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

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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, ida10, we identified another conserved 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, TW11, 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.
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 represent 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 dyneins a, f/I1, and g were almost normal or only slightly reduced.

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).
The mean number of outer arm dyneins per cross section was 6.8 ± 1.2 in \textit{ida10}, whereas it was 7.7 ± 1.0 in wild type. The \textit{ida10} locus codes for \textit{MOT48}, a protein conserved among organisms having motile cilia and flagella. The \textit{ida10} mutation was mapped near the \textit{MOT48} gene on linkage group X. The \textit{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 \textit{MOT48} revealed that \textit{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 \textit{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 \textit{MOT48} gene in \textit{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 protein (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).

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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/11 (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 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.
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 pre-assembly 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 x 10^{-11}), PIH1 domain containing 2 (2 x 10^{-6}), and PF13/KTU (1 x 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/H1, and g; i.e., dyneins whose assembly does not involve MOT48 or PF13/KTU (Fig. 5 B).
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 TWISTTER. This observation raises the possibility that the 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 axonal 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 MgSO4, 1 mM dithiothreitol, and 1 mM EGTA, Kamiya (1992). Axonemes were precipitated by centrifugation, suspended in HMDE (Witman, 1986). For SDS-PAGE of axonemal dyneins, each possibly functioning for a subset of a few dynein species.

**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 (Ikedo 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 zebrastraw Twist 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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