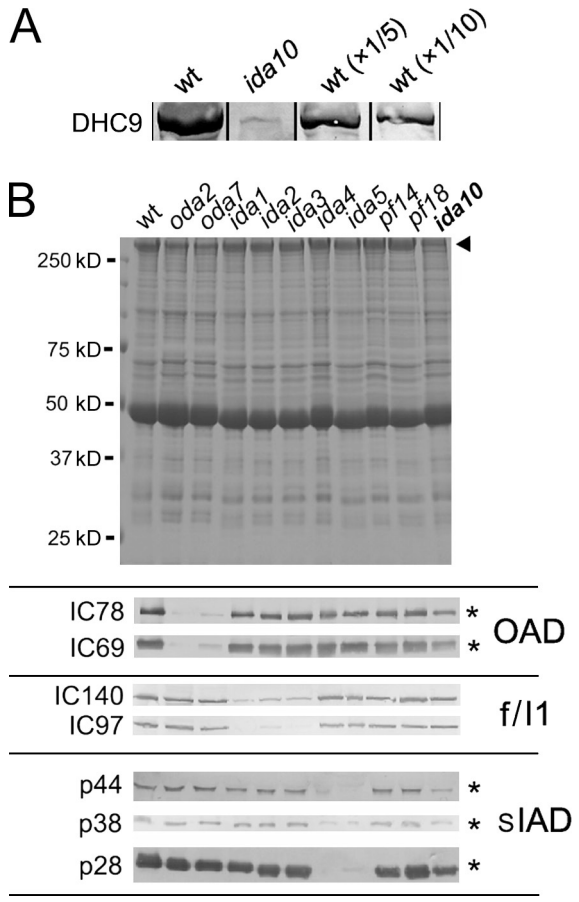

Figure 2. **Immunoblot analyses of an *ida10* axoneme.** (A) The amount of the dynein c heavy chain (DHC9) in the *ida10* axonemes was estimated by densitometry using diluted wild-type axonemes as standards. Equal amounts of axonemes of wild type (wt, lane 1) and *ida10* (lane 2), and wild-type axonemes at two different dilutions (lanes 3 and 4), were immunoblotted with anti-DHC9 antibody. All lanes are from the same blot. The amount of dynein c in the *ida10* axonemes was estimated to be much less than 10% of that in the wild-type axonemes. Black lines indicate that intervening lanes have been spliced out. (B) Immunoblot analyses on equal amounts of various mutant axonemes using dynein subunit antibodies. In the *ida10* axoneme, p28, p38, and p44, which are subunits of single-headed inner arm dyneins, are significantly reduced; and IC78 and IC69, which are intermediate chains of outer arm dynein, are slightly reduced (asterisks). In contrast, IC140 and IC97 (subunits of dynein f/I1) are present, apparently in normal amounts. Note also that the intensity of dynein heavy chain bands in the CBB-stained gel is weaker in *ida10* than in wild type (arrowhead). OAD, outer arm dynein; sIAD, single-headed inner arm dynein.

