Structure and function of outer dynein arm intermediate and light chain complex

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ABSTRACT The outer dynein arm (ODA) is a molecular complex that drives the beating motion of cilia/flagella. Chlamydomonas ODA is composed of three heavy chains (HCs), two ICs, and 11 light chains (LCs). Although the three-dimensional (3D) structure of the whole ODA complex has been investigated, the 3D configurations of the ICs and LCs are largely unknown. Here we identified the 3D positions of the two ICs and three LCs using cryo–electron tomography and structural labeling. We found that these ICs and LCs were all localized at the root of the outer-inner dynein (OID) linker, designated the ODA-Beak complex. Of interest, the coiled-coil domain of IC2 extended from the ODA-Beak to the outer surface of ODA. Furthermore, we investigated the molecular mechanisms of how the OID linker transmits signals to the ODA-Beak, by manipulating the interaction within the OID linker using a chemically induced dimerization system. We showed that the cross-linking of the OID linker strongly suppresses flagellar motility in vivo. These results suggest that the ICs and LCs of the ODA form the ODA-Beak, which may be involved in mechanosignaling from the OID linker to the HCs.

INTRODUCTION

Cilia and flagella are conserved motile organelles that play important roles in cellular motility and development of vertebrates (Gibbons, 1981; Hirokawa et al., 2006). The beating motions of cilia and flagella are driven by the outer and inner dynein arms (ODAs and IDAs, respectively). The Chlamydomonas ODA is an ∼2-MDa protein complex composed of three heavy chains (HCs), two ICs, and 11 light chains (LCs; Sakato and King, 2004). The three-dimensional (3D) structure of the ODA complex and the nucleotide-dependent conformational changes in the HCs have been intensively studied by cryo–electron microscopy and tomography (Nicastro et al., 2006; Ishikawa et al., 2007; Oda et al., 2007; Movassagh et al., 2010; Ueno et al., 2012; Lin et al., 2014), and the molecular interactions among ICs and LCs have been investigated using genetics and chemical cross-linking (King et al., 1991, 1995; Mitchell and Kang, 1993; Dibella et al., 2004, 2005). However, the 3D architecture of ICs and LCs remains to be fully elucidated. Although we roughly determined the 3D positions of IC1 and IC2 in the ODA-microtubule cross-bridging complex in a previous study (Oda et al., 2013), it is necessary to locate the ODA subunits in situ in order to determine the precise molecular architecture of ODA in cilia and flagella.

In this study, we found that the ICs and LCs constitute the root of the outer-inner dynein (OID) linker, using cryo–electron tomography and structural labeling. We also investigated the molecular mechanisms responsible for OID linker–mediated regulation of flagellar motility using the rapamycin-based cross-linking of FK506-binding protein 1A (FKBP) and FKBP-rapamycin binding domain (FRB). Our results suggest a possible mechanosignaling pathway from the OID linker to the HCs through a complex of ICs and LCs.

RESULTS AND DISCUSSION

Biotinylation tagging of ICs and LCs

To investigate the 3D configuration of ODA ICs and LCs, we labeled four positions in IC1, three positions in IC2, two positions in LC2, and one position in LC7a and LC10 with acetyl-CoA carboxylase biotin carboxyl carrier protein (BCCP) tags (Figure 1A and Table 1; Furuta et al., 2009; Oda et al., 2013; Oda and Kikkawa, 2013).
Positions of the tags on ICs were determined based on the domain organization; IC1 is divided into the amino-terminal (N-terminal) domain and carboxyl-terminal (C-terminal) WD-repeat domain (King et al., 1995; Wilkerson et al., 1995), and IC2 is divided into the N-terminal domain, the middle WD-repeat domain, and the C-terminal coiled-coil domain (Mitchell and Kang, 1991; Ogawa et al., 1995; DiBella et al., 2005). Biotinylation of and streptavidin-binding to BCCP tags were confirmed using immunoblotting and immunofluorescence (Figure 1B and Supplemental Figure S1A). The varied signal intensities of the immunoblots and immunofluorescence among the rescued strains suggest substoichiometric expression and/or labeling of tagged ICs and LCs (Figure 1B and Supplemental Figure S1A). However, wild-type motility of the rescued strains (Table 1) suggests that the expression of tagged ICs and LCs functionally restored defects of IC- and LC-missing mutants.

