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A New *Tetrahymena* Actin-Binding Protein Is Localized in the Division Furrow[1]

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Using an F-actin affinity column, a 60 kDa fragment of a 71 kDa F-actin-binding protein was partially purified from *Tetrahymena pyriformis*. After digestion of the 60 kDa fragment with cyanogen bromide, the N-terminal 21-amino acid sequence of one of the resulting peptides was found to show sequence similarity to a region near the actin-binding site (amino acid residues 260-281) of yeast fimbrin. An antibody prepared against a synthesized 21-mer oligopeptide reacted with the 71 kDa proteins in *T. pyriformis* and *T. thermophila* cell extracts, suggesting that the 60 kDa fragment was produced from the 71 kDa protein through partial digestion occurring during isolation. The 60 kDa fragment bound to *Tetrahymena* F-actin as well as to rabbit skeletal muscle F-actin, and induced the bundling of *Tetrahymena* F-actin. Indirect immunofluorescence revealed colocalization of the 71 kDa protein and actin in the oral apparatus and the deep fiber bundles in *T. pyriformis*. On the other hand, in *T. thermophila*, the 71 kDa protein was localized in the oral apparatus and the contractile vacuole pores during the interphase. During cytokinesis, the 71 kDa protein was localized in the division furrow. Therefore, the 71 kDa protein seems to associate with the actin cytoskeleton, and to regulate the actin filament organization during phagocytosis and cytokinesis in *Tetrahymena*.

Key words: actin, actin-binding protein, cytokinesis, phagocytosis, *Tetrahymena*.

Cytokinesis in animal cells takes place through constriction of the equatorial cell cortex. This cortical region is called the cleavage furrow, and the contractile machinery in this region comprises the contractile ring, which is mainly composed of actin filaments and myosin. The force for the cleavage is generated through the actin-myosin interaction (*1-4*).

In nonmuscle cells, actin is a very dynamic protein, filaments being formed from its monomers and a three-dimensional cytoskeleton from the filaments. These dynamic changes of the supra-molecular assembly of actin are under the control of various actin-modulating proteins. The formation and disappearance of the contractile ring are also considered to be due to the actions of actin-modulating proteins. Some of these proteins, for example, α-actinin (*5-7*), tropomyosin (*8*), and profilin (*9*), have been reported to be present in the cleavage furrow region.

The ciliate, *Tetrahymena*, is one of the best experimental systems for studying the mechanisms of cell division in eukaryotic cells (*10*). For the first time in eukaryotic cells, *Tetrahymena* cells exhibited synchronous division induced by heat treatment (*11*). The contractile ring structure in *Tetrahymena* is like that in animal cells and is composed of several division-associated structures (*12, 13*). On the basis of ultrastructural observations of various stages of dividing *Tetrahymena* cells, a model for the molecular basis of division furrow constriction was proposed, as follows (*14*): (i) The contractile force is presumably generated by the sliding contractile ring microfilaments *via* the ATP-dependent action of myosin. (ii) The lateral stripes, which bundle the contractile ring microfilamens, may be involved in the contractile ring organization. (iii) The linkers, which connect the lateral stripes and the epiplasm, may function in transmission of the contractile force from the contractile ring to the epiplasmic layer just below the membrane. (iv) Cytokinesis presumably progresses through contraction of the contractile ring and subsidence or cutting off of the surface layer. In this phenomenon, the functions of the lateral stripes and the linkers may be essential, and these structures may be composed of a kind of F-actin-binding protein. Therefore, to understand the dynamic properties of the contractile ring, it is important to elucidate the biochemical properties and the biological roles of the actin-binding proteins found in the division furrow.