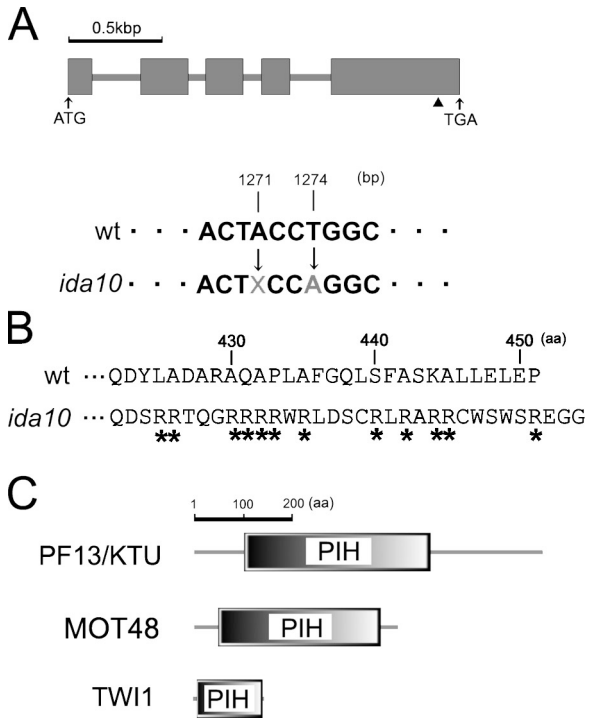

Figure 3. ***ida10* has a mutation in the gene encoding MOT48, a PIH protein.** (A) Structure of the MOT48 gene and the mutation in *ida10*. Boxed areas indicate exons contained in the MOT48 cDNA. There is a one-base deletion (A → X) and a one-base substitution (T → A; arrowhead) in *ida10*, which results in a frame-shift mutation. These mutations occurred at 1,271 and 1,274 bp, respectively, from the initiation codon of MOT48 cDNA. (B) C-terminal sequences of MOT48 proteins in wild type and *ida10*. The calculated pI of MOT48 protein is greatly increased, from 5.94 to 8.98, because of the addition of many arginine residues (asterisks). (C) Domain structures of *C. reinhardtii* MOT48, PF13/KTU (MOT45), and TWI1. These proteins are all predicted to be PIH family proteins by SMART and/or Pfam analyses. PF13/KTU has a PIH region in the N-terminal half, whereas MOT48 and TWI1 have PIH regions in the middle of the entire sequence.

(Fig. 1 C, D), although the amounts were somewhat variable from one culture to another; the amounts of several dyneins tended to slightly decrease in young cultures.

Western blot analyses using antibodies against dynein subunits yielded results consistent with the aforementioned observations; the light chains of single-headed dyneins, p28, p38, and p44 (LeDizet and Piperno, 1995; Yamamoto et al., 2006; Yamamoto et al., 2008) were greatly reduced, and the two intermediate chains of outer arm dynein (King et al., 1985, 1986) were slightly reduced. In contrast, the intermediate chains of dynein f/I1 (Yang and Sale, 1998) were apparently normal (Fig. 2 B). Electron microscopy also showed decreased or absent electron density in the inner arm dynein region and in some outer arm regions in axonemal cross sections (Fig. 1 B). The mean number of outer arm dyneins per cross section was 6.8 ± 1.2 in *ida10*, whereas it was 7.7 ± 1.0 in wild type.

The *ida10* locus codes for MOT48, a protein conserved among organisms having motile cilia and flagella

The *ida10* mutation was mapped near the MOT48 gene on linkage group X. The MOT48 gene codes for a protein specifically present in organisms having motile cilia and flagella (Merchant et al., 2007), and, like other flagella-associated proteins, it is upregulated upon deflagellation (Stolc et al., 2005). In fact, the cDNA and genomic DNA sequences of MOT48 revealed that *ida10* has a one-base deletion (A → X) and a one-base substitution (T → A) near the C terminus of the open reading frame of this gene, resulting in a frame-shift mutation (Fig. 3 A). The cDNA of wild-type MOT48 is predicted to encode a 451–amino acid protein with a molecular weight of 47,282.62 and an isoelectric point (pI) of 5.97, whereas the mutated MOT48 gene in *ida10* cells is predicted to encode a 454–amino acid protein with a molecular weight of 48,238.67 and a pI of 8.98. The increase in pI is caused by the addition of many arginine (R) residues (Fig. 3 B). Such an extremely large change of pI is likely to result in a loss of normal function or rapid degradation of the