**ICs and LCs form the ODA-Beak complex**

Next we identified the 3D positions of the BCCP tags on ICs and LCs, using cryo–electron tomography and structural labeling (Figure 2, A and B; Oda et al., 2013; Oda and Kikkawa, 2013). Surprisingly, all of the ICs and LCs were located around the root of the OID linker (Figure 2C, green). We designated this region the ODA-Beak complex, based on the bouquet structure of the isolated ODA (Figure 2, D and E, modified from Figure 2a of Goodenough and Heuser, 1984).

The position of IC1 is of interest because IC1 reportedly binds to the outer doublet microtubules (DMTs; King et al., 1991, 1995). Although we were unable to detect densities bridging between the IC1 and DMT (Supplemental Figure S2A and Supplemental Movie S1), the label densities of IC1-M248 were located on the DMT-facing side of the ODA-Beak (Figure 2B, IC1, yellow). Because the junction between the N-terminal domain and WD-repeat domain of IC1 is predicted to be a possible DMT-binding region (King et al., 1995), the ODA-Beak can be connected to DMT via the middle segment of IC1, which is not visible on our electron density map probably because the DMT-binding domain of IC1 is either flexible or thin.

The N-terminal domain of IC2 is reported to be essential for the assembly of LC2, LC6, and LC9, as the oda6-r88 mutant, which has a sequence alteration in residues 31–54 of IC2 (Mitchell and Kang, 1993), forms an ODA that lacks the three LCs (DiBella et al., 2005). In agreement with these previous biochemical and genetic analyses, labels on LC2 were located in close proximity to the N-terminus of IC2 (Figure 2B, Supplemental Movie S2, IC2, red; Oda et al., 2013; Oda and Kikkawa, 2013). Considering the LC deficiency in oda6-r88 (DiBella et al., 2005), our results suggest that the ODA-Beak is composed of IC1, IC2, LC2, LC6, LC7a, LC9, and LC10.

In contrast, the densities of IC2-M549 were located away from the ODA-Beak and were observed on the outer surface of the ODA (Figure 2B, IC2, yellow). Because the C-terminal domain of IC2 is predicted to form a coiled-coil (Lupas et al., 1991), the C-terminal domain of IC2 is likely to take an extended conformation from the ODA-Beak to the tail domains of α and β HCs (Figure 2, C and D; Movassagh et al., 2010; Lin et al., 2014). This model is intriguing because the OID linker was previously shown to modulate ODA activity (Oda et al., 2013). Our results suggest that the ODA-Beak transmits signals from the OID linker to HCs via the C-terminal coiled-coil domain of IC2.

We attempted to investigate the role of the C-terminal domain of IC2 by generating a partial deletion mutant of IC2, but we were unable to rescue the motility and ODA-assembly defects of the oda6-IC2-deficient mutant) strain by expressing the coiled-coil-deleted IC2 (unpublished data), suggesting that the C-terminal coiled-coil of IC2 is essential for ODA assembly.

Because one ODA contains one copy of IC1, IC2, LC2, and LC7a and two copies of LC10 (King and Witman, 1989; King and Kamiya, 2009; King, 2011; Bowman et al., 1999; DiBella et al., 2004), there should be at least four label densities within one 96-nm repeat of DMT (e.g., labels on IC2 in Figure 2B). However, some labels on IC1...
To examine whether these variations in labeling result from substoichiometric expression of tagged IC and LC and, we quantified the occupancy of ODAs along DMTs of wild-type and rescued strains (Table 1). Because absence of IC and LC results in ODA assembly defects (Kamiya, 1988; Mitchell and Kang, 1999; Wilkerson et al., 1995; Pazour et al., 1999; DiBella et al., 2004), insufficient expression of tagged ICs and LCs must be detected as gaps in the arrays of ODAs in tomograms. However, we observed few gaps in the rescued strains, as well as in wild-type cells. These results suggest that tagged ICs and LCs were sufficiently expressed to rescue ODA assembly defects.

and LCs were invisible in a subset of ODAs, which seems to depend on the relative position to OID linkers (Figure 2B). For example, the label densities of IC1-M31 (Figure 2B, IC1, red) and LC2-N (Figure 2B, LC2, red) appeared on the distal side of the ODA-Beaks that were not connected to OID linkers. On the other hand, the label densities of LC2-C (Figure 2B, LC2, yellow) appeared on the proximal side of the ODA-Beaks that were connected to the OID linkers. The label densities of LC7a-C and LC10-C (Figure 2B, LC7a, LC10, yellow) appeared only on the distal side of the ODA-Beak that were connected to the ODA-IDA f linker (the OID linker 1; Bui et al., 2012).

