学位論文

Studies on protein interactions required for the outer arm dynein assembly on the axonemal microtubule in *Chlamydomonas*

（外腕ダイニンのクラミドモナス軸糸微小管上への
構築に必要な蛋白質間相互作用の研究）

平成 25 年 7 月博士(理学)申請

東京大学大学院理学系研究科

生物科学専攻

井手隆広

Studies on protein interactions required for the outer arm dynein assembly on the axonemal microtubule in *Chlamydomonas*

Doctoral Dissertation

Submitted to the Graduate School of Science, University of Tokyo

Takahiro Ide

July, 2013

CONTENTS

Abbreviations

ATP:	adenosine 5’-triphosphate
BMH:	bismaleimidohexane
BSA:	bovine serum albumin
CBB:	Coomassie brilliant blue
DMSO:	dimethyl sulfoxide
DTT:	dithiothreitol
EGTA:	ethylene glycol bis (β-aminoethylether)-*N,N,N’,N’*,-tetraacetic acid
GTP:	guanosine 5’-triphosphate
HC:	heavy chain
HEPES:	*N*-2-hydroxyethyl-piperazine-*N’*-2-ethanoesulfonic acid
HPLC:	high pressure liquid chromatography
IC:	intermediate chain
IFT:	intraflagellar transport
LC:	light chain
OAD*:	outer arm dynein
ODA-DC*:	outer dynein arm docking complex
PIPES:	piperazine-*N,N’*-bis-(2-ehtanesulfonic acid)
QCM:	quarts crystal microbalance
SDS-PAGE:	sodium-dodecyl sulfate polyacrylamide gel electrophoresis
TAP:	tris acetic acid phosphate
Tris:	tris (hydroxymethyl) aminomethane

*: In this manuscript, “OAD” represents outer arm dynein that does not contain the ODA-DC. “Outer dynein arm” represents the structure that is observed on the outer surface of the doublet by electron microscopy.

Abstract

Motile cilia and flagella have a conserved structure from unicellular organisms to higher vertebrates. They are composed of more than 250 proteins. It is largely unknown how these components are assembled into the elaborate organelle. The outer arm dynein (OAD) of cilia and flagella is bound to specific loci on outer-doublet microtubules. Previous studies showed that the OAD-microtubule binding is achieved by interactions at two sites: intermediate chain 1 (IC1) and the outer dynein arm docking complex (ODA-DC) located at the base of OAD. Studies using *Chlamydomonas* mutants have suggested that the two sites have rather weak affinities to microtubules, and therefore strong OAD attachment to microtubules is achieved by their cooperation. To test this hypothesis, I examined interactions between IC1, IC2 (the other intermediate chain) and the ODA-DC using recombinant proteins. The recombinant IC1 and IC2 were found to form a 1:1 complex, and the IC1-IC2 complex further associates with the ODA-DC in vitro. Binding assays of IC1 to the axoneme suggested that there are specific binding sites for IC1 other than the ODA-DC. Judging from the dissociation constants, the binding affinities of the ODA-DC and the IC1-IC2 complex to the cytoplasmic microtubules are more robust than that of IC1. The affinity of OAD co-purified with the ODA-DC was slightly higher than that of the ODA-DC. These data support the above hypothesis and suggest a novel model that IC1 and the ODA-DC cooperate to make the OAD-doublet binding sufficiently strong and stable.

General Introduction

Eukaryotic cilia and flagella are hair-like organelles that project from the cell surface. Recent studies suggest that various types of cells in a wide range of organisms have either motile or non-motile cilia (Gibbons, 1981). Whereas non-motile cilia perform sensory functions, motile cilia and flagella function to produce cellular movements or fluid flow over a stationary cell surface (Kobayashi and Takeda, 2012). Motility defects in human cilia and flagella frequently result in a syndrome collectively called primary ciliary dyskinesia (PCD), which involves a variety of deficits such as infertility, respiratory/liver defects and left-right axis randomization (Nonaka et al., 1998; Pazour and Rosenbaum, 2002; Hirokawa et al., 2006; Zariwala et al., 2007). Correct assembly of functional cilia and flagella is essential for the development and viability of humans and other vertebrates.

The core structure of cilia and flagella, the axoneme, displays a highly conserved nine-fold symmetrical arrangement of doublet microtubules with two singlet microtubules at the center. It is composed of ~250 proteins and called the 9+2 structure (Gibbons, 1981; Dutcher, 1995). Along each outer doublet microtubule, several discrete components such as inner arm dynein, outer arm dynein, radial spokes and inter-doublet links are aligned at a unit repeat length of 96 nm (Goodenough and Heuser, 1985; Mastronarde et al., 1992). The mechanism of the regular arrangement of these components, as well as that of the formation of the 9+2 structure is largely unknown. In the present study, I explored how OAD is periodically arranged on the outer doublets using *Chlamydomonas reinhardtii*, a model organism of cilia/flagella studies. OADs are arranged in a single row with a 24 nm spacing (Fig.1; Yagi et al., 2009), whereas seven major types of inner arm dyneins, called subspecies a ~ g, are arrayed within the 96 nm periodic unit along the length of the A-tubule. These

dyneins hydrolyze ATP and transiently interact with adjacent microtubules to generate force.

OAD consists of three heavy chains (HCs: α, β, and γ), two intermediate chains (ICs: IC1 and IC2), and 11 light chains (LCs: LC1~LC10 containing LC7a and LC7b) (Fig. 2). It is an assembly having three globular "heads" composed of the C-terminal regions of HCs and a "tail" composed of the N-terminal regions of HCs, ICs and LCs. At the base of the tail, a complex called the outer dynein arm docking complex (ODA-DC) is present and mediates the binding of OAD to the doublet (Takada and Kamiya, 1994; Takada and Kamiya, 1997). The ODA-DC is composed of three subunits, DC1, DC2 and DC3 (Fowkes and Mitchell, 1998; Wakabayashi et al., 2001). In a recently elucidated pathway of OAD assembly in the cytoplasm, ICs and some LCs are first assembled to form an IC-LC complex, which then serves as the platform on which HCs and the other LCs are assembled with the help of Ktu/PF13 protein (Omran et al., 2008). In parallel, three DC proteins assemble into the ODA-DC in the cell body. The pre-assembled OADs and the ODA-DCs are transported to flagella by intraflagellar transport (IFT) independently and combined with each other on the outer doublets (Hou et al., 2007).

Recent studies have raised an interesting possibility that OAD has two sites for the connection with the axonemal microtubules. These are intermediate chain 1 (IC1), and the ODA-DC. However, analyses of mutants lacking these sites have indicated that the affinity of each interaction is apparently insufficient for the axonemal attachment of OAD. Therefore I hypothesized that a multiple weak interaction sites, such as IC1 and the ODA-DC, associate with each other and cooperate to produce a sufficiently strong affinity. To test this hypothesis, I carried out qualitative and quantitative analyses using recombinant proteins.

In Part 1, I described the results of qualitative analysis. The interactions of components at the base of OAD were analyzed by immunoprecipitation and chemical

crosslinking *in vitro*. Because the base of dynein is observed to form a cluster by electron microscopy, direct protein-protein interactions have not been examined in detail. My results have revealed direct interactions between the ICs and the ODA-DC. In Part 2, I described the results of quantitative analysis. To quantify the interactions between molecules, determination of dissociation constants (K_D) is essential. The binding affinity of recombinant proteins to axonemes or cytoplasmic microtubules was measured using co-precipitation assays and a Quarts Crystal Microbalance (QCM). From the results of these two parts, I presented a novel model of OAD-doublet binding in General Discussion.

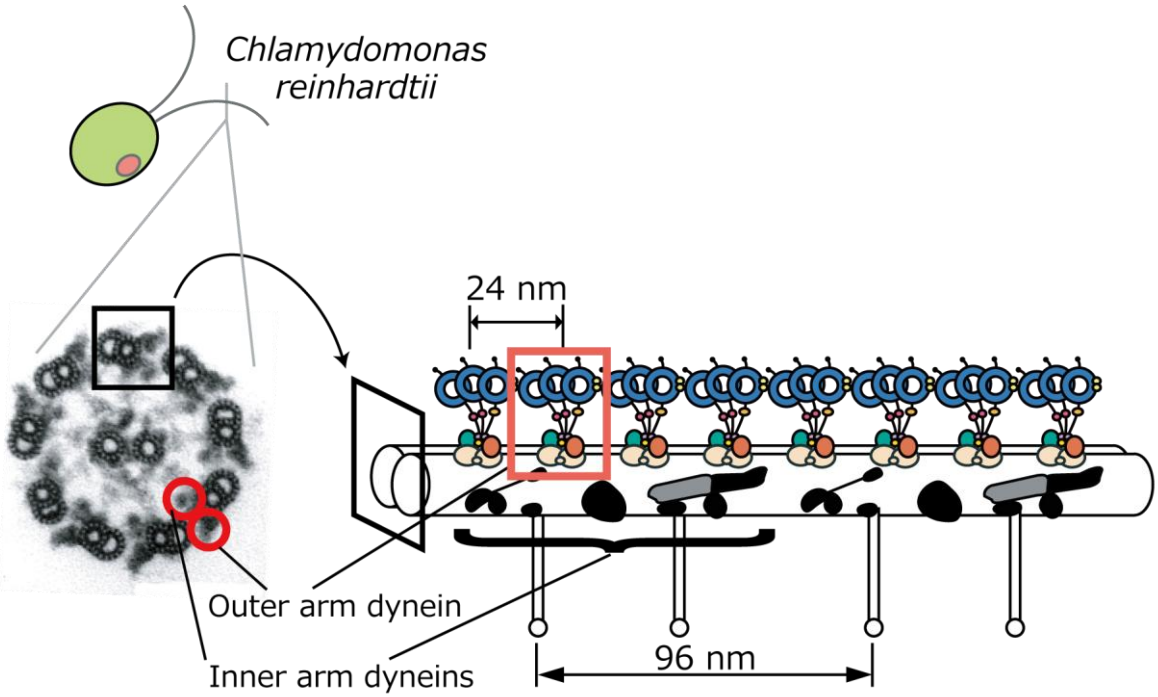

Fig. 1 | The diagram of the *Chlamydomonas* doublet microtubule. OADs are arranged in a single row with a 24 nm spacing. Seven inner arm dyneins are arrayed within the 96 nm periodic units along the length of the A-tubule.

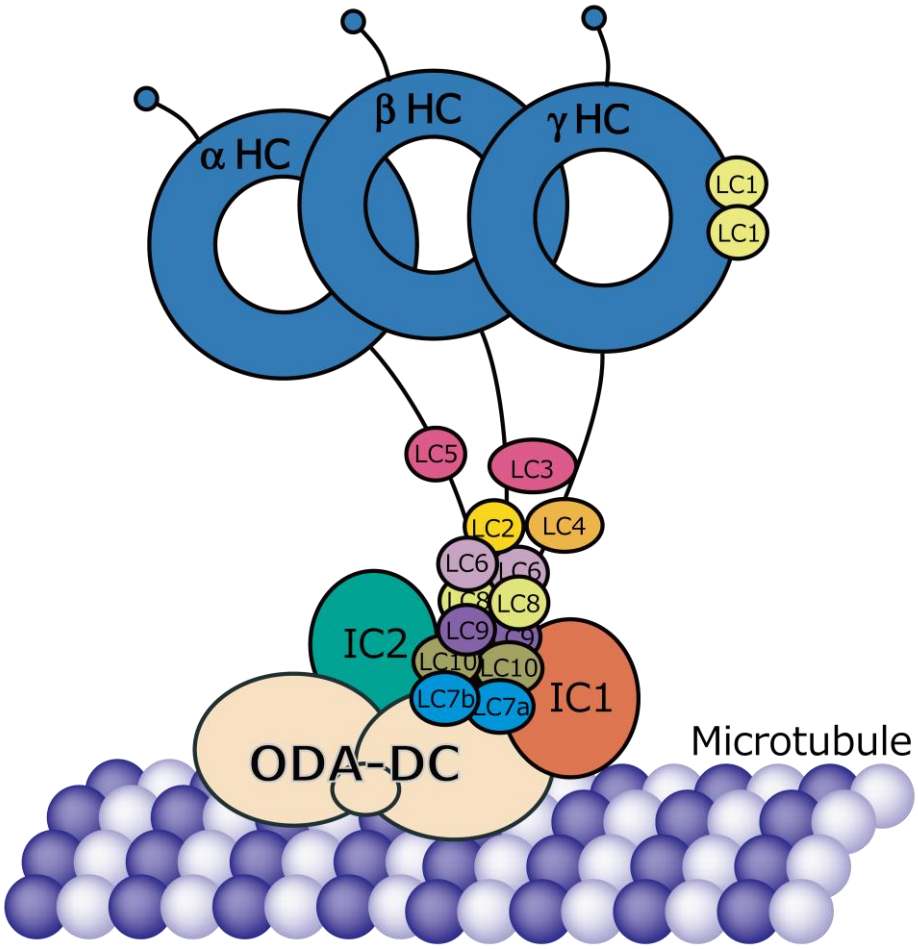

Fig. 2 | The model of the outer arm dynein. Three rings with projections represent heavy chains, and small circles represent light chains. OAD consists of three heavy chains (HCs: α, β, and γ), two intermediate chains (ICs: IC1 and IC2), and 11 light chains (LCs: LC1~LC10 containing LC7a and LC7b).