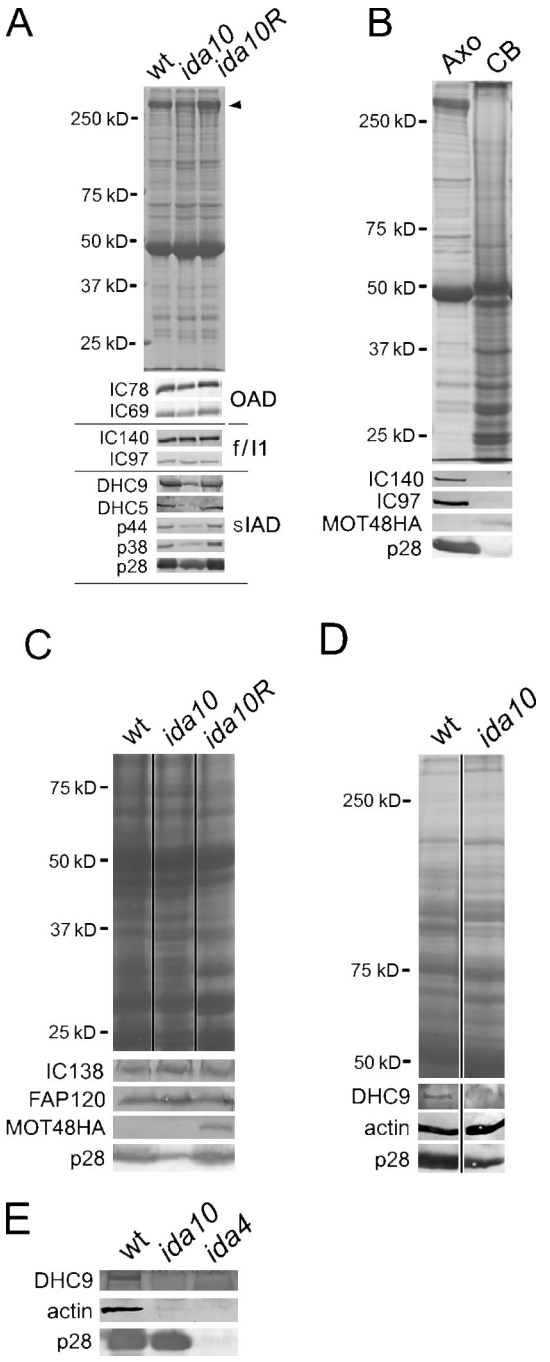

Figure 4. **MOT48 is a cytoplasmic protein required for the preassembly of axonemal dyneins.** (A) Patterns of SDS-PAGE of equal amounts of axonemes from wild type, *ida10*, and *ida10R* stained with CBB, and Western blotted with the antibodies against various proteins related to axonemal dyneins. IC78, IC69, DHC9 (dynein c heavy chain), DHC5 (dynein b heavy chain), p44, p38, and p28 are diminished in *ida10* compared with in wild type (wt), and are recovered in *ida10R*. Note that the band intensity of axonemal dyneins in the CBB-stained gel is also recovered in *ida10R* (arrowhead). (B) SDS-PAGE and Western blot analysis of equal amounts of axoneme and cell body samples from *ida10R*. Although IC140, IC97, and p28 are present mostly in the axoneme, almost all MOT48 is present in the cell body. (C) Western blot of equal amounts of cell bodies from wild type, *ida10*, and *ida10R* using antibodies to IC138, FAP120, HA tag, and p28. The amount of p28 is decreased in *ida10* compared with that in wild type or *ida10R*. The HA tag antibody detects the tagged MOT48 in *ida10R*. The CBB-stained lanes shown were taken from the same gel. (D) Western blot of equal amounts of cytoplasmic extracts from wild type and *ida10* using antibodies to DHC9, actin, and p28. The amount of p28 and dynein heavy chain c is decreased in *ida10* compared with that in wild type, whereas that of actin is slightly increased. Lanes were taken from the same gel and blots. Black lines indicate that intervening lanes have been spliced out. (E) Immunoprecipitation analysis on the cytoplasmic extracts from wild type, *ida4*, and *ida10* cells using anti-p28 antibody. Dynein heavy chain c (DHC9) and actin were precipitated in addition to p28 from the wild type extract, whereas few or none of them were precipitated from the *ida10* extract. This indicates that dynein c is preassembled in wild-type cytoplasm, and that *ida10* has a defect in this process. *ida4* was used as a no-antigen control.

protein in the cytoplasm. Although the actual state of the mutated MOT48 in *ida10* remains to be studied, the possible instability or "leakiness" of the mutation may be responsible for the variable phenotype of *ida10*. It is possible that a completely null mutant may show a more severe dynein-deficient phenotype.

