Cooperative binding of the outer arm-docking complex underlies the regular arrangement of outer arm dynein in the axoneme

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The authors declare no conflict of interest.


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Significance

In eukaryotic cilia and flagella, outer arm dyneins (OADs) are bound to axonemal doublet microtubules every 24 nm; however, how this regular arrangement is produced remains unknown. To approach this problem, we studied the properties of the OAD-docking complex (ODA-DC), a three-subunit complex that functions as the OAD-docking site on the doublet. Using recombinant ODA-DC, we found that the ODA-DC has an ~24-nm-long ellipsoidal shape and cooperatively binds to the axoneme in an end-to-end manner. These and other results indicate that cooperative association of the ODA-DC underlies the periodic OAD arrangement at specific positions on the doublets. These findings provide insight into how the regular axonemal repeat structure is produced.


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proteins, and observed how it and OAD are incorporated in vivo into axonemes lacking one or both structures. We found that the ODA-DC itself is 24 nm in length and that it produces 24-nm periodicity by end-to-end association on the doublet microtubules. We propose that this periodicity determines the ordered arrangement of the OAD.

Results and Discussion

Properties of Recombinant ODA-DC. Recombinant ODA-DC (which is hereafter referred to as “DC1–2–3”) to distinguish it from the native ODA-DC isolated from the axoneme was prepared by combining bacterially expressed DC3 (20) with the DC1–DC2–6xHis complex produced by coexpression of these proteins in cultured Sf21 insect cells (25), followed by purification on a Ni-NTA agarose column. The eluates contained DC1, DC2–6xHis, and DC3, verifying that DC1–2–3 forms a single stable complex (Fig. 1A).

By several criteria, the structure and functional properties of DC1–2–3 are identical to those of the native ODA-DC extracted from oda2 axonemes. First, the stoichiometry of the three subunits in DC1–2–3 estimated from the band intensities in the SDS/PAGE gel (assuming that all subunits have the same affinity for Coomassie brilliant blue) was 1:1:1, which is the same as that of the native ODA-DC (19). Second, the elution profile of DC1–2–3 from a gel filtration column is similar to that of the native ODA-DC, suggesting the structural similarity of the recombinant and native complexes (Fig. 1B). Third, DC1–2–3 rescued the phenotype of the oda3ida1 mutant (lacking the ODA-DC, OAD, and inner arm dyneins a, c, and d; nonmotile) when introduced by electroporation-mediated protein delivery: A few hours after introduction of proteins, ~19% ($n = 2,735$) of cells became motile whereas all control cells (pulse applied without proteins) remained nonmotile ($n = 862$) (Movies S1 and S2). This motility recovery rate was much higher than when DC1–2 was used (~2%) (25), suggesting that DC3 may stabilize the ODA-DC. Finally, when DC1–2–3 was added to isolated axonemes of the oda1 mutant lacking the ODA-DC and OAD (Fig. 1D), it bound to the correct site on the doublet microtubules as judged by thin-section electron microscopy; the addition resulted in the appearance of projections (Fig. 1E–G, arrows) identical to those observed in oda6 axonemes that lack ODA but retain the ODA-DC (Fig. 1C, arrows) (17).

To obtain information about the shape of DC1–2–3, we carried out analytical ultracentrifugation and electron microscopy. Calculation of the molecular mass from the sedimentation velocity data resulted in an estimate of ~152 kDa (Fig. 2A and Fig. S1). This value is close to the sum of the predicted molecular masses of DC1, DC2, or DC3 (~167 kDa), providing further evidence that DC1–2–3 is a heterotrimer. Low-angle rotary shadowing electron microscopy after fixation with 0.1% glutaraldehyde revealed that DC1–2–3 takes on an ellipsoidal shape (Fig. 3A and B). The major axis length of these particles was normally distributed with a mean of ~28 nm (Fig. 3C). Assuming that the thickness of the platinum coating was 2 nm on all sides, the actual mean length of DC1–2–3 is estimated to be ~24 nm.