To ascertain the functions of the actin-binding proteins and to characterize them, we have been trying to isolate a

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Abbreviations: BB, Bromophenol Blue; CBB, Coomassie Brilliant Blue R-250; CNBr, cyanogen bromide; DAPI, 4′,6-diamidino-2-phenylindole; EF-1α, elongation factor 1α; F-actin, filamentous actin; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; MBS, M-maleimidobenzoyl-*N*-hydroxysuccimide; NP-40, Nonidet P-40; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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contractile ring structure protein from the among *Tetrahymena* F-actin-binding proteins. We obtained a 60 kDa fragment of a 71 kDa F-actin-binding protein from a *Tetrahymena* lysate, using an F-actin affinity column. In this paper, we report that the partial amino acid sequence of the 60 kDa fragment is similar to one of yeast fimbrin (ABP67) (*15*). Immunoblotting indicated that the 60 kDa fragment is a digestion product of the 71 kDa protein. The 60 kDa fragment binds to *Tetrahymena* actin as well as to rabbit skeletal muscle actin. Furthermore, the 71 kDa protein is colocalized with actin in the oral apparatus in interphase cells, while in dividing cells, this protein is colocalized with actin in the division furrow.

MATERIALS AND METHODS

Cell Culture—Cultivation of *Tetrahymena pyriformis* (Strain W) and *T. thermophila* (B24964WT) was performed as described previously (*16*).

Electrophoresis and Immunoblot Analysis—SDS-PAGE was carried out according to the method of Laemmli (*17*). To examine the specificity of the antibody, immunoblot analysis was carried out according to the method of Towbin *et al.* (*18*), using alkaline phosphatase conjugated goat anti-rabbit IgG (Tago, Burlingame, USA) as a secondary antibody.

Preparation of F-Actin—Rabbit skeletal muscle actin was purified from acetone powder of rabbit skeletal muscle by the method of Pardee and Spudich (*19*). Purification of *Tetrahymena* actin was performed by the method of Hirono *et al.* (*20*).

Preparation of F-Actin Affinity Columns—F-Actin affinity columns were prepared as described by Miller and Alberts (*21*). Rabbit skeletal muscle F-actin (2 mg/ml) stabilized with phalloidin was immediately mixed with a 1:1 mixture of AFFIGEL 10 (Bio Rad, Hercules, USA) and Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden). A control column was prepared with bovine serum albumin. The column beds were packed in disposable plastic syringes (Telmo, Tokyo). The bed size was 3 ml and the protein content was 2 ml/mg for both the F-actin affinity and control columns, and they were equilibrated with buffer F (50 mM HEPES, 0.1 M KCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 5 mM $MgCl_2$, pH 7.5) at 4°C.

Affinity Chromatography of Tetrahymena Extracts—All procedures were carried out at 4°C. *T. pyriformis* cells were harvested by centrifugation at 3,000×*g* for 3 min. The cell pellet was washed twice with an NKC solution (0.2% NaCl, 0.008% KCl, 0.012% $CaCl_2$), and then resuspended in 3 volumes of E buffer (5 mM HEPES, 0.05% NP-40, 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, pH 7.5). The suspension was sonicated twice for 30 s each time at an output setting of 7 using a cell distributor (Ultrasonics Inst., Tomy Seiko, Tokyo). The sonicated suspension was centrifuged at 100,000×*g* for 1 h. Twenty-five-milliliter portins of the supernatant were each applied to an F-actin or control column, at the flow rate of 1 column volume/h. After loading, each column was rinsed with buffer A (50 mM HEPES, 0.05% NP-40, 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, 10% glycerol, pH 7.5) at 1-2 column volume/h for 12 h. Elution was then carried out in four steps with buffer A containing 1 mM ATP, 0.1 M KCl, 0.5 M KCl, and 1.0 M KCl, respectively.

Actin Filament Co-Sedimentation Assay—The 0.5 M KCl fraction eluted from the F-actin affinity column was pre-centrifuged at 200,000×*g* for 30 min to remove F-actin dissociated from the column. After centrifugation, the fraction was dialyzed against buffer S (5 mM HEPES [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 100 mM KCl, 2 mM ATP) and then mixed with *T. pyriformis* actin (200 μg/ml) or skeletal muscle actin (400 μg/ml). After incubation for 10 min at room temperature in buffer S, the mixture was centrifuged at 200,000×*g* at 4°C for 30 min. The supernatant and pellet were analyzed by SDS-PAGE.

Negative Staining Electron Microscopy—The 60 kDa fragment and *Tetrahymena* F-actin were mixed and incubated as in the co-sedimentation experiment. The mixture was mounted on a carbon-coated grid and then negatively stained with 4% uranyl acetate. The grids were examined under a JEOL 100CX electron microscope (JEOL, Tokyo) at an accelerating voltage of 80 kV.