The phenotype of *ida10* can be rescued by transformation with the wild-type MOT48 cDNA containing a 3×HA tag at its C terminus. The swimming velocity and the beat frequency of the rescued cells (*ida10R*) were ∼130 µm/s and ∼60 Hz, respectively, both being close to those of wild-type cells. The axonemal amounts of the single-headed dynein subunits—DHC9 (dynein c heavy chain), DHC5 (dynein b heavy chain), p44, p38, and p28—were also recovered almost to the wild-type level (Fig. 4 A).

MOT48 functions in the cytoplasm

Western blots of cell bodies and axonemes of the rescued strain, *ida10R*, using an anti-HA antibody showed that MOT48 is mostly localized in the cell body and that little, if any, is present within flagella (Fig. 4 B). This pattern is distinct from that of intraflagellar transport components. Together with the fact that MOT48 is not contained in the flagellar proteome database (Pazour et al., 2005), these results indicate that MOT48 is a cytoplasmic protein working in the assembly or maintenance of axonemal dyneins. Consistent with this idea, the amount of p28, a light chain associated with dyneins a, c, and d (LeDizet and Piperno, 1995; Yanagisawa and Kamiya, 2001), was reduced in both the cell body and the cytoplasmic extract of *ida10* compared with those of wild type and *ida10R*, whereas the amounts of IC138 and FAP120, subunits associated with dynein f/I1 (Hendrickson et al., 2004; Ikeda et al., 2009), were apparently normal (Fig. 4, C and D). In the cytoplasmic extract from *ida10*, the heavy chain of dynein c (DHC9) was also greatly reduced, whereas actin was slightly increased. These results suggest that, in the cytoplasm of *ida10*, DHC9 and p28 undergo rapid degradation, whereas actin accumulates. Such instability of some dynein subunits and accumulation of some other subunits in assembly-blocked cytoplasm has been observed in the *C. reinhardtii* mutant *pf13* (Omran et al., 2008). MOT48 is likely to be needed for the stability of the heavy chains and p28 in the cytoplasm as a factor that aids the preassembly of inner arm dynein complex. It may well directly promote subunit preassembly, or, alternatively, indirectly promote it by functioning as a co-chaperone required for the proper folding of the DHC9 polypeptide.

Immunoprecipitation experiments using anti-p28 antibody precipitated the dynein c heavy chain and actin from the cytoplasmic extract of wild type, whereas it precipitated only a little of them from the *ida10* extract (Fig. 4 E). This result

strongly supports our hypothesis that dynein c is preassembled in the wild-type cytoplasm, but not in the *ida10*.

MOT48 contains a PIH domain

Sequence analysis using the programs SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) indicated that MOT48 contains a PIH domain in the middle of its structure (Fig. 3 C). A protein with this domain was first identified in *Saccharomyces cerevisiae* as a co-chaperone possibly involved in preribosomal RNA processing. This protein, NOP17 (also known as PIH1), is required for either nucleolar retention or correct assembly of the box C/D small nucleolar RNP, but is not essential for the viability of the organism (Gonzales et al., 2005; Boulon et al., 2008; Zhao et al., 2008). As stated in the Introduction, a PIH domain was also found in PF13/KTU (also called MOT45), a conserved protein involved in the cytoplasmic preassembly of axonemal dyneins (Omran et al., 2008). Defects in the homologue of this protein in *C. reinhardtii* cause loss of outer arm dynein and inner arm dynein subspecies c as indicated by analysis of the mutant *pf13* (Fig. 1 C; Omran et al., 2008). Both PF13/KTU and MOT48 show weak similarities to NOP17. Judging from their structural similarity as well as from the similar dynein defects in the *pf13* and *ida10* mutants, the two PIH proteins are likely to play similar roles in axonemal dynein preassembly. *C. reinhardtii* apparently uses PF13/KTU mainly for outer arm dynein and MOT48 mainly for inner arm dyneins, although their functions are somewhat redundant and not strictly specific.