**TABLE 1:** Strains used in this study.

| Strain         | Abbreviation | Mutated gene | Missing structure | Swimming speed (μm/s) | Beat frequency (Hz) | ODA occupancy (%) | Reference |
|---------------|--------------|--------------|-------------------|-----------------------|---------------------|--------------------|-----------|
| Wild-type CC-125 |              |              |                   | 174.8 ± 12.4          | 60 ± 7              | 98.9               |           |
| oda4-s7       |              | IC2-ODA      |                   | 65.3 ± 8.1           | 35 ± 4              | ND                 |           |
| oda6          |              |              |                   | 60.9 ± 5.6           | 29 ± 3              | 0                  |           |
| oda9          |              | IC1-ODA      |                   | 62.1 ± 6.4           | 30 ± 4              | ND                 |           |
| oda12-1       |              | LC2, LC10-ODA (reduced) |   | 64.9 ± 4.2          | 31 ± 4              | ND                 |           |
| oda12-2       |              | LC2-ODA (reduced) |                   | 81.5 ± 6.1           | 40 ± 5              | ND                 |           |
| oda15         |              | LC7a-ODA (reduced), IDA f (reduced) | | 73.8 ± 5.8           | 36 ± 4              | 19*                |           |
| ida6          |              | DRC2-N-DRC (partial) |                   | 77.6 ± 4.7           | 71 ± 8              | ND                 |           |
| oda9-IC1-M31BCCP | IC1-M31      |              |                   | 170.2 ± 15.1         | 60 ± 7              | 99.3               |           |
| oda9-IC1-M125BCCP | IC1-M125     |              |                   | 176.4 ± 13.3         | 60 ± 6              | 99.0               |           |
| oda9-IC1-M248BCCP | IC1-M248     |              |                   | 175.1 ± 15.9         | 61 ± 6              | 99.5               |           |
| oda9-IC1-M648BCCP | IC1-M648     |              |                   | 171.7 ± 19.2         | 60 ± 7              | 99.1               |           |
| oda6-IC2-NBCCP | IC2-N        |              |                   | 98.1 ± 10.6          | 74 ± 7              | 99.4               | Oda et al. (2013) |
| oda6-IC2-M482BCCP | IC2-M482     |              |                   | 169.5 ± 14.2         | 60 ± 5              | 99.0               |           |
| oda6-IC2-M549BCCP | IC2-M549     |              |                   | 171.3 ± 15.5         | 60 ± 5              | 99.3               |           |
| oda12-2-LC2-NBCCP | LC2-N        |              |                   | 167.8 ± 11.4         | 59 ± 6              | 98.8               |           |
| oda12-2-LC2-CBCCP | LC2-C        |              |                   | 170.9 ± 18.7         | 61 ± 5              | 99.3               |           |
| oda15-LC7a-CBCCP | LC7a-C       |              |                   | 178.0 ± 12.4         | 60 ± 5              | 99.1               |           |
| oda12-1-LC2-LC10-CBCCP | LC10-C      |              |                   | 170.6 ± 16.4         | 60 ± 5              | 99.4               |           |
| oda6-IC2-NFRB | IC2-NFRB     |              |                   | 120.7 ± 11.0         | 65 ± 6              | ND                 |           |
| ida6-DRC2-MFKBP | DRC2-MFKBP   |              |                   | 181.0 ± 19.7         | 60 ± 5              | ND                 |           |
| oda6-IC2-NFRB ida6-DRC2-MFKBP | IC2-NFKBP/ DRC2-MFKBP | | 116.9 ± 13.9       | 65 ± 6              | ND                 |           |
| oda6-IC2-NFKBP | IC2-NFKBP    |              |                   | 115.6 ± 10.2         | 65 ± 6              | ND                 |           |

Swimming speed: means ± SEM were calculated from 20 cells. Beat frequency: means ± SEM were calculated from >500 cells. ODA occupancy: presence of ODAs along DMTs was examined in tomograms, and ODA occupancy was calculated as (number of ODA)/(number of ODA + number of ODA-missing gaps). The total of >5-μm-long axonemes was examined for each strain. ND, not determined.

