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アクチン遺伝子に変異を持つクラミドモナスダイニン内腕部分欠損変異株の単離と解析

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Feb., 1998

CONTENTS

INTRODUCTION

Eukaryotic motility systems are broadly classified into two categories by the protein polymers used as tracks; microtubule-based systems with dynein and kinesin as motors, and actin-based systems with myosin motors. The study presented in this thesis was aimed at revealing the mechanisms of eukaryotic flagellar motility, which is produced by precision machinery based on the coordinated interaction between microtubules and dyneins (see Appendix 1). Flagellar dynein is a large protein complex containing one to three heavy chains of ~500 kDa and several intermediate and light chains. There are two functionally and structurally different dynein arms, outer and inner dynein arms. Outer-arms, forming the outer protrusions of the "9 + 2" structure of the axoneme, mainly contribute to the high beat frequency of flagella. Inner-arms, forming the inner protrusions, are suggested to be important for the bending initiation and the regulation of waveform (reviewed in Kamiya, 1995).

Our present knowledge about the structure and function of eukaryotic cilia and flagella owes much to a biflagellate green algae, *Chlamydomonas*, the organism employed in this study. In this organism, outer-arm dynein exists as a single kind of molecular assembly containing three heavy chains (α, β, and γ) while inner-arm dynein exists as seven different subspecies (*a-g*). All the inner-arm subspecies except *f* have single heavy chains and contain actin as one of their subunits; *f* has two heavy chains and no actin (Piperno and Luck, 1979; Kagami and Kamiya, 1992; Sugase et al., 1996). Such a complicated molecular composition of flagellar dynein poses some interesting questions; why do the flagella adopt such many kinds of dynein arms, or, in other words, how do those dynein arms functionally differ?; and, what is the role of actin in inner-arms? One of the most effective experimental approaches to these questions is to isolate a gallery of mutants lacking particular dynein subspecies, determine their genetic backgrounds, and compare their characters with each other or with that of wild type. Under this strategy, more than 10 mutants lacking the outer dynein arm have been isolated (Kamiya and Okamoto, 1985; Mitchell and Rosenbaum, 1985; Kamiya, 1988), and six of them have been identified as having mutations in particular subunits of the arm (Kamiya, 1988; Mitchell

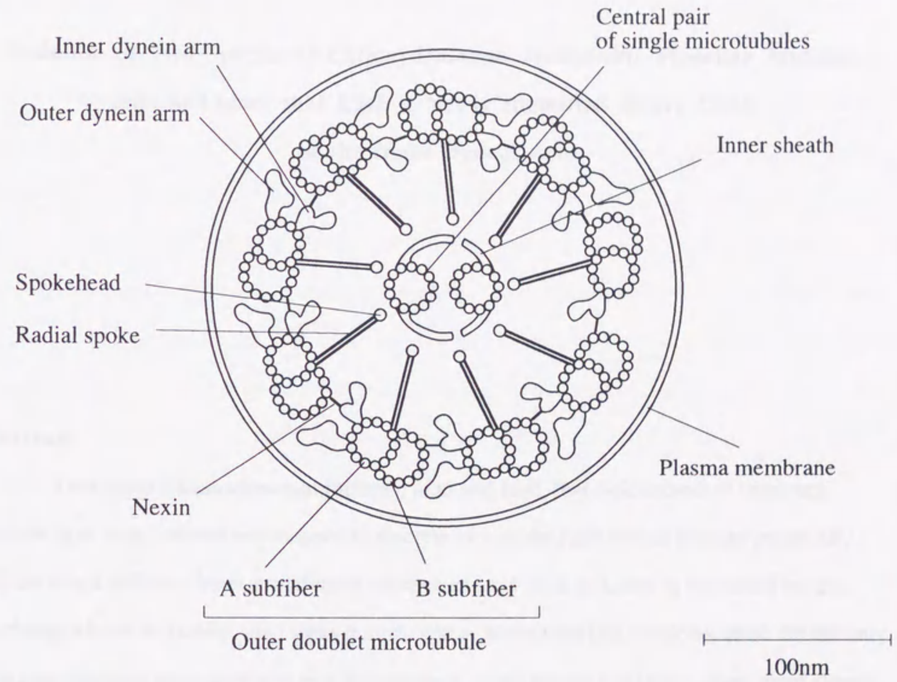
and Kang, 1991; Sakakibara et al., 1991; Wilkerson et al., 1994; Wilkerson et al., 1995; Koutoulis et al., 1997). Fewer mutants with defects in the inner dynein arm have been isolated although the inner arm is structurally more complicated than the outer (Kamiya, 1995). The mutants *ida1*, *ida2*, and *ida3* were isolated as mutants lacking subspecies *f* (Kamiya et al., 1991; Kagami and Kamiya, 1992). It has recently been shown that *ida1* (*pf9-3*) is a null mutant of the gene that encodes one of the two heavy chains of subspecies *f* (Myster et al., 1997). The mutant *ida4* was identified as defective in subspecies *a*, *c*, and *d* (Kagami and Kamiya, 1992). A recent study has identified it as having mutations in the gene of a 28 kD protein (p28), a light chain of inner-arms (LeDizet and Piperno, 1995). Because *ida4* lacks p28 and all of the three subspecies (*a*, *c*, *d*) that normally contain p28 as subunits, p28 appears to be necessary for the assembly of these subspecies on the outer doublet microtubules. In other words, a single component is essential to the formation of dynein subspecies.

Under these backgrounds, I tried to isolate more inner-arm-deficient mutants and succeeded in isolating two novel strains, which I designated *ida5* and *ida6*. The isolation and initial characterization of these mutants are described in Chapter 1. Biochemical analysis has revealed that *ida5* is missing the inner-arm subspecies *a*, *c*, *d*, and, *e*, and *ida6* is missing *e* alone. These are the first mutants that lack subspecies *e*. Their slow swimming phenotypes indicated that subspecies *e* is important but not essential for the flagellar motility. In Chapter 2, I describe analyses of the axoneme and genome of *ida5*, which led to the conclusion that it is a nonsense mutant of the conventional actin gene. This finding is surprising because the gene of conventional actin has been shown to be present in a single copy in the *Chlamydomonas* genome (Sugase et al., 1996). However, it was also found that *ida5* expresses a novel actin-like protein (NAP) which is apparently significantly different from ordinary actin. The inner-arm dynein subspecies *b* and *g* present in this mutant were found to contain NAP instead of actin. Thus NAP can apparently substitute for actin as dynein subunit in the inner-arm subspecies *b* and *g*, but not in the subspecies *a*, *c*, *d*, and *e*. It was also found that the mating-type-plus gamete of *ida5* is deficient in the formation of fertilization tubule, an actin-containing structure that facilitates mating. Because of this deficiency, mating efficiency was greatly reduced in this mutant. It is thus clear that the absence of conventional actin causes dysfunction

of various cellular processes. However, the growth rate of the mutant *ida5* was found not to differ from that of wild type. Hence, this mutant raises the striking possibility that *Chlamydomonas* can live without conventional actin. In particular, it necessitates reinvestigation as to whether actin plays an essential role in the cytokinesis in this organism as generally believed. Finally, in Chapter 3, I describe the determination of a partial amino-acid sequence of NAP isolated from the mutant *ida5*, showing that it is identical with a sequence recently reported as that of an actin-related protein (Lee et al., 1997). This protein has only a 64% amino acid identity with the conventional actin. Thus, these findings have established that *Chlamydomonas* has both conventional actin, with 89% amino-acid identity with skeletal muscle actin, and an actin-related protein (NAP) with a significantly lower similarity.

I have thus established that *ida5* is a null mutant of the conventional actin gene. In no other organisms, have null mutants of conventional actin been isolated, because conventional actin genes are usually present in multiple copies in other organisms, and more importantly, actin is essential for their growth. My study has shown that conventional actin is not essential to the growth of *Chlamydomonas*. Whether NAP is essential remains an important future question. At any event, the actin-null mutant *ida5* should become a powerful experimental material with which to study the function of actin, NAP and their internal structural domains.

Appendix 1. The "9 + 2" Structure of Chlamydomonas Flagella



CHAPTER I

Isolation of Two Species of *Chlamydomonas reinhardtii* Flagellar Mutants, *ida5* and *ida6*, that Lack a Newly Identified Heavy Chain of the Inner Dynein Arm

Abstract

Two novel *Chlamydomonas* mutants, *ida5* and *ida6*, that lack subsets of inner-arm dynein have been isolated and mapped to discrete loci on the right arm of linkage group XIV. Of the seven different inner-arm dynein subspecies (*a*, *b*, *c*, *d*, *e*, *f*, and *g*) identified by ion-exchange chromatography, *ida5* lacks *a*, *c*, *d*, and *e*, while *ida6* lacks *e* alone; these are the only mutants that have been shown to lack subspecies *e*. Both strains can swim, albeit more slowly than the wild type. Hence, subspecies *e* must contribute to flagellar movement although it is unnecessary for the generation of undulating movement.

Introduction

Inner and outer dynein arms in cilia and flagella are molecular assemblies responsible for force production. Both arms contain several heavy chains (Mr > 400,000) that have ATP-hydrolyzing activities. Although the two kinds of arms are similar in gross appearance in the cross section of the axoneme, recent studies using *Chlamydomonas* mutants have revealed that they differ rather strikingly in composition and organization. In particular, it has been proposed that the inner arm comprises three or more heterologous species each containing multiple heavy chains, whereas the outer dynein arm comprises a single species composed of two or three heavy chains and several smaller polypeptides. Piperno et al. (1990) suggested that the inner arms of *Chlamydomonas* comprise three subforms (I1, I2, and I3), each containing two heavy chains, and the unit of the three subforms repeats every 96 nm along the outer doublet. Moreover, the I2 and I3 species possibly contain different heavy chains depending on their position along the flagellum (Piperno and Ramanis, 1991). These studies have identified a total of six different inner-arm heavy chains. Kagami and Kamiya (1992), on the other hand, found that ion-exchange chromatography can separate the inner-arm dynein into seven discrete subspecies designated *a-g*, and that the total number of different inner-arm heavy chains is as many as eight, rather than six.

The classification of the inner arms into I1, I2, and I3, however, has gained little support from recent studies. The classification is based on the analysis of inner-arm composition by sucrose density gradient centrifugation and electron microscopic observation on mutant axonemes lacking subsets of inner arms (Piperno et al., 1990). However, recent electron microscopic studies have indicated that the inner arms are arranged in a more complex manner than has previously been thought; the inner arms do not appear to be grouped in three entities within the repeating unit distance of 96 nm (Mastronarde et al., 1992; Muto et al., 1991). More specifically, while both biochemical and ultrastructural data support the existence of the I1 species, they fail to define I2 and I3 as discrete assemblies (Kagami and Kamiya, 1992; Mastronarde et al., 1992).

For elucidation of the structure and function of the inner-arm dynein subspecies, mutants lacking particular subspecies are of great value. Kamiya and collaborators thus tried to

isolate various mutants deficient in the inner arm and so far have isolated four genetically different species named *ida1*, *ida2*, *ida3*, and *ida4*. Of these, the first three belong to a single type (idaA type) that lacks the I1 species (subspecies *f*), while the last species *ida4* belongs to another type (idaB type) that lacks three subspecies *a*, *c*, and *d* (Kagami and Kamiya, 1992). Interestingly, a recent image analysis of mutant axonemes has demonstrated that three separate densities are lacking within the 96 nm repeating distance in the *ida4* axoneme (Mastronarde et al., 1992). In the present study, I have isolated two novel inner-arm mutants lacking subspecies *a*, *c*, *d*, and *e* (*ida5*) or subspecies *e* alone (*ida6*). The isolation of these strains, as well as of *ida4*, suggests that subspecies *e* constitutes a discrete inner-arm subspecies, and that it has some connection with subspecies *a*, *c*, and *d*, which have been thought to constitute the subform I2 (Piperno et al., 1990). My study also shows that the subspecies *e* is dispensable for motility, since both *ida5* and *ida6* are able to swim, albeit slowly.

Materials and Methods

Strains

The following *Chlamydomonas reinhardtii* strains were used: 137c (wild type), *oda2*, *oda4* (Kamiya, 1988), *ida1* and *ida4* (Kamiya et al., 1991). Other strains used for genetic analyses were obtained from Dr. E. Harris of *Chlamydomonas* Genetics Center (Department of Botany, Duke University, Durham, North Carolina).

Mutant Isolation

Wild-type cells were mutagenized by UV light and screened for poor motility. The procedures have been described elsewhere (Kamiya, 1991). Isolated mutants were genetically analyzed using standard techniques (Harris, 1989; Levine and Ebersold, 1960).

