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Association of a 66 kDa Homolog of *Chlamydomonas* DC2, a Subunit of the Outer Arm Docking Complex, with Outer Arm Dynein of Sperm Flagella in the Ascidian *Ciona intestinalis*

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We previously identified a 66 kDa axonemal protein (Ci-Axp66.0) in sperm of the ascidian *Ciona intestinalis*. Here we found that Ci-Axp66.0 shows sequence similarity to the DC2 subunit of the *Chlamydomonas* outer arm docking complex. Analysis of secondary structure of Ci-Axp66.0 suggested that the N-terminal two-thirds of the molecule is rich in coiled coil structure, as in *Chlamydomonas* DC2. Immunogold localization revealed that it is located in the vicinity of outer arm dynein. Ci-Axp66.0 was partly extracted from the axonemes by a high salt solution and co-purified with outer arm dynein. This co-purification was not affected by the absence of Mg^{2+} in isolation buffer, indicating that Ci-Axp66.0 is associated with outer arm dynein. These results suggest that Ci-Axp66.0 is a component of the outer arm dynein docking complex in the axonemes of *Ciona* sperm.

Key words: dynein, flagella, sperm motility, axoneme, ascidian

INTRODUCTION

Flagellar axonemes are structures that have been well conserved during evolution. They are highly organized and contain approximately 250 proteins (Inaba, 2003). The power stroke for flagellar bending is exerted by molecular motor dyneins. Axonemal dyneins are divided into two categories according to their position on the outer doublet microtubule: the outer and inner arm dyneins. The molecular structure of outer arm dynein has been better studied than that of inner arm dynein. Outer arm dynein is composed of three polypeptide groups classified on the basis of size as the dynein heavy, intermediate, and light chains (King, 2000; Inaba, 2003). The heavy chains are members of the AAA protein family and consist of a ring-like head domain with ATP hydrolytic sites, and a flexible stem extending towards the base of the dynein molecule. Intermediate chains (ICs) and light chains (LCs) associate to form a complex at the dynein base and are involved in the assembly or regulation of dyneins.

Outer arm dynein from *Chlamydomonas* has two WD-repeat ICs, IC78 and IC69, both of which are members of the WD repeat family, along with the cytoplasmic intermediate chain, IC74 (Ogawa *et al*., 1995). Sea urchin outer arm dynein contains an intermediate chain with both thioredoxin and nucleoside diphosphate kinase domains, as well as two WD-repeat intermediate chains with sequence similarities to *Chlamydomonas* IC78 and IC69 (Ogawa *et al*., 1996). The outer arm dyneins of *Ciona*, salmonid fish, and molluscs contain another two or three intermediate chains; however they have not been characterized at the molecular level (Ogawa *et al*., 1996; Padma *et al*., 2001).

Outer arm dyneins are arranged on each doublet microtubule at intervals of 24 nm. It has been suggested that this periodical binding of outer arm dyneins is achieved by the outer dynein arm-docking complex (ODA-DC), which is located at the outer-arm-binding site on the doublet microtubule. The ODA-DC of *Chlamydomonas* contains equimolar amounts of three polypeptides, DC1 (83 kDa), DC2 (62

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kDa), and DC3 (21 kDa) (Takada and Kamiya, 1994; Wakabayashi *et al.*, 2001; Takada *et al.*, 2002). DC1 and DC2 were predicted to be coiled-coil proteins, and it has been suggested that they function in the positioning of outer arm dynein (Takada *et al.*, 2002; Koutoulis *et al.*, 1997). DC3 is a novel member of the EF-hand superfamily of calcium-binding proteins and is probably important for the assembly and regulation of both the outer dynein arm and the ODA-DC (Casey *et al.*, 2003). Although the ODA-DC potentially plays an important role in the positioning of outer arm dynein in *Chlamydomonas* flagella, it has not been identified in the axonemes of metazoan cilia and flagella.

We have isolated a cDNA encoding an axonemal protein with a molecular mass of 66 kDa (Ci-Axp66.0) from a testis cDNA library from the ascidian *Ciona intestinalis* (Padma *et al.*, 2003). By detailed sequence analysis, we show here that it is a homolog of *Chlamydomonas* DC2. Immunolocalization revealed its position at the base of the outer arm dynein, suggesting that it functions as a subunit of the ODA-DC in *Ciona* axonemes. Unlike *Chlamydomonas* DC2, Ci-Axp66.0 can be co-purified with the outer arm dynein even in the absence of Mg^{2+}.

MATERIALS AND METHODS

Isolation of outer arm dynein from *Ciona* axonemes

The ascidian *Ciona intestinalis* was collected near the Education and Research Center of Marine Bio-Resources, Tohoku University, or was cultured at the Maizuru Fisheries Research Station of Kyoto University or the Otsuchi Marine Research Center, University of Tokyo. Sperm were collected by dissecting the sperm duct and were diluted with approximately three volumes of filtered seawater. They were filtered through 62-μm nylon mesh and centrifuged at 8,000×*g* for 10 min at 4°C. The sperm pellet was washed with filtered seawater by suspension followed by centrifugation. Sperm flagella and axonemes were prepared from the sperm pellet