We attempted to detect interaction between MOT48 and heat shock protein 70 (HSP70) in the cytoplasm because PF13/KTU has been suggested to function as a cofactor of HSP70. However, our immunoprecipitation assays using the cytoplasm from the rescued strain (*ida10R*) and an anti-HA antibody did not detect any interaction of MOT48 with HSP70 or other proteins. Thus, the mechanism of MOT48 function remains to be elucidated. Because MOT48 and other PIH family proteins do not contain apparent DNA-binding motifs, it seems unlikely that they function in transcription levels, although we cannot completely rule out this possibility.

The double mutant *ida10pf13* was found to produce extremely short flagella (~1 µm) but was still viable. The reason for the flagellar growth defects is not understood, except that mutants lacking both outer arm dynein and certain inner arm dyneins frequently have similar defects.

PIH proteins are present in organisms having motile cilia and flagella

A Basic Local Alignment Search Tool (BLAST) search with the *C. reinhardtii* MOT48 sequence identified putative homologues in various organisms with motile cilia and flagella, such as human, zebrafish, *Tetrahymena*, and *Trypanosoma* (Figs. 5 A and S1 A). In contrast, no obvious homologues were found in organisms with only immotile cilia, such as *Caenorhabditis elegans*. A BLASTp amino acid sequence search of nonredundant human protein sequences with the *C. reinhardtii* MOT48 sequence, using default parameters, detected three putative homologous proteins: NOP17 (BLAST E value = 5×10^{-11}), PIH1 domain containing 2 (2×10^{-6}), and PF13/KTU (1×10^{-5}). In *C. reinhardtii*, PF13/KTU was most similar to MOT48. In addition, a sequence search in the *C. reinhardtii* genome database detected another conserved PIH protein, TWI1 (Figs. 3 C and S1 B). This protein is a putative homologue of zebrafish TWISTER protein, which was shown to be responsible for a polycystic kidney and curved body (Sun et al., 2004). Intriguingly, these phenotypes of fish can be caused by defects in ciliary motility (Kramer-Zucker et al., 2005). In addition, like MOT48, TWI1 is strongly up-regulated upon deflagellation (Stolc et al., 2005). Thus, all the PIH proteins that we found in *C. reinhardtii* appear to be closely related to the function of flagella (Table S1). It is tempting to speculate that TWI1 functions in the preassembly of dyneins a, f/I1, and g; i.e., dyneins whose assembly does not involve MOT48 or PF13/KTU (Fig. 5 B).

