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Highly Divergent Actin Expressed in a *Chlamydomonas* Mutant Lacking the Conventional Actin Gene

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The *Chlamydomonas* mutant *ida5* is deficient in the conventional actin gene and its axoneme lacks a subset of inner dynein arms that contain actin as a subunit. However, this mutant retains some other inner dynein arms because a novel protein (NAP) is expressed as a substitute for actin. In this study, we show by sequence analysis that NAP is identical to a putative actin-related protein, the cDNA sequence of which has recently been reported and shown to have 64% amino acid identity with conventional actin. A polyclonal antibody raised against a synthetic polypeptide corresponding to the NH_2-terminal sequence of this protein specifically reacted with the spot corresponding to NAP in two-dimensional electrophoresis patterns. NAP apparently can substitute for conventional actin in some, but not all, cellular functions, and therefore can be regarded as a highly divergent actin. This unconventional actin appears to be expressed only when conventional actin is absent.

The biflagellate green alga *Chlamydomonas* and the related alga *Volvox* [1] contain only a single gene of conventional actin, which has ~89 % sequence identity to mammalian skeletal muscle actin [2]. We have recently shown that the *Chlamydomonas ida5* mutant lacking some species of inner-arm dynein has a nonsense mutation in the conventional actin gene. Analysis of this and another allele of *ida5* has indicated that the gene produces no functional protein [3]. Since actin is a subunit of some inner arm dynein species [4], the loss of dyneins in *ida5* should be a direct consequence of the absence of actin. It is, however, puzzling that this mutant retains two inner dynein arm subspecies that also contain actin. More strikingly, this mutant undergoes normal cytokinesis, a process in which actin is thought to play an essential role [5]. However, two-dimensional electrophoresis of the *ida5* axoneme has shown that this mutant contains a novel protein (NAP) that shares some common antigenicity with actin. In fact, NAP was found to have replaced actin in some inner arm subspecies in *ida5*. Hence NAP has been speculated to act as a substitute for actin and perform essential cellular functions in this mutant. In this study, in order to clarify the molecular nature of NAP, we determined a partial amino-acid sequence of this protein and obtained a partial cDNA by PCR. The sequence of the cDNA revealed that NAP is identical with a putative actin-related protein, the sequence of which has been recently reported and shown to have 64% sequence identity with conventional actin [6]. Although no information has previously been obtained regarding the function of this protein, our present study suggests that it can be regarded as a highly divergent actin that is expressed as a substitute for the conventional actin when it is absent. Presence of such a divergent actin in *Chlamydomonas* is unexpected, since actins with as low as 64% sequence identity to conventional actin have only been found in some ciliates [7, 8]. *Chlamydomonas* provides a striking example of coexistence of conventional and unconventional actins within the same cell.

MATERIALS AND METHODS

Strains and cell culture. *Chlamydomonas reinhardtii* actin-less mutants, *ida5* [9] and *ida5-t* [3], were used. The latter is an allelic strain lacking almost the entire region of the actin gene. Cells were grown in Tris-acetic acid-phosphate medium (TAP) [10].

Peptide sequencing. The axonemes of *ida5* were prepared using standard methods [11]. Dynein was extracted with 0.6 M KCl from the axoneme and purified by centrifugation on a 5-20% sucrose density gradient [12]. Fractions that sediment at about 11 S and containing inner arms were collected and precipitated by addition of methanol [13]. The inner dynein fraction was separated by SDS-PAGE [14] and blotted onto a polyvinylidene difluoride membrane

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Lee et al.:     MTSGLPDTDTAIVCDNGSGVVKAGFCGEDAPRVMFASVTGRPRHSMAMVGMAAKQLYVGE  60
Present study: MTSGLPDTDTAIVCDNGSGVVKAGFCGEDAPRVMFASVTGRPRHSMAMVGMAAKQLYVGE

               EAQAKRGVLSLSHPIEHGVVTNWDDMEAIWRHTFEDQLRVDTSERPVMLTEAPRNPKQNR 120
               EAQAKRGVLSLSHPIEHGVVTNWDDMEAIWRHTFEDQLRVDTSERPVMLTEAPRNPKQNR