Isolation of Axonemes and Fractionation of Dynein

Culture of cells and isolation of flagella, preparation of crude dynein extracts, and fractionation of dynein subspecies by high-pressure liquid chromatography on a Mono-Q column have been described previously (Kagami and Kamiya, 1992).

Electrophoresis

The composition of dynein heavy chains was analyzed by SDS-PAGE using the method of Laemmli (1970), as modified by Jarvik and Rosenbaum (1980) and Pfister et al. (1982). A 3% to 5% polyacrylamide gradient and a 3 M to 8 M urea gradient were used. For analysis of the intermediate chains and low molecular weight chains, a Laemmli SDS-PAGE system with a 5% to 20% acrylamide gradient was also used. All gels were stained with silver (Blum et al., 1987).

Electron Microscopy

Axoneme samples were fixed with 2% glutaraldehyde in the presence of 1% tannic acid. Specimens were post-fixed with 1% OsO_4, dehydrated through a series of ethanol solutions, and embedded in Epon 812. Golden thin sections (~100 nm thick) were used. Sections were double-stained with uranyl acetate and lead citrate, and observed with a JEM100C microscope (Jeol Co., Tokyo).

To examine the shape of dynein arms in mutants, images of outer-doublet microtubules in axonemal cross sections were averaged using an Excel image processor (Avionics, Tokyo) as described (Kamiya et al., 1991; Sakakibara et al., 1991).

Motility Assessment

The swimming velocities of the mutant and wild-type cells were measured with a dark-field microscope, a video recording system, and a personal computer. A red filter (cutoff wavelength: 630 nm) was placed under the condenser lens to facilitate the measurement by suppressing the cells' response to light; when illuminated by white light, *Chlamydomonas* cells frequently display irregular swimming paths, which make measurement difficult. The velocity in a given sample was the average of 50 separate measurements.

Flagellar beat frequencies in swimming cells were measured by a fast Fourier transform (FFT) method (Kamiya and Hasegawa, 1987). This method analyzes the light intensity

fluctuation in the microscopic image of a population of swimming cells and yields an average beat frequency. All motility measurements were carried out at 25 °C.

Results

Isolation of New Inner Dynein Arm Mutants

In my attempts to isolate mutants deficient in flagellar dynein, I select those cells that swim slowly, rather than those without motility. This is because mutants lacking the entire outer arms or part of the inner arms have been shown to be slow swimmers (Kamiya et al., 1989). Although inner-arm mutants and outer-arm mutants are similar in terms of their slow swimming speeds, they can be distinguished because outer-arm mutants have lower flagellar beat frequencies than those of inner-arm mutants; inner-arm mutants swim slowly because of a decrease in flagellar bend angle rather than a decrease in beat frequency (Brokaw and Kamiya, 1987).

I isolated two strains that display phenotypes characteristic of inner-arm mutations but are genetically distinct from known *i*nner *d*ynein *a*rm-deficient mutants *ida1*-*ida4*. These two strains were found to be genetically linked but different from each other. Because they were actually found to have deficiencies in the inner arm, as shown below, I named them *ida5* and *ida6*. Tetrad analysis indicated that both strains result from single-site mutations that are linked with *ac206* on linkage group XIV: *ida5* x *ac206* = 21:0:24 (PD:NPD:T); *ida6* x *ac206* = 26:0:46; and *ida5* x *ida6* = 68:0:9. The location of the *ida5* site on linkage group XIV was confirmed by its linkage with *agg1*: *ida5* x *agg1* = 32:0:5. These results indicate that both *ida5* and *ida6* are on the right arm of linkage group XIV, *ida5* and *ida6* being about 6 cM and 12 cM from the centromere, respectively.

Deficiency in Inner Arms

The SDS-PAGE pattern of the *ida5* axoneme, in the region of dynein heavy chain bands, was similar to that of the *ida4* axoneme, whereas the pattern of the *ida6* axoneme was indistinguishable from that of the wild-type axoneme (Fig. 1). I further examined the *ida5* axoneme in the absence of the outer-arm dynein in double mutants with *oda2* and found that it

lacks two bands (no.3 and no.5) (Fig. 1). Thus, the deficiency in *ida5* again appears to be similar to that in *ida4*, which also lacks no.3 and no.5 inner-arm bands. I noticed, however, the *ida5* axoneme showed a novel type of heavy-chain deficiency when examined with the background of *ida1* that lacks the I1 inner-arm subspecies; a faint band between the beta and gamma outer-arm heavy chains normally present in the *ida1* pattern is weaker (almost absent) in the double mutant *ida5* x *ida1*. The same kind of defect was observed also in *ida6* x *ida1* (data not shown). These findings indicated that the two strains lack a heavy chain that had not been demonstrated to be lacking in any known mutants.

These observations prompted me to examine the mutant dyneins by a different method. I thus extracted dyneins from the mutant axonemes and fractionated them by ion-exchange chromatography on a Mono-Q column. This approach has been shown to be effective in the analysis of dynein subspecies (Goodenough et al., 1987; Kagami and Kamiya, 1992). The elution profile of the high-salt extract from wild-type, *ida5* and *ida6* axonemes (Fig. 2), together with the SDS/urea-PAGE patterns of their peak fractions in a high molecular weight range (Fig. 3), demonstrated that *ida5* lacks subspecies *a*, *c*, *d*, and *e*, while *ida6* lacks subspecies *e* alone. This conclusion was further supported by SDS-PAGE analyses of lower molecular weight proteins in each fraction (Fig. 4). In particular, a 42 kD band, possibly the band for actin (Piperno and Luck, 1979), is present in all of the *a*, *b*, *c*, *d*, and *e* subspecies in wild-type extract, whereas it is absent from fractions corresponding to *a*, *c*, *d*, and *e* in *ida5* and from that corresponding to *e* in *ida6*. Because the heavy chain in subspecies *e* is overlapped with that of the lower band of the I1 (subspecies *f*) heavy chains, these findings agree with the above observation that a novel type of deficiency was detected in these mutants with the background of I1 deficiency.

Electron Microscopic Observation

Kamiya et al. (1991) have shown that the inner-arm dynein in cross-sectional electron micrographs of wild-type axonemes appears to have two projections. This observation suggested that dynein subspecies are aligned in two rows on an outer doublet: one (outer row) pointing to the adjacent outer doublet and the other (inner row) to the inside of the axoneme.

Moreover, the density in cross section of the outer row is weak in mutants *ida1*, *ida2* and *ida3* that lack the inner arm I1, whereas that of the inner row is weak in *ida4*. More recently, Mastronarde et al. have examined the structural defects in *ida4* and an I1-missing mutant, *pf9*, using an image-processing technique. They concluded that the defects in the I1-lacking mutant occur mostly (but not entirely) in the outer row, whereas those in *ida4* are in the inner row.

Fig. 5 shows the cross-sectional and averaged doublet images in *ida5*, *ida6*, and wild-type axonemes. As expected from the deficiencies detected by SDS-PAGE, the cross-sectional images of the inner arms in *ida5* are similar to those of *ida4* axonemes in that the inner row is weak. However, the averaged doublet image of the *ida5* appears to lack the inner row to a greater extent than *ida4*; this suggests that the subspecies *e* is located on the inner row. On the other hand, the images of *ida6* axonemes are similar to those of the wild-type axonemes except that the density of their inner row appears somewhat weaker than that of wild type, agreeing with the above speculation that the subspecies *e* is present within the inner row.

Motility

Both *ida5* and *ida6* swim slowly. It is likely that the slow swimming speed in *ida6* results mostly from its reduced flagellar bend angle, as shown to be the case in *ida1* (Brokaw and Kamiya, 1987), since its beat frequency is slightly higher than that in wild type. In *ida5*, however, the power spectrum from the vibrating cell bodies in microscopic images suggested a significant variation in the flagellar beat frequency ranging from 27 to 52 Hz; therefore, the beat frequency in this mutant can be very low, although it can be as high as the wild-type frequency also. In this respect, the *ida5* mutation differs from other known inner-arm mutants. The motility of *ida5* lacking inner-arm subspecies *a*, *c*, *d*, and *e* is somewhat poorer than that of *ida4* lacking *a*, *c*, and *d* only. Thus subspecies *e* appears to contribute to the flagellar motility (Table I).

Unexpectedly, the flagellum of *ida6* was slightly shorter than that of *ida5*. In addition, its swimming velocity was as low as *ida5*, despite the apparently smaller deficiency in dynein subspecies. These observations suggest that the lack of subspecies *e* is not the sole deficiency in *ida6*. Conceivably, this mutant may have a second defect that has not been detected by my

analyses using chromatography and SDS-PAGE. In agreement with this idea, the double mutant *ida5* x *ida6* displayed poorer motility than that of *ida5* (Table I).

Like the previously isolated inner-arm mutants *ida1-ida4*, the new mutants lost flagella almost completely when combined with mutations that cause the loss of the outer arm. Thus the double mutants *ida5* x *oda4* and *ida6* x *oda2* did not grow flagella. Similarly, the double mutants *ida5* x *ida1* rarely grew flagella; it was only on very rare occasions that a small population of this double mutant was observed to grow flagella, but the flagellar movement in those cells was so weak that the cells were not able to swim in the medium. These results support the view that the function of different dynein subspecies is interdependent and axonemes lacking a large part of inner-arm subspecies cannot function properly (Kamiya et al., 1989).

Discussion

In this study I have isolated two mutant strains (*ida5* and *ida6*) that lack subsets of inner-arm dynein. Of the seven inner-arm subspecies *a-g* identified by biochemical analyses (Kagami and Kamiya, 1992), *ida5* lacks *a*, *c*, *d*, and *e* while *ida6* lacks *e* alone. This is the first isolation of mutants that lack subspecies *e*. The observation that *ida5* displays poorer motility than *ida4* indicates that *e* actually contributes to the strength of flagellar movement, and yet it is dispensable for the generation of motility. Since the mutants *ida1-ida3* and *pf9* lacking subspecies *f* are motile, all but *b* and *g* inner-arm subspecies have been shown to be dispensable for motility. Whether the subspecies *b* and *g* are essential to flagellar movement remains to be determined.

The isolation of *ida5* and *ida6* indicates that, while subspecies *e* can occur independently, it has some connection with subspecies *a*, *c*, and *d*, i.e., subspecies that have been thought to be components of inner-arm subform I2 (Piperno et al., 1990) and lacking in the mutant *ida4*. Piperno and Ramanis have suggested that an inner-arm heavy chain termed 3'(possibly corresponding to *b* (7)) occurs only in the proximal region of the axoneme, while another species termed 2'(possibly corresponding to *d*) occurs predominantly in the distal region. It seems possible that the location of the *e* subspecies in the axoneme is also biased

along the length of an axoneme: it could occur only in the proximal or distal region, or even in only one of the two axonemes of a cell which have been shown to be functionally different (Kamiya and Witman, 1984).

An unexpected observation is that *ida6*, despite its relatively small deficiency, has shorter flagella than those of *ida1*-*ida5* and displays as poor a motility as that of *ida5*. From this observation, I speculate that *ida6* has a second defect in addition to the lack of the subspecies *e*, although the lack of *e* is the only defect I found in the SDS-PAGE pattern of *ida6* axoneme. A more careful examination may clarify this point. Whatever the second defect is, it probably differs from the defect in *ida5*, since the double mutant *ida5* x *ida6* displayed poorer motility than that in either of the parent strains.

Recent studies on inner-arm dynein with biochemical and molecular biological techniques have revealed the presence of more than 10 inner-arm subunits including light chains. In theory, therefore, we may obtain more than 10 genetically distinct inner-arm mutants. The present study has added two novel mutants to the list of inner-arm mutants which includes *pf23*, *pf9* (*ida1*), *ida2*, *ida3*, and *ida4* (Huang et al., 1979; Kamiya et al., 1991; Porter et al., 1992). Since the variety of mutants available at present is still less than expected, attempts to isolate still new kinds of inner-arm mutants are important for our understanding of the function of various kinds of inner-arm dynein.

Table I. *Motility and Flagellar Length in Dynein-Arm Mutants*

	Flagellar length (μm)	Swimmimg velocity ($\mu m/s$)	Beat frequency (Hz)	Inner-arm deficiency
wt [a]	11.1 ± 1.9	155.0 ± 23.3	63	-
oda1 [a]	11.4 ± 2.0	58.0 ± 7.5	29	-
ida1 [a]	10.9 ± 1.6	82.1 ± 13.8	50	*f*
ida4 [a]	9.4 ± 1.6	102.2 ± 10.7	62	*acd*
ida5	12.4 ± 1.2	70.0 ± 5.6	27-52	*acde*
ida6	8.1 ± 1.4	69.6 ± 13.9	71	*e*
ida5 x *ida1*	-	Flagellaless [b]	-	*acdef*
ida6 x *ida1*	6.0 ± 0.9	20.7 ± 8.1	34	*ef*
ida5 x *ida4*	11.1 ± 1.5	50.8 ± 13.8	23	*acde*
ida6 x *ida4*	6.4 ± 1.0	28.8 ± 9.4	62	*acde*
ida5 x *oda4*	-	Flagellaless	-	*acde*
ida6 x *oda2*	-	Flagellaless	-	*e*
ida5 x *ida6*	6.2 ± 1.1	36.5 ± 8.9	55	*acde*

[a] From Kamiya et al. (1991)

[b] A small population bears paralyzed flagella.