Amino Acid Sequencing—The 60 kDa fragment in the 0.5 M KCl fraction was subjected to SDS-PAGE and then gel slices containing only the 60 kDa fragment were excised from the gel. After dialysis against a sample buffer (0.125 M KCl, 0.5% SDS, 10% glycerol, 0.02% BB), the 60 kDa fragment was digested with CNBr according to the method of Jahnen *et al.* (*22*). The N-terminal amino acid sequences of several fragments of the 60 kDa fragment were determined by automated Edman degradation with an Applied Biosystems gas-phase sequencer.

Antibody Preparation—From the N-terminal amino acid sequence of one of the fragments of the 60 kDa fragment, a 21-mer oligopeptide was synthesized. The synthesized peptide was crosslinked with KLH using MBS as described by Liu *et al.* (*23*). The KLH-crosslinked oligopeptide (200 μg) was emulsified with an equal volume of complete Freund's adjuvant and then injected weekly into rabbits. After five injections, the antiserum was collected. The

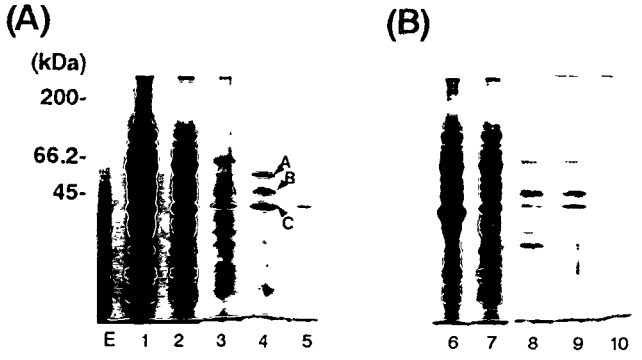

Fig. 1. **SDS-PAGE analysis of the proteins eluted from an F-actin affinity column (A) and a BSA control column (B) which were loaded with a *Tetrahymena* cell extract.** Lane E, *Tetrahymena* cell extract. Lanes 1 and 6, flow-through fraction; lanes 2 and 7, 1 mM ATP-eluted fraction; lanes 3 and 8, 0.1 M KCl-eluted fraction; lanes 4 and 9, 0.5 M KCl-eluted fraction; lanes 5 and 10, 1.0 M KCl-eluted fraction. Lane 4 shows the 0.5 M KCl fraction the contained 60, 49, and 42 kDa proteins, that were reproducibly dissociated from the F-actin affinity column, but the 60 kDa fragment was not eluted from the BSA control column. Arrowheads A, B, and C indicated the 60, 49, and 42 kDa proteins, respectively. The apparent molecular weights (kDa) are shown on the left.

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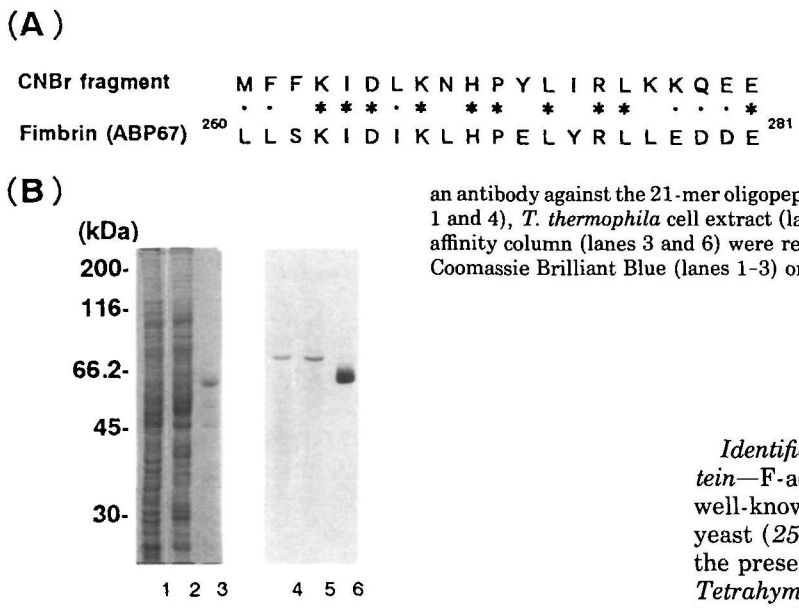