               ERATEIMMETFRVPAMYVAIQAVLSLYASGRTTGVVLDIGDGVSHAVPVYEGFSMPHAVK 180
               ERATEIMMETFRVPAMYVAIQAVLSLYASGRTTGVVLDIGDGVSHAVPVYEGFSMPHAVK

               RLDVAGRDMTQYLSRLLTEAGTRLTNSAEMEIVRDIKERLSYVALDYDTELATARSSSVV 240
               RLDVAGRDMTQYLSRLLTEAGTRLTNSAEMEIVRDIKERLSYVALDYDTELATARSSSVV

               SKDYTLPDGQSIAVGEERFRCAELLFDPSPLGHEKGEGIHTMLHDAVSACDIDVRKELLY 300
               SKDYTLPDGQSIAVGEERFRCAELLFDPSPLGHEKGEGIHTMLHDAVSACDIDVRKELLY

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               NVVLSGGTTMTQGIAAR

               WVTAEEYNEYGPGIVHRKCF                                          380
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FIG. 1. Amino acid sequence of NAP deduced from cDNA sequence. Upper row, the deduced amino acid sequence of a putative actin-related protein [6] retrieved from EMBL/Genbank/DDBJ under Accession No. U68060. Lower row, the deduced amino acid sequence of NAP deduced from the cDNA sequence determined in this study. Underlined sequences were directly determined from tryptic fragments of NAP.

(Immobilon P^{sq}; Millipore Corp., Woburn, MA)[15]. The 43 kDa 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. A partial cDNA was obtained by RT-PCR using two oligonucleotides designed based on the peptide sequence HPIEHG[V] and ELLYNV[V] as degenerate primers. To avoid misamplification of conventional actin, mRNA from *ida5-t,* which lacks the entire actin gene, was used as the template. mRNA was prepared while cells were re-growing flagella after deflagellation, 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 [16] (Life Technologies, Inc., Gaithersburg, MD). Products were subcloned into pBluescript vector (Stratagene, La Jolla, CA) and sequenced for both strands using an Applied Biosystems ABI PRISM 310 genetic analyzer at the NIBB Center for Analytical Instruments (Okazaki, Japan).

Antibody production and Western blotting. A 12-amino-acid synthetic peptide corresponding to the deduced NH_2-terminal sequence of NAP was conjugated with a carrier protein (keyhole limpet hemocyanin) [17] and used to immunize a rabbit. The collected serum was affinity-purified by binding to a protein A column[18]. The anti-actin antibody used was a polyclonal antiserum specific for the NH_2-terminal, 12-amino-acid sequence of *Chlamydomonas* actin [2]. Western blotting analysis was performed as described previously [3].

Northern blotting. For detection of the actin and NAP messages, the 3′-untranslated regions (3′-UTR) of respective genes were used as probes. For the actin message, the probe used was a 539 bp fragment starting at 36 bp downstream from the stop codon, amplified by PCR from the cloned genomic actin gene pA7 [2]. For the NAP message, the probe was a 524 bp fragment starting at 2 bp upstream of the stop codon, amplified by RT-PCR from the total RNA of *ida5.* Both fragments were subcloned into pT7Blue T-vector (Novagen, Madison, WI) and their sequences were confirmed by sequencing. The inserts were purified and radio-labeled to similar specific activities (6.4 and 6.0×10^8 cpm/μg DNA for actin and NAP, respectively) using a BcaBest labeling kit (Takara, Otsu, Japan). Total RNA from vegetative cells, grown for 3 days under a 12h/12h light/dark condition, was loaded on a 1.2% agarose/formaldehyde gel, and transferred to a Hybond-N+ membrane (Amersham Intl., Amersham, UK). The condition for hybridization was as described previously [3] except that the probes were ^{32}P-labeled.