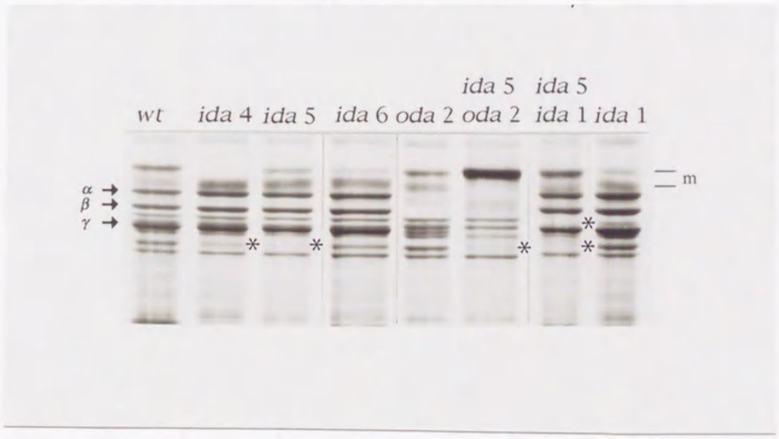


Figure 1. SDS/PAGE patterns of wild-type and mutant axonemes on a 3-5% polyacrylamide gel stained with silver. Only the high molecular weight region where dynein heavy-chain bands appear is shown. *wt*, wild type axonemes; *ida5/oda2*, axonemes of recombinant between *ida5* and *oda2*; *ida5/ida1*, those between *ida5* and *ida1*. Asterisks on the right side of lanes indicate the inner-arm bands that are missing in mutant axonemes. α, β, and γ indicate outer-arm heavy chains. m; bands with contaminated membrane proteins.

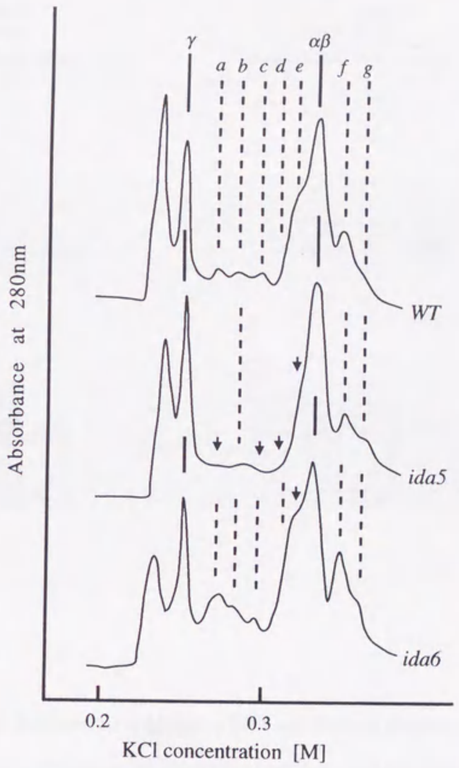


Figure 2. Elution patterns of high-salt extracts from axonemes of wild type, *ida5* and *ida6* in high-pressure liquid chromatography on a Mono-Q column. Vertical dotted lines indicate the peaks of inner-arm subspecies *a-g*, and vertical solid lines the peaks of outer-arm subunits, γ and $\alpha\beta$. Arrows show positions where particular subspecies are lacking in mutants. A shoulder appearing near an arrow in *ida6* is due to a three-headed species of outer-arm dynein (containing the α, β and γ heavy chains) originating from incomplete dissociation of the outer-arm dynein.

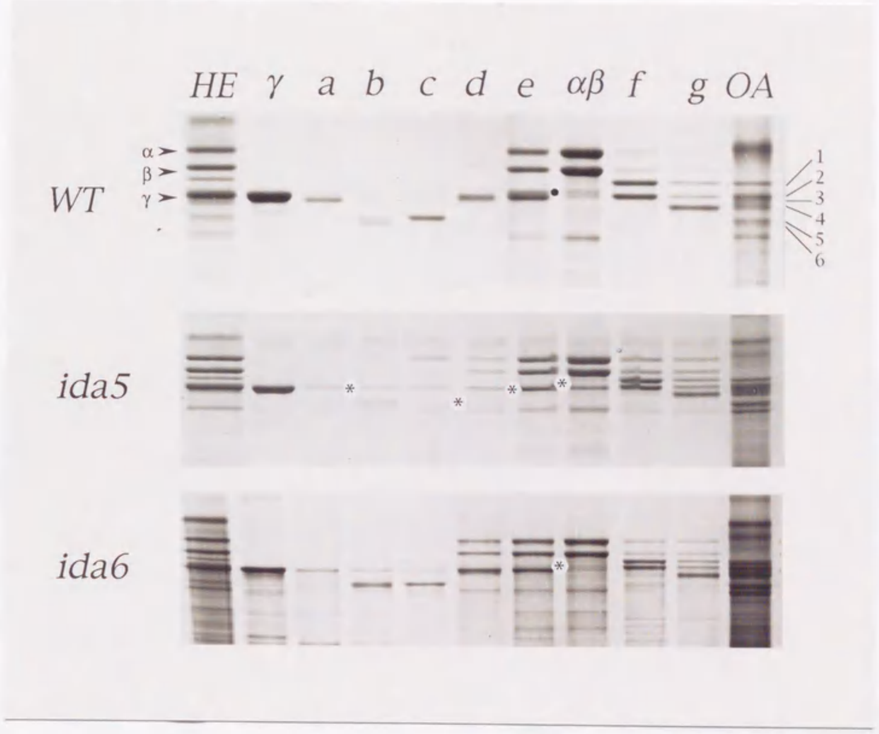


Figure 3. Dynein fractions in wild-type (*WT*) and mutant axonemes analyzed by SDS/PAGE. Fractions containing outer-arm and inner-arm subspecies (α-γ, *a*-*g*) in Fig. 1 were analyzed. Only high molecular weight regions are shown. Bands α-γ and 1-6 indicate outer-arm and inner-arm heavy chains, respectively. Lane HE, high-salt extract; OA, *oda2* axoneme. Bands appearing above the a heavy chain are those of membrane proteins. The band for subspecies *e* is marked by a filled circle in *WT*; this band occurs near the γ chain contained in a three-headed species of outer arm, which is present in a small amount and also eluted in fraction *e*. Note that the inner-arm band *e* is absent in *ida5* and *ida6*. Bands missing in mutants are marked by asterisks on the right side of each lane. In the *ida5* pattern, faint bands present near the asterisks in lanes *a* and *d* are those of the γ heavy chain of outer-arm dynein.

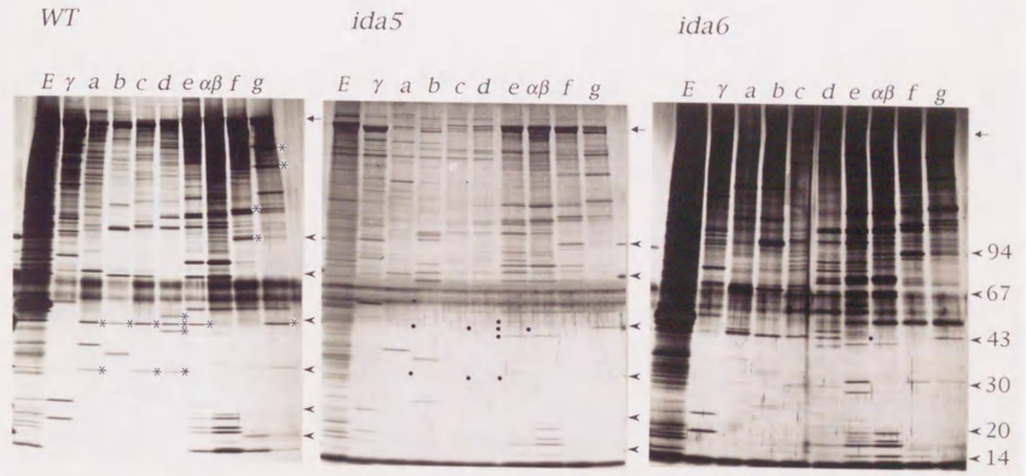


Figure 4. Intermediate and light chains associated with dynein subspecies. Crude high-salt extract (E) and chromatography fractions (γ-g) used in Fig. 2 were run on 5-20% acrylamide gradient gels. Asterisks in the wild-type pattern show the bands of putative dynein intermediate and light chains. Filled circles: bands lacking in the mutants. A faint band present in lane *e* of *ida6* is due to an incomplete separation of this species from species *d*. Arrows: positions of heavy-chain bands. Arrowheads with numbers: positions of molecular weight standards shown in Mr x 10^{-3}.

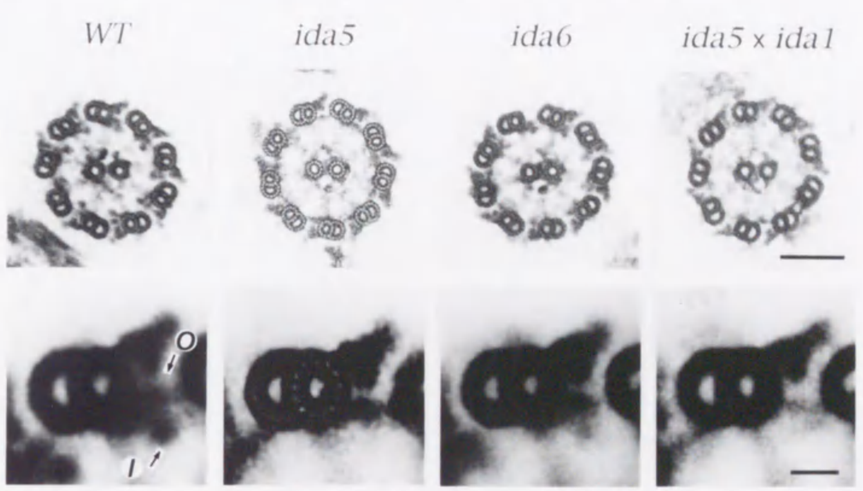


Figure 5. Cross-sectional electron micrographs of wild-type and mutant axonemes and the averaged images of their outer doublets. *WT*, wild type; *ida5* x *ida1*, double mutant between *ida5* and *ida1*. *I* and *O*: two projections of the inner dynein arm, the inner and outer rows. Bars: 0.1 μm (upper panel) and 0.02 μm (lower panel). Note that the image intensity of the inner row is weaker than normal in the averaged image of outer doublet of *ida6* and very weak in that of *ida5*. Note also that both inner and outer rows are very weak in the double mutant between *ida5* and *ida1*, an *ida*A-type inner-arm mutant (Kamiya et al., 1991).

CHAPTER II

Chlamydomonas Inner-Arm Dynein Mutant, *ida5*, Has a Mutation in an Actin-encoding Gene

Abstract

Chlamydomonas flagellar inner-arm dynein consists of seven subspecies (*a*-*g*), of which all but *f* contain actin as subunits. The mutant *ida5* and a new strain, *ida5-t*, lack four subspecies (*a*, *c*, *d*, and *e*). These mutants were found to have mutations in the conventional actin gene, such that its product is totally lost; *ida5* has a single-base deletion that results in a stop codon at a position about two-thirds from the 5' end of the coding region and *ida5-t* lacks a large portion of the entire actin gene. Two-dimensional gel electrophoresis patterns of the axonemes and inner-arm subspecies *b* and *g* of *ida5* lacked the spot of actin (isoelectric point [*p*I] = ~5.3) but had two novel spots with pIs of ~5.6 and ~5.7 instead. Western blot with different kinds of anti-actin antibodies suggested that the proteins responsible for the two novel spots and conventioal actin are different but share some antigenicity. Since *Chlamydomonas* has been shown to have only a single copy of the conventional actin gene, it is likely that the novel spots in *ida5* and *ida5-t* originated from another gene(s) that codes for a novel actin-like protein(s) (NAP), which has hitherto been undetected in wild-type cells. These mutants retain the two inner arm subspecies *b* and *g*, in addition to *f*, possibly because NAP can functionally substitute for the actin in these subspecies while they cannot in other subspecies. The net growth rate of *ida5* and *ida5-t* cells did not differ from that of wild type, but the mating efficiency was greatly reduced. This defect was apparently caused by deficient growth of the fertilization tubule. These results suggest that NAP can carry out some, but not all, functions performed by conventional actin in the cytoplasm and raise the possibility that *Chlamydomonas* can live without ordinary actin.