Part 1

Identification of the protein-protein interactions in outer arm dynein

Abstract

Outer arm dynein (OAD) of cilia and flagella is bound to specific loci on outer-doublet microtubules. Previous studies showed that the OAD-microtubule binding is achieved by interactions at two sites: intermediate chain 1 (IC1) and the outer dynein arm docking complex (ODA-DC) located at the base of OAD. The interaction between OAD and the ODA-DC is known to occur between LC7b, a light chain of OAD, and DC2, an ODA-DC subunit. Though intermediate chains of OAD and the ODA-DC seemed to be in the same proximity by electron microscopy, interactions between these proteins have not been found. In this part, I expressed recombinant intermediate chain proteins and tested if they interact with the ODA-DC recombinant subunits DC1 and DC2. Immunoprecipitation and chemical crosslinking revealed that IC1 and IC2 interact directly. This IC1-IC2 complex makes complex with DC1-DC2 complex, presumably via interactions between IC1-DC1 and IC2-DC1. From these results, I renewed the interaction map between the proteins at the base of OAD.

Introduction

Axonemal dyneins in cilia and flagella are attached to the doublet microtubules and generate force against the adjacent doublet microtubule. Outer arm dynein (OAD), which generates ~70% of total propulsive force in the axoneme (Minoura and Kamiya, 1995), binds to specific sites on the A-tubule with a regular spacing of 24 nm. How binding to specific axonemal sites is achieved remains a fascinating unanswered question.

IC1 and the ODA-DC, a protein complex located at the base of OAD, are considered to be important for OAD-doublet association from following two studies: IC1 was shown to directly bind to α-tubulin by chemical crosslinking (King et al., 1991); mutants lacking the ODA-DC lack OAD in the axoneme, even though a complete OAD complex is assembled in the cytoplasm (Takada and Kamiya, 1994; Fowkes and Mitchell, 1998). Binding of the ODA-DC and OAD involves an interaction between an LC (LC7b) and DC2 (DiBella et al., 2004). A study on a mutant lacking DC3 indicates that DC1 and DC2 are responsible for the binding of OAD to the ODA-DC (Casey et al., 2003).

Despite the postulated importance of IC1 and the ODA-DC for OAD attachment to the doublet microtubule, the available data indicate that both IC1 and the ODA-DC have rather weak affinity for axonemal doublet microtubules. First, OAD cannot bind to the doublets in mutant axonemes that lack the ODA-DC; this suggests that the IC1-doublet microtubule interaction is not very strong. Second, the ODA-DC binding to the doublet appears to be incomplete without OAD, because the amount of the ODA-DC attached to outer-doublets is reduced in the axoneme of mutants that cannot assemble OAD (such as *oda2* (ΔHCγ), *oda4* (ΔHCβ) and *oda6* (ΔIC2)) (Takada and Kamiya, 1994). Thus, the ODA-DC cannot bind to the doublet strongly enough without OAD, while OAD cannot bind to the

doublet without the ODA-DC. The inter-dependence of OAD and the ODA-DC in their microtubule binding suggests that there must be some unknown protein-protein interaction(s) between OAD, the ODA-DC and the doublet that strengthen OAD docking and assembly.

In this part, to further explore the mechanism of OAD - ODA-DC interaction(s), I performed protein-protein interaction analyses between IC1, IC2, DC1 and DC2 using recombinant proteins.

Materials and Methods

Strains and culture of Chlamydomonas reinhardtii *cells*

A *Chlamydomonas reinhardtii* wild-type strain (CC124) and the following mutants lacking outer dynein arms (Kamiya, 1988) were used: *oda1*, with a mutation in the structural gene of DC2, an ODA-DC subunit with predicted mass of 62.5 kDa but Mr of 70 k (Takada et al., 2002); *oda6*, with a mutation in the structural gene of IC2, the 63.4 kDa intermediate chain of OAD (Mitchell and Kang, 1991); *oda9*, with a mutation in the structural gene of IC1, the 76.5 kDa intermediate chain of OAD (Wilkerson et al., 1995). A double mutant of *ida4* (Kamiya et al., 1991) × *oda6*; *ida4* × *oda9* was produced by standard procedure (Harris, 1989). All cells were grown in Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) with aeration at 25°C , on a 12h/12h light/dark cycle (Gorman et al., 1965).

Preparation of Chlamydomonas axonemes

Flagellar axonemes were isolated from *Chlamydomonas reinhardtii oda1* strain by the method of Witman (1986). In short, *Chlamydomonas* cells were vigorously suspended in HMS (10 mM HEPES pH 7.4, 5 mM $MgSO_4$, 4% sucrose) containing 5 mM dibucaine-HCl. After removal of cell bodies by centrifugation at 3,000 ×g for 5 min at 4°C , detached flagella were harvested by centrifugation at 25,000 ×g for 10 min. Flagella were demembranated with HMDEK containing 0.1% Nonidet-P40. Axonemes were resuspended with HMDEK (30 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM EGTA, and 50 mM K-acetate).

Preparation of recombinant IC1, IC2, DC1 and DC2

IC1 and/or IC2 cDNAs were cloned into a vector pFastBacDual (Invitrogen,

Carlsbad, CA) with or without a 6×His-tag for either single- or co-expression. IC1 was tagged with 6×His at the N-terminus (for IP) or the C-terminus (for other experiments), and not tagged for experiments that assayed co-purification with IC2. Recombinant baculoviruses were generated with these constructs using the Bac-to-Bac protocol following the manufacturer's recommendations (Invitrogen) using *Sf21* insect cells. Recombinant viruses to co-express DC1 and DC2 were provided by Dr. Ken-ichi Wakabayashi (Wakabayashi, 2001).

For protein expression and purification, *Sf21* cells were infected with the recombinant viruses for three days. Recombinant proteins were purified by Ni-NTA agarose (QIAGEN, Hilden, Germany) as described by the manufacturer, with slight modifications (0.6 M NaCl was added to all the solutions to obtain greater amount of proteins).

Preparation of porcine brain tubulin and polymerization of cytoplasmic microtubules

Tubulin was purified from porcine brain by cycles of assembly and disassembly in vitro in a high-morality PIPES buffer (Castoldi and Popov, 2003). Purified tubulin was mixed with microtubule assembly buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP) containing ~1/10 volume of DMSO and incubated at 37˚C for 30 min. Polymerized microtubules were diluted to 1:100 in HMDEK containing 10 μM paclitaxel (SIGMA, St. Louis, MO, USA) and 0.1% DMSO, and ultracentrifuged at 400,000 ×g for 30 min at 4°C to remove unpolymerized tubulin. The precipitates of microtubules were suspended with HMDEK containing paclitaxel.

Immunoprecipitation

For immunoprecipitation of DC2 and IC2, protein A-agarose beads (Roche, Basel, Switzerland) were washed three times with blocking buffer (TBS, pH 7.2, 3% BSA (w/v),

1%Triton-X100 (v/v)) and incubated with the anti-DC2 antibody (Wakabayashi et al.,2001) and anti-IC2 antibody (SIGMA) for 1 hour at 4°C. The beads were then washed twice with blocking buffer and once with HMDEK buffer with addition of 0.1 M KCl (to obtain greater amount of DC1-2 complex), and incubated with purified recombinant proteins for 1 hour at 4°C . The resultant beads were washed three times with HMEK buffer (HMDEK without DTT) and resuspended with SDS sample buffer.

Chemical crosslinking of immunoprecipitated products

Recombinant proteins were mixed in HME buffer (HMDEK without DTT or K-acetate) with 0.1 M KCl at a final concentration of 0.3 mg/ml, and treated with a chemical crosslinker bismaleimidohexane (BMH) (Pierce Chemical, Rockford, IL, USA) for 1 h at room temperature (King et al., 1991). Reactions were terminated by the addition of SDS-PAGE sample buffer containing 2-mercaptoethanol. The resultant samples were analyzed by western blotting.

Electroporation

Electroporation was carried out after Hayashi et al. (2001). The mutant *ida4ida6* or *ida4ida9* cells were digested in an autolysin solution prepared from mating gametes, as described in Harris (1989), to remove the cell wall. The digested cells were washed with HMKCaS buffer (30 mM HEPES pH 7.4, 5 mM $MgSO_4$, 50 mM K-acetate, 1 mM Ca-acetate, 60 mM sucrose) and suspended in same solution (final concentration: 1×10^8 cells/ml) with recombinant IC1 (*ida4ida9*) or IC2 (*ida4ida6*) at appropriate concentrations (0.2 ~ 1.0 mg/ml). A 125 μl aliquot of the mixture was transferred to the electroporation cuvette (Model 620, BTX, San Diego, CA) and subjected to an electric pulse in an ECM600 electroporation

apparatus (BTX). Electroporation was carried out at a variable voltage, with the resistance set to 24 Ω and the conductance to 600 μF. The overall time constant used in standard experiments was about 15 msec. After pulse application, the cuvette was placed at 15°C for 30 min and stirred every 5 min. Then, cells were suspended in 500 μl of TAPS (TAP medium with 60 mM sucrose) (Shimogawara et al., 1998) after washing with the same solution and then left at room temperature. Electroporated cells were observed and recorded under a dark-field microscope. The recovery rate was calculated using flagellated cells that beat at least twice in 20 seconds. Motility measurements were performed at room temperature.

Results

Expression and purification of recombinant IC1, IC2, DC1 and DC2

I used a baculovirus system to obtain protein samples. This system yielded much greater amounts of recombinant IC1 and IC2 than that in vitro translation system used in a previous study (King et al., 1995) and allowed me to perform quantitative biochemical studies. Recombinant IC1 and IC2 were successfully expressed in insect culture cells, with ~50% of the produced proteins being soluble (Fig. 3A). Recombinant IC1 and IC2 tagged with 6×His were partially purified with Ni-NTA agarose beads (Fig. 3B). When IC1 without a His-tag and His-tagged IC2 were co-expressed, they could be co-purified with Ni-NTA (Fig. 3B), suggesting that these proteins are associated with each other in the cultured cell. This idea was further supported by the observation that anti-IC2 antibody immunoprecipitated both IC2 and IC1 from the mixture of these proteins (Fig. 4A). Densitometry of Coomassie blue-stained gels of co-purified IC1-IC2His indicated that the stoichiometry of IC1 and IC2 is 1:1; this assumes that they have equal affinity for the dye. As one copy of each of these proteins is present in the native OAD (King et al., 1989), I conclude that they form a heterodimer; however, this must be verified in future studies. I hereafter refer to the recombinant protein complex of IC1 and IC2 as “IC1-2”.