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**Fig. 1.** Expression of functional recombinant ODA-DC (DC1–2–3) in vitro. (A) SDS/PAGE of DC1–2–3 stained with Coomassie brilliant blue. DC1 and DC2–6xHis coexpressed in Sf21 insect cells were mixed with bacterially expressed GST–DC3 after digestion with protease to cleave off GST, and then purified using a Ni-NTA column. The resultant DC1–DC2–6xHis–DC3 complex (termed DC1–2–3) contains equimolar amounts of the three proteins (DC1:DC2:DC3 = 1.0:0.89:0.92). (B) Gel filtration column chromatography fractions of DC1–2–3 (Upper, Coomassie brilliant blue-stained gel) and the native ODA-DC extracted from oda2 axonemes (Lower, Western blot probed with antibodies for each subunit). For each sample, fractions from 21 to 35 min were subjected to SDS/PAGE or Western analysis. The elution patterns of DC1–2–3 and native ODA-DC were almost the same, indicating the structural similarity of the two complexes. (C–G) Thin-section electron microscopy images of axonemes: (C) oda6, lacking OAD but having the ODA-DC; (D) oda1, lacking both OAD and the ODA-DC; and (E–G) oda1 mixed with DC1–2–3. C–G, Lower show representative doublets. The projections (arrows) were observed at the same site on the doublets as the native ODA-DC on the oda6 axoneme (C, arrows).

**Fig. 2.** ODA-DC is a heterotrimer. (A) Sedimentation coefficient distribution function c($s$) was obtained from sedimentation velocity data by SEDFIT (36). c($s$) was converted to c($M$) to estimate the molecular mass of DC1–2–3 to be ~152 kDa. (B) There were two minor peaks in A; 2.74% of the solutes had an S value of 3.04S (64.5 kDa), which is close to the molecular mass of DC2 (62,204 Da). This peak most likely reflects DC2–6xHis that was purified by Ni-NTA but did not form a complex with DC1 or DC3; 0.43% of the solutes had an S-value of 8.915 (325 kDa), which is close to twice the sum of the molecular masses of DC1, DC2, and DC3 (333,850 Da). This peak suggests that a small fraction of the DC1–2–3 complexes formed dimers.
togram of the major axis length of DC1
Typical images of DC1
croscopy images of DC1

End-to-End Association of ODA-DC on Outer Doublets. To examine subunit-subunit interactions either within the ODA-DC or between ODA-DCs, we used chemical cross-linking with 1,6-Bismaleimido-hexane (BMH), a sulfhydryl–sulfhydryl cross-linker, under various conditions.

First, we treated isolated oda6 axonemes with BMH; the cross-linked products were then solubilized and immunoprecipitated with anti-DC1 antibody. The SDS/PAGE pattern of the immunoprecipitates showed three or four major bands above the band of DC1. Mass spectrometry revealed that the band at ~230 kDa contained peptides from only DC1 and DC2 (Fig. 4A, arrow; and Fig. S2). Although the predicted molecular masses of DC1 and DC2 are 83 and 62 kDa, they migrate in SDS/PAGE with apparent masses of ~105 and 70 kDa, respectively. Therefore, this product is most likely composed of one DC1 and two DC2 molecules (the combined molecular masses would be 207 kDa with an apparent mass in SDS/PAGE of ~245 kDa). In support of this possibility, BMH reacts with Cys residues, and there are two Cys in DC1 and one Cys in DC2 (Fig. S24); hence, DC1 can be cross-linked to two adjoining DC2s, and those DC2s then cannot be cross-linked to another protein. Western analysis of the high-salt extract from the cross-linked axonemes confirmed that the ~230-kDa product contained both DC1 and DC2 but not α- or β-tubulin (Fig. 4B, arrow).

Second, a mixture of DC1–2–3 and porcine brain microtubules was cross-linked with BMH. In Western analysis of the high-salt extract of the specimen, a band pattern similar to that in Fig. 4B was observed (Fig. 4C, arrow). The resultant ~240-kDa product reacted with anti-DC1 and -DC2 antibodies but not with anti-tubulin antibodies. Quantitative mass spectrometry of this product revealed that it was mostly composed of DC1 and DC2 and that the ratio of DC1 to DC2 was 0.6. Small amounts of DC3 (~4%) and β-tubulin (~1%) were also present (Table S1). From these results, it seems most likely that this ~240-kDa product contains one DC1 and two DC2s.