Introduction

Since the first discovery by Piperno and Luck (1979) from studies on *Chlamydomonas* flagella, the inner-arm dynein of cilia and flagella in various organisms has been shown to contain actin as a subunit (Pratt, 1986; Muto et al., 1994). Immunological evidence (Pratt, 1986; Muto et al., 1994; Sugase et al., 1996) strongly suggests that the actin contained in the inner-arm dynein is a true actin, not an actin-related protein that has been shown to be associated with dynactin, an activator of cytoplasmic dynein (Lees-Miller et al., 1992; Paschal et al., 1993). A recent study has indicated that *Chlamydomonas*, like *Volvox* (Cresnar et al., 1990), has only a single gene for actin, suggesting that the same actin is used for both cytoplasmic and axonemal functions (Sugase et al., 1996). The deduced sequence of *Chlamydomonas* actin indicated that it is a typical conserved actin, sharing ~90% homology to rabbit skeletal muscle actin.

The inner-arm dynein of *Chlamydomonas* consists of several distinct subspecies, each containing one or two heavy chains (Piperno et al., 1990; Kagami and Kamiya, 1992). Using ion exchange chromatography, Kagami and Kamiya (1992) have shown that the inner-arm dynein from outer arm-missing mutants can be separated into seven different subspecies, named *a-g*, containing eight distinct heavy chains altogether. Actin is associated with all subspecies except *f*. Several inner arm-deficient mutants were found to lack distinct sets of inner-arm subspecies. For example, *ida1* lacks subspecies *f* (containing two heavy chains) and *ida4* lacks subspecies *a*, *c*, and *d* (Kagami and Kamiya, 1992; for another terminology system of inner-arm subspecies, see Piperno [1995]). The mutant *ida4* has recently been shown to have a mutation in the structural gene for a 28-kD component of the subspecies *a*, *c*, and *d* (LeDizet and Piperno, 1995).

In the present study I further investigated the structural defects in the mutant *ida5*, which lacks subspecies *a*, *c*, *d*, and *e* and displays slow swimming (Kato et al., 1993). Here, I report an unexpected finding that *ida5* has a mutation in the actin-encoding gene that results in conventional actin not being expressed at all. Instead, a novel protein, previously undetected in wild type, appears to substitute for the ordinary actin as a subunit of the inner-arm subspecies *b* and *g*.

Materials and Methods

Strains and Cell Culture

Chlamydomonas reinhardtii 137c (wild type), *nit1/cw15*, *ida5* (Kato et al., 1993), and a new *ida5* strain, *ida5-t*, were used. The mutant *ida5* was produced by mutagenesis with UV light, while *ida5-t* was produced by insertional mutagenesis (see below). *nit1/cw15* was obtained from Dr. E. Harris of the *Chlamydomonas* Genetic Center (Department of Botany, Duke University, Durham, NC). Cells were grown either in liquid Tris-acatic acid-phosphate (TAP) medium (Gorman and Levine, 1965) or on TAP/agar plates.

Isolation of an ida5 Allele by Insertional Mutagenesis

An *ida5* allele, *ida5-t*, was obtained using a gene-tagging technique as described by Tam and Lefebvre (1993). Briefly, a nitrate reductase-encoding plasmid, pMN24, was introduced into the mutant *nit1/cw15* lacking nitrate reductase by vigorous stirring of the cells and plasmid with glass beads (Kindle, 1990). Transformants were selected by growing the cells on SGII/agar plates (Sager and Granick, 1953) containing nitrate as the sole source of nitrogen. The mutant *ida5-t* was one of the mutants deficient in inner arm dyneins obtained by screening ~6,500 transformants for slowswimming phenotypes. Southern blot analyses indicated that this mutant carries four plasmid insertions in the genome.

Isolation of Axoneme and Dynein

Flagella were detached from the cell body using dibucaine and purified by differential centrifugation (Witman, 1986). Dynein was obtained by extracting the demembranated flagellar axoneme with a buffer solution containing 0.6 M KCl, and fractionated by chromatography on a Mono-Q column, as described previously (Kagami and Kamiya, 1992).

Cell Body Extract

About 10^9 cells in 100 ml of TAP medium were deflagellated as above. The cell bodies were collected by centrifugation, resuspended in 5 ml of a solution containing 0.5 mM ATP, 0.1 mM $CaCl_2$, 10 mM imidazole, 0.01% β-mercaptoethanol, and 1 mM PMSF (Hirono et al.,

1989), and sonicated with a model US50 ultrasonic generator (Nihon Seiki Ltd., Tokyo, Japan) at 0 °C for 2 min. The sonicated material was centrifuged at 200,000 *g* for 1 h, and the supernatant was used as the cell body extract. The final protein concentration was ~1mg/ml.

Electrophoresis

One-dimensional SDS-PAGE was performed by the method of Laemmli (1970). Two-dimensional gel electrophoresis was performed using Immobiline DryStrip gel (Pharmacia LKB Biotechnology, Uppsala, Sweden), which covered a pH range of 4.0-7.0. Isoelectric focusing was performed at 2,300 V for 16 h, in the presence of 5 M urea and 2 M thiourea (Nakamura et al., 1989), followed by SDS-PAGE in the second direction. Gels were stained with silver or colloidal gold (AuroDye; Amersham Intl., Amersham, UK), or used for immunoblot analysis.

Immunoblot

Western blotting of axonemes and cell body extracts was performed by the method of Towbin et al. (1979). Samples were electrophoresed, transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) and probed with antibodies. Antibodies used were a polyclonal antiserum raised against an 11 amino-acid peptide corresponding to the NH_2-terminal sequence of *Chlamydomonas* actin (Sugase et al., 1996), an anti-*Physarum* actin polyclonal antibody (IgG fraction obtained by ammonium sulfate fractionation; a gift from Dr. K. Owaribe [Nagoya University, Japan]); and two commercially available anti-chicken gizzard actin mAbs, C4 (Lessard, 1988 ; ICN Biomedicals, Costa Mesa, CA) and N350 (Lin, 1981; Amersham Intl.). Immunoreactive spots were visualized with an alkaline phosphatase-conjugated secondary antibody (Cappel Research Products, Durham, NC) with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) as the chromogen.

Isolation and Sequence Analysis of ida5 Actin cDNA

Total RNA of *ida5* and *ida5-t* was prepared by acid guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). I used cells that were in the process of flagellar regeneration after being deflagellated by pH shock (Luck et al., 1977), since I speculated that the actin gene transcription may be activated during flagellar growth (see Sugase et al., 1996). Poly(A)$^+$ RNA was purified using oligotex-dT30 (Nihon Gousei Gum Co., Tokyo, Japan). 5 µg of poly(A)$^+$ RNA was used for the construction of Uni-ZAP XR cDNA library (Stratagene, La Jolla, CA). For screening for actin cDNA, a wild-type actin cDNA (Sugase et al., 1996) was used as a probe. One of the two positive clones obtained from the *ida5* library was sequenced over its entire length. The sequence difference observed between the mutant and wild type was verified by sequencing a segment of cDNA containing the possible mutation site. For this purpose, a 200-bp sequence starting at nucleotide 730 (in the cDNA sequence registered in EMBL/DDBJ/GenBank under accession number D50839) was amplified by reverse transcription (RT)-PCR from both wild-type and *ida5* total RNA using 5'-GGCCACCGCGCTGTCCAGT-3' and 5'-TACTGGCGCACTCAAAAGCG-3' as primers. A first-strand cDNA synthesis kit (Clontech, Palo Alto, CA) was used. The fragments were cloned into pBluescript vector (Stratagene) and sequenced for both strands.

DNA and RNA Blotting

DNA was isolated by the method of Weeks et al. (1986). For Southern blot analysis, DNA was digested with restriction enzymes, loaded on a 1% agarose gel, transferred to a Hybond-N+ membrane (Amersham Intl.), and probed with various fragments from genomic and cDNA clones of wild-type actin. As a control, the same samples were probed with a fragment of α-1 tubulin gene (Brunke et al., 1984) (obtained from the *Chlamydomonas* Genetics Center). The probes were labeled using a DIG DNA labeling kit (Boehringer Mannheim GmBH, Mannheim, Germany). The membranes were hybridized at 67 °C overnight in 5 x SSPE (1 x SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.2% SDS, 5 x Denhardt's reagent (1 x Denhardt's reagent is 0.02% Ficoll, 0.02%

polyvinylpyrrolidone, and 0.02% BSA), and 0.5 mg/ml salmon sperm DNA, and then washed with 0.1 x SSPE containing 0.1% SDS at 67 °C.

For Northern blot analysis, total RNA, prepared as above, was separated on a 1.5% agarose/formaldehyde gel, and transferred to a Hybond-N+ membrane. Hybridization was performed at 65°C overnight in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% SDS, 2% blocking reagent (Boehringer Mannheim GmBH), and 0.5 mg/ml salmon sperm DNA, and the mebmranes were washed with 0.2 x SSC (1 x SSC is 150 mM NaCl and 15 mM Na_3 citrate, pH 7.0) containing 0.5% SDS at 65 °C.

Mating Efficiency

Gametes were produced by incubating cells for 4-5 h in the nitrogen-free medium (see Harris, 1989). For the determination of mating efficiency, equal numbers of plus mating type (mt^+) and minus mating type (mt^-) gametes (density: 1 x 10^7 cells per ml) were mixed together, and the numbers of biflagellate cells (*B*) and quadriflagellate cells (*Q*) were counted after fixing the cells with 0.5% formaldehyde at different times. The percentage of the gametes that underwent cell fusion was calculated as 100 x $2Q/(2Q + B)$.

Observation of Fertilization Tubules

Gametes (mt^+) of wild type or *ida5* were incubated in the presence of 10 mM dibutyryl-cAMP and 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 1 h to induce fertilization tubule growth (Pasquale and Goodenough, 1987). For optical microscopy, gametes were fixed with 3.6% formaldehyde and observed using differential-contrast optics. For EM, gametes were fixed with 1% glutaraldehyde and processed after the method of Detmers et al. (1983). A 100CX microscope (Jeol Co., Tokyo, Japan) was used for the observation.

Results

Altered Actin in ida5

Fractionation by chromatography of the highsalt extract of *ida5* axonemes showed that its axonemes lack four (*a*, *c*, *d*, and *e*) of the seven (*a*-*g*) inner-arm dynein subspecies normally present in the wild-type axoneme (Kato et al., 1993). SDS-PAGE patterns of the dynein fractions indicated that its subspecies *b* and *g* had a 43-kD band at a position similar to that of the actin band in the wild-type counterparts (Kato et al., 1993). However, two-dimensional gel electrophoresis of the axoneme revealed a striking difference (Fig. 1A, B); the *ida5* axoneme lacked the spot corresponding to that of actin (isoelectric point [pI] = ~5.3), but had two novel spots at apparent pIs of ~5.6 and ~5.7. The same change in the actin spot was observed with the dynein fractions *b* (data not shown) and *g* (Fig. 1B). In one-dimensional SDS-PAGE, the 43-kD band in *ida5* had a slightly larger mobility than that in wild type (Fig. 1C).

Sequence Analysis of ida5 Actin cDNA

The above finding, together with the recent mapping of the actin gene to a locus near that of *ida5* (right arm of linkage group XIV) by C. Silflow and P. Lefebvre (personal communication), suggested that the mutant *ida5* might have a mutation in the actin-encoding gene itself. I thus analyzed its actin gene. Actin cDNA was isolated from an *ida5* cDNA library using the wild-type actin cDNA as the probe. One of the two clones obtained was sequenced over its entire length. The sequence indicated that a base (C) had been deleted from the CCCC sequence starting at nucleotide 838 (in the cDNA nucleotide sequence registered in EMBL/DDBJ/GenBank). The same deletion was found in a cDNA fragment obtained by RT-PCR from total *ida5* RNA (see Materials and Methods). This deletion should cause a frame shift and produce a stop codon at the amino acid position 268, i.e., at about two-thirds of the total of 377 amino acids (Fig. 2). Hence, no functional actin should be produced in this mutant since the COOH-terminal 110 amino acid sequence is essential to the actin assembly and function (Kabsch et al., 1990). However, although Northern blotting detected the presence of full-length actin mRNA (data not shown), Western blot analysis using an antibody against

NH_2-terminal 11 amino acid sequence did not detect any truncated actin (see Fig. 4). Truncated products, if any, may be degraded quickly in the cytoplasm.