The recombinant proteins thus obtained were assayed for their functional activity using an electroporation-mediated protein delivery method (Hayashi et al., 2001; Kohno et al., 2011). Recombinant IC1 or IC2 were introduced into mutant cells that lacked the respective proteins and an inner arm dynein, *ida4oda9* and *ida4oda6*, and were thus immotile. These non-motile mutants become motile if the OAD is restored. After introduction of the recombinant proteins and incubation for a few hours, ~0.1% of cells did display flagellar

motility (Table 1), albeit slower than the *ida4* mutant. The small number of motile cells and the low level of recovered motility are most likely due to inefficiency in the delivery of sufficient proteins of such high molecular weight (Kohno et al., 2011). However, as some motility was restored, the recombinant ICs must be functional as the subunits of OAD.

As described in a previous study, recombinant DC1 and DC2 were solubilized in a buffer under physiological conditions only when co-expressed (Wakabayashi, 2001). I followed his co-expression method using baculovirus and insect culture cells. DC1 and DC2 were co-purified by Ni-NTA chromatography even when only DC2 was His-tagged, suggesting they form a complex (Fig. 5). I hereafter refer to the DC1-DC2His complex as "DC1-2". I confirmed that the recombinant DC1-2 was functional in the same way as ICs.

Interactions between IC1-2 and DC1-2 complexes

The tail domain of OAD has been shown to be in close apposition to the ODA-DC on the doublet microtubules (Bui et al., 2009). However, thus far, the only protein-protein interaction between OAD and the ODA-DC that has been biochemically identified is between a dynein light chain (LC7b) and the ODA-DC subunit DC2 (DiBella et al., 2004); whether or not ICs are involved is unclear. I therefore tested whether there is any interaction between IC1-2 and DC1-2.

When mixed, IC1-2 and DC1-2 were found to be co-immunoprecipitated upon treatment with anti-DC2 antibody (Fig. 4B). Conversely, DC1-2 was co-immunoprecipitated with IC1-2 when treated with anti-IC2 antibody (Fig. 4B). These data suggest that ICs and the ODA-DC directly interact with each other. To assess which subunits are responsible for their association, IC1-2 and DC1-2 complexes were mixed and chemically crosslinked with several crosslinkers with different reaction groups and spacer lengths. The results using BMH, which

reacts with –SH groups and has a spacer length of 13 Å, showed interactions between two complexes as identified by western blotting using four antibodies. IC1-DC1 and IC2-DC1 are crosslinked with each other and therefore they are within 13 Å of each other (Fig. 4C, Table 2). The crosslinked product #1 in Fig. 4C (containing IC1 and DC1) is apparently ~250,000 Da. Because the molecular weight of IC1 is 78,000 and that of DC1 is 83,000 (although it appears to be ~ 105,000 in SDS-PAGE), the #1 product may be composed of either 1 IC1 and 2 DC1 or 2 IC1 and 1 DC1 molecules. The crosslinked product #8 is apparently ~180,000 Da, which is close to the sum of the molecular weights of IC2 and DC1. These data suggest that the IC1-2 complex interacts with the DC1-2 complex via IC1-DC1 and IC2-DC1 interactions. The bands #3, #4, and #5 contain three subunits. These bands may involve a novel interaction (either IC1-DC1 or IC2-DC1) in addition to a known interaction between DC1 and DC2. Both #2 and #6 bands contain only DC1 and DC2. The two bands may reflect different stoichiometries or different crosslinking sites between the two proteins.

Discussion

Association of outer arm dynein intermediate chains in vitro

In this study, I examined biochemical properties of OAD intermediate chains (IC1 and IC2) using recombinant proteins. These ICs are responsible for both assembly and doublet microtubule binding of OAD. In a recently elucidated pathway of OAD assembly in the cytoplasm, ICs and some LCs are first assembled to form an IC-LC complex, and then HCs and the other LCs are assembled on the IC-LC complex with the help of the Ktu/PF13 protein (Omran et al., 2008). ICs are thus thought to be the "core" on which OAD assembly occurs; defining protein-protein interactions involving ICs is thus the key to understanding the mechanism of OAD assembly.

The result that IC1 and IC2 form a 1:1 complex in cultured cells is important since it implies that they can associate with each other without LCs. In the case of *Drosophila melanogaster* cytoplasmic dynein, ICs form a homodimer only when LC7 (DYNLRB) and LC8 (DYNLL) or Tctex1 (DYNLT) are bound to the IC (Nyarko and Barbar, 2011). Of these, LC8 is known to be a dimerization enhancer (Barbar et al., 2008). In *Chlamydomonas* OAD, several LCs have been shown to bind the ICs and form an IC-LC complex (DiBella et al., 2005; Tanner et al., 2008). Three of them (LC6, LC8, and LC10) belong to the LC8/DYNLL family. The data raise the possibility that IC1 and IC2 can heterodimerize without the dimerization enhancers. A previous study also proposed the direct interaction between IC1 and IC2 by stepwise dissociation of OAD subunits by detergent or by immunoprecipitation of recombinant proteins expressed using an in vitro translation system (Mitchell and Rosenbaum, 1986; King et al., 1995).

However, our SDS-PAGE analysis of cell lysates revealed another aspect of ICs: IC1,

IC2, and even IC1-2 are unstable and only ~50% of the recombinant proteins are soluble (Fig. 3A). In support of this observation, a previous study showed that quadriflagellated temporary dikaryons formed between *oda6* (ΔIC2) and *oda9* (ΔIC1) gametes do not readily recover normal motility, suggesting that these proteins are quickly degraded when not associated (Fowkes and Mitchell, 1998). LCs may stabilize the IC1-IC2 interaction in vivo, and the stabilized IC complex may act as the core for OAD assembly (Omran et al., 2008).

IC complex interacts with the ODA-DC

The tail domain of OAD and the ODA-DC are localized next to each other. Furthermore, OAD and the ODA-DC can be co-purified in a single "23S dynein" particle when extracted from axonemes with high-salt buffers containing Mg^{2+} (Takada et al., 2002). However, the only interaction thus far found between OAD and the ODA-DC is between LC7b and DC2 (DiBella et al., 2004). My immunoprecipitation experiments demonstrated that IC1-2 directly binds DC1-2, most likely through interactions between IC1-DC1 and IC2 -DC1. Since OAD cannot stably bind to the axoneme without the ODA-DC, the binding between OAD and the ODA-DC must be strong. I suggest this strong binding is accomplished by multiple interactions, at least in part, between LC7b-DC2, IC1-DC1 and IC2-DC1. A new model of OAD-outer doublet binding that represents my findings in Part 1 is shown in Fig. 6.

Table 1 | Recovery of motility after induction of recombinant proteins.

Protein	Strain	Recovery rate[a]	
IC1H	*oda9ida4*	7 / 2819	0.25%
-	*oda9ida4*	1 / 4537	0.02%
IC2H	*oda6ida4*	9 / 2345	0.38%
IC1IC2H	*oda6ida4*	10 / 1631	0.68%
-	*oda6ida4*	0 / 2547	-

a: Number of motile cells / total cells

Table 2 | The table of crosslinked products in Fig. 4C with calculated molecular weights.

	Component				Calculated MW	Relative Mobility
	IC1	IC2	DC1	DC2		
#1	1		2		243.1	263
	2		1		236	
#2			1	2	207.8	227
			2	1	229	
#3	1		1	1	221.9	209
#4		1	1	1	209.1	196
#5		1	1	1	209.1	179
#6			1	1	145.6	167
#7	1	1			139.8	145
#8		1	1		146.9	125

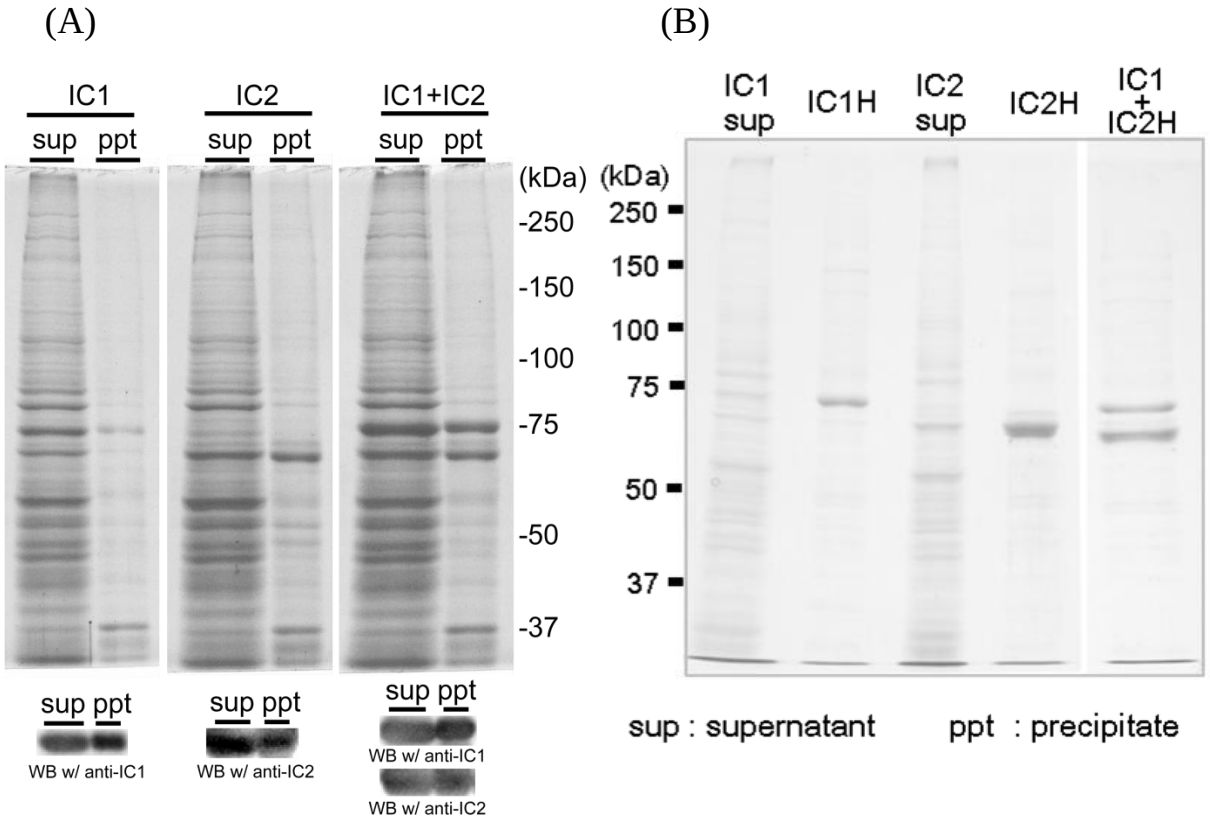

Fig. 3 | Expression and purification of IC1 and IC2. All gels were stained with CBB. (A) Supernatants and precipitates from the lysate of *Sf21* cells expressing IC1-6×His (Mol. Wt 76,305), IC2-6×His (Mol Wt. 63,534) or co-expressing IC1 (not tagged) and IC2-6×His. All proteins were solubilized by 50-60%. (B) IC1-His and IC2-His were partially purified by Ni-NTA chromatography. Co-expressed IC1 and IC2-6×His were co-purified by Ni-NTA chromatography as well (IC1+IC2H).

