ADF/Cofilin Is Not Essential but Is Critically Important for Actin Activities during Phagocytosis in *Tetrahymena thermophila*

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ADF/cofilin is a highly conserved actin-modulating protein. Reorganization of the actin cytoskeleton *in vivo* through severing and depolymerizing of F-actin by this protein is essential for various cellular events, such as endocytosis, phagocytosis, cytokinesis, and cell migration. We show that in the ciliate *Tetrahymena thermophila*, the ADF/cofilin homologue Adf73p associates with actin on nascent food vacuoles. Overexpression of Adf73p disrupted the proper localization of actin and inhibited the formation of food vacuoles. *In vitro*, recombinant Adf73p promoted the depolymerization of filaments made of *T. thermophila* actin (Act1p). Knockout cells lacking the *ADF73* gene are viable but grow extremely slowly and have a severely decreased rate of food vacuole formation. Knockout cells have abnormal aggregates of actin in the cytoplasm. Surprisingly, unlike the case in animals and yeasts, in *Tetrahymena*, ADF/cofilin is not required for cytokinesis. Thus, the *Tetrahymena* model shows promise for future studies of the role of ADF/cofilin *in vivo*.

Dynamic reorganization of the actin cytoskeleton is associated with membrane deformations such as phagocytosis, endocytosis, ameboid movement, and cytokinesis in eukaryotes. ADF (actin-depolymerizing factor)/cofilin (AC) is the principal actin-modulating protein involved in these events, and its biochemical activities have been well studied in animals, yeasts, and higher plants (1). By binding to F-actin, canonical AC induces a twist in the arrangement of subunits (2), which produces distortion at the boundary between twisted and nontwisted polymer regions (3, 4). Consequently, F-actin is severed, and dissociation of monomeric actin (G-actin) from the pointed end of filaments is accelerated (5). On the other hand, at higher concentrations, AC increases the rate of actin nucleation and induces abnormal actin structures, called bars or rods, probably because AC binding bridges longitudinal contacts between actin subunits (3, 6, 7). Thus, it has been considered that AC exerts distinct effects on F-actin, depending on the AC-actin stoichiometry. In addition, AC binds preferentially to ADP-G-actin and inhibits nucleotide exchange to form assembly-competent ATP-actin (8). This activity may be involved in maintaining the G-actin pool, cooperatively with other G-actin-binding proteins, such as thymosin β4 and profilin.

Protists also show dynamic reorganization of the actin skeleton accompanying unique cell behaviors, but neither their fundamental molecular mechanisms nor regulatory systems have been uncovered. One exception is the malaria parasite, a lineage of the Apicomplexa, which expresses an AC-like protein, ADF1, with unusual biochemical properties (9). ADF1 does not bind or sever F-actin but strongly stimulates nucleotide exchange on G-actin to promote rapid actin polymerization (10). These unique properties of ADF1 could coevolve with a malaria-specific actin molecule that is extremely unstable as a polymer compared with conventional actins of other eukaryotes (11). Their functional relationship probably enables the malaria parasite to rapidly turn over F-actin in gliding motility during host invasion (9).

Ciliates form the phylogenetic group of alveolates, together with apicomplexans and dinoflagellates, and are highly diverged from the animal and yeast lineage of opisthokonts (12). *Tetrahymena thermophila* is a well-studied model ciliate with a sequenced genome (13). This unicellular organism vigorously internalizes extracellular solutions and particles as nutrients by forming food vacuoles (FVs) at the funnel-shaped oral apparatus, located in the anterior region of the cell cortex (14). Electron microscopic observations revealed that FVs are formed along the deep fiber, a fibrous microtubule-rich structure that protrudes from the bottom of the oral apparatus (14). After absorption of nutrients, undigested materials are egested at an organelle called the cytoproct, located in the posterior region of the cell (14). The *Tetrahymena* actin protein (Act1p) (15) and actin-bundling proteins, including fimbrin (16) and eEF1A (15), all localize near the deep fiber and nascent FVs. Moreover, strong accumulation of actin is seen around the cytoproct (17). Consistently, actin-binding drugs block the formation of FVs in the oral apparatus (18), as well as membrane retrieval after FV ejection in the cytoproct (17). Thus, the actin cytoskeleton may play an important role in the deformation of membranes associated with the formation and ejection of FVs. Meanwhile, profilin, which promotes nucleotide exchange on G-actin and hence is generally involved in accelerating actin turnover, is not visibly associated with FV formation (19). Overall, the exact function of actin dynamics accompanying the FV cycle remains poorly understood. In addition, the *Tetrahymena* actin is