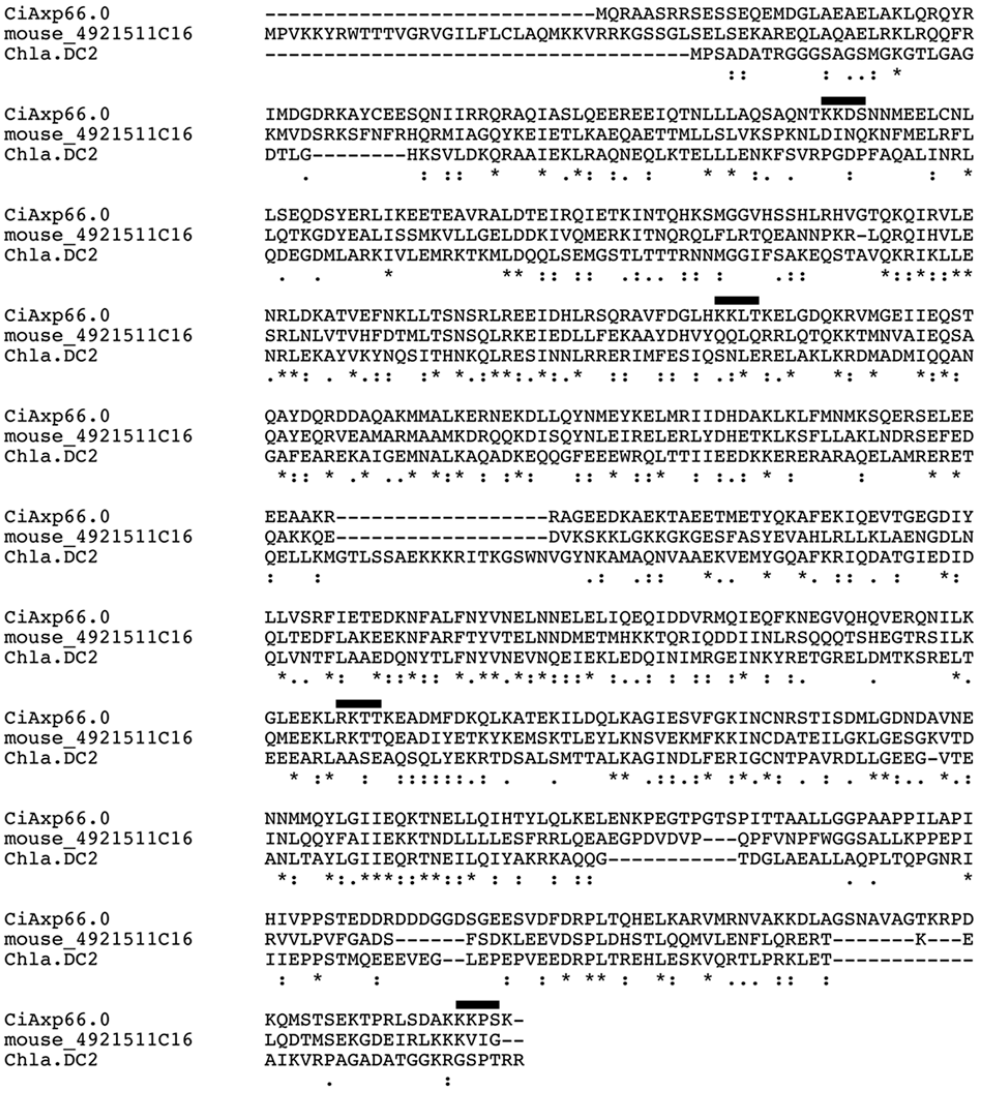


Fig. 1. Sequence comparison of *Ciona* Ci-Axp66.0 with homologs in mouse (4921511c16, accession number AK029513) and *Chlamydomonas* ODA-DC DC2 (accession number AY039618), aligned by CLUSTALW. Asterisks, colons, and dots indicate residues identical in all sequences in the alignment, conserved substitutions, and semi-conserved substitutions, respectively. Motifs for phosphorylation by cAMP-dependent protein kinase ([Arg/Lys]-[Arg/Lys]-X-[Ser/Thr]) are indicated by bold bars.

as described previously (Inaba and Mohri, 1989).

Outer arm dynein was isolated according to the method described previously by Inaba and Mohri (1989). Briefly, axonemes were treated with a high-salt buffer (0.6 M KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris-HCl pH 8.0, 0.2 mM DTT) and kept for 30 min on ice. The suspension was centrifuged at 12,000×*g* for 15 min and the supernatant was further centrifuged at 100,000×*g* for 30 min. The final supernatant was applied to a Superdex 200 gel-filtration column (10×300 mm, Amersham Biosciences). Proteins were separated at a flow rate of 0.2 ml/min and fractions of 0.25 ml each were collected. Further separation was conducted usisng a Poros HQ anion exchange column (4.6×100 mm; Applied Biosystems, Foster City, CA) with a BioCAD HPLC Workstation (Global Medical Instrumentation, Albertville, MN). Activity of ATP hydrolysis in each fraction was determined by the method of Taussky and Schorr (1953).

Fractions rich in Ci-Axp66.0 were pooled and dialyzed against Mg^{2+} buffer (0.15 M KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 10 mM Tris-HCl, pH 8.0, 0.2 mM DTT) or Mg^{2+}-free buffer (0.15 M KCl, 2 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl pH 8.0, 0.2 mM DTT). Samples were applied to a 5–20% sucrose density gradient made with the same buffer and centrifuged in a HITACHI Himac CP65β centrifuge with P28S2 rotor at 26,000 rpm for 21 hrs. Fractions of 500 μl each were collected.