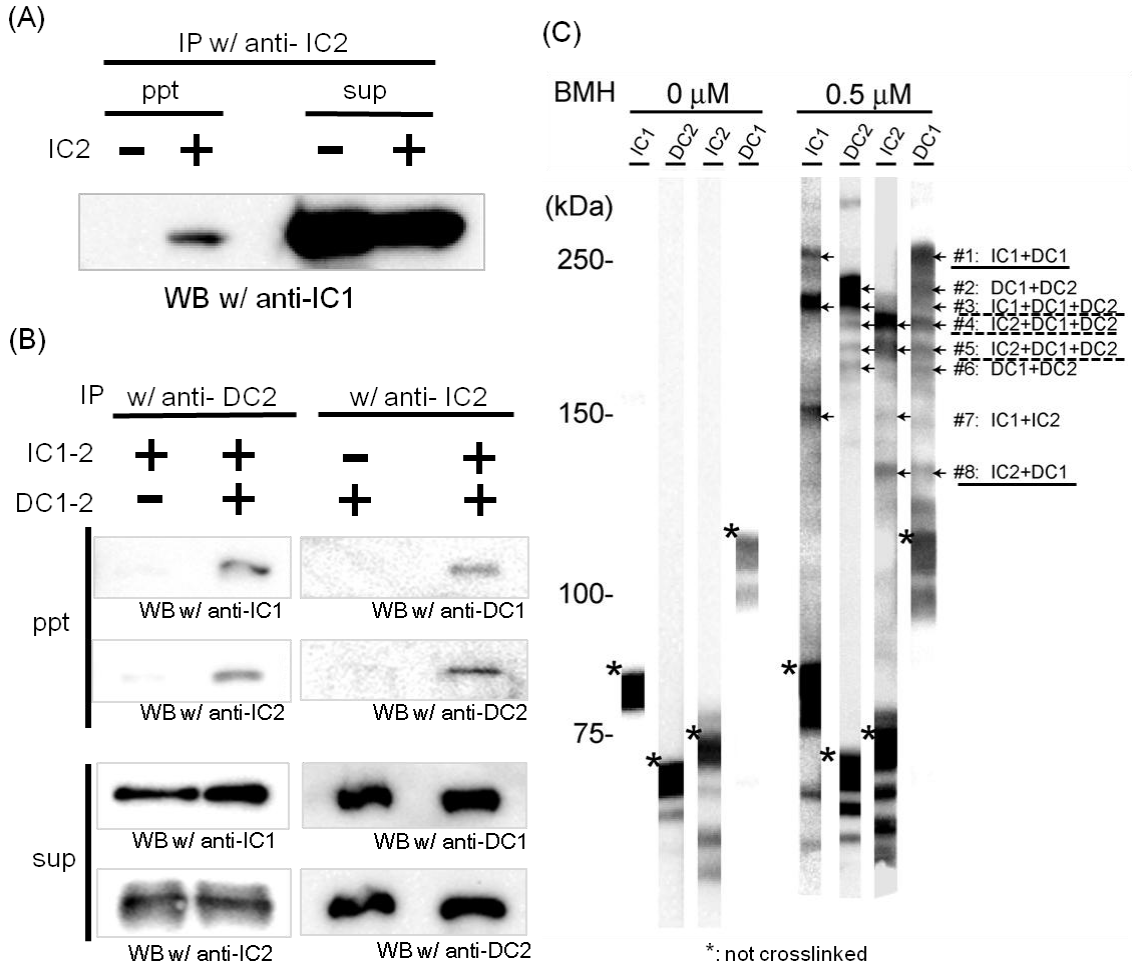

Fig. 4 | Association between IC1, IC2, DC1 and DC2. (A) IC1 associates with IC2. His-IC1 sample alone (IC2 -) or mixed with IC2 (IC2 +) was immunoprecipitated with anti-IC2 antibody and the precipitate and supernatant fractions were assayed by western blotting for the presence of IC1. (B) IC1-2 associates with DC1-2. IC1-2 alone (DC1-2 -) or mixed with DC1-2 (DC1-2 +) was immunoprecipitated (IP) with anti-DC2 antibody. Also, DC1-2 alone (IC1-2-) or mixed with IC1-2 (IC1-2+) was immunoprecipitated with anti-IC2 antibody. Western blotting (WB) with antibodies against IC1, IC2, DC1 or DC2 indicates both IC1-2 and DC1-2 were precipitated from the mixture with either antibody, demonstrating that IC1-2 and DC1-2 form a complex. (C) Chemical crosslinking of mixed "IC1-2 + DC1-2" complex

with BMH. Western blotting against mixed proteins with or without chemical crosslinking using four antibodies is shown. The four blot strips in each sample were probed independently and placed immediately adjacent to each other so that the alignment of the crosslinked product bands could be assessed directly. The bands detected with multiple antibodies were marked with arrows and numbered. The bands #1 and #8 revealed new interactions (underlines). The bands #3, #4 and #5 show the combination of a known interaction (DC1-DC2) and new interactions (#1 or #8). The asterisks represent the proteins that are not crosslinked. For details, see Table 2.

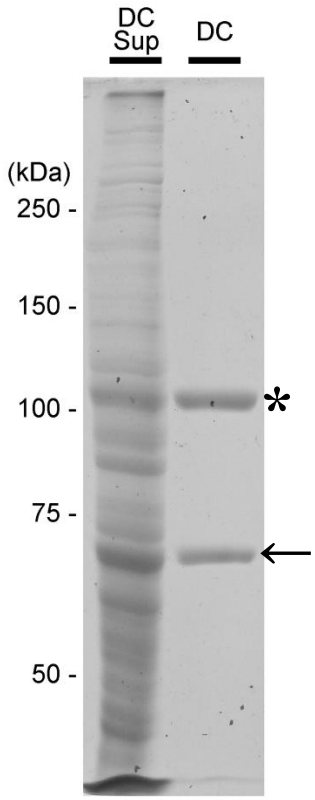

Fig. 5 | Expression and purification of DC1 and DC2. The Gel was stained with CBB. The supernatant purified from the lysate of *Sf21* cells expressing DC1 (Mol. Wt. 83,381 (migrates at Mr~105,000)) and DC2-6×His (Mol. Wt. 62,234). DC1 (asterisk) and DC2 (arrow) were co-purified by Ni-NTA chromatography even when only one component was His-tagged.

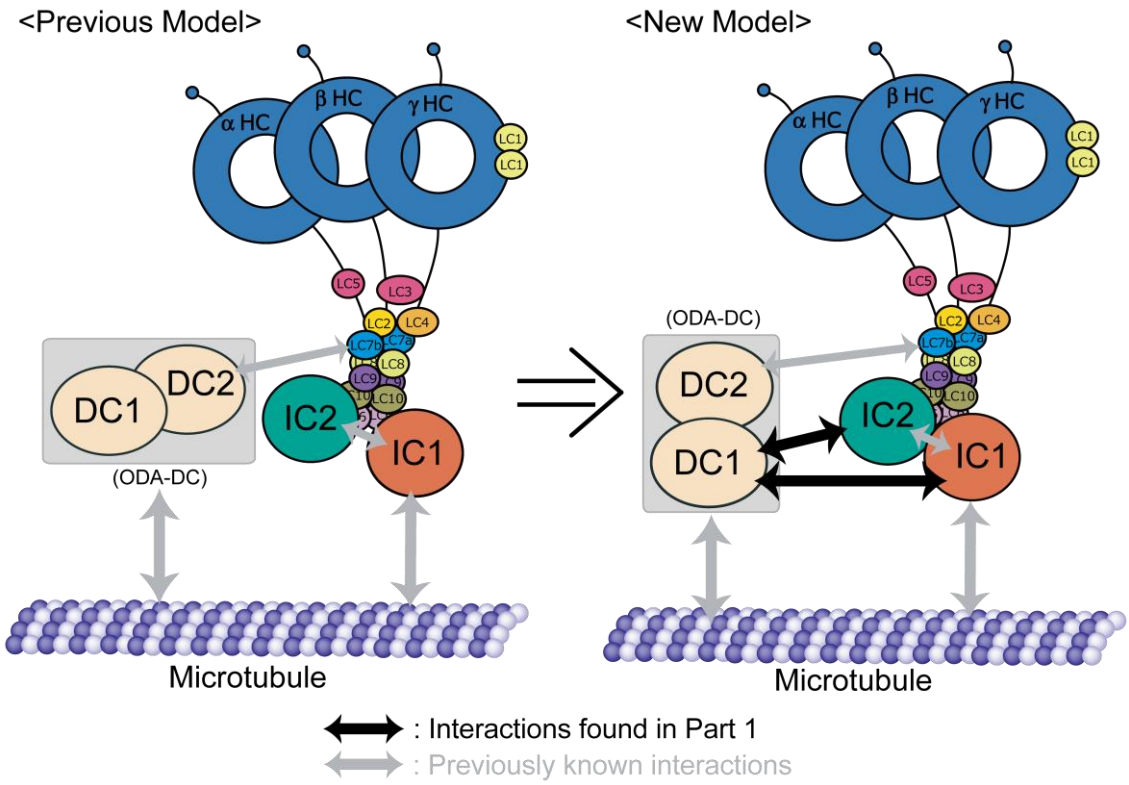

Fig. 6 | The model of IC-DC binding from Part 1. Previously known interactions are drawn as gray arrows. Interactions found in Part 1 are drawn as black arrows.

Part 2

Quantification of the protein-protein interactions in outer arm dynein

Abstract

Axonemal outer arm dynein (OAD) is bound to doublet microtubules at specific sites with a regular spacing of 24 nm. As shown in Part 1, this binding is achieved by multiple protein-protein interactions. The next question is how multiple interactions are necessary for the OAD-microtubule binding. To answer it, in Part 2, I quantified the binding affinities of these protein interactions by the co-precipitation assay and Quarts Crystal Microbalance (QCM) using recombinant OAD/ODA-DC subunits and cytoplasmic or axonemal microtubules. Results showed that IC1 is bound to axonemes at specific site other than the ODA-DC, and that IC2 does not bind to axonemes or cytoplasmic microtubules. Calculation of dissociation constants to cytoplasmic microtubules showed that the binding affinity of the ODA-DC or that of IC1-IC2 complex is more robust than IC1. The affinity of purified native OAD with the ODA-DC was slightly stronger than that of ODA-DC. From these results, the IC1 and the ODA-DC have rather weak affinities to microtubules, and therefore strong OAD attachment to microtubules is achieved by their cooperation.

Introduction

The results of studies described in Part 1 revealed that IC1 directly interacts with IC2, and the IC1-IC2 complex interacts with the ODA-DC. These data help to understand how the ODA-DC on the outer doublet captures OAD. The next question is how OAD is tightly bound to axonemal microtubules.

Previous studies showed that IC1 directly binds to α-tubulin (King et al., 1995) and the ODA-DC binds to axonemal microtubules (Takada and Kamiya, 1994). Together with the results in Part 1, the available data indicate that OAD has multiple sites to bind with axonemal microtubules. However, phenotypic analyses of mutants lacking these subunits suggest that each interaction is not sufficiently strong for stable binding of OAD to the axoneme (Kamiya, 1988). Thus, I propose that each of the IC1-tubulin interaction and the ODA-DC-tubulin interaction is rather weak, but these interactions congregate to enable sufficiently robust and stable OAD binding to microtubules.

To verify my hypothesis, I tested whether the binding affinities of these OAD components to microtubules increase by protein interactions between the components. For the quantification of the affinity between molecules, dissociation constants must be measured. I carried out co-sedimentation assays and Quarts Crystal Microbalance (QCM) assays. Previous co-sedimentation assays and chemical crosslinking studies showed that IC1, but not IC2, is a microtubule-binding protein, although the microtubule binding activities of IC2 has not been examined quantitatively. Because IC proteins are now available in sufficient amounts for quantitative affinity measurements, I carried out experiments to determine the binding affinities of intermediate chains to axonemal microtubules or to porcine brain cytoplasmic microtubules by conventional co-precipitation assays. QCM is a method to assess

protein-protein interactions without labeling by measuring mass shifts of sensors resulting from protein binding (Matsuno et al., 2004; Mitomi et al., 2007; Lord et al., 2008). I used a NAPiCOS equipment (Nihon Denpa Kogyo, Tokyo, Japan) with a 30 MHz quartz crystal oscillator, which is highly sensitive and more accurate than conventional models. From these assays, I determined the affinities of protein-protein interactions, which led me to propose a model as to how OAD is tightly bound to the axoneme.