Loss of the Actin Gene in an ida5 Allele Produced by Insertional Mutagenesis

I isolated a mutant similar to *ida5* by screening a population of cells mutagenized by plasmid insertion. Its axoneme lacked the same inner-arm subspecies as those missing from *ida5* and had the same two novel spots in two-dimensional gels as observed with *ida5* (Fig. 1B). Sexual crossing of this mutant with *ida5* did not produce cells with a wild-type phenotype in 40 tetrads. In addition, temporary dikaryons between them were not rescued. From these observations, I concluded that this mutation is an *ida5* allele. I named it *ida5-t*.

Unexpectedly, Southern blot analyses of the *ida5-t* genome using four different fragments from the wild-type actin genomic clone as probes did not detect bands that hybridized with any of the probes. With the parent strain *nit1/cw15*, on the other hand, the same four probes clearly detected bands at positions exactly as predicted from the sequence (Fig. 3A). These findings indicate that a large portion of the actin-encoding region is missing from *ida5-t*. Such loss of a gene upon insertional mutagenesis has often been observed before, although its exact mechanism is unknown (Tam and Lefebvre, 1993).

In agreement with the major loss of the actin gene, Northern analysis detected no actin mRNA in *ida5-t*, while the same analysis detected actin mRNA in the parent strain *nit1/cw15* (Fig. 3B). Thus the findings with *ida5* and *ida5-t* both indicate that these mutants do not express conventional actin. It is likely that the two novel spots in the two-dimensional gel are products of gene(s) other than the known actin-encoding gene. The proteins responsible for these two spots will be tentatively called novel actin-like proteins (NAPs). (It is not certain whether NAP is a single protein or two, because a single protein frequently appears as two or three spots in my two-dimensional gel system for unknown reasons.)

Western Blot Analysis of NAP

Although the above findings suggest that NAP is not the product of the conventional actin gene, I speculated that it is somehow related to actin. This is because NAP has an

apparent molecular weight similar to that of actin and can substitute for actin as the subunit of inner-arm dynein subspecies *b* and *g*. To assess the similarity of NAP to conventional actin, its cross-reactivity with four different anti-actin antibodies was examined by Western blotting (Fig. 4A). All these antibodies were found to react with the actin in the SDS-PAGE pattern of wild-type axonemes, although most of them also reacted with a few other bands of unidentified origins. In *ida5* axonemes, the polyclonal antibody against the NH_2-terminal 11 amino acid sequence of *Chlamydomonas* actin did not react with NAP as stated before, and neither did a monoclonal anti-smooth muscle actin antibody (C4). However, a polyclonal antibody against *Physarum* actin and another monoclonal anti-smooth muscle actin antibody, N350, did react with it, although less intensely than with actin in the wild-type axoneme. In two-dimensional electrophoresis patterns of the axoneme, the *Physarum* antibody reacted with the actin spot in wild type and both of the two spots of NAP in *ida5* and *ida5-t,* while the *Chlamydomonas* antibody reacted only with the actin in wild type (Fig. 4B).

To detect actin and NAP in the cell body, extracts from deflagellated cells of wild type and *ida5* were subjected to two-dimensional electrophoresis and Western analysis. Both of the *Chlamydomonas* and *Physarum* antibodies used above revealed the presence of actin in wild type but not in *ida5* (Fig. 4C). Rather unexpectedly, NAP spots were not detected with the *Physarum* antibody in the *ida5* cell body. This suggests that NAP may be present in the cell body of this mutant in a much smaller amount than actin in the wild-type cell body.

Effects of Actin Mutation in Cell Growth and Fertilization

I expected that the actin mutation in *ida5* would cause some defects in its growth or cell division. However, I found no difference in the gross growth rate between the mutants and wild type (Fig. 5). Time-lapse observations of the cytokinesis process revealed that the mutants form cleavage furrows similar to those observed during the cell division in the wild-type cells (data not shown).

During the sexual conjugation of *Chlamydomonas*, mt^+ gametes produce a process of a few micrometers in length at the apical end of the cell body and thereby facilitate the cell fusion process (Goodenough and Weiss, 1975). This process, called the fertilization tubule, has been

shown to contain an F-actin bundle (Detmers et al., 1983). I speculated that *ida5* gametes may be deficient in fertilization because they cannot produce the fertilization tubule normally. Just as I expected, the efficiency of cell conjugation greatly decreased when mt^+ *ida5* was mated with mt^- wild type or *ida5*. Interestingly, conjugation efficiency did not decrease when mt^- *ida5* was mated with mt^+ wild type (Fig. 6). These results suggest that the gamete of mt^+ *ida5* is deficient in fertilization tubule growth.

To confirm the above prediction, I induced fertilization tubule growth by treating gametes with dibutyryl-cAMP and IBMX (Pasquale and Goodenough, 1987) and observed them with a differential-interference microscope. After about an hour of treatment, fertilization tubules were clearly seen in > 70% of the mt^+ wild-type gametes, whereas no fertilization tubules were found in mt^+ *ida5* gametes. EM indicated that a small fraction of *ida5* gametes in fact produced fertilization tubules of abnormal shapes (Fig. 7). In contrast with the slender fertilization tubules of wild type, the *ida5* fertilization tubules were round and stubby. Appendices of round appearance were also observed in mt^- *ida5* gametes treated with dibutyryl-cAMP and IBMX, as well as in the mt^- wild-type gametes thus treated (Pasquale and Goodenough, 1987).

Southern Blot Analyses at Different Stringencies

Sugase et al. (1996) showed that *Chlamydomonas* has only a single copy of actin-encoding gene (Sugase et al., 1996). However, the above finding that the mutants *ida5* and *ida5-t* with serious deletions in the actin gene grow normally has raised the possibility that another protein(s), such as NAP, substitutes for actin in important functions in these mutants, because actin has been believed to be essential for cellular function. The substituting protein, if any, may be structurally related to actin. Thus, I examined if *Chlamydomonas* has other genes that become hybridized with a fragment of the conventional actin gene at low stringency. Fig. 8 shows the results of such an experiment. Under the conditions shown in Fig. 3, probe 2 did not hybridize with any band in *ida5-t*, whereas in the parent (*nit1/cw15*) it hybridized with one or two bands, at positions exactly as expected from the restriction map of the known actin gene. When the stringency was slightly decreased by lowering the temperature from 67° to 65°C, an

additional band appeared in each sample of the DNA fragments of both strains. This band cannot be explained from the sequence of the conventional actin gene. With additional lowering of stringency, it was obscured by many other nonspecific bands.

Discussion

I have shown that the *Chlamydomonas* mutants *ida5* (Kato et al., 1993) and *ida5-t*, lacking four subspecies of inner-arm dynein, have mutations in an actin-encoding gene such that its gene product is totally lost. The axoneme of these mutants contain NAP instead of conventional actin, as evidenced by the two novel spots in two-dimensional electrophoresis patterns. These findings are unexpected since *Chlamydomonas* and a related species, *Volvox*, have been shown to have a single gene for actin, which is ~90% homologous to rabbit skeletal muscle actin (Cresnar et al., 1990; Sugase et al., 1996). The fact that *ida5* and *ida5-t* grow normally suggests either that NAP or other proteins may be able to substitute for conventional actin in important functions, or that actin is not essential for the growth of *Chlamydomonas*.

The molecular identity of NAP remains to be studied, but it appears to be somehow related to actin. This is because NAP and actin have similar molecular weights, can serve as the subunit of some inner-arm dynein, and share some antigenicity; although NAP did not cross-react with the antibody against the NH_2-terminal sequence of *Chlamydomonas* actin or a mAb C4, it weakly cross-reacted with two other anti-actin antibodies. NAP may be another actin that is only moderately homologous to conventional actin and has hitherto been undetected. The Southern analysis in the present study under low stringency conditions revealed, in addition to the band expected from the known actin gene sequence, a weakly hybridizing band that has not been detected by Sugase et al. (1996). This additional band cannot be explained by the known actin sequence and may well have originated from the gene of NAP. Whatever the gene is, it may not be highly homologous to conventional actin, because it cannot be detected at higher stringencies; if the two genes were members of a gene family of conventional actin, like the multiple actin genes in other organisms such as *Dictyostelium* (Romans and Firtel, 1985) and mouse (Minty et al., 1983), the two sets of bands might well have appeared even under high stringency conditions. It is likely that conventional actin is totally missing in the *ida5* mutants.

The association of actin with axonemal inner-arm dynein (Piperno and Luck, 1979; Piperno, 1990; Muto et al., 1994; Sugase, 1996) and that of an actin-like protein (dynactin, Arp1) with an activator of cytoplasmic dynein, dynactin complex, have been established (Lees-Miller et al., 1992; Paschal et al., 1993; Schafer et al., 1994). The dynactin complex probably serves to bind dynein to intracellular membranes to facilitate their movement on microtubules (see Schroer, 1994). By analogy, the actin in inner-arm dynein may function to facilitate binding of dynein to the A-tubule of the outer doublet. This idea is supported by the present finding that the actin mutation in *ida5* results in the loss of four inner-arm subspecies. The fact that the mutant axoneme has NAP in place of actin and retains inner-arm subspecies *b* and *g* suggests that NAP can functionally replace actin in these subspecies. However it is not understood why NAP is absent from the wild-type axoneme (Fig. 1B). It may be that, in wild-type cells, the association of NAP with the inner-arm dynein is prevented by the abundance of conventional actin. Alternatively, the cell may regulate the expression of NAP so that it is produced in a significant quantity only when the expression of conventional actin is blocked. Whether the wild-type cell also expresses NAP will be made clear when its specific antibody or DNA clone is obtained.

I have found that *ida5* and *ida5-t* have a serious defect in the fertilization tubule growth. My observation is in good agreement with that of Detmers et al. (1983) who showed that the mating efficiency is greatly reduced if mt^+ gametes, but not mt^-, gametes have been preincubated with cytochalasin D. They showed that cytochalasin-treated mt^+ gametes produce short fertilization tubules that can make contact with mt^- gametes, permitting the gametes to fuse with low efficiency. Thus my observation that mt^+ *ida5* gametes with stubby round fertilization tubules can undergo cell fusion at low efficiency is compatible with the idea that the gamete does not express polymerizable actin at all.

Harper et al.(1992) have examined the actin distribution during the cell cycle of *Chlamydomonas* using mAbs C4 and N350, which I also used in this study. They found that actin surrounds the nucleus in interphase cells and undergoes dynamic reorganization during mitosis and cytokinesis, including relocalization to the cleavage furrow during cytokinesis. From these observations they suggested that actin may play a role in the movement of basal

bodies and cytokinesis. It is important to note, however, that the actin in the cytoplasm could not be visualized by staining with FITC-phalloidin, nor could the cytokinesis be blocked with cytochalasin B or cytochalasin D. Hence they also suggested the possibility that the change they observed may be a change in the distribution of G-actin and not that of F-actin (Harper et al., 1992). It is possible that F-actin is present in wild-type *Chlamydomonas* only in the fertilization tubule, which has been demonstrated to be clearly stained with fluorescence-labeled phallacidin (Detmers et al., 1985).

Although a large number of actin mutants have been isolated in other organisms (for review see Sheterline and Sparrow, 1994), all the null mutants, including those of yeast (Shortle et al., 1982), have been found to be lethal. The fact that the *ida5* mutations are null alleles yet nonlethal opens the way for experiments in which functions of conventional actin are investigated by transforming the mutants with modified constructs of the gene. In fact, Ohara et al. (Ohara, A., Kato-Minoura, T., Kamiya, R. and Hirono, M., manuscript in preparation) have recently succeeded in rescuing the *ida5* phenotype by transforming it with a wild-type actin gene clone. Initial experiments aimed at elucidation of the functional domains of actin that are important for the assembly of dynein are under way.