Preparation of antibody

The open reading frame of Ci-Axp66 was amplified by polymerase chain reaction. Sense and antisense primers used were 5'-CGCGGGATCCATGCAGCGAGCAGCAAGCA and 5'-CGCGCAATTCAAATGTATGGTGTTCGCA, respectively. The amplified cDNA and pET32a vector were cleaved with EcoRI and BamHI, ligated to each other after removal of small DNA fragments by an S-400 spin column (Amersham Biosciences), and transformed into AD494 host cells. Expression of Ci-Axp66.0/thioredoxin fusion protein was induced by the addition of 2 mM IPTG and was checked by SDS-PAGE on a 10% polyacrylamide gel (Laemmli, 1970). Bacterial cells expressing the protein were harvested by centrifugation at 10,000×g for 5 min at 4°C. The protein was extracted with 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0, 6 M urea, and 0.5% Triton X-100, and the recombinant protein was purified in a column with Ni^{2+}-immobilized His-Bond metal chelation resin (Novagen, Madison, WI). The fractions containing the fusion protein were assessed by SDS-PAGE, pooled, and dialyzed against PBS. The purified protein was emulsified with Freund's complete adjuvant, and mice were given three subcutaneous injections at intervals of 10 days. Finally a booster dose was given 1 week before the collection of serum. The antiserum was divided into 20-μl aliquots and stored at –80°C until use.

Immunological analysis

Immunofluorescence microscopy and immunogold electron microscopy were carried out according to the methods described previously (Padma *et al*., 2003). Anti-Ci-Axp66.0 antiserum was diluted 1:100 for both immunofluorescence and immunogold electron microscopy. Immunoprecipitation with anti-Ci-Axp66.0 antibody was carried out as previously described (Satouh *et al*., 2005).

Sequence analysis

The cDNA insert in λZAP II vector was subcloned into pBluescript by in vivo excision and sequenced using a BigDye terminator sequencing kit with an ABI310 DNA sequencer. Translation of the DNA sequence, calculation of molecular mass, and estimation of isoelectric points were conducted by GENETYX-Mac software. Multiple sequence alignment was carried out by CLUSTALW. Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used for prediction of functional sites or domains in the amino acid sequence. The prediction of coiled-coil structure was performed using COILS (Lupas *et al*., 1991). Searches in the *Ciona* genomic and cDNA databases were performed on-line databases at http://ghost.zool.kyoto-u.ac.jp/indexr1.html and http://genome.jgi-psf.org/*Ciona*4/*Ciona*4.home.html.

Two-dimensional gel electrophoresis

For the first dimension, 70 μg of axonemal proteins solubilized in a solution containing 8 M urea, 2 M thiourea, 10% isopropanol, 0.1% Triton X-100, 50 mM dithiothreitol, 4% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate, and 5% IPG buffer (Amersham Biosciences), were incubated for 20 min at room temperature.

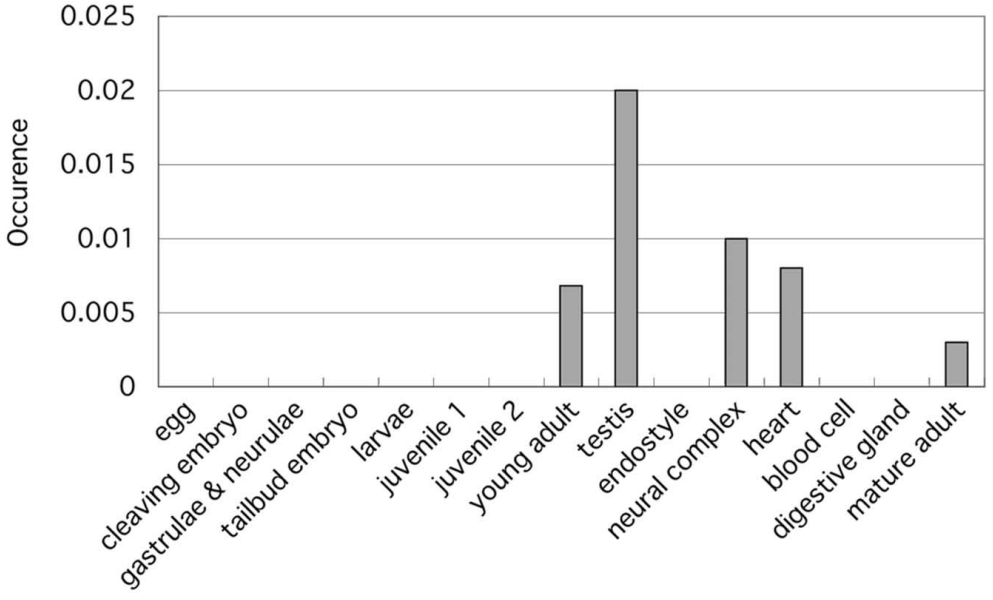


Fig. 2. Expression profile of Ci-Axp66.0 in *Ciona* tissues. The cDNA for Ci-Axp66.0 was grouped in cluster 09552 in the *Ciona* cDNA database (http:// ghost.zool.kyoto-u.ac.jp/indexr1.html). The expression profile was based on the EST count in each tissue. The values represent the EST count / number of total cDNA clones in each tissue.