Materials and Methods

Strains and culture of Chlamydomonas reinhardtii *cells*

Chlamydomonas reinhardtii mutants *ida4* (Δp28: for preparation of outer-arm dyneins) and *oda1* (ΔDC2: for preparation of axonemal microtubules) were used (Kamiya, 1988). The method of the culture is as described in Part 1.

Co-sedimentation assay of recombinant proteins with Chlamydomonas *axonemes or porcine brain cytoplasmic microtubules*

The purified proteins at concentrations of 0-0.2 mg/ml were mixed with axonemes (0.75 mg/ml) or microtubules (0.01 mg/ml) in HMDEK with modifications (10% sucrose, 0.1% Tween 20 and 10 μM paclitaxel (microtubules) were added) and incubated for 20 min at 4°C (axonemes) or room temperature (microtubules). The samples were centrifuged at 20,000 ×g for 12 min at 4°C (axonemes) or at 120,000 ×g for 20 min at 15°C (microtubules). Precipitates were washed with the same buffer, and then resuspended with SDS-PAGE sample buffer for western blotting. Signals were detected by chemiluminescence using Chemi-Lumi One (Nacalai, Kyoto, Japan) and Atto Light Capture (Atto, Tokyo, Japan). Luminescence intensity was quantified using Image J software (http://rsb.info.nih.gov/ij/). The amount of ICs was calculated from a calibration curve determined with known amounts of purified ICs, and the values were expressed relative to the amount of native ICs in wild-type axonemes.

Purification of outer arm dynein with the ODA-DC

Flagella were isolated from *ida4* (Δp28) to minimize the contamination of inner arm dyneins, and demembranated with 0.2% Nonidet P-40 in HMEK solution (Witman, 1986).

High-salt extracts of axonemes (with 0.6 M KCl in HMEK) containing dyneins were fractionated into individual dynein species by anion-exchange chromatography (Kagami and Kamiya, 1992) with modifications: to achieve better separation of OAD from other proteins, I used Uno-Q column (Bio-Rad, Hercules, CA, USA) instead of Mono-Q column (GE Healthcare, Wauwatosa, WI, USA). The fraction that contains OAD together with the ODA-DC (so-called 23S dynein) was detected by western blotting.

Measurement of dissociation constants using QCM

To measure dissociation constants between OAD components and microtubules, NAPiCOS (Nihon Denpa Kogyo, Tokyo, Japan), an instrument for QCM, was used. NAPiCOS is equipped with 30 MHz twin sensors (EXS00A-SX00007, Nihon Denpa Kogyo) that have an AT-cut quartz crystal with a fundamental frequency of 30 MHz. This instrument has 50 μl cells equipped with a sensor plate with the temperature control system. Sensors were treated with 100 μl of 1.36 mg/ml polymerized cytoplasmic microtubules in HMDEK with paclitaxel, and reference sensors were treated with 100 μl of 1% BSA in HMDEK. After incubation for 10 min, sensors were washed with the same solution without proteins and installed into the instrument. According to the manufacturer's manuals, HMDEK was flown until frequency became stabilized. Blocking non-specific binding, 100 μl of BSA was injected several times. Then, 100 μl of purified proteins were injected for analysis. Frequency decay was analyzed with the software NAPiCOS Analysis V2 by Affinity methods (for dissociation constants) or Kinetic methods (for association rate constant (k_+) and dissociation rate constant (k_-)). For calculation of k_+, the value of molecular weight of the immobilized protein is necessary. I applied 100,000 for IC1 and IC1-2 as approximate molecular weight of a tubulin

dimer. I applied 300,000 for ODA-DC and 23S dynein as approximate molecular weight of three tubulin dimers, because they bind to axonemes every 24 nm (three tubulin dimers).

Results

Purification of outer arm dyneins together with the ODA-DC

To obtain OAD containing the ODA-DC, I carried out anion-exchange chromatography on the high-salt extract from axonemes using a Mg^{2+}-containing buffer (Takada et al., 2002) (Fig. 7A). Western blotting of the fractions showed that OAD was co-purified with the ODA-DC, as expected (Fig 7B). The peak fraction that contained both DC1 signal (representing the ODA-DC) and IC2 signal (representing OAD) was used as 23S dynein (OAD - ODA-DC complex).

IC1 binds to cytoplasmic microtubules.

Previous studies using co-sedimentation assays and chemical crosslinking showed that IC1, but not IC2, is a microtubule-binding protein (King et al., 1995). However, only qualitative assessment has been made for IC2 binding. Because I now have sufficient amounts of IC proteins, I reassessed the microtubule-binding activities of both proteins. I examined cosedimentation with porcine brain microtubules to determine whether or not ICs directly bind tubulin. I found that IC1 bound with cytoplasmic microtubules, whereas IC2 did not (Fig. 8). These results are consistent with the previous report by King et al. (1995).

Although qualitatively the same results were always obtained, the amount of IC1 molecules pelleting with microtubules varied greatly from one experiment to another, preventing quantitative binding assessments. Despite repeated experiments using various conditions, I was unable to determine the cause of this variation; currently I suppose that the use of ultracentrifugation, which is necessary to recover IC1-bound microtubules, might have interfered with the assay. For these technical problems, it was difficult to estimate the

dissociation constant from this experiment.

Possible docking site for IC1 on the doublet

I then examined the binding of IC1 and IC2 to the flagellar axonemes of the *oda1* mutant, which lack both OAD and the ODA-DC. From a mixture of axonemes, IC1 and IC2, IC1/IC2-bound axonemes were precipitated by low-speed centrifugation. This allowed assessment of the amount of bound proteins. As observed in the above qualitative experiments, IC1 precipitated with the *oda1* axoneme, while IC2 did not (Fig. 9A).

The binding curves of both IC1 and IC2 reached plateaus equally at ~2.0 μM ICs. IC1 bound to axonemes in a dose-dependent manner, and saturated at 136 % of the amount of native IC1 in the wild-type axoneme. In contrast, IC2 binding saturated at only 28% of the level in the wild type axoneme. From these curves, the dissociation constants of IC1 and IC2 were calculated as 1.08 μM, 1.50 μM, respectively. The Hill's coefficients were 1.025, 1.021, suggesting there is no significant cooperativity in these bindings.

Structural considerations led to estimation that, at the saturating level of IC1, at least eight molecules could bind to the microtubule wall per 24 nm. However, IC1 binding to the axonemes was saturated at almost the same level as that of native IC1 in the wild-type axoneme, i.e. one molecule per 24 nm (Fig. 9A). This finding suggests that IC1 preferentially bind to a specific site on the axoneme, not indiscriminately to all available microtubule walls.

IC2 did not seem to bind to cytoplasmic microtubules, because the saturated amount of bound IC2 was only about one fifth of that of IC1 (Fig. 8). The small amount of IC2 molecules bound to microtubules may be due to those bound nonspecifically or via unknown protein(s) on the axoneme with a low affinity.

Binding analysis with QCM

I next quantified the OAD-microtubule binding by QCM. Solutions containing proteins for analysis were applied to the crystal oscillator ("sensor") on which cytoplasmic microtubules had been immobilized. If the protein binds to the immobilized microtubules, the mass of the sensor increases and the frequency of the quartz oscillation decreases. The proteins examined were IC1, IC2, IC1-2, the ODA-DC (all were recombinant proteins) and native 23S dynein purified from *Chlamydomonas* axonemes (Fig. 7). Note that 23S dynein contains IC1, IC2, the ODA-DC and other components such as HCs and LCs (Takada et al., 2002). (IC1-IC2-DC1-DC2 complex was not examined in this experiment because the complex was unstable.) .

Figures 10A and 10B show representative data of the frequency change observed when the ODA-DC and IC1-2 were applied. In the experimental protocol used, samples were applied to the equipment for 2 minutes by perfusion, followed by perfusion of the buffer alone. In both cases, the frequency quickly decreased with protein application, and then the frequency slowly increased as the solution was exchanged with buffer. The two traces indicated that dissociation of the ODA-DC upon perfusion with buffer alone caused a faster increase in frequency than the dissociation of IC1-2. This suggests that the ODA-DC dissociates from microtubules faster than IC1-2.

Association rate constants (k_+) and dissociation rate constants (k_-) were estimated for all proteins except IC2 (which tended to attach to the sensor without bound microtubules) using a curve fitting software provided by the manufacturer. The data are shown as a map in Fig. 10C. Both k_+ and k_- were higher in the ODA-DC than in other proteins, suggesting association and dissociation of the ODA-DC to/from microtubules are faster than other proteins tested. The on-off rate map suggests that each protein has distinct properties in the

interaction with microtubules. Importantly, though IC1-2 and IC1 do not differ greatly in k_-, IC1-2 has a much greater k_+ than IC1. Co-sedimentation assay of IC2 and cytoplasmic microtubules showed that IC2 does not bind with microtubules by itself. This suggests that IC2 stabilizes the microtubule binding activity of IC1 and that IC1-2 binds with microtubules more readily than IC1.

K_Ds were calculated from modified Scatchard plots (Table 3). Judging from these values, the protein that most strongly binds microtubule is 23S dynein, followed in order by the ODA-DC, IC1-2 and IC1. It is to be noted that 23S dynein has two microtubule binding sites of IC1 and the ODA-DC. These data are consistent with my hypothesis that IC1 and the ODA-DC congregate and make sufficiently strong binding.

Discussion

In Part 2, I carried out several quantitative experiments to understand how OAD binds to axonemes tightly and stably.

Among the 16 subunits of OAD, IC1 is the only subunit that has been shown to have a microtubule binding activity. My results confirmed that IC1 is a microtubule binding protein and IC2 is not. The saturated amount of bound IC1 was almost the same as the amount of IC1 in native axonemes, suggesting that IC1 bound to specific sites on the axoneme. This is quite surprising because IC1 may well bind to anywhere on the bare microtubule surface. It is possible that the surface structure of the binding site for IC1 in the outer-doublet microtubules differs from cytoplasmic microtubules (Konno et al., 2012).

It is difficult to interpret the saturated amount of bound IC2 to the axoneme (~30% of IC2 in the wild type axoneme). If this binding is specific, the data might suggest that one IC2 molecule is bound to axonemes every 96 nm. Recently, IC2 has been shown to interact with DRC4, a subunit of dynein regulatory complex that binds to the axoneme every 96 nm (Oda et al., 2013). It is possible that the saturated amount represents the IC2-DRC4 binding. It is also possible that IC2-axoneme binding is nonspecific. Further analyses are necessary to understand the manner of IC2-axoneme binding.

The QCM analysis revealed that IC1 and IC2, both localized at the base of OAD, have different microtubule binding properties. The ODA-DC associates to and dissociates from microtubules faster than other OAD proteins. Also, IC1-2 has a greater k_+ than IC1. This suggests that microtubule binding of IC1 is somehow facilitated by IC2. However, the relevance of the k_+ value in this analysis is uncertain, because the k_+ of 23S dynein and IC1 are close.

The QCM analyses enabled me to quantitatively compare the affinity of OAD to microtubules with other microtubule associating proteins (MAPs). K_Ds of several other microtubules binding proteins have been calculated in previous studies: e.g. MAP2, 1.1 μM (Coffey and Purich, 1995); LC8, 2.6 μM (Asthana et al., 2012); tau, 16 nM (Makrides et al., 2004) or 0.45 ~ 0.9 μM (Butner and Kirschner, 1991). These proteins play various roles in modifications and stabilization of cytoplasmic microtubules. The K_D of purified OAD was estimated in this study to be 0.18 μM, suggesting that OAD has a rather high affinity among various MAPs. This high affinity is likely to facilitate stable binding of OAD to the axoneme even in the presence of shear stress between adjacent doublet microtubules.