Immunofluorescence microscopy. Vegetative cells were fixed with cold methanol and processed following the method of Sanders and Salisbury [19]. The antibodies described above and an FITC-conjugated anti-rabbit IgG antibody (ZYMED laboratories, South San Francisco, CA) were used as the first and second antibodies, respectively. The sample was observed with a fluorescence microscope equipped with a cooled CCD video camera (Hamamatsu Photonics, Hamamatsu, Japan).

RESULTS

For sequence analysis of NAP, we first prepared a sample enriched for inner-arm dyneins from *ida5* axonemes, because the inner arm subspecies (designated *b* and *g*) in this mutant have been shown to contain NAP as subunits. A band corresponding to NAP was excised from SDS-PAGE gels, digested with trypsin, and analyzed for amino-acid sequence. Five peptide sequences of 9 to 21 residues were obtained (Fig. 1, underlined). PCR was performed with primers that were designed on the basis of these sequences, and yielded a partial cDNA. When the partial amino-acid sequence of NAP was determined from the cDNA sequence, it was found to be perfectly identical to the recently reported sequence of a putative actin-related protein [6] (Fig. 1). In agreement with our prediction [3], this protein shares low (64%) amino-acid identity with *Chlamydomonas* conventional actin [6].

To determine whether the protein thus identified actually corresponds to either one or both of the two NAP spots with isoelectric point (pI) values of 5.6 and