A band at ~180-kDa was detected only by anti-DC2 antibody (Fig. 4C, arrowhead). Mass spectrometry showed that the product was composed mostly of DC2 (with small amounts of DC1 (~1%), DC3 (~1%), and β-tubulin (~0.1%) (Table S2). These results suggest that the ~180-kDa band contains at least two DC2s. A similar ~180-kDa band was also detected by the anti-DC2 antibody in cross-linked axonemes (Fig. 4B, arrowhead), although its amount relative to other products was less than that in Fig. 4C. The differences may be due to the fact that binding of the ODA-DC is constrained to a specific site on the doublet microtubules, whereas it can bind to multiple sites around cytoplasmic microtubules.

Intriguingly, upon cross-linking the DC1–2 complex with BMH in the absence of microtubules, a product containing one DC1 and two DC2s was also detected (Fig. 4D, arrow). This result indicates that a small fraction of the DC1–2 complex dimerizes even in solution. The slight differences in apparent masses of the 1:2 complex of DC1–2 in Fig. 4A–D might be caused by different gel sizes (SI Materials and Methods). Consistent with this idea, in the sedimentation distribution of DC1–2–3, there was a minor peak of ~325 kDa, suggesting that a small fraction of DC1–2–3 forms dimers in solution (Fig. 2B).

Because the sedimentation analysis (Fig. 2) showed that the ODA-DC is a heterotrimer (i.e., containing only one molecule each of DC1, DC2, and DC3), the appearance of cross-linked products containing two DC2s indicates that ODA-DCs associate with each other on the doublet. Furthermore, these results also suggest that self-association of the ODA-DCs does not require the presence of axonal components other than microtubules.

Quantitative Binding Assay. The apparent self-association of the ODA-DCs suggests potential cooperative binding to microtubules. To explore this possibility, we quantified ODA-DC–microtubule binding by assaying cosedimentation of DC1–2–3 with either porcine brain cytoplasmic microtubules or three kinds of Chlamydomonas axonemes: axonemes from wild type (in which the binding sites for the ODA-DC are fully occupied), from oda6 (~70% occupied; Fig. S3), and from oda1 (all sites empty).

The amount (B) of DC1–2–3 bound to microtubules and axonemes shows a dependence on the DC1–2–3 concentration that can be approximated by the Hill equation $B = B_{\text{max}} \times F^\delta (K_A + F)^\delta$, where $F$ is the concentration of free DC1–2–3, $B_{\text{max}}$ is the saturated amount of bound DC1–2–3, $n$ is the Hill coefficient (a measure of cooperativity), and $K_A$ is the apparent dissociation constant (Fig. 5A). The saturated amounts of bound DC1–2–3 were in the order of cytoplasmic microtubules > oda1 axonemes > oda6 axonemes > wild-type axonemes, reflecting the availability of “empty” sites for the ODA-DC. From Scatchard plots of the data, the number of ODA-DCs that bind to a 24-nm segment of microtubule were estimated to be ~0.46 for wild type, ~0.71 for oda6, and ~2.0 for cytoplasmic microtubules (Table 1 and Fig. S4A–D). We may consider the binding to wild-type axonemes as nonspecific and subtract this value from all data. With this correction the amount of ODA-DC bound to the oda1 axoneme would be close to 0.9, therefore ODA-DC binding to oda1 axonemes saturates at the wild-type level (in
Chlamydomonas, one of the nine doublets has no ODA-DC and OAD, hence the number of native ODA-DC molecules on wild-type axonemes is ~0.9 per 24 nm (26). The results are consistent with the electron microscopy observations that the ODA-DC preferentially binds to a specific site on the doublet microtubules. This site presumably is specified by some additional structure(s) on the microtubules.

A previous study showed by electron microscopy of microtubule cross-sections that up to about four rows of dynein arms become attached to a cytoplasmic microtubule upon addition of high-salt extracts from axonemes (containing OADs and ODA-DCs) (27). In contrast, in our experiments, the saturating amount of DC1–2–3 bound to microtubules was about two per 24 nm. Although the reason for this apparent discrepancy is not understood, it may be that the saturating level of DC1–2–3 was underestimated in our experiments because it was technically difficult to measure microtubule binding with high concentrations of DC1–2–3. Moreover, a mixture of OADs and ODA-DCs is likely to have a much higher microtubule-binding affinity than ODA-DCs alone, because the OAD intermediate chain IC1 binds directly to tubulin (28–30).