*ODA occupancy of oda15 was estimated from the biochemically determined value in DiBella et al. (2004).
Cross-linking of ODA-Beak and nexin-dynein regulatory complex using the FKBP-rapamycin-FRB system

To investigate the role of the ODA-Beak in the regulation of flagellar motility, we focused on the OID linker between ODA-Beak and nexin-dynein regulatory complex (N-DRC; Figure 2C, arrowhead, OID linker 3a; Bui et al., 2012). We examined our previous electron microscopy data and found that the N-terminus of IC2 is located in close proximity to the middle segment of DRC2 (Figure 3A; Oda et al., 2013, 2015; Oda and Kikawa, 2013). We hypothesized that we could manipulate the signal transmission from N-DRC to ODA-Beak in vivo if we cross-linked IC2 and DRC2 using rapamycin-based cross-linking of FKBP and FRB (Rivera et al., 1996).

We inserted human FKBP (Harding et al., 1989) after His-245 of DRC2 and added the FRB of human rapamycin target 1 (RAPT1; Chiu et al., 1994) to the N-terminus of IC2. We generated the IC2-NFRB/DRC2-MFKBP strain, in which rapamycin cross-links IC2 and DRC2. As a negative control, we also generated the IC2-NFKBP/DRC2-MFKBP strain, which has FKBP tags on both IC2 and DRC2, so that rapamycin treatment does not cross-link IC2 and DRC2 (Figure 3B and Table 1). To verify that ODA and N-DRC were cross-linked by the FKBP-rapamycin-FRB ternary complex (Choi et al., 1996), we extracted ODAs after cross-linking IC2-NFRB and DRC2-MFKBP with rapamycin (Figure 3, C and E, and Supplemental Figure S3A). We found that one ODA every 96 nm remained attached to the DMT (Figure 3D, arrowhead). These results indicate that we successfully cross-linked ODA-Beak and N-DRC by IC2-NFRB-rapamycin-DRC2-MFKBP heterodimerization.

In vivo cross-linking of ODA-Beak and N-DRC suppressed flagellar motility

We examined the effects of ODA-Beak and N-DRC cross-linking on cell motility by treating live cells of IC2-NFRB/DRC2-MFKBP with rapamycin (Figure 4A). Although rapamycin is known to suppress the growth of Chlamydomonas (Crespo et al., 2005), it did not affect the motility of wild-type and IC2-NFKBP/DRC2-MFKBP cells within 5–30 min of observation (Figure 4A). Of interest, rapamycin treatment suppressed the motility of IC2-NFRB/DRC2-MFKBP cells in a dose-dependent manner. At 1 μM rapamycin, swimming speed

FIGURE 2: Structural labeling of ICs and LCs. (A) The 3D structures of the axoneme. Left, tip-to-base view of the 9+2 structure. Right, enlarged view of one of the DMTs. The 90°-rotated views of the DMT are shown on the left of B. (B) The 3D localizations of the labels on ICs and LCs. Arrowheads indicate positions of slices on the right. Colored densities indicate positions of streptavidin labels. Colors of the label densities correspond to colors of arrowheads in Figure 1A. Position of IC2-M549 differed from that in our previous result (Oda et al., 2013). We believe that our previous localization of the C-terminus of IC2 was an artifact due to flexibility of the ODA-microtubule complex. N-DRC and IC-LC complex of IDA f are indicated (gray).