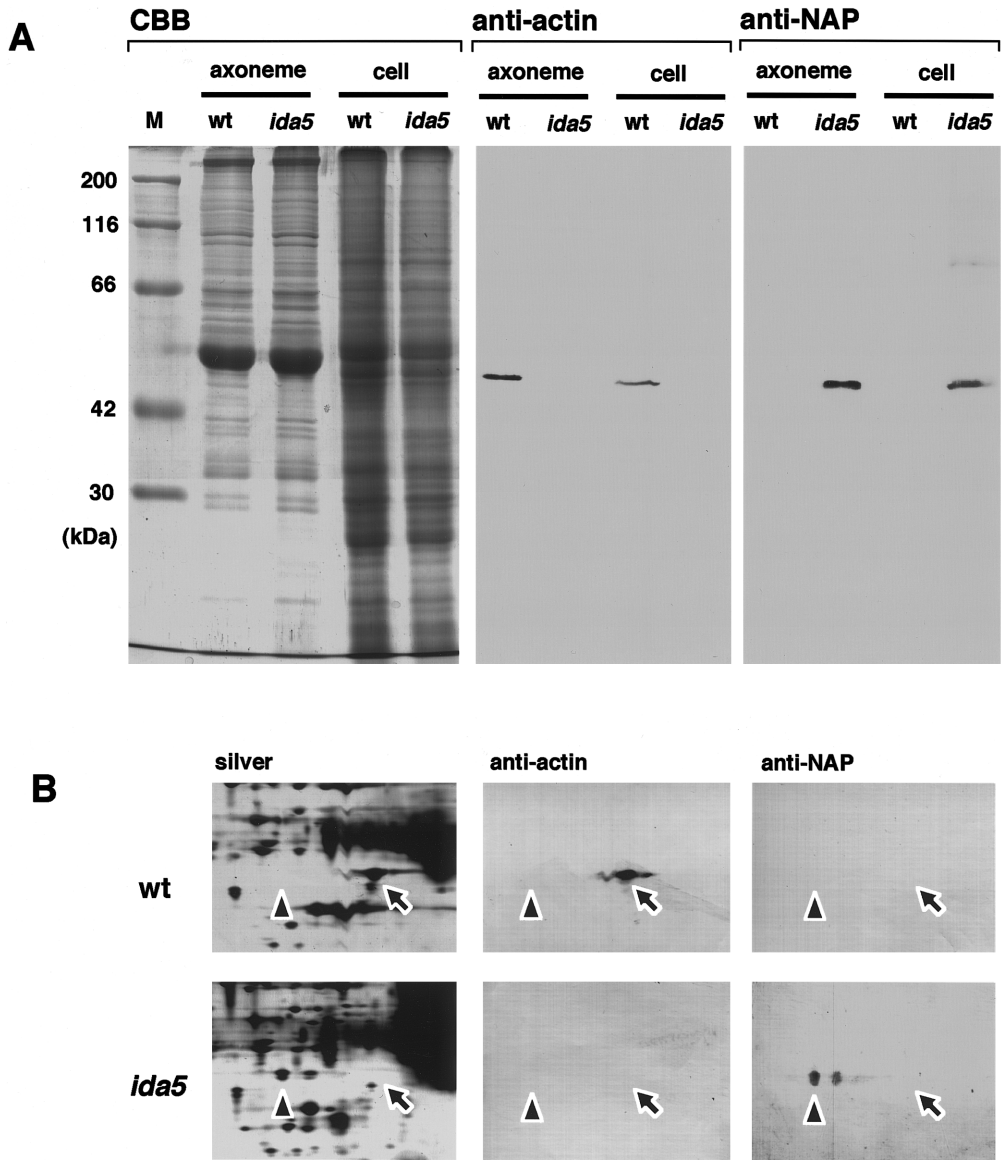

FIG. 2. Western blot analysis of actin and NAP in the axoneme and cytoplasm. (A) One-dimensional SDS–PAGE of axonemes and whole cell lysates of wild type (wt) and *ida5*, and (B) part of the two-dimensional electrophoresis patterns of axonemes. Total cell protein of wild type and *ida5* was obtained by adding methanol and chloroform to the cell suspension, retaining the precipitate at the chloroform/water interface, and dissolving it in 1% SDS [13]. Samples were separated by SDS–PAGE, blotted to membranes, and probed with the anti-conventional actin antibody (anti-actin) or the anti-NAP antibody (anti-NAP). CBB, stained with Coomassie brilliant blue. Silver, stained with silver. The oblique arrows and arrowheads in (B) indicate the positions of actin and NAP, respectively.

5.7 appearing in the two-dimensional gel electrophoresis patterns of *ida5* axonemes [3], we carried out Western blot analyses using a specific antibody. For this purpose, we raised a polyclonal antibody against the NH_2-terminal 12 amino-acid sequence of NAP by injecting rabbits with a corresponding synthetic peptide. As shown in Fig. 2, this antibody detected a single band in both the cytoplasm and axoneme of *ida5*. In most cases, the antibody did not react with any band in the axonemes or cytoplasm of wild type (Fig. 2A), although on rare occasions a very faint band was detected in the axoneme. Thus NAP appears to be present in wild-type axonemes in a very small concentration, if present at all. In contrast, the actin-specific antibody reacted with one band in both the cytoplasm and axoneme of wild type, but with no bands in the axonemes or cytoplasm of *ida5* (Fig. 2 and [3]). This is consistent with the fact that *ida5* lacks conventional actin. Immuno-blots of the two-dimensional electrophoresis patterns of axonemes clearly showed that the two antibodies are specific to either conventional actin or NAP, and that actin is only detectable in wild type whereas NAP is only detectable

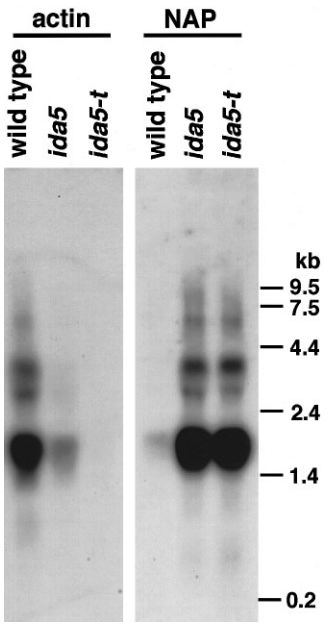

FIG. 3. Northern blot analysis of actin and NAP. The total RNA from wild type, *ida5*, and *ida5-t* (17 μg each) was loaded on each lane and probed with the 3′-UTR fragments of actin and NAP genes. After washing, membranes were exposed for 3 days for the actin probe or for 1 day for the NAP probe.

in *ida5* (Fig. 2B). Furthermore, the NAP antibody reacted with both of the two NAP spots, indicating that both spots are those of NAP (Fig. 2B).