K_Ds in the interactions of IC1-cytoplasmic microtubules and IC1-axonemes were 7.7 μM and 1.1 μM, respectively, indicating that IC1 binds stronger to axonemes than to cytoplasmic microtubules. There may be an unknown protein(s) that provides specific and strong binding sites for IC1 on the axoneme. From the K_D in IC1- cytoplasmic microtubule binding estimated by QCM, the co-sedimentation assay shown in Fig.7 is likely to report dose-dependent binding of IC1 up to at least ~10 μM. At 10 μM, the number of IC1 molecules expected to bind microtubules should be much greater than one per 24 nm. Thus, the observation that only ~one IC1 molecule was bound per 24 nm also suggests the existence of specific binding site for IC1 on the axoneme.

A new model of OAD-outer doublet binding based on my analyses in Part 2 is shown in Fig. 11. In brief, the K_Ds between OAD components and microtubules are now available and the affinity is in the order of 23 S dynein>ODA-DC>IC1-2>IC1. There must be unknown protein(s) on the axonemes that specified sites for IC1.

Table 3 | Dissociation constants of interactions between the proteins and porcine brain cytoplasmic microtubules measured by QCM.

Proteins	K_D
23S dynein (containing ODA-DC)	1.82×10^{-7} M
ODA-DC	3.01×10^{-7} M
IC1-IC2	5.92×10^{-7} M
IC1	7.65×10^{-6} M

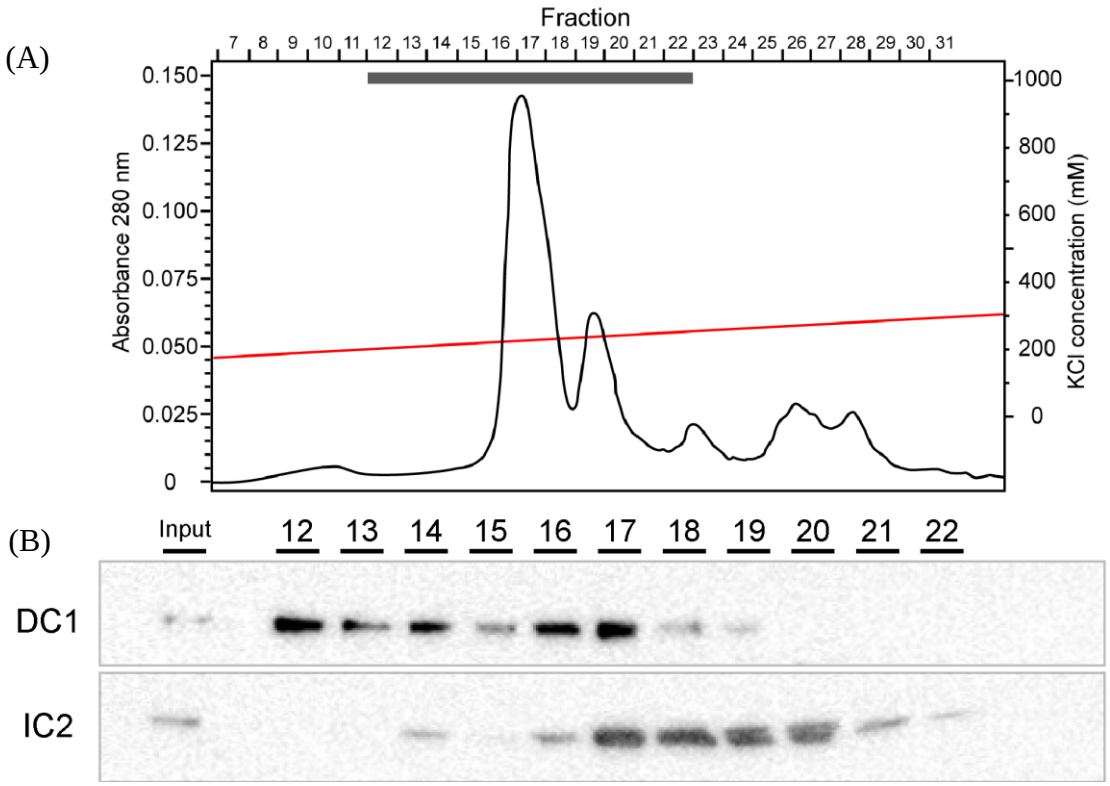

Fig. 7 | Purification of OAD with the ODA-DC from *ida4* axonemes. (A) Anion-exchange chromatography of the extract from *ida4* axonemes. Trace of 280 nm absorbance is shown as the black line, and salt conditions are shown in the red line. Gray bar indicates the fractions analyzed by immunoblotting in (B). (B) Immunoblotting against the fractions between #12 and #22 with anti-DC1 and anti-IC2 antibodies. In this purification, fraction-#17 contained 23S dynein, a complex of OAD and the ODA-DC.

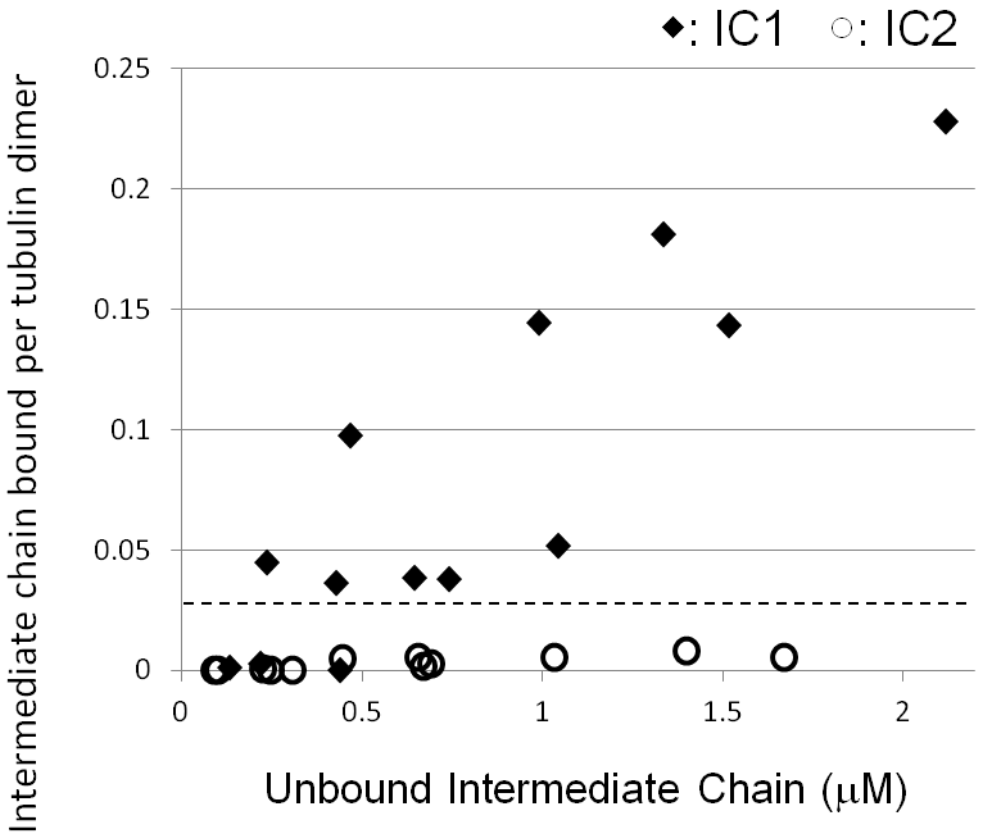

Fig. 8 | Binding of IC1 and IC2 to porcine brain cytoplasmic microtubules. Cytoplasmic microtubules (0.01 mg/ml) were mixed with recombinant IC1 or IC2 at concentrations of 0-0.2 mg/ml in the HMDEK buffer with modifications: 10% sucrose, 0.1% Tween 20 and 10 mM paclitaxel were added. The samples were centrifuged at 120,000 ×g for 20 min at 15°C. IC1 bound to microtubules, although the amount of IC1 bound varied greatly. IC2 did not show marked binding in this concentration range. Both IC1 and IC2 tended to pellet by ultracentrifugation in the absence of microtubules at concentrations >~2.5 mM. Dotted line (= 0.026) represents the amount of native ICs present in wild-type axonemes (i.e. binding at one locus around a microtubule with a regular spacing of 24 nm (one IC molecule per 39 tubulin dimers)). These data suggest that IC1 binds to all available sites on the cytoplasmic microtubule wall.

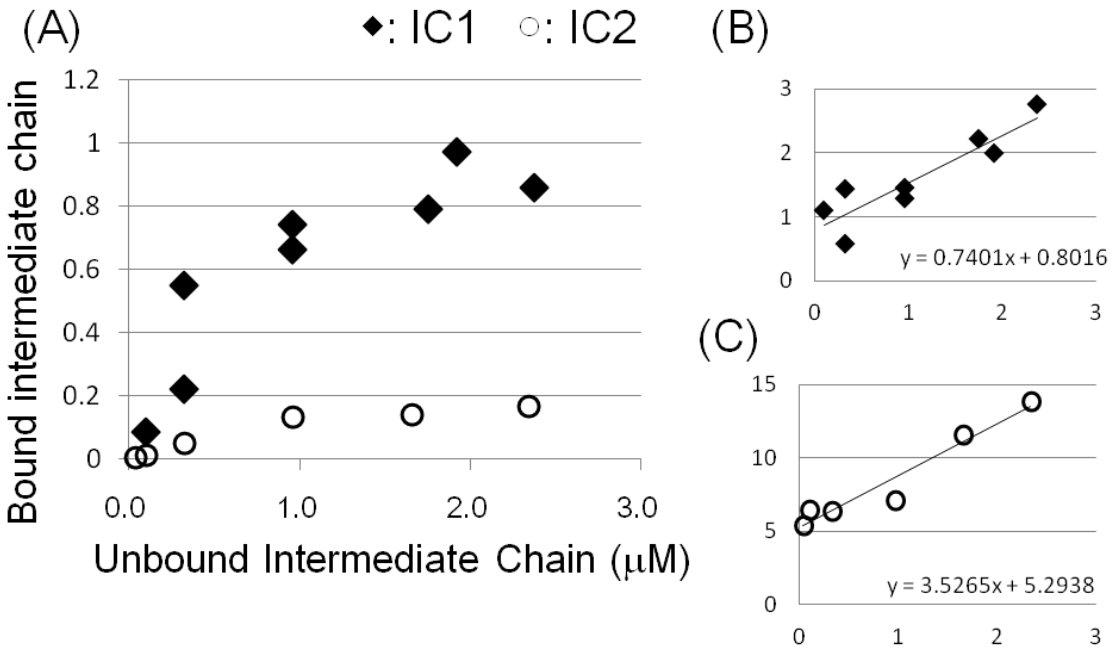

Fig. 9 | Binding of IC1 and IC2 to *Chlamydomonas oda1* axonemes. (A)Axonemes (0.75 mg/ml) were mixed with recombinant IC1 or IC2 at concentrations of 0-0.2 mg/ml in HMDEK buffer with modifications: 10% sucrose and 0.1% Tween 20 added. The samples were centrifuged at 20,000 ×g for 12 min at 4°C. The amount of IC in the pellet, i.e., the amount of IC bound was determined by western blotting; values were expressed relative to the amount of native IC in wild-type axonemes. IC1 binding saturated at almost the same level as in wild-type axonemes. IC2 did not show marked binding in this concentration range. These results suggest that IC1 preferentially binds to specific sites on the doublet microtubules. (B,C) Hanes Woolf plot of IC1 (B) and IC2 (C) used for calculation of K_D.