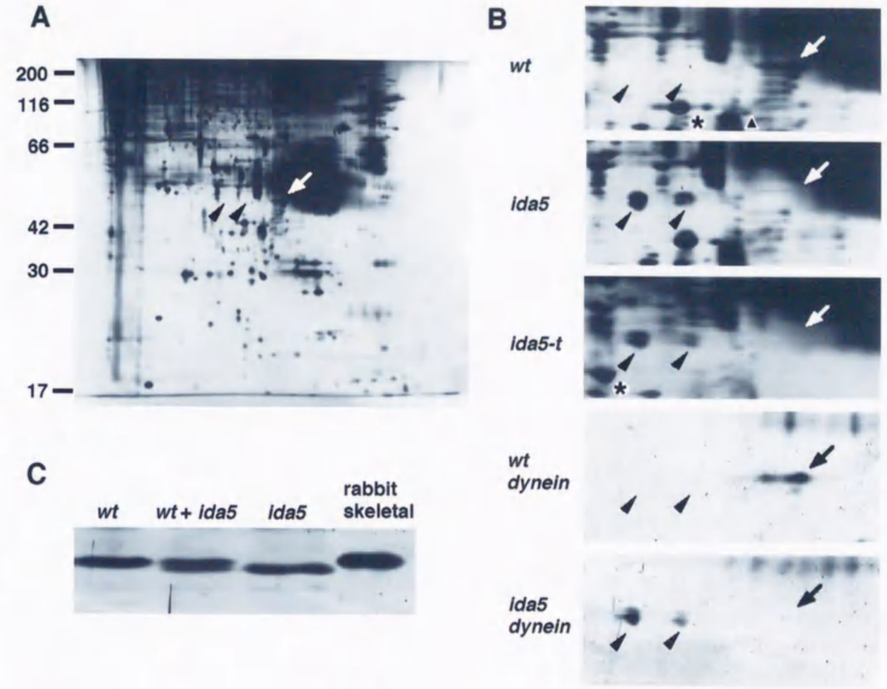


Figure 1. Electrophoresis patterns of wild-type and mutant axonemes. (A) Two-dimensional SDS-PAGE pattern of wild-type axonemes. pH range: 4.0-7.0. (Left) Basic polypeptides. (Bars with numbers) Positions of molecular mass standards shown in Mr x 10^{-3}. (B) Portions of two-dimensional electrophoresis showing spots of actin and NAP appearing in the mutants. (*wt, ida5*, and *ida5-t*) Axonemes of wild type and mutants. (*wt dynein* and *ida5 dynein*) Inner-arm subspecies *g* separated by chromatography. Arrows in A and B indicate the position of actin; arrow heads indicate those of NAP. In *ida5-t,* two spots of unidentified origins were shifted by ~0.2 pH unit to more alkaline positions (*). This shift may be caused by another gene disruption event in this mutant. The smear (triangle) is an artifact of silver staining. The faint spots seen in the dynein patterns (shown in a series) are those of tubulins with various degrees of posttranslational modification. (C) One-dimensional SDS-PAGE of subspecies *g*. Samples from wild type, *ida5*, and their mixture were loaded on the same 11% polyacrylamide gel. (Lane rabbit skeletal) Rabbit skeletal muscle actin. Only a portion near the actin band is shown. All gels were stained with silver.

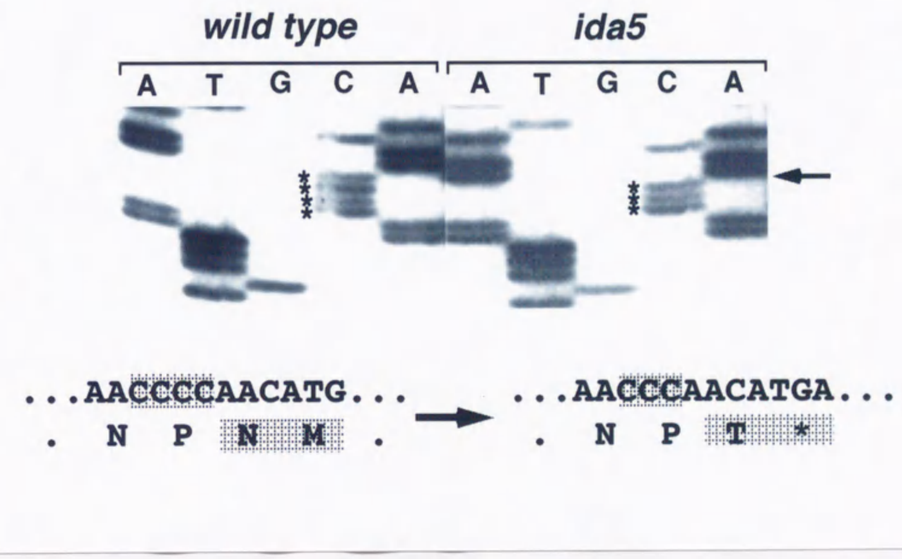


Figure 2. Sequence analysis of the *ida5* mutation. Sequencing patterns of the partial cDNA clones obtained from wild type and *ida5* by RT-PCR were shown side by side. (Arrow, right) Position of a base (C) deletion (asterisks). The altered cDNA and amino acid sequences are shown at the bottom. The leftmost asparagine is at amino acid 265. The deletion in *ida5* results in a stop codon TGA at position 268.

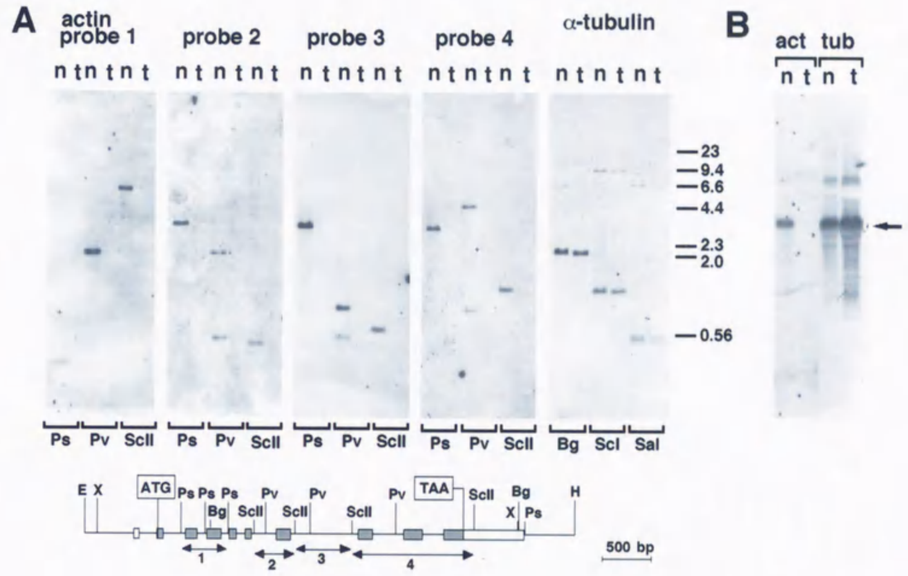
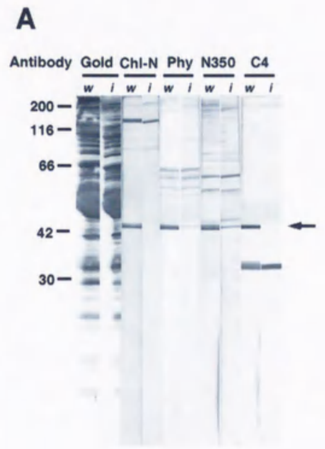


Figure 3. Southern (A) and Northern (B) hybridization analyses of *ida5-t*. Along with the actin gene, tubulin gene and message were analyzed as controls. (A) Genomic DNAs from *nit1/cw15* (n) and *ida5-t* (t) were digested with a restriction enzyme shown at the bottom, and probed with the probes indicated in the restriction map (Sugase et al., 1996). The solid and open boxes indicate coding exons and transcribed UTR. Pv, PvuII; ScII, SacII; Ps, PstI; Bg, BglII; ScI, SacI; Sal, SalI; E, EcoRI; X, XhoI; H, HindIII. The membranes were hybridized at 67 °C overnight. (Bars with numbers) Positions of the λHindIII markers shown in bp x 10^{-3}. (B) Total RNA was probed with either wild-type actin cDNA (act) or TubA1 fragment (tub). Hybridization was performed at 65°C. (Arrow) Bands of actin and α-tubulin mRNAs (almost the same size).


A
Antibody
Gold
Chl-N
Phy
N350
C4
w
i
200
116
66
42
30

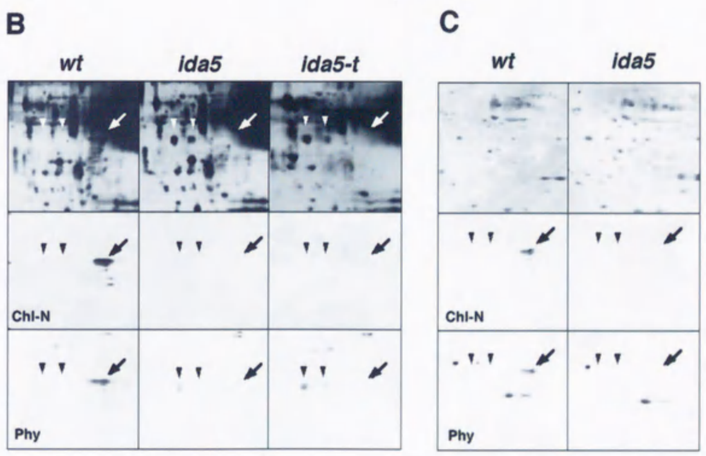

B
wt
ida5
ida5-t
Chl-N
Phy
C
wt
ida5
Chl-N
Phy

Figure 4. Western blot analyses. (A) axoneme of wild-type (*w*) and *ida5* (*i*) was loaded on 11 % polyacrylamide gel, transferred, and stained with colloidal gold (Gold), or probed with anti-*Chlamydomonas* actin (Chl-N), anti-*Physarum* actin (Phy), N350, or C4 antibodies. (Arrow) The 43-kD band of actin or NAP. (B) Two-dimensional gel electrophoresis patterns and Western blot of wild-type (*wt)* and mutant axonemes. Only a region of Mr 30-66 kD and pH 5.1-5.9 is shown. (Arrows) Positions of actin. (Arrowheads) Positions of NAP. (Top rows) Silver-stained gel patterns; (middle and bottom rows) blot with anti-*Chlamydomonas* actin (Chl-N) and anti-*Physarum* actin (Phy) antibodies. (C) Two-dimensional gel electrophoresis patterns of cell body extracts. Cell bodies from wild type (*wt*) and *ida5* were collected immediately after deflagellation. Apparent Mr range: 24-66 kD. pH range: 5.1-5.9. (Top row) Stained with colloidal gold; (middle and bottom rows) Western blot with anti-*Chlamydomonas* actin (Chl-N) and anti-*Physarum* actin (Phy) antibodies.

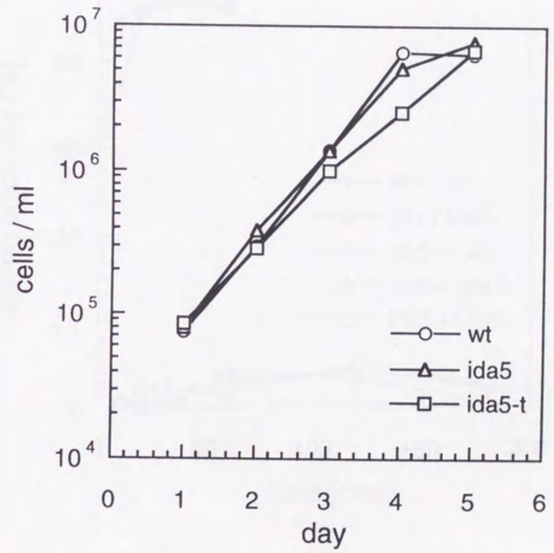


Figure 5. Proliferation rate of wild type and mutants. Cells cultured in the liquid TAP medium were sampled every 24 h and their numbers were counted.

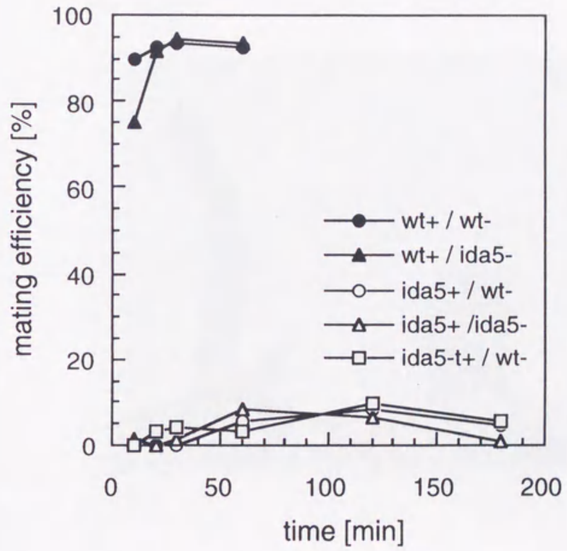


Figure 6. Mating efficiency. (Ordinate) Percentage of cells that underwent cell fusion and became quadriflagellate. (Abscissa) Time after the onset of mating. The combinations of mating pairs are shown with a slash. + and - denote the mating type of the gametes used. Since the flagella of zygotes were gradually resorbed with time, the apparent mating efficiency decreased after ~2 h of mating.