The dissociation constant between DC1–2–3 and oda1 axonemes was calculated to be 7.8 × 10⁻³⁰, which is nearly 20 times lower than that between DC1–2–3 and cytoplasmic microtubules, 1.6 × 10⁻¹⁷ (Table 1 and Fig. S4 A–D). Therefore, the microtubule–ODA-DC affinity must be strengthened by yet unidentified factors on the doublet. The Hill coefficient of the binding curve was 2.8 for the oda1 axonemes and 1.4 for cytoplasmic microtubules, indicating that the binding is positively cooperative (Fig. S4 E and F).

### Table 1. Parameters of DC1–2–3 binding to microtubules

<table>
<thead>
<tr>
<th>Microtubules used for cosedimentation</th>
<th>$K_{d}$, M</th>
<th>$B_{max}$, μM (no. of molecules per 24 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oda1 axonemes</td>
<td>7.8 × 10⁻⁹</td>
<td>0.069 (1.3)</td>
</tr>
<tr>
<td>oda6 axonemes</td>
<td>6.1 × 10⁻⁸</td>
<td>0.037 (0.71)</td>
</tr>
<tr>
<td>Wild-type axonemes</td>
<td>2.5 × 10⁻⁷</td>
<td>0.024 (0.46)</td>
</tr>
<tr>
<td>Cytoplasmic microtubules</td>
<td>1.6 × 10⁻⁷</td>
<td>0.26 (2.0)</td>
</tr>
</tbody>
</table>

In Vitro Cooperative Binding to Microtubules Observed by Microscopy. Cooperative binding between DC1–2–3 and microtubules could be directly observed by light microscopy. In the experiment shown in Fig. 5B, rhodamine-labeled cytoplasmic microtubules were mixed with different concentrations of Alexa-488-labeled DC1–2–3 and observed by fluorescence microscopy. At 0.1 μM DC1–2–3, some microtubules were labeled whereas others were not at all. At 0.3 μM, more microtubules were labeled but some remained unlabeled. At 0.6 μM, almost all microtubules were labeled. (B, Upper) The lengths of the arrows represent the fluorescence intensity, and microtubules marked with the same size of arrows have similar intensities. Shortest arrows are ~30 a.u., mid-size arrows ~60 to ~70 a.u., and the longest arrow ~87 a.u.

ODA-DC Binding to Axonemes in Vivo. The process by which the native ODA-DC binds in vivo was observed by the immunofluorescence microscopy of Chlamydomonas zygotes. We first mated oda1oda6 with a transformant of oda1oda6 expressing HA-tagged DC2 to examine the manner of binding of DC2-HA to the oda1oda6 axonemes that initially lacked the ODA-DC. We found that the signal of DC2-HA first appeared in the proximal part and then proceeded to the distal part of the oda1oda6 flagella (Fig. 6A4). The unidirectional extension of the bound portion indicates that newly transported ODA-DCs add next to preexisting ODA-DCs along the flagellum.

![Fig. 5. Cooperative binding of DC1–2–3 to microtubules. (A) Binding between DC1–2–3 and axonemes or cytoplasmic microtubules was analyzed by cosedimentation assay. Purified DC1–2–3 was mixed with axonemes of oda1, oda6, and wild-type cells, and with porcine brain cytoplasmic microtubules. The amount of bound DC1–2–3 was calculated as the number of molecules bound to microtubules per 24 nm (y axis), and plotted against total DC1–2–3 (x axis). The saturated amount of bound DC1–2–3 was in the order of cytoplasmic microtubules > oda1 > oda6 > wild type, reflecting the available sites for ODA-DC binding. (B) Different concentrations of Alexa-488-labeled DC1–2–3 were mixed with rhodamine-labeled microtubules and observed by fluorescence microscopy. At 0.1 μM DC1–2–3, some microtubules were labeled whereas others were not at all. At 0.3 μM, more microtubules were labeled but some remained unlabeled. At 0.6 μM, almost all microtubules were labeled. (B, Upper) The lengths of the arrows represent the fluorescence intensity, and microtubules marked with the same size of arrows have similar intensities. Shortest arrows are ~30 a.u., mid-size arrows ~60 to ~70 a.u., and the longest arrow ~87 a.u.](image-url)
orange) in the experiments of patterns of labeling for the ODA-DC (green) and the OAD (shades of oda1). The axoneme assembly in the nemesis; hence, ODA assembly was constrained to follow the pattern of ODA-doublets of the axoneme. The difference is that the ODA-DC was already present on the contrast, binding of OAD gradually increased over the whole length of the axoneme. (∆oda1oda6) demonstrated that when a high-salt extract from Chlamydomonas axonemes contains both OAD and ODA-DCs. Our present results suggest that the ODA-DC is based on the size of the ODA-DC. Consequently OAD is arranged on the doublet from the proximal part of the axoneme to the distal part. Subsequently, OAD binding of the ODA-DC. The ODA-DC cooperatively binds to the outer doublet microtubule. The ODA-DC molecules cooperatively bind to the doublet in an end-to-end manner and establish a 24-nm periodicity. Subsequently, OAD binds to the outer doublet through its association with the ODA-DCs on the doublet, and this interaction is stabilized through the direct interaction of IC1 with the microtubule, thereby establishing the 24-nm periodicity in the OAD arrangement (24, 25, 29, 30).