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highly divergent from conventional actin in its amino acid sequence and shows unique biochemical characteristics (20).

After completion of the macronuclear genome project on *T. thermophila* (13), it was revealed that this organism has only one gene encoding an AC homolog, namely, ADF73. Previously, we purified the gene product, Adf73p (21). Biochemical studies using rabbit muscle actin showed that Adf73p has F-actin-severing activity, which was confirmed by the consequences of overexpression and deletion. We showed that Adf73p is required for phagocytosis, likely via its localization and the role in the reorganization of the actin cytoskeleton during FV formation by severing and depolymerizing F-actin in *Tetrahymena thermophila*. Surprisingly, unlike animal cells, *Tetrahymena* cells do not require AC for cytokinesis.

**MATERIALS AND METHODS**

**Cell culture.** Wild-type *Tetrahymena thermophila* was cultured in NEFF (0.25% protease peptone, 0.25% yeast extract, 0.55% t-(+)-glucose, 33 μM FeCl3) or SPP (1% protease peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA-ferric sodium salt) (22) medium at 30°C. To study the effects of ADF73 deletion, wild-type and adf73 knockout (adf73KO) strains were cultured in MEPP (2% protease peptone, 0.06% sodium citrate - 2·H2O, 0.027% FeCl3·6H2O, 0.0003% CuSO4·5H2O, and 0.0001% folic acid) (23) medium, which promotes the growth of cells lacking the ability to form FVs. The nuclear division index was defined as the percentage of cells undergoing micro- and macronuclear division relative to the total number of cells. Nuclear DNA was observed microscopically by staining cells with 1 μM 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) after fixation with 1% formaldehyde (Wako).

**Antiserum.** To produce anti-Adf73p antibodies, recombinant GST-Adf73p was purified, and Adf73p was separated from glutathione S-transferase (GST) by use of thrombin (21). Adf73p was dialyzed in phosphate-buffered saline (PBS) overnight at 4°C, mixed with adjuvant, and emulsified by sonication (UD-201; Tomy). For the first injection and Freund’s incomplete adjuvant (Wako Pure Chemical Industries, Ltd.) for the first injection and Freund’s complete adjuvant (Wako Pure Chemical Industries, Ltd.) for the following injections. The antigen was hypodermically injected into the back of a rabbit. One month after the first injection, additional injections were performed once every 2 weeks. After the third additional injection, antiserum was collected, diluted 10 times with PBS, and incubated with a polyclonal antibody against GST (Roche Diagnostics). Mouse antichicken α-tubulin antibody was purchased from Roche Diagnostics.

**Immunoblotting.** *T. thermophila* cells cultured in NEFF medium at 30°C were collected by centrifugation for 3 min at 750 × g and washed twice with NKC solution (34 mM NaCl, 1 mM KCl, 1 mM CaCl2). The cells were suspended in NKC containing 1 mM ATP, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK) and then smashed with a sonicator (UD-201; Tomy). After adding a one-third volume of the loading buffer (0.25 mM Tris [pH 6.8], 4% SDS, 40% glycerol, and 8% β-mercaptoethanol) followed by incubation at 95°C for 5 min, the cell lysate was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with Tris-buffered saline (TBS; 20 mM Tris-HCl, 0.9% NaCl, pH 7.5) containing 0.5% Tween 20 (TTBS) with 1% skim milk and incubated with 0.1% affinity-purified anti-Adf73p antiserum for 2 h. After washing with TTBS with 1% skim milk, the membrane was incubated with 1% goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Biosource International) for 1 h. Immunoblots were developed using a BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) phosphate substrate kit (Kirkgaard & Perry Laboratories, Inc.) and/or 1% fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Immune Research Laboratories, Inc.) in PBS containing 1% skim milk for more than 6 h at room temperature. After washing with PBS 3 times, cells were observed by use of an LSM510 confocal laser scanning microscope (Carl Zeiss, Inc.). Immunofluorescence staining with an-α-tubulin was performed by the method of Wogla et al. (24).