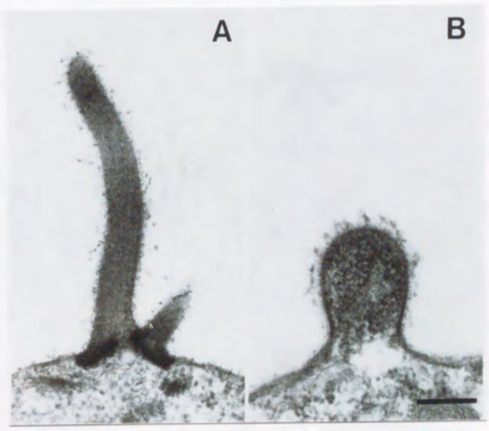


Figure 7. Fertilization tubules in wild-type (A) and *ida5* (B) mt^+ gametes produced in response to a 1-h exposure to 10 mM dibutyryl-cAMP and 1 mM IBMX. Bar, 0.3 µm.

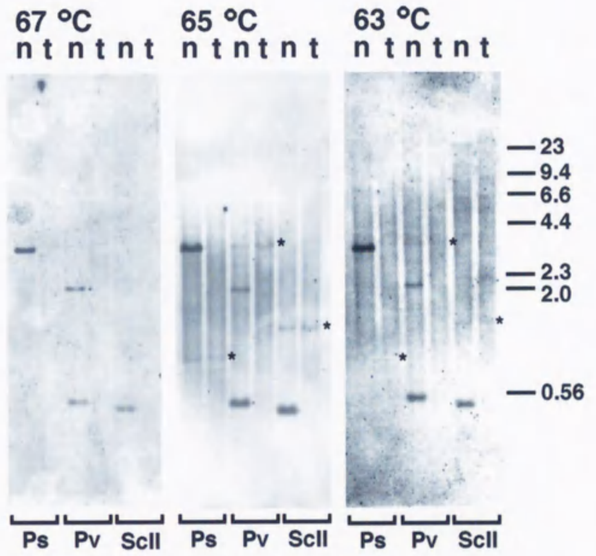


Figure 8. Southern hybridization analyses at different stringencies. Probe 2 in Fig. 3 was used. n, Restriction fragments from *nit1/cw15* (parent strain); t, fragments from *ida5-t.* For abbreviations of restriction enzymes, see Fig. 3 legend. Experimental procedure was the same as in Fig. 3A except for the temperature at which hybridization and wash were performed. At lower stringencies, an additional band (*) appears in each lane of both samples.

CHAPTER III

A Highly Diverged Actin Expressed in the Null Mutant of Conventional Actin of *Chlamydomonas*

Abstract

The *Chlamydomonas* mutant *ida5* has a nonsense mutation in the conventional actin gene and lacks four of the six subspecies of inner-arm dyneins that normally contain actin as subunits. The remaining two inner-arm subspecies in this mutant has been shown to contain a novel actin-like protein (NAP). In this study, NAP was purified from the inner-arm dynein fraction from the *ida5* axoneme and subjected to direct sequencing. Sequences of five tryptic fragments were determined. Four of them were found to have homologies to part of known actin sequences. By reverse transcription (RT)-PCR with primers designed after these sequences, partial cDNA clones including one covering the 5'-untranslated region (5'UTR) were isolated. Database search showed that their sequences agreed with the sequence recently reported as that of a gene encoding a *Chlamydomonas* actin-related protein. This protein, with a 64% amino acid identity with the conventional actin, may be considered to be a highly diverged actin. It is thus concluded that *Chlamydomonas* has both conventional actin and NAP, and NAP can function as a subunit of some, but not all, inner-arm dynein subspecies. Functional difference of these two actin proteins in the cytoplasm of *Chlamydomonas* remains an interesting future problem.

Introduction

With the advance in molecular biology, numerous actins and actin-related proteins (Arps) have been discovered (for reviews, see Mullins et al., 1996; Sheterline et al., 1996). Except for the classical protein actin, however, studies on their function are still behind those on their primary structures. The phylogenetical analyses of those sequences, on the other hand, enabled estimation of their functional differences. So far, the actin superfamily is categorized into four major subgroups; actin, Arp1, Arp2, and Arp3, and several minor branches with unique sequene profiles. Actin is a highly conserved protein among species and constitutes the cytoskeletal filaments that are essential for the force production by myosin. Besides this major function in the cytoplasm, it is contained in a subset of flagellar inner-arm dynein as a subunit (Piperno and Luck, 1979; Muto et al., 1994; Sugase et al., 1996). Arp1 is also associated with dynein; the dynactin complex, an activator of cytoplasmic dynein, has been shown to contain Arp1 (Lees-Miller et al., 1992; Paschal et al., 1993).

A biflagellate green algae, *Chlamydomonas*, has only a single copy of the conventional actin gene (Sugase et al., 1996). This is also true with a closely related species, *Volvox* (Cresnar et al., 1990). In my previous study, I have shown that a mutant that lacks a subset of inner-arm dyneins, *ida5*, has a nonsense mutation in this actin gene (Kato-Minoura et al., 1997). Indeed, no actin was detected in the axoneme or cytoplasm of this mutant. However, two-dimensional electrophoresis patterns of the axoneme revealed two novel spots with molecular weights similar to that of actin and isoelectric point(s) (pI[s]) values different from that of actin. These spots were referred to as novel actin-like proteins (NAPs), since they reacted with two kinds of antibodies against actin. Furthermore, Southern blot analysis at a low stringency using part of actin gene as a probe suggested the existence of an actin-related sequence; in the mutant *ida5-t* which is missing the almost entire actin gene, faint bands of an unknown origin were detected although the bands corresponding to the actin gene were totally absent. From these observations, I postulated that *Chlamydomonas* has a hitherto undetected gene that encodes some actin-related proteins, and that NAP may be the product of this gene. To explore this possibility, I tried to determine the primary structure of NAP. Direct sequencing analysis performed in this study has shown that NAP is identical with the sequence

recently reported as that of an actin-related protein (Lee et al., 1997). NAP was not a conventional actin but a novel actin-related protein having a strikingly low homology to actin. Hence it was confirmed that *Chlamydomonas* has a single species of conventional actin and at least one species of unconventional actin or actin-related protein, NAP.

Materials and Methods

Strains and Cell Culture

Chlamydomonas reinhardtii actin-less mutants, *ida5* (Kato et al., 1993) and *ida5-t* (Kato-Minoura et al., 1997), were used. *nit1/cw15* was obtained from Dr. E. Harris of the *Chlamydomonas* Genetic center (Department of Botany, Duke Univercity, Durham, NC). For large-scale preparation of NAP, cells were grown on Tris-acetic acid-phosphate (TAP) (Gorman and Levine, 1965)/1.3% agar plates. Cells were allowed to flagellate by suspending in liquid TAP medium two hours before preparation.

Axoneme Isolation and Dynein Purification

The axonemes of *ida5* were prepared using standard methods (Witman, 1986). Dynein was extracted with 0.6 M KCl from the axoneme and purified by centrifugation on a 5-20% sucrose density gradient (Takada et al., 1992). Fractions containing inner arms were collected and precipitated by adding methanol (Wessel and Flugge, 1984).

Peptide Sequencing

The inner dynein fraction was concentrated and separated by electrophoresis in a 5-20% acrylamide gradient gel (Laemmli, 1970) and blotted to polyvinylidene difluoride membrane (Immobilon Psq; Millipore Corp., Woburn, MA) in standard Tris-Glycine-SDS buffer (Towbin et al., 1979). The 43-kD band of NAP was identified by staining with amidoblack, excised, and digested in situ with trypsin. Peptides eluting from the membrane were isolated by capillary HPLC. Five peptides were sequenced using an Applied Biosystems 492A sequencer (Foster City, CA) in the Protein Chemistry Facility at the Worcester Foundation for Biomedical Research (Shrewsbury, MA).

cDNA Cloning

To extend the sequences, RT-PCR was performed using two oligonucleotides (5'-CACCCSATYGAGCAYGGYGT-3' ; based on the peptide sequence HPIEHG[V] and 5'-ACSACGTTGTASAGSAGCTC-3'; based on ELLYNV[V]) as degenerated primers. To avoid miss-amplification of actin, mRNA from *ida5-t*, an allelic strain lacking almost entire region of actin gene (Kato-Minoura et al., 1997), was used as a template. mRNA was prepared during the re-flagellation, and converted into cDNA using a first-strand cDNA synthesis kit (Clontech, Palo Alto, CA). The 5' region of NAP cDNA was further extended using a 5' rapid amplification of cDNA ends (5'-RACE) system (Frohman et al., 1988) (Life Technologies, Inc., Gaithersburg, MD). For the initial extension from mRNA, a gene specific primer (5'-TAGTCCTTGCTGACCACA-3'; based on [S]VVSKD[Y], a fragment which has the lowest melting temparature (Tm) within the sequence available at that time of designing the primer) was used. For the second and final amplification, 5'-ATCTCCGTGGCGCGCTCCCTGTTCT-3'; based on [Q]NRERATE[I], and 5'-GTSAGCATSACSGGGCGCTC-3'; based on ERPVML[T] were used as the reverse primers, respectively. Products were subcloned into pBluescript vector (Stratagene, La Jolla, CA) and sequenced for both strands using an Applied Biosystems ABI PRISM 310 genetic analyzer (Foster City, CA) in NIBB Center for Analytical Instruments (Okazaki, Japan).

Results

Using a fraction enriched for NAP from the *ida5* axoneme as the starting material, I obtained five peptide sequences of 9 to 21 residues (Fig. 1, shaded boxes). Partial cDNAs were obtained by using PCR with primers designed after these sequences. Their sequences including 5'UTR region were determined (Fig. 1, underlined). Database search revealed that the sequence was identical with that of a gene recently reported by Lee et al. (1997) as a gene encoding an actin-related protein. From my previous study, NAP has been predicted to have some sequence similarity to actin for the following reasons; first, two kinds of anti-actin antibodies reacted with NAP. Second, faint bands corresponding to the fragments of the putative NAP gene were detected by Southern blotting performed under low stringency

conditions using part of the actin gene as a probe (Kato-Minoura et al., 1997). In agreement with those data, NAP was found to be a highly diverged actin sharing only a low amino acid identity of 64% with *Chlamydomonas* actin (Sugase et al., 1996). Southern blot analysis using part of the NAP cDNA as a probe at low stringency detected three strong bands corresponding to the fragments of the NAP gene, including one at a position exactly the same as that of the faint band detected by actin probe (Fig. 2, leftward arrow), as well as another faint band corresponding to the actin gene fragment (Fig. 2, rightward arrow). The observation that the probe for actin gene can weakly hybridize with the NAP gene, and the probe for NAP gene can weakly hybridize with the actin gene confirmed that these two genes have a certain similarity.

Discussion

I have shown that NAP, expressed in the *Chlamydomonas* actin-less mutant *ida5*, is a highly diverged actin, sharing a low (64%) amino acid identity (Sugase et al., 1996; Lee et al., 1997). Since NAP is the only protein that can be detected by Western blot analyses on the *ida5* axoneme and cytoplasm with various kinds of anti-actin antibodies (Kato-Minoura et al., 1997), it is unlikely that *Chlamydomonas* has a third protein that is more closely related to the conventional actin. Hence *Chlamydomonas* must have only a single kind of conventional actin. The protein NAP with a 64% homology to actin may well be regarded as an unconventional actin, although whether it has the basic properties of actin remains to be studied.

Two-dimensional gel electrophoresis of the *ida5* axoneme has revealed two spots with apparent pIs of ~5.6 and ~5.7 (Kato-Minoura et al., 1997). However, it is not clear whether one or both of those two spots correspond to the NAP identified in this study, as a single protein can be separated into the two spots of different pIs because of some protein modifications. Lee et al. (1997) showed that *Chlamydomonas* has only a single copy of gene encoding their actin-related protein (corresponding to NAP). Also, my Southern blot analyses at a low stringency using NAP/actin probe failed to detect any bands other than those actin and NAP bands (Kato-Minoura et al., 1997 and Fig. 2). These observations favor the idea that the *Chlamydomonas* axoneme contains only a single species of NAP, and the two spots in the two-

dimensional gel appeared not because of any genetic difference but because of some post-translational modification or artifacts in electrophoresis.

The growth rate of *ida5* and its cytokinesis pattern are apparently normal (Kato-Minoura et al., 1997 and unpublished results). These facts suggest that *Chlamydomonas* does not need conventional actin for its growth. Actin may not be important for these cellular functions of *Chlamydomonas*, or NAP may be able to substitute for actin in important cellular functions. If NAP does function by substituting actin in *ida5*, it must be considered different from other known Arps, which have been reported to have properties significantly different from those of actin. Examination of the localization of NAP within wild-type and mutant cells using anti-NAP antibody should be most important for elucidating this point.