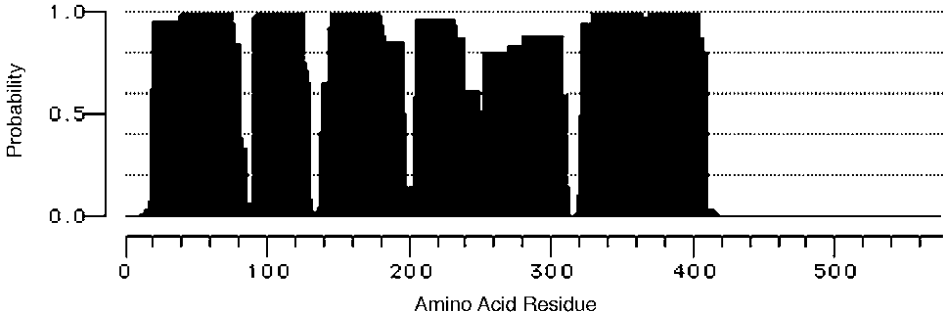


Fig. 3. Prediction of coiled-coil structure in Ci-Axp66.0 by COILS. The search showed a high probability for coiled-coil structure in the N-terminal two-thirds of Ci-Axp66.0.

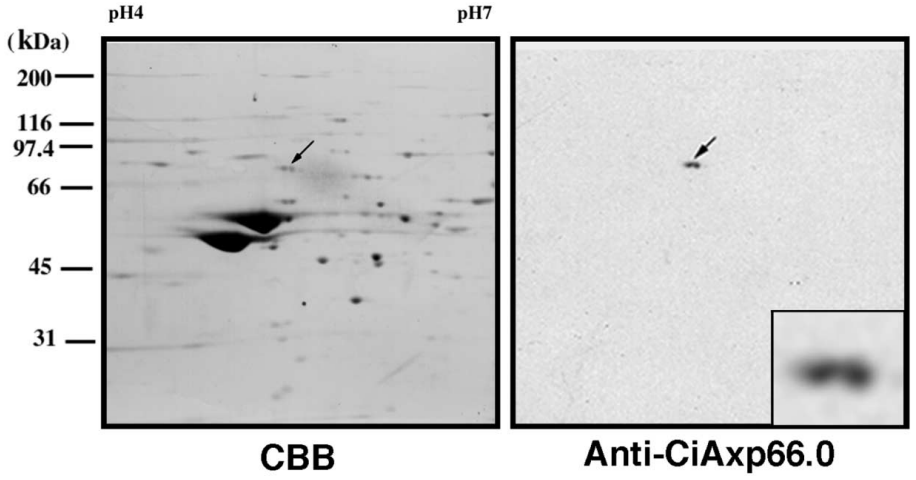
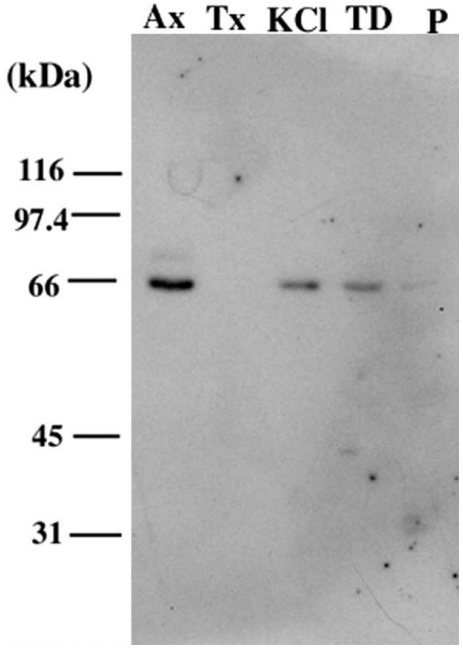


Fig. 4. Two-dimensional gel electrophoresis of axonemal proteins (left) and the corresponding immunoblot with anti-Ci-Axp66.0 antibody (right). Arrows indicate the position of Ci-Axp66.0. Ci-Axp66.0 was separated into two spots with an isoelectric point of approximately 5.1. The inset shows the Ci-Axp66.0 spots at higher magnification.



Proteins were separated on an 11-cm dry strip with an immobilized pH gradient gel (Amersham Biosciences). Separation by isoelectric focusing and subsequent SDS-PAGE were carried out by the method of Hozumi *et al.* (2004). Immunoblotting was carried out according to the method of Padma *et al.* (2003).

RESULTS

We selectively collected cDNAs encoding axonemal proteins using antiserum against axonemal proteins in *Ciona* sperm and obtained 76 positive cDNA clones for axonemal proteins (Padma *et al.*, 2003). Among the positive clones, three showed sequence similarity to *Drosophila melanogaster* CG14905, whose function is unknown. The full sequence of the longest cDNA clone revealed that the open reading frame encodes a polypeptide of 575 amino acids

Fig. 5. Western blot of several fractions from chemical dissection of sperm axonemes. Sperm flagella were fractionated into Triton X-100 extract (TX) and the axonemes (Ax). The axonemes were subsequently fractionated into an 0.6 M KCl extract (KCl), an extract obtained by dialysis against Tris-EDTA solution (TD), and a final residual pellet (P). Ci-Axp66.0 was detected in both the KCl and the TD axoneme fractions.