**Gene overexpression.** A plasmid vector for overexpression of ADF73, pMTT1-GFP-ADF73, was obtained by inserting ADF73 cDNA into the BamHI-HindIII sites of pMTT1-GFP (25). CU522 cells were transformed with pMTT1-GFP-ADF73 as described previously (26). Overexpression of ADF73 was induced by the addition of 2.5 μg/ml CdCl2 to the medium for 3 h.

**Gene knockout.** To obtain a targeting plasmid for knockout of ADF73, 1.5-kb DNA fragments of 5′- and 3′-untranslated regions of the ADF73 gene were amplified by PCR and cloned into the Apal-Smal sites and Pstl-Sacl sites of pNeo4 (27), respectively. Log-phase growing B2086 and CU428 cells in SPP were washed, starved in 10 mM Tris-HCl (pH 7.4) for 16 to 24 h at 30°C, and mixed for conjugation. pNeo4-ADF73 was introduced into micronuclei of the conjugating cells by biolistic transformation (28). Germ line transformants were selected by the addition of 10 μg/ml paromomycin, 15 μg/ml 6-methylpurine, and 1 μg/ml CdCl2. Disrupted loci in the micronuclei were eliminated by phenotypic assortment in the absence of paromomycin (29). Heterozygous heterokaryons were mated with a “star” strain (A*III) to make homozygous heterokaryon strains with double-disrupted loci in the micronucleus and wild-type genes in the macronucleus by uniparental pronuclear transfer. Two heterokaryons were mated to obtain homozygous homokaryon strains (adf73KO).

**Isolation and fractionation of cell bodies and cilia.** Cell bodies and cilia were isolated by the method of Ueno et al. (30).

**Cosedimentation assay.** Purification of recombinant Adf73p and the cosedimentation assay were performed as described by Shiozaki et al. (21).

**Swimming behavior of cells.** To investigate swimming behavior, 20 μl cell culture was placed in a basic chamber assembled from a slide glass with two slits of thin vinyl tape arranged in parallel as a spacer and covered with a cover glass. In this chamber, cells could swim around freely. Swimming behavior was recorded at 15 Hz for 30 s by using a charge-coupled device (CCD) camera (DFK41AU02; Argo) under an inverted microscope (Olympus IX70) equipped with a 10× objective lens. Swimming velocity was determined using a manual tracking plug-in tool in ImageJ software. To record the ciliary beating of swimming cells at 1 kHz for 1 s, a 100× objective lens and a high-speed CCD camera (Focuscoppe SV-200i; Photron Limited) were used. All observations were performed at room temperature.

**RESULTS**

Adf73p is a component of F-actin structures associated with FVs. In *T. thermophila*, dynamic reorganization of the actin cyto-
skeleton accompanies the course of phagocytosis (see Fig. S1 in the supplemental material). AC is known as the major actin-modulating protein (1). The macronuclear genome contains only a single gene putatively encoding an AC homologue (13, 21). To investigate the cellular localization of its gene product, Adf73p, we prepared an antiserum against the recombinant protein. After affinity purification, immunoblotting against the total lysate of *T. thermophila* cells showed that the serum most strongly recognized a band of 15 kDa, corresponding to the estimated molecular mass of Adf73p (Fig. 1A). This signal was increased by Adf73p overexpression and diminished by *ADF73* gene knockout (see Fig. 2A and 4C). Under immunofluorescence microscopy with an affinity-purified anti-Adf73p serum, a strong signal colocalized with F-actin protruding from the oral apparatus, with actin dots located on nascent FVs (small arrows) near the oral apparatus (OA) and to an actin clump near a cytoproct (CP) (large arrow). The midsize arrow indicates an actin cable protruding from the OA. This cable-like structure seems to be formed transiently during phagocytosis, since it is found only in limited populations of cells forming FV. Adf73p associates with this structure as well. Cells shown in the bottom two rows were treated with dimethyl sulfoxide (solvent only; control) or the actin polymerization inhibitor Lat-B (final concentration, 10 μM) for 10 min. Note that localization of Adf73p was diminished in cells incubated with Lat-B. (C) Cells transiently expressing GFP-Adf73p under the control of the exogenous promoter of *MTT1* were stained with an anti-actin antiserum and anti-GFP antibody. The right column shows merged images (red, actin; green, Adf73p). GFP-Adf73p showed localization with Adf73p similar to that observed in panel B. Bars, 10 μm.