The Western analysis described above indicates that the expression of NAP is suppressed in wild type cells. To see whether the expression suppression takes place at the level of transcription or translation, we next carried out Northern blot analysis using probes specific for the message of conventional actin and that of NAP. As shown in Fig 3, the actin message was detected in wild-type cells and the mutant *ida5* but not in *ida5-t*. This result is reasonable since *ida5* has an actin gene with a single-base deletion, while *ida5-t* is lacking the actin gene almost entirely. The message of NAP, on the other hand, was found to be weak in wild-type cells but greatly enhanced in both *ida5* and *ida5-t*.(Fig. 3). A simple comparison of the band densities suggests that the amount of the NAP message in *ida5* or *ida5-t* is greater than the amount of the actin message in the wild type, although precise quantification remains to be carried out. At any rate, the Northern analysis thus clearly indicates that the expression regulation of NAP takes place at the level of transcription.

Immuno-fluorescence microscopy revealed that the localization of NAP in vegetative *ida5* cells is similar to that of actin in the wild type (Fig. 4). Namely, actin is localized in the mid-portion of the cell in the interphase, whereas it is localized predominantly at the dividing plane in dividing cells as reported previously [5]; likewise, NAP is localized in the mid portion in interphase cells of *ida5*, whereas it is localized at the cleavage furrow in dividing cells (Fig. 4D). As expected from the results of Western blot, fluorescent staining was too weak to show any specific localization when wild type cells were stained with anti-NAP antibody, or when the *ida5* cells were stained with anti-actin antibody (data not shown).

DISCUSSION

We have shown that the novel actin-like protein, NAP, expressed in the *Chlamydomonas* mutant *ida5* lacking the conventional actin gene is identical with an actin-related protein the DNA sequence of which has recently been identified. This protein shares 64% amino-acid identity with actin [6], but its relationship with true actin has been unknown. As discussed below, the present study has indicated that it can function as a substitute of conventional actin and may therefore be regarded as a highly divergent actin. Southern blot analyses of genomic DNA using probes specific for the coding regions of either the conventional actin gene or NAP gene showed some cross-hybridization between the two genes under low-stringency conditions [6, and our unpublished results]. The fact that no other bands were detected in the Southern analyses, as well as the observation that 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 [3], makes it unlikely that *Chlamydomonas* has a third protein that is more closely related to conventional actin. Hence *Chlamydomonas* must have just two kinds of actin: a single conventional actin and NAP, a protein that may well be regarded as an unconventional actin. Since Southern blot analyses have indicated that each of the two proteins is encoded by a single-copy gene [2, 6], *Chlamydomonas* can be regarded as having two genes for actin altogether. The two spots of NAP appearing in two-dimensional gel electrophoresis patterns of the *ida5* axoneme [3] must have originated from the same NAP gene. The finding that both of these spots can react with an antibody raised against the NH_2-terminal sequence of NAP supports this idea. The difference in pI between these spots may be due to some post-translational modification.

Conventional actin and NAP appear to partially overlap in function. For example, both can be the subunit of the inner arm dynein subspecies *b* and *g*, although only conventional actin can be the subunit of other inner arm dynein subspecies [3]. Conventional actin seems to be essential for the growth of the fertilization tubule [3], an actin-containing structure produced in gametes of the plus mating type [20]. On the