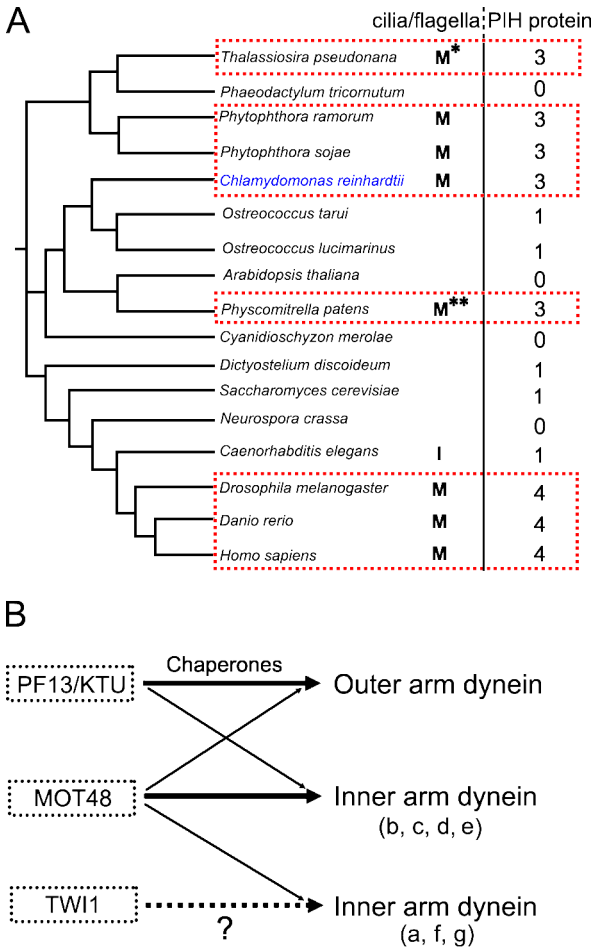

Figure 5. **PIH proteins are present in organisms having motile cilia and flagella.** (A) A phylogenetic tree modified from Merchant et al. (2007). Organisms are marked as having motile (M) or immotile (I) cilia and/or flagella. A BLAST search identified 3–4 PIH proteins in the genome of organisms that have motile cilia and flagella (marked with red boxes), but only 0–1 such protein in organisms without motile cilia or flagella. This suggests conserved roles of the PIH proteins in the formation of motile cilia and flagella. The number on the right side of each species indicates the number of PIH proteins found in the genome database. The male gametes of *Thalassiosira pseudonana* (M*) have motile axonemes without inner arm dyneins, and those of *Physcomitrella patens* (M**) have motile flagella without outer arm dynein. (B) A proposed model of three axonemal dynein preassembly systems. The three *C. reinhardtii* PIH proteins—PF13/KTU, MOT48, and TWI1—may function for different subsets of dyneins, although there is some redundancy.

The genome databases of ciliated eukaryotes that we examined have three to four genes coding for PIH proteins, including potential homologues of MOT48, PF13/KTU, and TWI1, whereas those of nonciliated organisms typically contain no, or only one, such protein (Fig. 5 A). Phylogenetic analyses of the PIH family proteins in nonciliated organisms showed that most of them also fall into families related to MOT48, PF13/KTU, or TWISTER. This observation raises the possibility that PIH family proteins in ciliated organisms may also function in preribosomal RNA processing, in addition to their function in dynein preassembly. This possibility remains to be studied in future studies. At any rate, although whether TWI1 actually functions in dynein preassembly in *C. reinhardtii* remains to be studied in future studies, the results presented in this report suggest that PIH proteins generally function in the preassembly of axonemal dyneins, each possibly functioning for a subset of a few dynein species.

Materials and methods

Strains and culture

The strains used in this study were *C. reinhardtii* wild-type 137c and the mutants listed in Table S2. The novel mutant, *ida10*, was produced by UV mutagenesis and selected as a slow-swimming mutant. The mutant *ida10* was crossed with the S1D2 strain for amplified fragment length polymorphism (AFLP) mapping (Kathir et al., 2003). All cells were grown in liquid Tris-acetate/phosphate medium (Gorman and Levine, 1965) with aeration on a 12 h/12 h light/dark cycle.

Preparation of axonemes, whole cell body and cytoplasmic extract

Flagella were obtained by dibucaine-induced deflagellation followed by centrifugation. The flagella were extracted with 0.2% Nonidet P-40 in HMDEK solution (30 mM Hepes, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM EGTA, and 50 mM potassium acetate, pH 7.4) and centrifuged to remove the membrane and matrix fraction, then the resultant axonemes were suspended in HMDEK (Witman, 1986). For SDS-PAGE of *C. reinhardtii* cell bodies, cells were deflagellated and treated with methanol and chloroform to remove DNA and RNA fractions. For SDS-PAGE and immunoprecipitation of the cytoplasmic extract, cells were deflagellated, sonicated in HMDEK, and ultracentrifuged to remove insoluble fractions.

Separation of axonemal dyneins

Dynein species in mutant axonemes were analyzed according to Kagami and Kamiya (1992). Axonemes were precipitated by centrifugation, suspended in HMDE (30 mM Hepes, 5 mM $MgSO_4$, 1 mM dithiothreitol, and 1 mM EGTA, pH 7.4) containing 0.6 M KCl, left on ice for 30 min, and then precipitated. The supernatant containing dynein was retained. The supernatant fractions containing dynein were diluted sixfold with HMDE solution to lower the final KCl concentration. The solution was centrifuged at 27,000 *g* for 15 min before applying to a Mono-Q column (GE Healthcare). High-pressure liquid chromatography was performed using a SMART system (GE Healthcare).