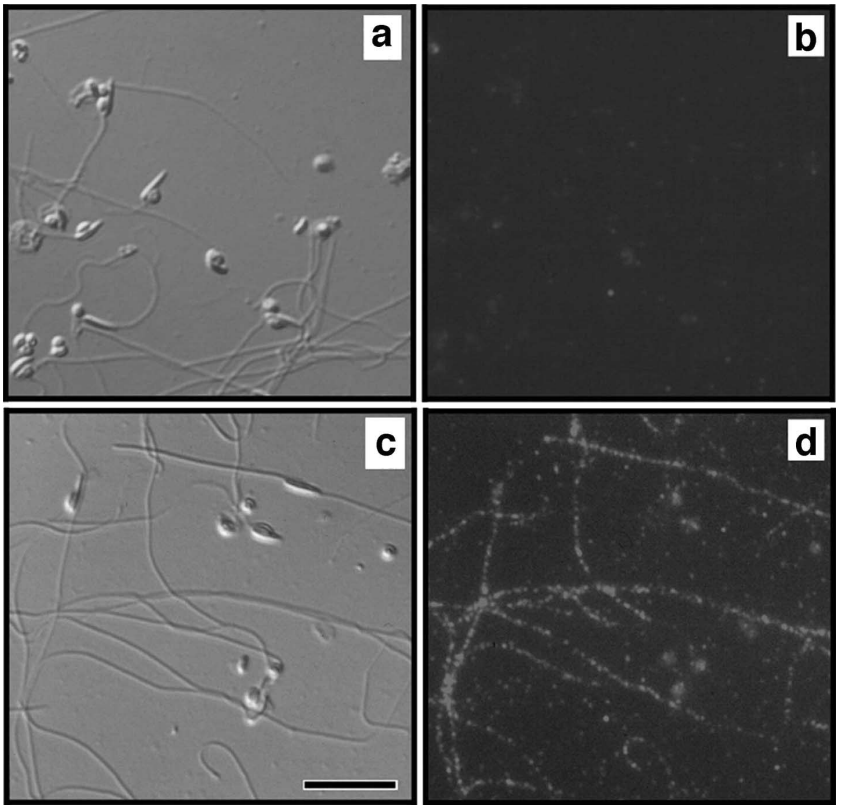


Fig. 6. Immunofluorescence microscopy showing the localization of Ci-Axp66.0 along sperm flagella. *Ciona* sperm were fixed, and treated with normal mouse serum (a, b) or anti-Ci-Axp66.0 (c, d). Differential interference contrast (a, c) and fluorescence images (b, d) are shown. Bar, 20 μm.

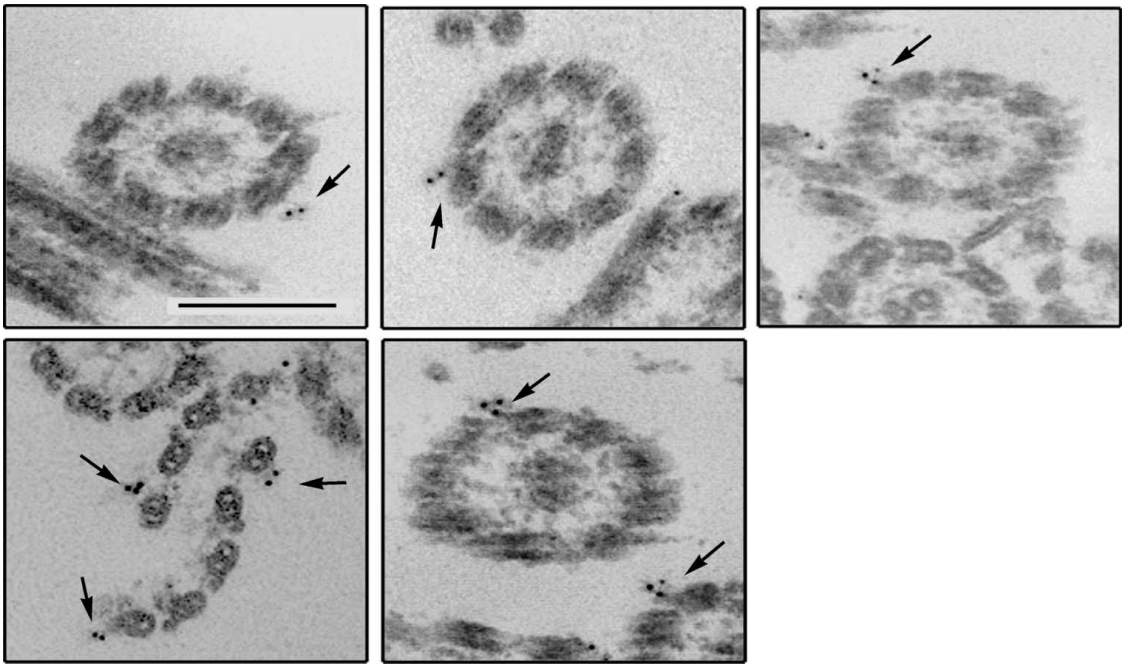


Fig. 7. Immunogold localization of Ci-Axp66.0 in the vicinity of outer arm dynein in the axonemes. Five typical images are shown. Experiments with control mouse serum as a primary antibody exhibit no gold labeling (not shown). Arrows show the position of gold particles. Bar, 200 nm.

with a predicted isoelectric point of 5.13 and a predicted molecular mass of 66.0 kDa; hence we named it "Ci-Axp66.0" (*Ciona intestinalis* axonemal 66.0 kDa protein, accession number AB083180).