Our study suggests that the ODA-DC functions not only as the docking site for OAD, but that its self-association during its assembly onto the axoneme forms a scaffold of 24-nm periodicity that ensures the ordered assembly of the OAD along the doublet microtubule. If the ODA-DC bound to doublet microtubules independently of other bound ODA-DCs, then the 24-nm ODA-DC would be able to bind to any three longitudinally aligned tubulin dimers, which together span 8 × 3 nm. In such a case, there would be a high probability of producing a gap of one or two tubulin dimers between adjacent ODA-DCs and hence between adjacent OADs. Ultimately, such gaps would perturb the regular arrangement of OAD in the 96-nm axonemal repeat and disrupt interactions between OAD and inner arm dyneins that are necessary for the coordination of force generation by the different dyneins (2, 32). The cooperativity in the ODA-DC binding would prevent such gap formation.

This study has yielded insight into the molecular mechanism that establishes the long-range periodicity of outer dynein arms along the doublet microtubules of the flagellar axoneme. A similar mechanism may underlie the periodic binding of other microtubules without any preferred position along the length. In other words, OAD binding on axonemes containing the ODA-DC does not proceed in a single direction; this is in strong contrast to the binding of OAD on mutant axonemes lacking the ODA-DC, which always proceeds from base to tip.

**A Model for Construction of OAD Periodicity.** Haimo et al. demonstrated that when a high-salt extract from Chlamydomonas wild-type axonemes is mixed with cytoplasmic microtubules, OAD orderly attaches to the microtubules with a periodicity of 24 nm (28). The 24-nm periodicity and the ordered arrangement were thought to be produced by interaction of adjacent OADs, which are ~24 nm long. However, it must be noted that a high-salt extract from the wild-type axonemes contains both OAD and ODA-DCs. Our present results suggest that the ODA-DC provides the basis for cooperative binding on the outer doublet. It is likely that the ODA-DC also was responsible for the ordered arrangement of OAD on the cytoplasmic microtubules.

The data obtained in our present and previous studies lead to a model that explains the periodic arrangement of OAD on the outer doublet (Fig. 7). First, OAD and the ODA-DC are separately assembled in the cytoplasm and separately transported to the flagella (12, 16). After entry into the flagella, the ODA-DC, 24 nm in length, starts binding to the appropriate site at the proximal end of the doublet microtubule. The ODA-DC molecules cooperatively bind to the doublet in an end-to-end manner and establish a 24-nm periodicity. Subsequently, OAD binds to the outer doublet through its association with the ODA-DCs on the doublet, and this interaction is stabilized through the direct interaction of IC1 with the microtubule, thereby establishing the 24-nm periodicity in the OAD arrangement (24, 25, 29, 30).

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This study has yielded insight into the molecular mechanism that establishes the long-range periodicity of outer dynein arms along the doublet microtubules of the flagellar axoneme. A similar mechanism may underlie the periodic binding of other
axonemal structures, such as the radial spokes and the projections of the central pair of microtubules.

Materials and Methods

Strains and Culture of Chlamydomonas Cells. A. C. reinhardtii wild-type strain (CC124) and the following mutants lacking outer dynein arms (17) were used: oda1, with a mutation in the structural gene of DC2, lacking the ODA-DC (19); oda2, with a mutation in the structural gene of the OAD γ-CH, lacking OAD; oda6, with a mutation in the structural gene of the ODA intermediate chain IC2, lacking OAD (33). Double mutants were produced by standard procedures (34). All cells were grown in Tris-acetate-phosphate medium (35) with aeration at 25 °C on a 12:12 h light:dark cycle.