Phenotypic rescue experiment of *ida10*

A plasmid vector, modified from pGenD (Fischer and Rochaix, 2001), was used for the rescue experiments. The *aphVIII* gene, which confers paromomycin resistance, and a DNA fragment coding for the 3×HA epitope were inserted into the plasmid so that the expressed product was tagged at the C terminus with a 3×HA tag. The coding region of the wild-type cDNA of MOT48 was amplified by PCR with the primers MOT48-NF2 (5′-GGATCCCATATGCTGATAACAGATAACATGCTAGGGAAACAAGAA-3′) and MOT48-NR2 (5′-GAATTCCGGCTCCAGCTCCAGCAGCGCCTTGCTCGCAAACGACAG-3′), which contained the recognition sites for BamHI–NdeI and EcoRI, respectively (underlined). The NdeI–EcoRI cDNA fragment of MOT48 was cloned into the pT7-Blue T-vector, ligated into the pGenD vector, and used for transformation of *ida10* cells by electroporation. The synthesis of cDNA and isolation of total wild-type RNA were performed as described previously (Yamamoto et al., 2008). The cDNA sequence of *C. reinhardtii* MOT48 has been deposited in the DNA Data Bank of Japan (DDBJ) under accession No. AB531999.

SDS-PAGE and immunoblotting

SDS-PAGE was performed using 8% or 10% acrylamide gels (Laemmli, 1970), or 3–5% acrylamide gradient gels with a 3–8 M urea gradient. Gels were used for immunoblot analysis, or stained with Coomassie brilliant blue (CBB) or silver. Immunoblot procedures were modified from those of Towbin et al. (1979). The primary antibodies used were as follows: anti-IC78 (1878A; King et al., 1985), anti-IC69 (1869A; King et al., 1986), anti-IC140 (provided by W. Sale, Emory University School of Medicine, Atlanta, GA; Yang and Sale, 1998), anti-IC138 (provided by W. Sale; Hendrickson et al., 2004), anti-IC97 (provided by W. Sale; Wirschell et al., 2009), anti-FAP120 (Ikeda et al., 2009), anti-actin (Kato-Minoura et al., 1997), anti-p44 (Yamamoto et al., 2008), anti-p38 (Yamamoto et al., 2006), anti-p28 (provided by G. Piperno, Mount Sinai School of Medicine, New York, NY; LeDizet and Piperno, 1995), anti-DHC9 (provided by T. Yagi, University of Tokyo, Tokyo, Japan; Yagi et al., 2009), anti-DHC5 (provided by T. Yagi; Yagi et al., 2009), and anti-HA-tag (rat monoclonal antibody, 3F10).

Immunoprecipitation

Immunoprecipitation from cytoplasmic extracts of the wild type or the *ida10* mutant was performed by the method of Fowkes and Mitchell (1998) using the anti-p28 antibody. Protein A beads (Roche) were added to the mixture of the antibody, the cytoplasmic extract, and immunoprecipitation (IP) buffer, and the mixture was left standing at 4°C for overnight. The precipitates were washed three times with IP buffer without BSA, and the samples were boiled and processed for SDS-PAGE and Western blotting with antibodies against DHC9, actin, and p28. Immunoprecipitation with DHC9 antibody did not yield clear results. This was most likely due to the low titer of the antibody.

Other methods

Flagellar motility was measured as described previously (Kamiya, 2009). In brief, the swimming velocities of cells were measured in video recordings of microscope images. More than 20 cell images were measured and averaged. Mean beat frequency was obtained by analyzing the vibration frequency of cell bodies in a population of swimming cells using a Fast Fourier transform analyzer (A&D Company). Electron microscopy was performed as described previously (Kamiya et al., 1991). Protein concentration was measured using the method of Bradford (1976). For the sequence comparison, data were aligned using ClustalW and the output was processed with Jalview (http://www.jalview.org/). Protein motifs were obtained using SMART and Pfam analyses.

Online supplemental material

Fig. S1 shows a sequence comparison of *C. reinhardtii* MOT48 homologues and zebrafish Twister homologues. Table S1 shows dynein species missing in *pf13* and *ida10*. Table S2 lists the mutants used. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201002081/DC1.

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