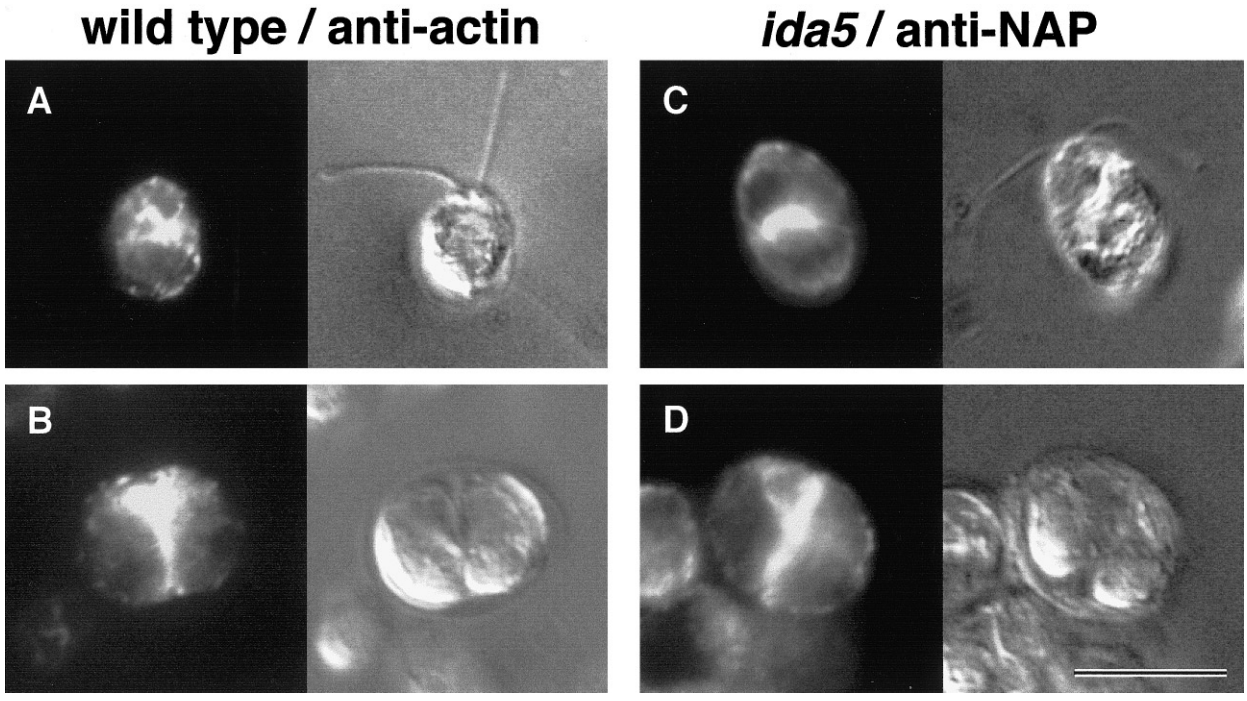

FIG. 4. Immunolocalization of actin and NAP in vegetative cells of wild type (A, B) and *ida5* (C, D). The upper photos (A, C) show interphase cells; the lower photos (B, D) dividing cells. The left photo in each panel is an immunofluorescence micrograph and the right one a phase-contrast micrograph of the same cells. Bar, 10 μm.

other hand, both conventional actin and NAP may be able to function in cytokinesis, a process in which actin has been assumed to play a key role [5], since the cytokinesis of *ida5* has been found to proceed quite normally despite the absence of conventional actin [3, and Hirono and Kato-Minoura, unpublished observation]. In fact, NAP is localized to the cleavage furrow during the cytokinesis of the *ida5* mutant, as is actin in the wild-type cell [5](Fig. 4). However, no evidence has been obtained as to whether actin or NAP takes a filamentous form in the cytoplasm [5]. For this reason, we still cannot rule out the possibility that neither actin nor NAP is necessary for cytokinesis; to clarify this matter, we may need to isolate a mutant deficient in both actin and NAP.

Finally, the question of how the expression of these two actins are regulated is certainly important. Our Western and Northern analyses indicate that the expression of NAP is suppressed in wild type cells in which conventional actin is expressed (Fig. 2), and that the suppression occurs at the level of transcription (Fig. 3). Also, quantification of actin in *ida5* cells transformed with wild-type actin genes indicates that the expression of NAP decreases with the amount of conventional actin expressed (Ohara et al., manuscript in preparation). The mechanism of expression regulation of conventional actin and NAP, as well as the specific functions of these proteins in the cytoplasm and axonemes, remain important subjects for future research.

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