Generation and Purification of Recombinant DC1–2–3 and DC1–2. Bacterially expressed GST–DC3 (20) was purified with glutathione Sepharose 4B (17-0756-01; GE Healthcare), and treated with PreScission Protease (27-0843-01; GE Healthcare) to cleave off GST. A mixture of GST–DC3 and protease was mixed with insect culture cell lysate containing DC1 and DC2–6His produced by the baculovirus system (25), followed by purification on a Ni-NTA agarose column. (For DC1–2 purification, GST–DC3 was not added.)

See SI Materials and Methods for further information.

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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-molarity Pipes buffer using the Castoldi and Popov’s method (1). Microtubute pellets were resuspended in HMEKCl (30 mM Hepes, 5 mM MgSO4, 1 mM EGTA, 0.1 M KCl, pH 7.4) containing 10 μM paclitaxel.

Cosedimentation of Recombinant Proteins with Axonemes and Microtubules. The binding of the recombinant proteins to axonemes and microtubules was assessed by centrifugation followed by SDS/PAGE and Western blotting. DC1–2–3 samples purified with Ni-NTA resin (cComplete His-tag Purification Resin; Roche) and an Uno Q1 column (Bio-Rad) were dialyzed against HMEKCl and mixed with axonemes or porcine brain cytoplasmic microtubules. After incubation at 4 °C (axonemes) or 25 °C (microtubules) for 60 min in HMEKCl containing 0.1% Tween 20, the mixture was centrifuged at 19,000 × g for 15 min at 4 °C for axonemes or 100,000 × g for 20 min at 25 °C for microtubules. The precipitates were resuspended in HMEKCl, mixed with an equal volume of 2x SDS-sample buffer, boiled for 3 min, and analyzed by SDS/PAGE and Western blotting with anti–His–tag antibody (MBL D291-3). The amount of bound DC1–2–3 was calculated from the band intensity of DC2–His in Western blots using a calibration curve generated from purified DC1–2–3 of known concentrations. The estimated molar ratio of the DC1–2–3 bound to the oda1 axonemes compared with the ODA-DC contained in the same amount of wild-type axonemes was multiplied by eight-ninths (this is because one of the nine outer doublets in the wild-type axoneme has no ODA-DC and OAD, whereas all nine outer doublets in the oda1 axoneme are capable of binding OAD) (2). For calculation of the amount of the ODA-DC contained in the wild-type axoneme, the ODA-DC and purified DC1–2–3 used for calibration were immunodetected with anti-DC2 antibody and their intensities were compared. The amount of DC1–2–3 bound to the cytoplasmic microtubule per 24 nm was estimated from the molar ratio of the bound DC1–2–3 to total tubulin monomers (α + β-tubulins); the molar ratio was multiplied by 78 because a cytoplasmic microtubule contains 78 tubulin molecules per 24 nm (6 tubulin monomers × 13 protofilaments).

Labeling and Fluorescence Microscopy. Purified DC1–2–3 was mixed with 10-fold molar excess of Alexa-488 maleimide (A10254; Sigma-Aldrich) for 1 h at room temperature. The reaction was quenched by addition of 5 mM Tris(2-carboxyethyl)phosphine-HCl, and then free Alexa-488 maleimide was removed by Ni-NTA purification. Immunofluorescence microscopy was carried out as previously described (3). Temporary dikaryons were allowed to adhere to a slide glass, demembranated with Igepal CA-630 (18986; Sigma), and fixed with 2% (wt/vol) paraformaldehyde. The antibodies used were anti-HA tag antibody (1:100, 1583816; Roche Applied Science) and anti-IC2 antibody (1:100, D6168; Sigma-Aldrich). The secondary antibodies used were anti-mouse IgG antibody conjugated with Alexa Fluor 594 (1:500, A11005; Invitrogen) and anti-mouse IgG antibody conjugated with Alexa Fluor 350 (1:200, A11045; Invitrogen). All fluorescence images were collected using an upright fluorescence microscope (Axioplan; Zeiss) equipped with a Plan-Apochromat (×63, 1.40 N.A.) objective and a CCD camera (COOL SNAP; Photometrics). The camera was controlled by Micro-Manager 1.4 (http://micro-manager.org). Fluorescence intensity of microtubules was measured with Image J (http://imagej.nih.gov/ij/).