A BLAST search showed the greatest hit with *Xenopus laevis* MGC68565 protein, with the E value of 9e-92. Other hits included proteins from human, mouse, and zebrafish; however, their functions have not been elucidated. The only protein with functional information showing sequence similarity to Ci-Axp66.0 was *Chlamydomonas* DC2 (Accession number AY039618, probability 9e-55), a subunit of the outer dynein arm-docking complex (ODA-DC). Ci-Axp66.0 showed similarity over its entire length (Fig. 1) with aligned *Chlamydomonas* DC2 and a mouse homolog, 4921511c16 (accession number AK029513). Ci-Axp66.0 showed 29% identity and 51% similarity with *Chlamydomonas* DC2, and 34% identity and 55% similarity with the mouse homolog. Ci-Axp66.0 and the mouse DC2 homolog both have an extended N-terminal region, and both lack the central region of *Chlamydomonas* DC2 (AA.307-325).

We compared gene expression of Ci-Axp66.0 among several developmental stages of embryos and adult tissues by means of the *Ciona* cDNA database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) (Satou *et al.*, 2002). C-Axp66.0 is highly expressed in the testis, but is also abundant in young and mature adults (Fig. 2). It is interesting that the level of expression is also high in the neural complex and heart.

A motif scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and a conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) showed that Ci-Axp66.0 has a Smc domain for chromosome segregation ATPases (AA.50-246, E-value, 5e-04). A search for phosphorylation sites revealed four sites for cAMP-dependent protein kinase consensus sequence [Arg/Lys]-[Arg/Lys]-X-[Ser/Thr]; amino acid residues 80–83, 191–194, 381–384 and 571–574) (Fig. 1). Ci-Axp66.0 also contains twelve potential phosphorylation sites for casein kinase II, nine for protein kinase C, and one for protein tyrosine kinase. A search by COILS (Lupas *et al.*, 1991) detected an overall coiled-coil region in the N-terminal two-thirds of Ci-Axp66.0 (Fig. 3). This is also characteristic of *Chlamydomonas* DC2 (Takada *et al.*, 2002).

To further characterize Ci-Axp66.0, we prepared an antibody against it. Immunoblotting of whole axonemal proteins on a two-dimensional gel showed two positive spots

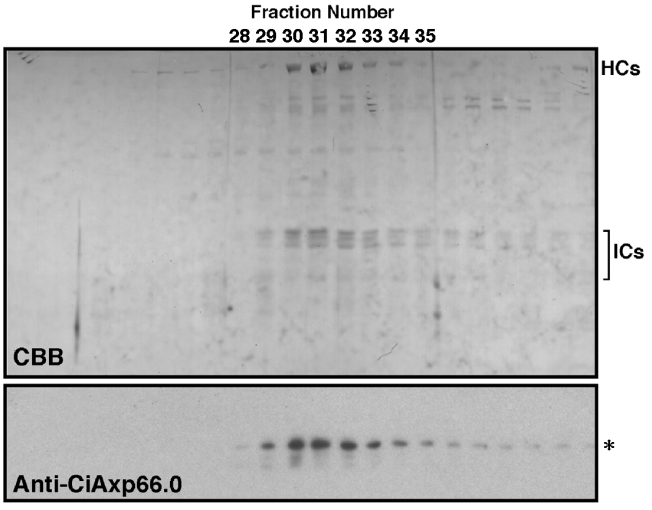


Fig. 9. Separation of fraction 9 from a Superdex200 column by an HQ anion exchange column. Ci-Axp66.0 was coeluted with the components of outer arm dynein in fractions 30–32. The lower panel shows an immunoblot of the fractions with anti-Ci-Ax p66.0. The asterisk indicates the position of Ci-Axp66.0.

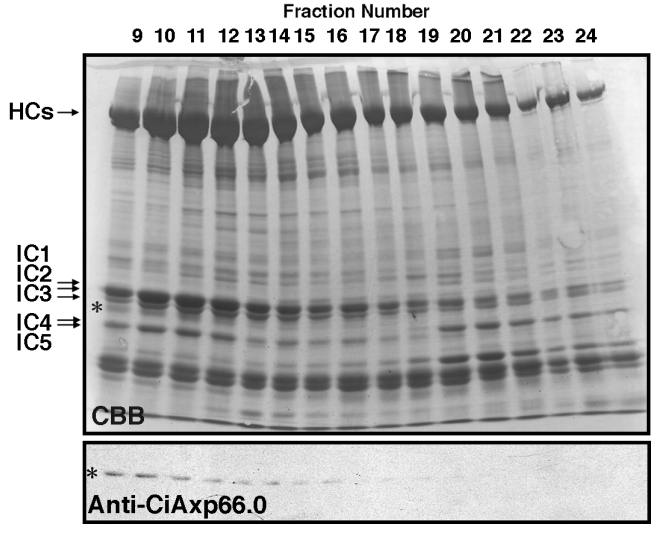


Fig. 8. Separation of the complex containing Ci-Axp66.0 by Superdex200 gel filtration of a KCl extract of the axonemes. Outer arm dynein composed of heavy chains (HCs) and intermediate chains (IC1, IC2, IC3, IC4 and IC5) was eluted with a peak in fraction 11, whereas the peak of Ci-Axp66.0 was in fractions 9–10. The lower panel shows an immunoblot of the fractions with anti-Ci-Ax p66.0 antibody. Asterisks indicate the position of Ci-Axp66.0.