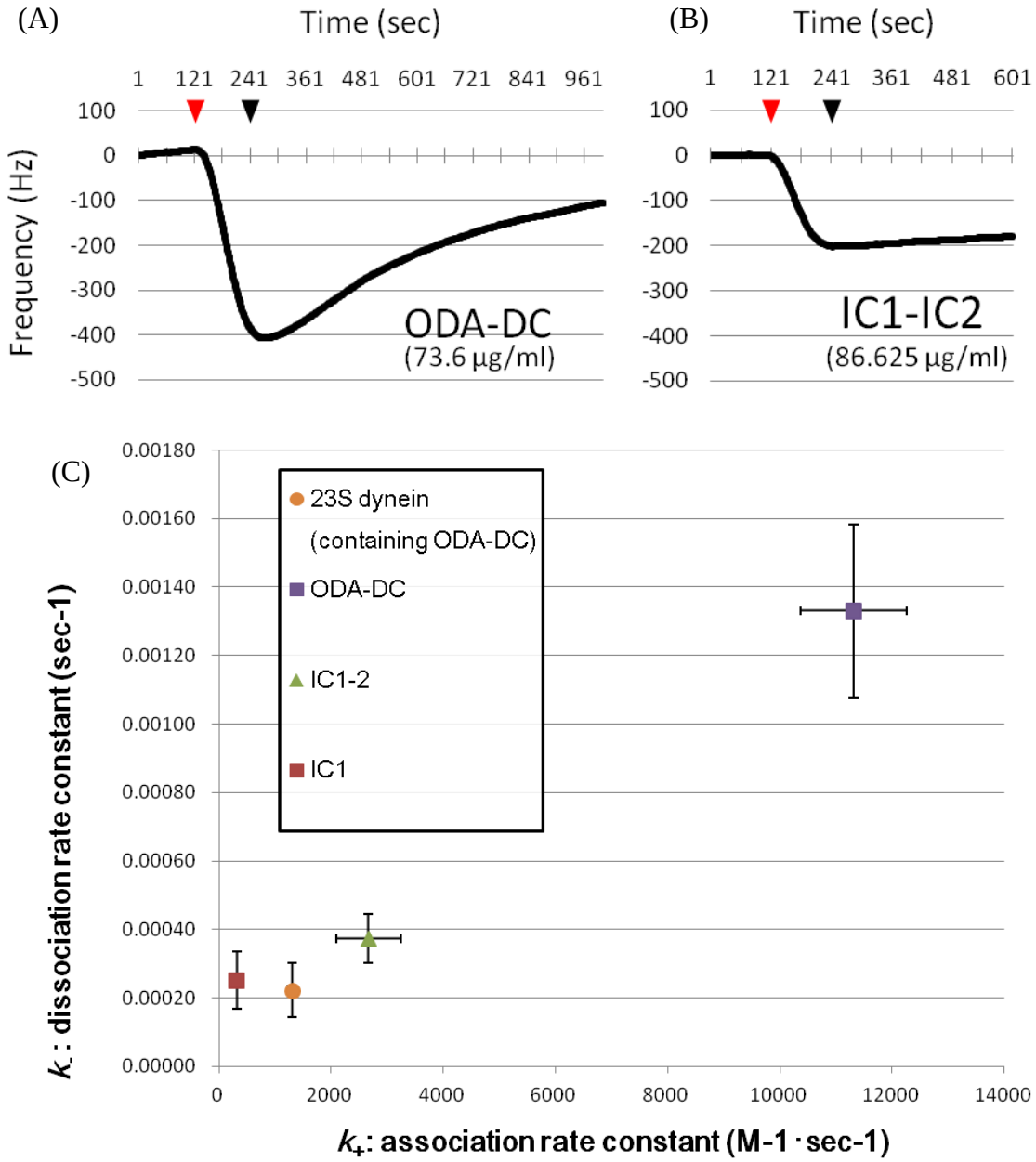

Fig. 10 | QCM analysis of OAD/ODA-DC subunits-microtubule binding. (A)(B) Frequency traces of the ODA-DC and IC1-2 to cytoplasmic microtubules. Microtubules (1.36 mg/ml) were immobilized on the sensor, and perfusion of protein solutions and washing buffers to the sensor started at 120 sec (red arrowheads) and 240 sec (black arrowheads), respectively. (C) The on/off-map showing association rate constant (k_+) vs dissociation rate constant (k_-).

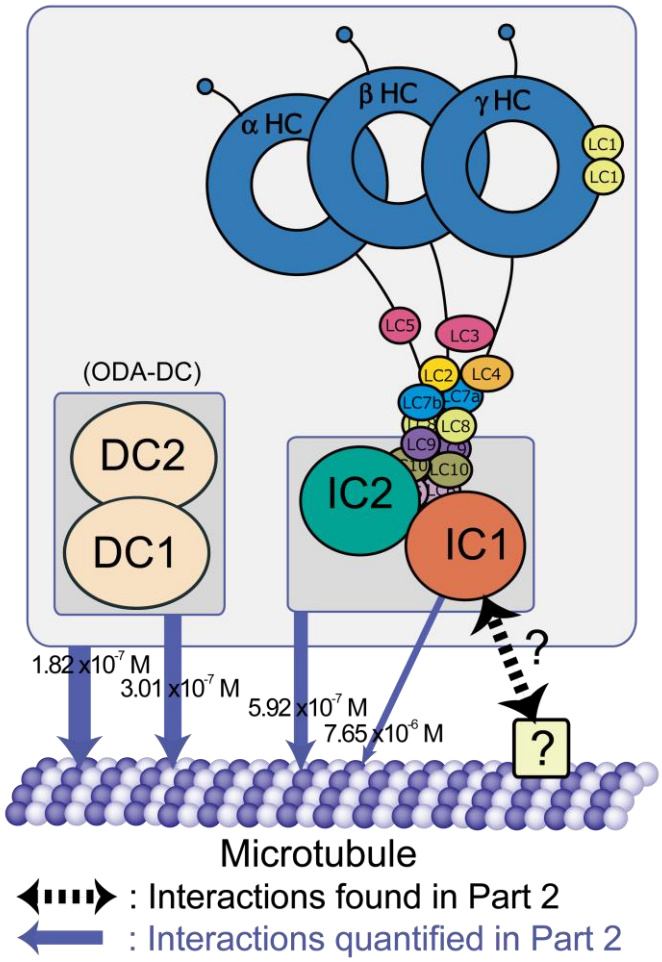

Fig. 11 | The model of OAD-outer doublet microtubule binding from Part 2. A box with a question mark represents the binding site for IC1 in the outer-doublet microtubules. An interaction found in Part 2 is drawn as the dotted black arrow, and interactions quantified in Part 2 are drawn as blue arrows with the dissociation constants.

General Discussion

I asked the question in this study: “How can OAD bind to axonemes tightly enough to endure the sliding forces?” To answer this question, I carried out several qualitative and quantitative experiments to assess interactions between OAD intermediate chains, the ODA-DC subunits and microtubules. The obtained results led me to propose a model of the OAD binding to axonemal microtubules, which is shown in Fig. 12. The most important feature is that while each subunit has only a weak affinity to microtubules, all subunits cooperate in microtubule binding to make the OAD-microtubule binding sufficiently robust and stable.

In Part1, I showed that two microtubules binding sites of 23S dynein interact: IC1 and the ODA-DC. I found that, OAD and the ODA-DC are connected via IC1-DC1 and IC2-DC1, in addition to the previously reported interaction between LC7b and DC2 (DiBella et al., 2004). The C terminus of IC2 is predicted to form a coiled-coil and DC1 is suggested to have at least three coiled-coil domains. Some of these coiled coils may form an interaction interface, which should be confirmed by further structural analyses.

In Part 2, I showed that the binding affinity increases significantly when IC1 and the ODA-DC are present in 23S dynein. The weak affinity of each site to microtubules possibly prevents OAD from binding to incorrect sites on the axoneme. Moreover, my results suggested some unknown protein(s) that associate with IC1. It is interesting because only the ODA-DC has been considered as the structure that determines the site of OAD docking. Chemical crosslinking studies using recombinant ICs and *oda1* axonemes may give clues to these postulated protein(s).

Figure 12 shows a new model of OAD-outer doublet binding based on new findings

on protein interactions at the base of OAD in this study. I believe that our understanding of how OAD subunits are assembled and how OAD is bound to axonemal microtubules has been significantly advanced by this study. The next challenge is to elucidate the mechanism of how the specific binding of IC1, as well as of the ODA-DC, is accomplished.

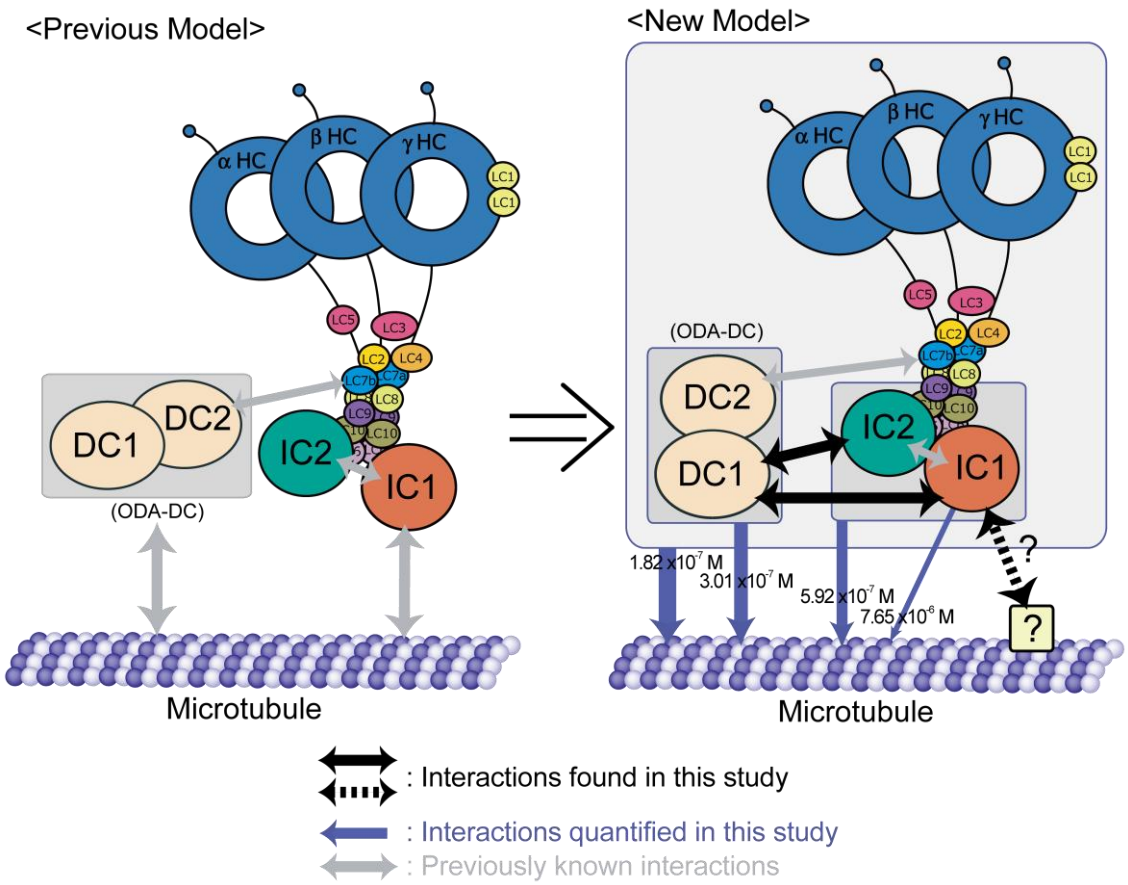

Fig. 12 | The model of OAD-outer doublet microtubule binding. Previously found interactions are drawn as gray arrows. Interactions found in this study are drawn as black arrows, and interactions quantified in this study are drawn as blue arrows with the dissociation constants. A box with a question mark represents the binding site for IC1 on the outer-doublet microtubules. The IC1-2 complex interacts with the ODA-DC via IC1-DC1, IC2-DC1. Also, stoichiometric binding of IC1 to the axoneme suggests a new interaction, between IC1 and a specific binding site on the doublet microtubule (dotted black arrow).

Acknowledgements

I would like to express my deepest gratitude to my supervisor Prof. Ritsu Kamiya (Gakushuin University) for generously giving me the opportunity to study in his laboratory over the past 3 years. I am truly grateful to him for continuously motivating and encouraging me to complete this study.