(C, D) Structural configuration of ODA and N-DRC. (C) Approximate positions of α, β, and γ HCs (orange) based on previous reports (Nicastro et al., 2006; Ishikawa et al., 2007; Oda et al., 2007; Movassagh et al., 2010; Ueno et al., 2012; Lin et al., 2014). Ovals and lines indicate head and tail domains of HCs, respectively. Arrowhead indicates one of the OID linkers (OID linker 3a) bridging between ODA and N-DRC (pink). (D) Possible 3D configuration of ICs and LCs. Red structure represents IC2, composed of WD repeat (yellow frustum) and coiled-coil (yellow rod). Yellow structure behind IC2 represents IC1, composed of WD repeat (red frustum) and the N-terminal domain (red rod). Three small ovals below IC2 represent LCs. (C, D) ICs and LCs form the ODA-Beak complexes (green). (E) Diagram modified from Figure 2a of Goodenough and Heuser (1984), showing the bouquet structure of the isolated ODA. Annotations of subunits were taken from the same figure, except for assignments of the α, β, and γ HCs.

We suppose that these variations in labeling can be attributed to flexibility of the labeled domains, substoichiometric labeling caused by steric hindrance, and/or limited signal-to-noise ratio of tomo-
and beat frequency decreased by 65 and 55%, respectively. The half-maximal inhibitory concentrations of rapamycin on the swimming speed and beat frequency were \( \sim 360 \) and \( \sim 460 \) nM, respectively. Rapamycin-dependent decreases in beat frequency suggest that cross-linking between ODA-Beak and N-DRC affected ODA activity (Kamiya and Okamoto, 1985; Brokaw and Kamiya, 1987). Next we analyzed the flagellar waveforms of IC2-NFRB/DRC2-MFKBP cells and found that rapamycin-treatment decreased the amplitude of beating (Figure 4B), suggesting that the cross-linking also affects IDA activity (Brokaw and Kamiya, 1987). These results agreed with our previous results that the OID linker works as a hub controller for ODA and IDA activities (Oda et al., 2013). We propose that the interaction between ODA-Beak and N-DRC needs to be dynamic in order to allow constitutive cross-linking of the two structures to disrupt the regulation of ODA and IDA activities.

Note that the N-terminal tagging of IC2 alone reduced the swimming speed by 33% and slightly increased beat frequency by 8% (Figure 4A, IC2-NFRB), probably due to changes in the interaction between ODA and N-DRC (Oda et al., 2013). We analyzed the effects of rapamycin on ATPase and microtubule-sliding activities of IC2-NFRB/DRC2-MFKBP axonemes, but both activities were up-regulated, regardless of rapamycin treatment (Figure 4, C and D, and Supplemental Figure S3B; Oda et al., 2013). These results suggest that the effects of cross-linking between the ODA-Beak and N-DRC are detectable in in vivo live-cell experiments, but, in contrast, the effects are masked by hyperactivation of ODA activity caused by the N-terminal tagging of IC2 on in vitro biochemical assays.

Remaining questions about the DMT-binding region of ODA

Localization of IC1, IC2, LC2, LC7a, and LC10 in ODA-Beak indicates that the rest of the ODA subdomains are composed of...
Although DC is reportedly to form a 24-nm-long, oval-shaped structure (Oda et al., 2013), addition of tags to the N-terminus of IC2 enhanced sliding disintegration. Rapamycin treatment did not affect elevated ATPase activities. Means ± SEM were calculated from 20 cells. (B) Waveforms of wild-type and IC2-NFRB/DRC2-MFKBP cells after dimethyl sulfoxide (DMSO) and rapamycin treatments. Rapamycin treatment reduced wave amplitude (red lines) compared with DMSO-treated control. (C) ATPase activities of axonemes. In accordance with previous results (Oda et al., 2013), addition of tags to the N-terminus of IC2 alone caused hyperactivation of axonemal ATPase activities. Rapamycin treatment did not affect elevated ATPase activities. Means ± SEM were calculated from 10 measurements. (D) Sliding disintegration assays. Axonemes were disintegrated by incubation with 1 mM ATP and 0.3 μg/ml nagarse for 1 min. In accordance with previous results (Oda et al., 2013), addition of tags to the N-terminus of IC2 enhanced sliding disintegration. Rapamycin treatment (1 μM) did not affect DMT sliding activity.