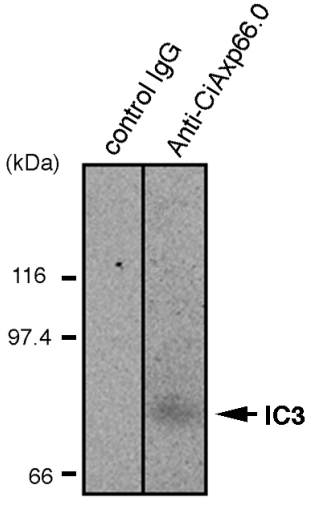


Fig. 10. Immunoprecipitation of outer arm dynein by anti-Ci-Axp66.0 antibody. The fraction from an HQ column was immunoprecipitated with IgG from normal mouse serum (left) or anti-Ci-Axp66.0 antibody (right). The immunoprecipitate was immunoblotted with an antibody against NDPK-related sequence (NDPKR) (Ogawa *et al.*, 1996) that detects the intermediate chain 3 (IC3) of outer arm dynein (Padma *et al.*, 2001).

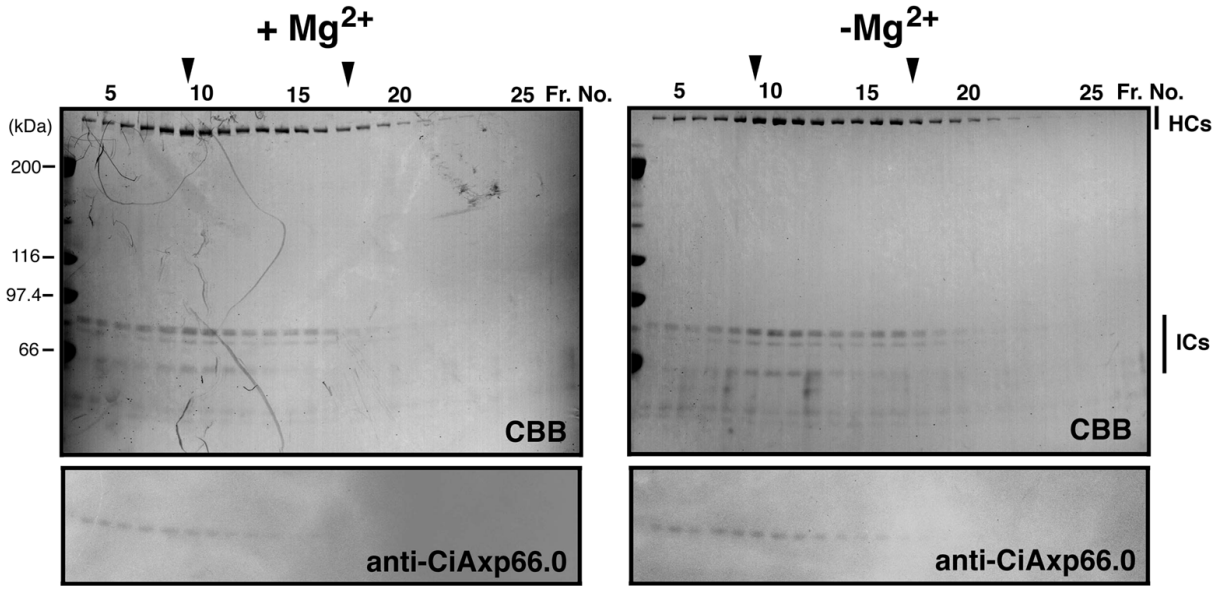


Fig. 11. Effect of Mg^{2+} on the interaction of outer arm dynein and Ci-Axp66.0. The fractions of outer arm dynein rich in Ci-Axp66.0 were dialyzed against Mg^{2+} buffer or Mg^{2+}-free buffer, followed by 5–20% sucrose density gradient centrifugation. The protein profiles in a 6% SDS gel and corresponding immunoblots with anti-Ci-Axp66.0 antibody are shown under Mg^{2+} (left) and Mg^{2+}-free (right) conditions. Fraction numbers are shown at the top of the gels. Arrowheads indicate the positions of markers for sedimentation coefficients (thyroglobulin for 19S, left; catalase for 11.3S, right).

with a molecular mass of 70 kDa and a pI of approximately 5.1, which agreed well with the values predicted from the amino acid sequence deduced from the cDNA sequence (Fig. 4).

We carried out a successive extraction of axonemes to obtain information on the localization of Ci-Axp66.0 (Fig. 5). Although part of the Ci-Axp66.0 molecule was extracted from the axonemes by 0.6 M KCl, a condition necessary for extracting outer arm dyneins, half of the Ci-Axp66.0 molecule could not be extracted with this solution. The remainder of the Ci-Axp66.0 protein could be efficiently extracted with a low-ionic-strength solution (Tris-EDTA solution, TD), with only a trace of it retained in the residue (Fig. 5). Immunofluorescence microscopy showed that Ci-Ax66.0 was distributed along sperm flagella (Fig. 6). Immunogold electron microscopy of the axoneme further showed that the Ci-Axp66.0 was localized in the vicinity of the outer arm dynein (Fig. 7).