**FIG 1** Adf73p colocalizes with F-actin in *T. thermophila*. (A) Western blotting with anti-Adf73p antiserum. Cell lysate of *T. thermophila* was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was slit and subjected to immunoblots with preimmune serum (slit 1) and anti-Adf73p antiserum before (slit 2) and after (slit 3) affinity purification. The arrow indicates a band consistent with the predicted size of Adf73p. Arrowheads indicate extra bands not eliminated after the process of affinity purification. (B) Immunofluorescence microscopy to detect cellular localization of Adf73p. Cells were processed for immunofluorescence staining after India ink was loaded on their FVs for 30 min. The right column shows bright-field microscopic images of cells merged with immunofluorescence images (red, actin; green, Adf73p). Blue dotted lines show the outline of the cell. In the top row, Adf73p is localized to actin dots located on nascent FVs (small arrows) near the oral apparatus (OA) and to an actin clump near a cytoproct (CP) (large arrow). The midsize arrow indicates an actin cable protruding from the OA. This cable-like structure seems to be formed transiently during phagocytosis, since it is found only in limited populations of cells forming FV. Adf73p associates with this structure as well. Cells shown in the bottom two rows were treated with dimethyl sulfoxide (solvent only; control) or the actin polymerization inhibitor Lat-B (final concentration, 10 μM) for 10 min. Note that localization of Adf73p was diminished in cells incubated with Lat-B. (C) Cells transiently expressing GFP-Adf73p under the control of the exogenous promoter of *MTT1* were stained with an anti-actin antiserum and anti-GFP antibody. The right column shows merged images (red, actin; green, GFP-Adf73p). GFP-Adf73p showed localization with Adf73p similar to that observed in panel B. Bars, 10 μm.

**FIG 2** Overexpression of *ADF73* disorganizes actin cytoskeleton in vivo. (A) Addition of Cd²⁺ to cell culture induces Adf73p overexpression in *MTT1-ADF73*-expressing strain (*MTT1-ADF73* strain). Immunoblotting of cell lysates from CU522 cells (parental cells) as the control and from *MTT1-ADF73* cells was performed using anti-Adf73p antiserum and anti-α-tubulin antibody. The expression of Adf73p in *MTT1-ADF73* cells was specifically increased by adding 2.5 μg/ml CdCl₂, to the medium. (B) Immunofluorescence images of CU522 (control) and *MTT1-ADF73* (Adf73p-OE) cells stained with anti-actin antiserum (left and green in merged images) and affinity-purified anti-Adf73p antiserum (middle and red in merged images). Several actin bars were produced by overexpression of Adf73p, while control cells showed normal localization of actin and Adf73p, as mentioned in the legend to Fig. 1. The arrow and arrowhead indicate a nascent FV and an actin cable, respectively. (C) FV formation was significantly suppressed in *ADF73*-overexpressing cells. Cells were incubated with 0.1% India ink for 1 h. Bars, 10 μm.

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thermophila cells appeared to possess high turnover rates of polymerization and depolymerization, because it disappeared upon a short period of incubation with Lat-B (Fig. 1B).