I am grateful to Dr. Ken-ichi Wakabayashi (Tokyo Institute of Technology) for providing various experimental methods, fruitful discussions and suggestions. I am also grateful to Dr. Masafumi Hirono (University of Tokyo) for his kind advices and encouragement throughout my 5 years. I am grateful to Drs. Manabu Yoshida (University of Tokyo) and Kaoru Yoshida (Toin University of Yokohama) for their help in QCM analysis, Prof. Stephen M. King (University of Connecticut Health Center) for his providing anti-IC1 antibody and cDNA clones of IC1 and IC2, Prof. Fumio Arisaka (Tokyo Institute of Technology) for analysis of IC1-IC2 dimerization, and Mr. Mikito Owa (University of Tokyo) for his providing DC1-2-3 and encouragement. I thank Drs. Toshiki Yagi (University of Tokyo) and Haru-aki Yanagisawa (University of Tokyo) for their technical supports, instructions, very helpful discussions and suggestions about my work.

Thanks are extended to the past and present members of the Laboratory of Molecular Physiology, Department of Biological Sciences, Graduate School of Science, University of Tokyo for their help and encouragement.

Last but not least, I thank my parents and sister for their encouragement and support.

References

Asthana J, Kuchibhatla A, Janab SC, Rayb K, Panda D. (2012) Dynein Light Chain 1 (LC8) association enhances microtubule stability and promotes microtubule bundling. *J Biol Chem.* **287**:40793-805.

Barbar E. (2008) Dynein light chain LC8 is a dimerization hub essential in diverse protein networks. *Biochemistry*. **47**:503-8.

Bui KH, Sakakibara H, Movassagh T, Oiwa K, Ishikawa T. (2009) Asymmetry of inner dynein arms and inter-doublet links in C*hlamydomonas* flagella. *J Cell Biol.* **186**:437-46.

Butner KA, Kirschner MW. (1991) Tau Protein Binds to Microtubules through A Flexible Array of Distributed Weak Sites. *J Cell Biol.* **115**:717-30.

Casey DM, Inaba, K, Pazour GJ, Takada S, Wakabayashi K, Wilkerson CG, Kamiya R, Witman GB. (2003) DC3, the 21-kDa subunit of the outer dynein arm-docking complex (ODA-DC), is a novel EF-hand protein important for assembly of both the outer arm and the ODA-DC. *Mol Biol Cell.* **14**:3650-63.

Castoldi M, Popov AV. (2003) Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr Purif.* **32**:83-8.

Coffey RL, Purich DL. (1995) Non-cooperative binding of the MAP-2 microtubule-binding region to microtubules. *J Biol Chem.* **270**:1035-40.

DiBella LM, Sakato M, Patel-King RS, Pazour GJ, King SM. (2004) The LC7 light chains of *Chlamydomonas* flagellar dyneins interact with components required for both motor assembly and regulation. *Mol Biol Cell.* **15**:4633-46.

DiBella LM, Gorbatyuk O, Sakato M, Wakabayashi K, Patel-King RS, Pazour GJ, Witman GB, King SM. (2005) Differential light chain assembly influences outer arm dynein motor function. *Mol Biol Cell.* **16**:5661-74.

Dutcher SK. (1995) Flagellar assembly in two hundred and fifty easy-to-follow steps. *Trends Genet.* **11**:398-404.

Fowkes ME, Mitchell DR. (1998) The Role of Preassembled Cytoplasmic Complexes in Assembly of Flagellar Dynein Subunits. *Mol Biol Cell.* **9**:2337-47.

Gibbons IR. (1981) Cilia and flagella of eukaryotes. *J Cell Biol.* **91**:107s-24s.

Goodenough UW, Heuser JE. (1985) Outer and inner dynein arms of cilia and flagella. *Cell.* **41**:341-2.

Harris EH. (1989) The *Chlamydomonas* Sourcebook, Academic Press, Inc., San Diego.

Hayashi M, Hirono M, Kamiya R. (2001) Recovery of flagellar dynein function in a *Chlamydomonas* actin/dynein- deficient mutant upon introduction of muscle actin by electroporation. *Cell Motil Cytoskeleton.* **49**:146-53.

Hirokawa N, Tanaka Y, Okada Y, Takeda S. (2006) Nodal flow and the generation of left-right asymmetry. *Cell*. **125**:33-45.

Hou Y, Qin H, Follit JA, Pazour GJ, Rosenbaum JL, Witman GB. (2007) Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella. *J Cell Biol*. **176**:653-65

Kagami O, Kamiya R. (1992) Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner-arm dynein. *J Cell Sci.* **103**:653-64.

Kamiya R, Kurimoto E, Muto E. (1991) Two types of *Chlamydomonas* flagellar mutants missing different components of inner-arm dynein. *J Cell Biol.* **112**:441-7.

Kamiya R. (1988) Mutations at twelve independent loci result in absence of outer dynein arms in *Chlamydomonas reinhardtii*. *J Cell Biol.* **107**:2253-8.

Kato T, Kagami O, Yagi T, Kamiya R. (1993) Isolation of two species of *Chlamydomonas reinhardtii* flagellar mutants, ida5 and ida6, that lack a newly identified heavy chain of the inner dynein arm. *Cell Struct Funct.* **18**:371-7.

King SM, Wilkerson CG, Witman GB. (1991) The M_r 78,000 Intermediate Chain of *Chlamydomonas* Outer Arm Dynein Interacts with α-Tubulin *in Situ*. *J Biol Chem.* **266**:8401-7.

King SM, Patel-King RS, Wilkerson CG, Witman GB. (1995) The 78,000-M(r) intermediate chain of *Chlamydomonas* outer arm dynein is a microtubule-binding protein. *J Cell Biol.* **131**:399-409.

Kobayashi D, Takeda H. (2012) Ciliary motility: the components and cytoplasmic preassembly mechanisms of the axonemal dyneins. *Differentiation.***83**:S23-9.

Kohno T, Wakabayashi K, Diener DR, Rosenbaum JL, Kamiya R. (2011) Subunit interactions within the *Chlamydomonas* flagellar spokehead. *Cytoskeleton.* **68**:237-46.

Konno A, Setou M, Ikegami K. (2012) Ciliary and flagellar structure and function--their regulations by posttranslational modifications of axonemal tubulin. *Int Rev Cell Mol Biol.* **294**:133-70

Koutoulis A, Pazour GJ, Wilkerson CG, Inaba K, Sheng H, Takada S, Witman GB. (1997) The *Chlamydomonas reinhardtii* ODA3 gene encodes a protein of the outer dynein arm docking complex. *J Cell Biol.* 137:1069-80.

Lord MS, Modin C, Foss M, Duch M, Simmons A, Pedersen FS, Besenbacher F, Milthorpe BK. (2008) Extracellular matrix remodelling during cell adhesion monitored by the quartz crystal microbalance. *Biomaterials.* **29**:2581-7

Makrides V, Massie MR, Feinstein SC, Lew J. (2004) Evidence for two distinct binding sites for tau on microtubules. *Proc Natl Acad Sci U S A.* **101**:6746-51

Marshall WF, Rosenbaum JL. (2001) Intraflagellar transport balances continuous turnover of outer doublet microtubules: implications for flagellar length control. *J Cell Biol.* **155**: 405-14.

Mastronarde DN, O'Toole ET, McDonald KL, McIntosh JR, Porter ME. (1992) Arrangement of inner dynein arms in wild-type and mutant flagella of *Chlamydomonas*. *J Cell Biol.* **118**:1145-62.

Matsuno H, Furusawa H, Okahata Y. (2004) Kinetic Study of Phosphorylation-Dependent Complex Formation between the Kinase-Inducible Domain (KID) of CREB and the KIX Domain of CBP on a Quartz Crystal Microbalance. *Chem Eur J.* **10**:6172-8.

Minoura I, Kamiya R. (1995) Strikingly different propulsive forces generated by different dynein- deficient mutants in viscous media. *Cell Motil Cytoskeleton.* **31**:130-9.

Mitchell DR, Rosenbaum JL. (1986) Protein-protein interactions in the 18S ATPase of *Chlamydomonas* outer dynein arms. *Cell Motil Cytoskeleton*. **6**:510-20.

Mitchell DR, Kang Y. (1991) Identification of *oda6* as a *Chlamydomonas* Dynein Mutant by Rescue with the Wild-type Gene. *J Cell Biol.* **113**:835-42.

Mitomo H, Shigematsu H, Kobatake E, Furusawa H, Okahata Y. (2007) IgG binding kinetics to oligo B protein A domains on lipid layers immobilized on a 27MHz quartz-crystal microbalance. *J Mol Recognit.* **20**:83-9.

Nonaka S, Tanaka Y, Okada Y, Takeda S, Harada A, Kanai Y, Kido M, Hirokawa N. (1998) Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell.* **95**:829-37.

Nyarko A, Barbar E. (2011) Light chain-dependent self-association of dynein intermediate chain. *J Biol Chem*. **286**:1556-66.

Oda T, Yagi T, Yanagisawa H, Kikkawa M. (2013) Identification of the outer-inner dynein linker as a hub controller for axonemal Dynein activities. *Curr Biol.* **23**:656-64

Omran H, Kobayashi D, Olbrich H, Tsukahara T, Loges NT, Hagiwara H, Zhang Q, Leblond G, O'Toole E, Hara C, Mizuno H, Kawano H, Fliegauf M, Yagi T, Koshida S, Miyawaki A, Zentgraf H, Seithe H, Reinhardt R, Watanabe Y, Kamiya R, Mitchell DR, Takeda H. (2008) Ktu/PF13 is required for cytoplasmic pre-assembly of axonemal dyneins. *Nature.* **456**:611-6.

Pazour GJ, Rosenbaum JL. (2002) Intraflagellar transport and cilia-dependent diseases. *Trends Cell Biol.* **12**:551-5

Shimogawara K, Fujiwara S, Grossman A, Usuda H. (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics.***148**:1821-8.

Takada S, Kamiya R. (1994) Functional reconstitution of *Chlamydomonas* outer dynein arms from alpha- beta and gamma subunits: requirement of a third factor. *J Cell Biol.* **126**:737-45.

Takada S, Kamiya R. (1997) Beat frequency difference between the two flagella of *Chlamydomonas* depends on the attachment site of outer dynein arms on the outer-doublet microtubules. *Cell Motil Cytoskeleton.***36**:68-75.

Takada S, Wilkerson CG, Wakabayashi K, Kamiya R, Witman GB. (2002) The Outer Dynein Arm-Docking Complex: Composition and Characterization of a subunit (Oda1) Necessary for Outer Arm Assembly. *Mol Biol Cell.* **13**:1015-29.

Tanner CA, Rompolas P, Patel-King RS, Gorbatyuk O, Wakabayashi K, Pazour GJ, King SM. (2008) Three Members of the LC8/DYNLL Family Are Required for Outer Arm Dynein Motor Function. *Mol Biol Cell.* **19**:3724-34

Wakabayashi K, Takada S, Witman GB, Kamiya R. (2001) Transport and arrangement of the outer-dynein-arm docking complex in the flagella of *Chlamydomonas* mutants that lack outer dynein arms. *Cell Motil Cytoskeleton.* **48**:277-86.

Wakabayashi K. (2001) Studies on the structure and function of the outer-dynein-arm docking complex in *Chlamydomonas* flagella. (Doctoral dissertation)

Wilkerson CG, King SM, Koutoulis A, Pazour GJ, Witman GB. (1995) The 78,000 M(r) intermediate chain of *Chlamydomonas* outer arm dynein is a WD-repeat protein required for arm assembly. *J Cell Biol.* **129**:169-78.

Witman GB. (1986) Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods Enzymol.* **134**:280-90.

Zariwala MA, Knowles MR, Omran H. (2007) Genetic defects in ciliary structure and function. *Annu Rev Physiol.* **69**:423-50.