Mass Spectrometry. Mass spectrometry analysis [LC tandem MS on a Q Exactive (Thermo Scientific)] of the cross-linked products was performed at the Proteomics and Mass Spectrometry Facility (University of Massachusetts Medical School). For quantitative analysis, raw data files were processed with Mascot Distiller (Version 2.5) (www.matrixscience.com/distiller.html) before searching with Mascot Server (Version 2.4) (www.matrixscience.com/server.html) against the Chlamydomonas index in the non-redundant database of the National Center for Biotechnology Information, NCBI nr. Search parameters used were fully tryptic with two missed cleavages, parent mass tolerances of 0.1 ppm, and fragment mass tolerances of 0.05 Da. Variable modifications of acetyl (protein N terminus), pyro glutamic for N-terminal glutamine, oxidation of methionine, and carbamidomethyl cysteine were considered. Mascot Distiller’s Average method was used to generate extracted ion chromatograms of precursors to determine relative protein abundances from the three most intense peptides of each protein (6).

Electron Microscopy. Isolated axonemes and axonemes mixed with DC1–2–3 were observed by electron microscopy following a previously described method (7). DC1–2–3 molecules were imaged by low-angle rotary shadowing electron microscopy using Matsui et al.’s method (8). All samples were observed and

Supporting Information
Owa et al. 10.1073/pnas.1403101111

SI Materials and Methods

Gel Filtration Chromatography. Gel filtration chromatography was used to compare the fluid dynamic properties of the native outer arm dynein-docking complex (ODA-DC) and recombinant ODA-DC (DC1–2–3). The protein samples were applied to a Superose 6 HR gel filtration column (Pharmacia Biotech) using a Biologic Chromatography System HPLC apparatus (Bio-Rad) at a flow rate of 0.2 mL/min.

Chemical Cross-Linking, Immunoprecipitation, and SDS/PAGE. Chemical cross-linking and immunoprecipitation were used to detect molecular interaction between different ODA-DC subunits. The axonemes of oda6, the mixture of DC1–2–3 and cytoplasmic microtubules, or DC1–2 were treated with 1,6-Bismaleimido-hexane (Thermo Scientific) for 60 min at room temperature. After the reaction was quenched with excess β-mercaptoethanol, the axonemal and DC1–2 samples were centrifuged at 19,000 × g for 15 min at 4 °C. The ODA-DC was released from the axoneme or cytoplasmic microtubules by suspending the pellets in a high salt buffer (30 mM Hepes, 5 mM MgSO4, 1 mM EGTA, 0.6 M KCl, pH 7.4). The suspension was incubated for 10 min at 4 °C (axonemes) or 25 °C (microtubules) and then centrifuged at 19,000 × g for 10 min at 4 °C (axonemes) or 25 °C (microtubules). The samples containing cytoplasmic microtubules underwent the same procedure at room temperature. The supernatants were mixed with sample buffer, boiled, and analyzed by SDS/PAGE (the length of the separating gel was 9.5 cm for axonemal samples, or 5.5 cm for DC1–2 and cytoplasmic microtubule samples) and Western blotting. The antibodies used were anti-DC1 antibody, anti-DC2 antibody, anti-DC3 antibody, anti-α-tubulin antibody (T6074; Sigma), and anti-β-tubulin antibody (T0198; Sigma). The cross-linked samples were immunoprecipitated with anti-DC1 antibody and anti–His–tag antibody (MBL D291-3) following a previously described method (4, 5). The apparent molecular masses of the detected bands were calculated with Image J.
photographed by a JEM-1011 electron microscope (JEOL). The length of DC1–2–3 was measured using Image J.

**Analytical Ultracentrifugation.** Sedimentation velocity experiments were performed in an Optima XL-1 analytical ultracentrifuge (50Ti rotor, 40,000 rpm, 20 °C; Beckman-Coulter). Moving boundaries were recorded at a wavelength of 390 nm without time intervals between successive scans. The sedimentation coefficient distribution function c(s) was calculated by the SEDFIT program (9). The molecular mass distribution c(M) was obtained by converting c(s) on the assumption that the frictional ratio f/f0 was common to all of the molecular species as implemented in SEDFIT. The protein partial-specific volume was calculated from the amino acid sequence; the buffer density and viscosity were calculated according to the solvent composition using the program SEDNTERP (10).