**Adf73p affects actin in vivo and in vitro.** To evaluate the activity of Adf73p against the actin cytoskeleton in vivo, we constructed a strain that overexpresses Adf73p under the control of the Cd²⁺-inducible MTT1 promoter. The levels of Adf73p were markedly elevated after the addition of Cd²⁺ (Fig. 2A). Overproduction of Adf73p diminished actin associated with FVs and frequently induced thick actin bars, probably because of the excessive amount of Adf73p associated with F-actin (Fig. 2B). Importantly, the formation of FVs was strongly suppressed by overproduction of Adf73p (Fig. 2C); thus, proper expression levels of Adf73p are important for organization of actin structures and phagocytosis in *T. thermophila*.

We previously studied the biochemical activities of Adf73p against actin derived from rabbit skeletal muscle; however, Tetrahymena actin isotypes are quite evolutionarily divergent from skeletal actin and possess unique biochemical features (20). *T. thermophila* has 4 actin genes in its genome (13), and Act1p is the most abundant actin expressed in vegetative growing cells (K. Nakano and O. Numata, unpublished data). Therefore, we investigated the biochemical activity of Adf73p on recombinant *T. thermophila* Act1p purified from an expression system using the slime mold *Dictyostelium discoideum* (see Fig. S2 in the supplemental material). Polymerized Act1p was precipitated by ultracentrifugation (Fig. 3). After incubation with Adf73p, the amount of Act1p in the supernatant was significantly increased. Thus, Adf73p directly depolymerizes F-actin made of Act1p.

**Adf73p is required for vigorous cell growth.** To investigate the cellular function of Adf73p, we made an *adf73* gene knockout strain by replacing the coding region with the neo marker in the micronucleus (Fig. 4A). Homozygous knockout cells were obtained as progeny of mating heterokaryons. Initially, we failed to obtain viable progeny of knockout heterokaryons in SPP, the standard medium for *T. thermophila*. However, putative gene knockout homoygotes were isolated in MEPP, a medium that allows the growth of cells that lack the ability to phagocytose (31). Gene replacement was confirmed by PCR using gene-specific primers (Fig. 4B). Moreover, the gene product was not detected in the *adf73* gene knockout strain (*adf73KO*) by immunoblotting (Fig. 4C). It is generally thought that AC is essential for cell viability in animals and yeast, probably because its activity is required for cytokinesis (31–35). In contrast, *Tetrahymena adf73KO* popula...

![FIG 3](image-url) Adf73p depolymerizes filaments of *T. thermophila* actin (Act1p). Act1p (2 μM) was polymerized, mixed with various amounts of Adf73p for 2 h at 25°C, and then centrifuged at 100,000 × g for 30 min. The supernatant (S) and pellet (P) were subjected to SDS-PAGE. Note that the amount of Act1p in the supernatant increased in the presence of Adf73p.

![FIG 4](image-url) ADF73 gene knockout severely affects cell growth. (A) Scheme of disruption of ADF73 gene by DNA homologous recombination. The open reading frame (ORF) of ADF73 was completely replaced with the NEO4 marker gene. UTR, untranslated region. (B) Confirmation of gene replacement by PCR. Genomic DNAs prepared from a parental strain (CU428) and two independent clones of ADF73 knockout cells (KO) were amplified using two sets of PCR primers (primers 1 and 2 or primers 1 and 3). Annealing sites of the primers are indicated in panel A. (C) Expression of Adf73p was abolished in the knockout strain. Immunoblotting of cell lysates was performed using affinity-purified anti-Adf73p serum. The membrane area corresponding to the molecular mass of Adf73p was trimmed and shown. The expression level of actin was unaffected by ADF73 gene knockout. (D) Immunofluorescence microscopy of *adf73KO* cells was performed using affinity-purified anti-Adf73p serum. The right image is rather overexposed to show the nonspecific fluorescence signal. DIC, differential interference contrast. Bar, 10 μm. (E) Cell growth of strain CU428 (wild type [WT]) and three independent clones of the *adf73* knockout strain. Each strain was cultured in MEPP medium at 30°C. Note that both the cell growth rate and maximum number of cells in the steady state were significantly reduced in all of the *adf73KO* strains.
After the phagocytic process, were abundant in the cultures of wild-type cells but not in adf73KO cultures after prolonged incubation with India ink (Fig. 5B). The result shown in Fig. 5C also suggests that phagocytic activity was markedly reduced by gene knockout of ADF73. Thus, ADF73 is required for efficient formation of FVs.