FIGURE 4: Effects of cross-linking between ODA-Beak and N-DRC. (A) Motility analyses of live cells. Only IC2-NFRB/DRC2-MFKBP cells showed rapamycin-dependent decreases in motility. Means ± SEM were calculated from 20 cells. (B) Waveforms of wild-type and IC2-NFRB/DRC2-MFKBP cells after dimethyl sulfoxide (DMSO) and rapamycin treatments. Rapamycin treatment reduced wave amplitude (red lines) compared with DMSO-treated control. (C) ATPase activities of axonemes. In accordance with previous results (Oda et al., 2013), addition of tags to the N-terminus of IC2 alone caused hyperactivation of axonemal ATPase activities. Rapamycin treatment did not affect elevated ATPase activities. Means ± SEM were calculated from 10 measurements. (D) Sliding disintegration assays. Axonemes were disintegrated by incubation with 1 mM ATP and 0.3 μg/ml nagarse for 1 min. In accordance with previous results (Oda et al., 2013), addition of tags to the N-terminus of IC2 enhanced sliding disintegration. Rapamycin treatment (1 μM) did not affect DMT sliding activity.
acid, 50 mM NaCl or 50 mM CH₃COOK, and 1× Protease Inhibitor Cocktail (Nacalai Tesque).

Electrophoresis and immunoblotting
Axonemal proteins were resolved by SDS–PAGE on 5–15% polyacrylamide gradient gels (Nacalai Tesque) and blotted onto polyvinylidene difluoride membranes. Blots were probed with streptavidin conjugated with horseradish peroxidase (Thermo Scientific, Rockford, IL) or the indicated primary antibodies.

Construction of the expression vectors
Expression plasmids for IC2, DRC2, and IC2-NBCCP were as described previously (Oda et al., 2013, 2015). Fragments spanning from the start codon to immediately before the stop codon for genes encoding IC1, LC2, LC7a, and LC10 were amplified with genomic PCR using genomic DNA from the wild-type strain CC-125 and then inserted into pIC2 plasmids (Oda et al., 2015). We inserted the tag sequence corresponding to amino acids 141–228 of Chlamydomonas BCCP in the middle of the sequences of IC1 (between Ile-31 and Pro-32, Asp-125 and Met-126, Val-248 and Pro-249, and Pro-648 and Glu-649) and IC2 (between Thr-482 and Gly-483, and Thr-549 and Thr-550) and added tags to the N-terminus of LC2 and the C-termini of LC2, LC7a, and LC10. For expression plasmids of IC2-NF and IC2-NFKBP, we added the codon-optimized cDNA sequence of human FKBP and FRB domain of human RAPT1 (residues 2021–2113), respectively, to the N-terminus of IC2. For expression plasmid of DRC2-MFKBP, we inserted the codon-optimized cDNA sequence of FKBP between His-245 and Arg-246 of DRC2. At the junctions between the DRC2 and FKBP tag sequence, we inserted eight-amino acid linker sequences (Lys-Gly-Ser-Gly-Ser-Gly-Ser-Gly and Lys-Ser-Ala-Lys-Ala-Ser-Ala-Ser).
and the SD was obtained from the shape of the peak, fitted with a Gaussian curve. In a typical experiment at a total magnification of 100×, ~500–1000 cells contributed to one FFT spectrum.

Waveform analysis
Chlamydomonas cells were observed using a dark-field microscope (BX53; Olympus), and images were captured using a high-speed digital camera (EXILIM EX-F1; Casio, Tokyo, Japan) at 600 frames/s. Cells whose flagella were clearly in focus were selected, and the shapes of flagella were manually traced using Illustrator (Adobe).

ATPase assay
The rate of phosphate release by axonomes was measured using Biomol Green reagent (Enzo Life Sciences, Farmingdale, NY). Axonomes (0.1 mg/ml) were incubated for 5 min in HMDEK buffer in the presence of 1 mM ATP. Released phosphate concentrations were calculated based on changes in absorbance at 620 nm.

Sliding disintegration of the axoneme
Axonomes were absorbed onto a glass slide, and sliding disintegration was initiated with HMDEK buffer containing 1 mM ATP and 0.3 μg/ml nagarse. Sliding of doublet microtubules was observed using a dark-field microscope (BX53; Olympus) equipped with a 40x oil-immersion objective lens and a 100-W mercury lamp. Image sequences were recorded using an electron multiplying CCD (ADT-33S; FLOVEL, Tokyo, Japan).

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