**Protein Electroporation.** Recombinant proteins were introduced into live *Chlamydomonas* cells by electroporation as described in ref. 11. Briefly, autolysin-treated *oda3ida4* cells were mixed with DC1–2–3 (1.2 mg/mL), and an electric pulse was applied with an ECM630 electroporation system (BTX). Cells were observed under a dark-field microscope and recorded using a video camera 2.5 h after pulse application.

**Generation of the *oda1oda6::ODA1–3×HA* Strain.** The *ODA1* gene was amplified from a BAC library (PTQ12942) of the *Chlamydomonas* genome, and an NruI site was inserted immediately in front of the stop codon. A 3×HA tag fragment was inserted at the NruI site and the construct, along with the plasmid pSI103, cotransformed into the nonmotile double mutant *ida2oda1* (12). Rescued strains that displayed the slow-swimming phenotype of *ida2* were isolated and mated with *oda1oda6. oda1oda6::ODA1–3×HA* was selected from the progeny as a clone that had an HA epitope and displayed the jerky swimming typical of an *oda* mutant.

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Fig. S2. (A) The amino acid sequences of DC1 and DC2. The peptides detected by mass spectrometry of the ∼230-kDa product in Fig. 5A are highlighted (yellow boxes represent where some peptides overlapped). The cysteine residues are highlighted in purple boxes. (B) All peptides were detected by mass spectrometry.

Fig. S3. The relative amounts of the ODA-DC in oda6 or oda9 axonemes compared with wild type. Values were determined from DC2 band intensities in the Western blot (Upper), and normalized based on the tubulin band intensities in the Coomassie brilliant blue (CBB)-stained gel (Lower).
Fig. S4. (A–D) Scatchard plot analyses of binding data between DC1–2–3 and (A) oda1 axonemes, (B) oda6 axonemes, (C) wild-type axonemes, and (D) porcine brain cytoplasmic microtubules. Bound (B)/free (F) was plotted against bound (B). $K_d$ and $B_{max}$ are (A) $7.8 \times 10^{-9}$ M and 0.069 μM, (B) $6.1 \times 10^{-8}$ M and 0.037 μM, (C) $2.5 \times 10^{-7}$ M and 0.024 μM, and (D) $1.6 \times 10^{-7}$ M and 0.26 μM. (E and F) Hill plot analyses of binding between (E) DC1–2–3 and oda1 axonemes, and (F) DC1–2–3 and porcine brain cytoplasmic microtubules. Log $B/(B_{max} - B)$ was plotted against log F. Hill coefficients derived from the fits are (E) 2.8 and (F) 1.4, suggesting positively cooperative interactions.

Table S1. Quantitative analysis of proteins in the ∼240-kDa product in Fig. 4C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Score*</th>
<th>Mass</th>
<th>Amount, %†</th>
</tr>
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<tbody>
<tr>
<td>DC2</td>
<td>7,782</td>
<td>62,223</td>
<td>100</td>
</tr>
<tr>
<td>DC1</td>
<td>5,226</td>
<td>83,440</td>
<td>60.943</td>
</tr>
<tr>
<td>DC3</td>
<td>815</td>
<td>21,556</td>
<td>3.813</td>
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<tr>
<td>β-Tubulin</td>
<td>147</td>
<td>50,157</td>
<td>0.137</td>
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*Score values represent the summation of individual Mascot scores for identified peptides.
†Amount values represent relative ratios, with the most abundant protein normalized to 100 by Mascot Distiller (method: Average).
Table S2. Quantitative analysis of proteins in the ∼180-kDa product in Fig. 4C

<table>
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<tr>
<th>Protein</th>
<th>Score*</th>
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<th>Amount, %†</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>DC1</td>
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<tr>
<td>DC3</td>
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*Score values represent the summation of individual Mascot scores for identified peptides.
†Amount values represent relative ratios, with the most abundant protein normalized to 100 by Mascot Distiller (method: Average).

Movie S1. Cells of oda3ida4 after electroporation-mediated introduction of DC1–2–3; ∼19% cells (n = 2,735) became motile.

Movie S2. Cells of oda3ida4 after application of pulse without proteins; no cells became motile (n = 862).