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Protein–protein interactions between intermediate chains and the docking complex of *Chlamydomonas* flagellar outer arm dynein

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ABSTRACT

Outer arm dynein (OAD) is bound to specific loci on outer-doublet-microtubules by interactions at two sites: via intermediate chain 1 (IC1) and the outer dynein arm docking complex (ODA-DC). Studies using *Chlamydomonas* mutants have suggested that the individual sites have rather weak affinities for microtubules, and therefore strong OAD attachment to microtubules is achieved by their cooperation. To test this idea, we examined interactions between IC1, IC2 (another intermediate chain) and ODA-DC using recombinant proteins. Recombinant IC1 and IC2 were found to form a 1:1 complex, and this complex associated with ODA-DC in vitro. Binding of IC1 to mutant axonemes revealed that there are specific binding sites for IC1. From these data, we propose a novel model of OAD-outer doublet association.

Structured summary of protein interactions:
IC2 physically interacts with **DC2** and **DC1** by anti bait coimmunoprecipitation (View interaction)
DC2 physically interacts with **IC2** and **IC1** by anti bait coimmunoprecipitation (View interaction)
IC2 and **IC1** physically interact by cross-linking study (View interaction)
IC2 and **DC1** physically interact by cross-linking study (View interaction)
DC2 and **DC1** physically interact by cross-linking study (View Interaction: 1, 2)
DC1 and **IC1** physically interact by cross-linking study (View interaction)
IC2 binds to **IC1** by anti bait coimmunoprecipitation (View interaction)
IC2, **DC1** and **DC2** physically interact by cross-linking study (View interaction)
DC2, **IC1** and **DC1** physically interact by cross-linking study (View interaction)

1. 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 [1], 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. Also, it is important for understanding the mechanisms underlying human diseases called primary ciliary dyskinesia (PCD), since they are mostly caused by defects in OAD assembly [2].

Chlamydomonas OAD consists of three heavy chains (HCs: α, β, and γ), two intermediate chains (ICs: IC1 and IC2), and 11 light chains (LCs). It is a complex macromolecular system with three globular "heads" composed of the C-terminal regions of the HCs, and a "tail" comprising the HC N-terminal regions, and the ICs and LCs. At the base of the tail, an additional structure called the outer-dynein-arm docking complex (ODA-DC) is present and mediates the binding of OAD to the doublet microtubule. The ODA-DC is composed of three subunits, DC1, DC2 and DC3. It is preassembled in the cytoplasm and transported into flagella independently of OAD [3,4].

IC1 and the ODA-DC are considered important for OAD-doublet association. IC1 was shown by chemical crosslinking to directly bind to α-tubulin [5]. The ODA-DC also must be important for OAD-doublet microtubule binding, since mutants lacking the

Abbreviations: BMH, bismaleimidohexane; IC, intermediate chain; LC, light chain; OAD, outer arm dynein; ODA-DC, outer dynein arm docking complex

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ODA-DC lack OAD in the axoneme, even though a complete OAD complex is assembled in the cytoplasm [4,6]. Binding of the ODA-DC and OAD involves interaction between a LC (LC7b) and DC2 [7]. Studies using a mutant lacking DC3 indicate that DC1 and DC2 are responsible for the binding of OAD to the ODA-DC [8].

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 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)) [6]. 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.

To further explore the mechanism of OAD–doublet microtubule association, in this study we performed protein–protein interaction analyses between IC1, IC2, DC1, DC2, and microtubules. We established an expression system for these proteins using insect culture cells and used the recombinant proteins for biochemical analyses. Our results suggest that, although individually IC1 and the ODA-DC attach OAD only weakly to the doublet microtubules, they associate with each other through multiple interactions, and that together this association strengthens OAD attachment to the doublets.

2. Materials and methods

For details see Supplementary information.

2.1. Strains and culture of Chlamydomonas reinhardtii cells

The following mutants of *Chlamydomonas reinhardtii* were used: *oda1* (ΔDC2) [9,10], *oda3* (ΔDC1) [9,11], *oda6* (ΔIC2) [9,12], *oda9* (ΔIC1) [9,13], *ida4* (Δp28) [14], and *ida5* (Δactin) [15]. Double mutants of *oda1ida5*, *oda3ida4*, *oda6ida4*, and *oda9ida4* were produced by the standard procedure [16]. All cells were grown in Tris–acetate-phosphate (TAP) medium with aeration at 25 °C, on a 12 h/12 h light/dark cycle [17].

2.2. Preparation of Chlamydomonas axonemes

Flagellar axonemes were isolated from *Chlamydomonas reinhardtii oda1* strain by the method previously described [18]. Axonemes were resuspended in HMDEK (30 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM EGTA, and 50 mM potassium acetate).

2.3. Preparation of recombinant IC1, IC2, DC1 and DC2

IC1, IC2, DC1 and DC2 were expressed in *Sf21* cells by baculovirus system. IC1 was tagged with 6 × His at the N-terminus (for IP) or the C-terminus (for electroporation experiments), and not tagged for experiments that assayed co-purification with IC2. Other proteins were 6 × His-tagged at the C-terminus except for DC1 or DC2 used in co-purification experiments. 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).

2.4. Protein electroporation

Electroporation was used to introduce recombinant proteins into live *Chlamydomonas* cells as described in [19]. Briefly, autolysin-treated cells were mixed with a recombinant protein (0.5–1.0 mg/ml), and an electric pulse was applied with an ECM600 electroporation system (BTX, Holliston, MA, USA). Cell images were observed under a dark-field microscope and recorded using a video camera.

2.5. 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 [20]. Microtubule pellets were resuspended in HMDEK containing paclitaxel.

2.6. Immunoprecipitation

Protein A-agarose beads (Roche) were washed with blocking buffer (TBS, pH 7.2, 3% BSA (w/v), 1%Triton-X100 (v/v)), incubated with the anti-DC2 antibody [3] or anti-IC2 antibody (sigma), and then incubated with purified recombinant proteins. The resultant beads were resuspended with SDS sample buffer.

2.7. Chemical crosslinking of immunoprecipitated products

Recombinant proteins were mixed and treated with the chemical crosslinker bismaleimidohexane (BMH) (Pierce Chemical, Rockford, IL, USA) for 1 h at room temperature [5]. Reactions were terminated by the addition of SDS–PAGE sample buffer containing 2-mercaptoethanol.

2.8. Co-precipitation assay of recombinant proteins with axonemes

The purified proteins were mixed with axonemes and incubated for 20 min at 4 °C. The samples were centrifuged at 20,000×*g* for 12 min at 4 °C. Pellets were washed with the same buffer, and then resuspended in SDS–PAGE sample buffer forimmunoblotting. Signals were detected by chemiluminescence. The amount of ICs was calculated from the luminescence intensity and a calibration curve determined with known amounts of purified ICs.

3. Results

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

We used a baculovirus system to obtain protein samples. This system yielded much greater amounts of recombinant IC1 and IC2 than the in vitro translation system used in a previous study [21] and allowed us 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. 1A). Recombinant IC1 and IC2 tagged with 6 × His were partially purified with Ni–NTA agarose beads (Fig. 1B). When IC1 without a His-tag and His-tagged IC2 were co-expressed, they could be co-purified with Ni–NTA (Fig. 1B), 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 immuno-precipitated both IC2 and IC1 from the mixture of these proteins (Fig. 3A). 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