What is the primary defect in phagocytosis induced by loss of ADF73p function? By examining the cellular localization of actin, we noticed large aggregates of actin in the posterior region of adf73KO cells that were not seen in wild-type cells, in which actin is mostly localized around a nascent FV near the oral apparatus, located in the anterior part of a cell (Fig. 6). It seems that remodeling of the actin cytoskeleton during FV formation (see Fig. S1 in the supplemental material) is severely affected in the absence of ADF73.

As mentioned previously, gene knockout of the T. thermophila ACT1 actin gene abrogated the ability for phagocytosis, potentially because of a dysfunction of ciliary motility that affects the undulation of the oral membranelles and produces a current of extracellular materials toward the bottom of the oral cavity (36). Actin is a component of the inner dynein arm of cilia in T. thermophila (37). Interestingly, a homologue of AC is found in flagella of Leishmania, where it is required for axoneme assembly (38). However, we did not detect any ADF73p signal in isolated cilia by immunoblotting, whereas actin was detected in both the cell and cilia (see Fig. S4 in the supplemental material). Also, adf73KO cells are motile (see Movies S1 and S2), in contrast to act1KO cells, which are paralyzed (36). Although the average swimming velocity was somewhat lower in adf73KO cells than in wild-type cells, the difference was not statistically significant (Table 1). Interestingly, adf73KO cells turn more frequently than wild-type cells.

**DISCUSSION**

Adf73p regulates the reorganization of the actin cytoskeleton required for formation of FVs. Here we show that ADF73p colocalizes with actin dots associated with nascent FVs and a fibrous

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**FIG 5** Phagocytosis activity is severely affected in adf73KO cells. (A) FV-forming ability is significantly reduced by gene knockout of ADF73. Wild-type and adf73KO cells were incubated with 0.1% India ink in MEPP medium at 30°C. At 0.5, 2, 4.5, and 7.5 h after the India ink was loaded, cells were fixed and the average number of FVs per cell was evaluated (>10 cells). (B) Microscopic images of cells after 2 h of incubation with India ink. Arrows indicate aggregates of India ink ejected from a cytoproct through the process of phagocytosis. (C) The colloid of India ink was significantly reduced by long incubation for wild-type Tetrahymena cells but not adf73KO cells. Microcentrifuge tubes containing cell culture with India ink as described for panel A were left to stand and observed. Note that almost all India ink settled into wild-type cells, but not adf73KO cells, after prolonged incubation for 24 h, since the colloid was mostly precipitated with cells as the FV contents and India ink aggregated as a result of phagocytosis.

**FIG 6** Abnormally large accumulation of actin in adf73KO cells. Immunofluorescence images are shown for the parental strain CU428 and the adf73KO strain, which were stained with anti-actin antiserum and anti-α-tubulin antibody for microtubules (MTs). Arrowheads indicate normal localization of actin dots surrounding a nascent FV located close to the oral apparatus (yellow arrows). White arrows indicate an abnormal large accumulation of actin in the cytoplasm, which was frequently observed in adf73KO cells but not in wild-type cells. Bar, 10 μm.
structure extending from the oral apparatus in *T. thermophila*. *Tetrahymena* internalizes extracellular liquid with particles into FVs every 1 to 2 min. The actin cytoskeleton is implicated in the formation of FVs, since actin inhibitors potentially block FV formation (18). Moreover, this study shows that the formation of FVs is greatly reduced in cells that assemble abnormal actin structures due to overexpression of GFP-Adf73p (Fig. 2). Taking into account that Adf73p has depolymerizing activity against filaments made of Act1p (Fig. 3), a major isoform of *T. thermophila* actin, it is conceivable that Adf73p-mediated turnover of the actin cytoskeleton is associated with the formation of FVs. Importantly, we found that the formation of FVs was significantly inhibited in cells lacking Adf73p (Fig. 5A) and that the organization of the actin cytoskeleton was abnormal in those cells (Fig. 6). Accordingly, the *adf73*KO cells failed to proliferate in standard SPP medium but grew in MEPP medium, which supported the growth of mutants lacking the ability to phagocytose (23). Thus, the phagocytosis-mediated uptake of nutrients is severely affected in cells lacking Adf73p, while the endocytosis-mediated uptake of nutrients is probably functional. Indeed, it has been demonstrated that endocytosis at the coated pits near basal bodies is not actin dependent in *T. thermophila* (39); however, in the natural environment, actin-based phagocytosis from the oral apparatus is unambiguously indispensable to *T. thermophila* for preying on microorganisms such as bacteria.