During mating of gametes, *ida5* of plus mating type cannot form the fertilization tubule, a cellular apparatus that contains an actin filament bundle and functions to facilitate mating (Kato-Minoura et al., 1997). It may be that NAP cannot substitute for actin in the formation of this structure, or the amount of NAP is not large enough to produce the fertilization tubule. It would be interesting to examine whether NAP can be polymerized to form filamentous actin *in vivo* and *in vitro*.

Of the seven inner arm dynein subspecies of *Chlamydomonas* (*a-g*), six subspecies except *f* have actin as a light chain (Piperno and Luck, 1979; Kagami and Kamiya, 1992; Sugase et al., 1996). However, the mutant *ida5* devoid of the conventional actin lacks only four of them (Kato et al., 1993); subspecies *b* and *g* are retained apparently because NAP can substitute for actin as their subunits. Interestingly, however, subspecies *b* and *g* contain actin but not NAP in wild type cells, which also appear to express NAP (unpublished observation). The lack of NAP in the wild-type inner-arm dyneins is probably because the expression of NAP is regulated in *Chlamydomonas* cells depending on the amount of conventional actin, and only a small amount of NAP is present in the wild type cell. The regulation of expression of conventional actin and NAP, as well as their specific functions in the cell and axoneme, remain an important subject of future studies.

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   1 AGTGACTCGTGTTTTCGGCGTTCGACGTGCAAGCATCGCAAGTAAACGCGGAGACGCTTT   60

  61 CCCCGTCGCTGGCTCGTAATAACAGCGCAGACCAAAATGACTTCCGGCCTTCCAGACACT  120
   1                                        M  T  S  G  L  P  D  T    8

 121 GATACTGCCATTGTTTGCGATAATGGCTCCGGTGTGGTGAAGGCGGGCTTCTGCGGCGAG  180
   9  D  T  A  I  V  C  D  N  G  S  G  V  V  K  A  G  F  C  G  E   28

 181 GACGCGCCCCGAGTCATGTTTGCCAGCGTGACGGGTCGGCCGCGCCACAGCATGGCCATG  240
  29  D  A  P  R  V  M  F  A  S  V  T  G  R  P  R  H  S  M  A  M   48

 241 GTGGGCATGGCCGCCAAGCAGCTGTACGTGGGCGAGGAGGCGCAGGCCAAGCGCGGCGTG  300
  49  V  G  M  A  A  K  Q  L  Y  V  G  E  E  A  Q  A  K  R  G  V   68

 301 CTGAGCCTGTCGCACCCCATCGAGCACGGCGTGGTGACCAACTGGGACGACATGGAGGCC  360
  69  L  S  L  S  H  P  I  E  H  G  V  V  T  N  W  D  D  M  E  A   88

 361 ATCTGGCGCCACACCTTTGAGGACCAGCTGCGGGTGGACACCTCCGAGCGGCCCGTCATG  420
  89  I  W  R  H  T  F  E  D  Q  L  R  V  D  T  S  E  R  P  V  M  108

 421 CTCACGGAGGCGCCGCGAAACCCCAAGCAGAACAGGGAGCGCGCCACGGAGATCATGATG  480
 109  L  T  E  A  P  R  N  P  K  Q  N  R  E  R  A  T  E  I  M  M  128

 481 GAGACGTTCCGTGTGCCCGCCATGTACGTGGCCATCCAGGCCGTGCTGTCCCTGTACGCC  540
 129  E  T  F  R  V  P  A  M  Y  V  A  I  Q  A  V  L  S  L  Y  A  148

 541 TCCGGCCGCACCACCGGAGTGGTGCTGGACATCGGCGACGGAGTCAGCCACGCCGTGCCC  600
 149  S  G  R  T  T  G  V  V  L  D  I  G  D  G  V  S  H  A  V  P  168

 601 GTGTACGAGGGTTTCTCCATGCCTCACGCCGTGAAGCGGCTGGACGTGGCGGGTCGCGAC  660
 169  V  Y  E  G  F  S  M  P  H  A  V  K  R  L  D  V  A  G  R  D  188

 661 ATGACGCAGTACCTGTCGCGCCTGCTCACCGAGGCCGGCACACGGCTGACCAACAGTGCC  720
 189  M  T  Q  Y  L  S  R  L  L  T  E  A  G  T  R  L  T  N  S  A  208

 721 GAGATGGAGATCGTGCGGGACATCAAGGAGCGGCTGTCGTACGTGGCTCTGGACTACGAC  780
 209  E  M  E  I  V  R  D  I  K  E  R  L  S  Y  V  A  L  D  Y  D  228

 781 ACGGAGCTGGCCACGGCGCGCAGCAGCAGTGTGGTCAGCAAGGACTACACGCTGCCGGAT  840
 229  T  E  L  A  T  A  R  S  S  S  V  V  S  K  D  Y  T  L  P  D  248

 841 GGGCAGAGCATCGCGGTGGGGGAGGAGCGGTTCAGGTGCGCGGAGCTGCTGTTCGACCCC  900
 249  G  Q  S  I  A  V  G  E  E  R  F  R  C  A  E  L  L  F  D  P  268

 901 TCGCCGCTGGGTCACGAGAAGGGCGAAGGCATCCACACCATGCTGCACGACGCCGTGTCC  960
 269  S  P  L  G  H  E  K  G  E  G  I  H  T  M  L  H  D  A  V  S  288

 961 GCATGCGACATCGACGTGCGCAAGGAGCTGCTGTACAACGTGGTGTTGTCCGGCGGCACC 1020
 289  A  C  D  I  D  V  R  K  E  L  L  Y  N  V  V  L  S  G  G  T  308

1021 ACCATGACGCAGGGCATCGCGGCGCGCCTGCACAAGGAGCTGACGGCCCTGGCGCCCTCC 1080
 309  T  M  T  Q  G  I  A  A  R  L  H  K  E  L  T  A  L  A  P  S  328

1081 GCCTGCACCGTGCGCGTGGTGGCGCCGCCGGAGCGCAAGTTCCTGGTGTGGATCGGCGGC 1140
 329  A  C  T  V  R  V  V  A  P  P  E  R  K  F  L  V  W  I  G  G  348

1141 AGCGTGCTGGCCTCGCTGGCCAGCTTCGCCAGCCAGTGGGTGACGGCGGAGGAGTACAAC 1200
 349  S  V  L  A  S  L  A  S  F  A  S  Q  W  V  T  A  E  E  Y  N  368

1201 GAGTACGGGCCGGGCATTGTGCACCGCAAGTGCTTCTGAGGAGGCGGTGGTGCAGGGGAG 1260
 369  E  Y  G  P  G  I  V  H  R  K  C  F  *                       380

1261 TGGAGGGGTGCCGGGGAGTGGCATTTTAATGGGGCTGTGAGGTGGGTTGGAGAGCGTGTG 1320
1321 TAGGGAGGAAGGGGCAGGGTAGGGGCGTGGGTTGATAGGGAGGGAGGCTGGGCGGGCGTG 1380
1381 GTGAAGGATTCTCGACCCATGGAGCCTGCGGGAAAGCGGATTCAGCGGGCTCGGCGGGCA 1440
1441 GCGGGACAGACGTGCGGAGTGGATCGCAGATGCTTTAAGGATCTCGGTGTTTGATTGTTG 1500
1501 GAGAGCGCGTCCGATGGGTACGATCTTGTGACGACCACAAGGGGTTGTCACGCCTGCACT 1560
1561 GTGTTGGACGGAAGGATAAGGATTGGAAGGTGCACGGCGGTACGGTGTTGTTTAGAAGAA 1620
1621 CTAGTGTCCAGCCATGGTAGGGATGTGTGGGATTTCCTGTGGCTGCGGCTGGTGTGATGA 1680
1681 GCCATCCGCTGTCATTTGGCGGCATGACGGTCACAATGAATGAGCAACTGCAATGATTAA 1740
1741 ATGTCTTCAAAGCCCAATAGCCAACGCCTGACATTCAAGGTCCATAACCTCACTGTAA   1798
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Figure 1. Nucleotide sequence of NAP cDNA with the deduced amino acid sequence. (Box) Residues identified by peptide sequencing. (Underline) The portion of the cDNA sequence determined in this study. The complete sequence was retrieved from EMBL/Genbank/DDBJ under accession number U68060 (Lee et al., 1997). (Broken arrows) The positions and directions of primers used in RT-PCR and 5' RACE (see Materials and Methods).

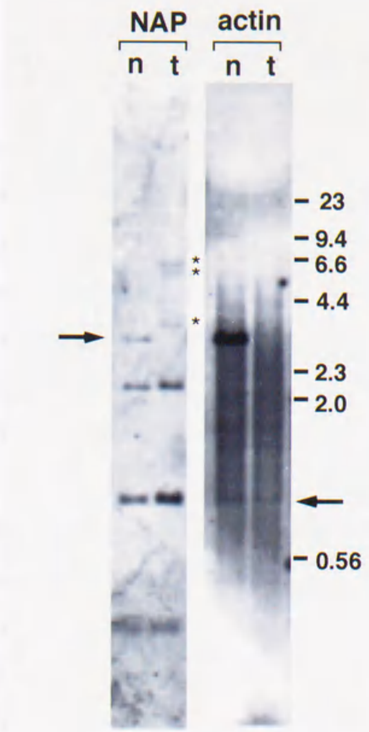

Figure 2. Sourthern hybridization analysis. Genomic DNAs from *nit1/cw15* (n) and *ida5-t* (t) were digested with PstI, and hybridized with actin or NAP probes. DNA isolation and hybridization procedure are the same as described in Kato-Minoura et al. (1997), except that hybridization and wash were performed at 65 °C. The actin probe is a 0.4 kb of SacII fragment of genomic DNA, which corresponds to the sequence from nucleotide 1760 to 2186 (in the genomic DNA sequence registered in EMBL/DDBJ/GenBank under accession number D50838). The NAP probe is a 0.3 kb of the product of PCR using primers of 5'-GAGCGCCCSGTSATGCTSAC-3', based on the peptide sequence ERPVML[T] , and 5'-AGCTCSGTSTCGTARTCSAG-3', based on ELLYNV[V]. These two probes are overapping but not identical in their corresponding amino acid sequences. The faint bands seen in *ida5-t* (*) are of unknown origins, but probably are those of the actin gene of which a large part appears to have been eliminated in this mutant (Kato-Minoura et al., 1997). (Bars with numbers) Positions of the λHindIII markers shown in bp x 10^{-3}.

ACKNOWLEDGMENTS

I would like to cordially thank Professor Ritsu Kamiya (University of Tokyo and National Institute for Basic abiology) for his kind guidance and encouragement throughout this study. I also thank Professor Hirokazu Hotani (Nagoya University), Emeritus Professor Sho Asakura (Nagoya University), and the late Professor Yasuo Imae (Nagoya University) for their support. I owe a great deal to Professor Ryoko Kuriyama (University of Minnesota) for introducing me to experiments of molecular cloning and antibody handling. Dr. Masafumi Hirono (University of Tokyo) gave me invaluable advice on the functional studies on actin. I am also grateful to Ms. Yasuko Sugase (University of Tokyo) for her technical advice about molecular work, Mr. Akinori Yoda (University of Tokyo) for his help in mutant isolation, Dr. Katsushi Owaribe (Nagoya University) for the Physarum actin antibody, Dr. John Leszyk (Worcester Foundation), for assistance with the protein sequencing, and Ms. Satomi Uryu for carrying out Sourthern blotting experiments of NAP. I also wish to thank Drs. Saeko Takada (The Institute of Physical and Chemical Research [RIKEN]), Osamu Kagami (National Institute for Basic Biology), Toshiki Yagi (University of Tokyo), and Mr. Itsushi Minoura (University of Tokyo) for helpful discussion, and Dr. Charlotte Omoto (Washington State University) for a critically reading of the manuscript. I should state that a part of this study was supported by the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. I thank all the members of the Third Division of Department of Molecular Biology (Nagoya University), Laboratory of Molecular Physiology (University of Tokyo), and National Institute for Basic Biology for making the research environment comfortable. Last but not least, I thank my son and my parents for their understanding and mental support.

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副論文

1. Isolation of two species of *Chlamydomonas reinhardtii* flagellar mutants, *ida5* and *ida6*, that lack a newly identified heavy chain of the inner dynein arm.

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   Cell Structure and Function, 18, 371-377 (1993)

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2. *Chlamydomonas* inner-arm dynein mutant, *ida5*, has a mutation in an actin- encoding gene.

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   The Journal of Cell Biology, 137, 649-656 (1997)

   （クラミドモナスダイニン内腕欠損株 *ida5* はアクチン遺伝子に変異を持つ）