Ci-Axp66.0 could be partially extracted from axonemes using a high-salt solution (Fig. 5). Gel filtration of the high-salt extract resulted in the isolation of outer arm dynein, with five intermediate chains of molecular mass 80, 78, 75, 65, and 64 kDa (Fig. 8), as described previously (Padma *et al*., 2001). Ci-Axp66.0 migrated into the same region as the outer arm dynein. However, the position of the peaks of Ci-Axp66.0 and outer arm dynein were slightly different: Ci-Axp66.0 eluted earlier than the major peak of outer arm dynein (Fig. 8). To confirm that Ci-Axp66.0 was associated with outer arm dynein, fraction 9 was further separated by HQ ion-exchange column chromatography (Fig. 9). Ci-Axp66.0 was co-eluted with the intermediate chains of outer arm dynein, indicating an association between the two. This result was confirmed by an immunoprecipitation experiment (Fig. 10). The fraction containing the outer arm dynein was immunoprecipitated with anti-Ci-Axp66.0 antibody. An antibody against the NDPK-related region in outer arm dynein (Ogawa *et al*., 1996; Padma *et al*., 2001) clearly detected outer arm dynein only in the immunoprecipitate with anti-Ci-Axp66.0 antibody.

In *Chlamydomonas*, the ODA-DC can be separated from outer arm dynein and ~7 S sediments in a sucrose density gradient in Mg^{2+}-free buffer (Takada and Kamiya, 1994). To examine the effect of Mg^{2+} on the interaction between Ci-Axp66.0 and outer arm dynein, the fraction of outer arm dynein rich in Ci-Axp66.0 was dialyzed against Mg^{2+}-free buffer and separated by sucrose density gradient centrifugation (Fig. 11). Our data showed that Ci-Axp66.0, in contrast to the *Chlamydomonas* ODA-DC, sedimented in almost the same position as outer arm dynein in the presence or absence of Mg^{2+}, indicating that Ci-Axp66.0 is associated with the outer arm dynein even in the absence of Mg^{2+}.

DISCUSSION

In this study, we isolated and characterized a protein that appears to function as a subunit of the ODA-DC in sperm flagella of the ascidian *Ciona intestinalis*. This is the first report in metazoan spermatozoa of a subunit of the complex for positioning outer arm dynein on the outer doublet microtubules. In *Chlamydomonas*, DC2 is essential for the assembly of outer arm dynein. Unlike DC2 in *Chlamydomonas* flagella, however, Ci-Axp66.0 and possibly the docking complex could not be completely extracted from axonemes, suggesting that interactions of outer arm dynein with the docking complex, and/or of the docking complex and outer doublet microtubules, are different between *Chlamydomonas* and *Ciona*. This difference is also supported by the fact that Ci-Axp66.0 is associated with outer

arm dynein even in the absence of Mg^{2+} (Fig. 11).

The ODA-DC of *Chlamydomonas* contains three polypeptides, DC1, DC2, and DC3 (Takada and Kamiya, 1994; Wakabayashi *et al*., 2001; Takada *et al*., 2002). The primary structures of IC4 and IC5 have not been elucidated, and these might be homologs of DC1 and DC3. A BLAST search of *Chlamydomonas* DC1 (oda3) (AF001309), however, resulted in no hits in the *Ciona* database. DC3, a calcium-binding protein in the docking complex in *Chlamydomonas* flagella (accession number, AY294291) hits one protein in the *Ciona* genome; however, this is related to *Ciona* calmodulin. We have recently identified a novel calcium-binding protein that is associated with outer arm dynein, and this may play a role similar to that of *Chlamydomonas* DC3 (Inaba and Padma, 2003).

Approximately half of Ci-Axp66.0 was isolated in association with outer arm dynein (Fig. 5), suggesting heterogeneity of Ci-Axp66.0 in terms of its interaction with outer arm dynein or with the doublet microtubule. Two-dimensional gel electrophoresis revealed two spots of Ci-Axp66.0 (Fig. 4). This may be due to post-translational modification affecting the net charge of the protein, and could also be related to phosphorylation state. Since the two spots appeared almost in equal amounts, it is possible that this modification may be related to partial extraction of Ci-Axp66.0 by salt from the axonemes (Fig. 5). Outer arm dynein is regulated by phosphorylation of the Tctex2-related dynein light chain (Inaba *et al*., 1999) by a cAMP-dependent protein kinase located at the base of outer arm dynein (Itoh *et al*., 2003). Ci-Axp66.0 contains four motifs for cAMP-dependent protein phosphorylation (Fig. 1). It is therefore possible that phosphorylation of these sites might modify the binding property of Ci-Axp66.0 to the outer arm dynein or the doublet microtubule.

In *Chlamydomonas*, outer arm dyneins purified from wild-type axonemes bind to axonemes of the oda1 mutant that lacks DC2. The beat frequency of oda1 is recovered by the reconstitution of outer arm dyneins, but the frequency imbalance in flagella between the cis- and trans-flagellum is not restored, suggesting that the ODA-DC is important in the regulation of flagellar beat frequency (Takada and Kamiya, 1997). We observed that ATPase activity in the fraction rich in Ci-Axp66.0 (fraction 9) was higher than that in the peak fraction of outer arm dynein (fraction11) (data not shown). Although further experiments should be carried out to examine the effect of Ci-Axp66.0 on dynein ATPase using purer samples, it is possible that Ci-Axp66.0 affects the function of outer arm dynein. To elucidate the functions of the ODA-DC in the architecture and regulation of outer arm dynein in *Ciona* axonemes, the complex needs to be purified from the axonemes and its precise molecular composition examined.

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