In other eukaryotic cells, such as macrophages and slime molds, the actin cytoskeleton promotes invagination or protrusion of the plasma membrane during phagocytosis (40). In contrast, it has not been understood exactly how actin structures such as actin dots and fibers induce the formation of FVs, in part because the source membrane for the nascent FV seems to be distinct from the plasma membrane (41). Uncovering how the actin cytoskeleton functions in phagocytosis in *T. thermophila* may shed light on the general question of how the actin cytoskeleton regulates the plasma membrane dynamics.

In addition, we found that Adf73p colocalized with actin to FVs near a cytoproct. Sugita et al. (17) previously showed that the cytoproct-associated “actin clump” is a transient structure engaged in membrane recycling from old FVs; therefore, Adf73p-mediated reorganization of the actin cytoskeleton may be involved in this process.

**Adf73p is not essential for cytokinesis in *T. thermophila***. In the cytokinesis of animal and yeast cells, the cleavage furrow is induced by the contraction of a ring made of actin within the division plane (42–45). Many actin-modulating proteins, including AC, are involved in the assembly and dynamics of the contractile ring. *Tetrahymena* divides by binary fission and assembles an actin-rich contractile ring (46). Several actin-modulating proteins, including fimbrin (16), eEF1A (47), and profilin (48), localize to the cleavage furrow. On the other hand, we failed to detect Adf73p in the cleavage furrow (see Fig. S3 in the supplemental material; our unpublished data). Supporting this observation, *adf73*KO cells multiplied without a major increase in the mitotic index (Fig. 4E); therefore, Adf73p is dispensable for cytokinesis in *T. thermophila*. Moreover, cells lacking Act1p can develop an advanced cleavage furrow (36) but fail to perform scission, the terminal stage of cytokinesis, by which the cell bridge linking the two daughter cells is broken by rotokinesis, a cilium-dependent rotation of daughter cells (49). This is probably because of the essential function of Act1p in ciliary movement in the inner arm dynein complex (36, 37). It is therefore likely that isotypes of actin distinct from Act1p are involved in the contractile ring and that their functions do not require Adf73p. Alternatively, it is possible that another type of actin-severing or depolymerizing protein dominantly functions in *Tetrahymena* cytokinesis. Very recently, it was reported that the cyclase-associated protein CAP, known as a synergistic factor for AC function, can sever F-actin without AC, although the activity is weak (50). We have found a gene encoding a CAP-homologous protein in the *T. thermophila* genome (our unpublished data), although the function of this gene remains to be uncovered. In addition, a myosin II heavy chain, an essential component of the contractile ring in animal and yeast cells, is not present in *T. thermophila* (51, 52). Therefore, the formation of the contractile ring in *Tetrahymena* is likely to involve divergent forms of actin and actin regulators. Interestingly, it has been demonstrated that *Giardia intestinalis*, an intestinal parasite whose genome lacks most of the genes encoding canonical actin-modulating proteins, including AC and myosin, requires actin function for cytokinesis (53). Further studies on protists such as *Giardia* and *Tetrahymena* will provide important evolutionary insights into the molecular mechanism of cytokinesis. In addition, the divergence of the cytokinetic apparatus creates an experimental opportunity in *Tetrahymena*, namely, regulators of important actin-dependent functions, such as AC, can be studied in vivo because their function is not required for survival.

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