Fig. 2. **Amino acid sequence of the 21-mer oligopeptide of the 60 kDa protein and the specificity of an antibody against the 21-mer oligopeptide.** (A) Comparison of the amino-terminal amino acid sequence of the CNBr fragment of the 60 kDa protein with residues 260-281 of yeast fimbrin. Asterisks indicate identity and dots similarity. (B) Specificity of an antibody against the 21-mer oligopeptide of the 60 kDa protein. *T. pyriformis* cell extract (lanes 1 and 4), *T. thermophila* cell extract (lanes 2 and 5), and the 0.5 M KCl fraction from the F-actin affinity column (lanes 3 and 6) were resolved by 10% SDS-PAGE, and then either stained with Coomassie Brilliant Blue (lanes 1-3) or analyzed by immunoblotting (lanes 4-6).

antiserum against the 21-mer oligopeptide was affinity purified using the purified 60 kDa fragment immobilized on Immobilon membrane filters (Millipore, Bedford, USA) (*24*). The affinity purified antibody was designated as the anti-71 kDa protein antibody. From the N-terminal amino acid sequence of *T. thermophila* actin, a 17-mer oligopeptide was synthesized. Using this 17-mer oligopeptide, a guinea pig antiserum against *T. thermophila* actin was prepared (Numata *et al.*, manuscript in preparation). The guinea pig antiserum against rabbit skeletal muscle actin was kindly provided by Dr. K. Sutoh (Tokyo University). Rabbit antiserum against *Tetrahymena* EF-1α had previously been prepared and characterized (Kurasawa *et al.*, manuscript in preparation), and rabbit antiserum against *T. pyriformis* actin was prepared according to Hirono *et al.* (*20*).

Indirect Immunofluorescence—Tetrahymena cells were quickly air-dried on a slide and then fixed with 0.2% formaldehyde in methanol for 30 min at 20°C. For staining with the guinea pig antiserum against *T. thermophila* actin, *T. thermophila* cells were fixed at −80°C in methanol for 30 min. The cells were washed in PBS and then preincubated in 3% nonfat dried milk in PBS for 30 min. The cells were incubated with either the anti-71 kDa protein antibody (diluted 1:100 in PBS), the rabbit antiserum against *T. pyriformis* actin (diluted 1:200 in PBS), or the guinea pig antiserum against *T. thermophila* actin (diluted 1:200 in PBS) for 1 h at room temperature. The cell specimens were washed with PBS, and then incubated with FITC-conjugated anti-rabbit IgG (KPL, Maryland, USA; diluted 1:100 in PBS) or FITC-conjugated anti-guinea pig IgG (KPL, Maryland, USA; diluted 1:100 in PBS) for 1 h at room temperature. After washing, the specimens were mounted with 50% glycerol in PBS containing 1μg/ml DAPI. The specimens was observed under a Karl Zeiss Axio fluorescence microscope and photographed with Tri-X Pan 400 film.

RESULTS

Identification of the Tetrahymena Actin-Binding Protein—F-actin columns were used previously to isolate both well-known and unidentified actin-binding proteins from yeast (*25*), *Drosophila* (*26*), and sea urchin eggs (*27*). In the present study, rabbit skeletal muscle actin instead of *Tetrahymena* actin was used to prepare an F-actin column, because *Tetrahymena* actin hardly interacts with phalloidin (*20*). *Tetrahymena* cell lysates, prepared in a low ionic strength buffer that favors disassembly of actin filaments, were passed through the F-actin column, and actin-binding proteins were eluted first with 1 mM ATP, and then with 0.1 M KCl, 0.5 M KCl, and 1.0 M KCl. The 0.5 M KCl fraction contained 60, 49, and 42 kDa proteins, that were reproducibly eluted from the F-actin column (Fig. 1A, lane 4). Since the 60 kDa protein was not eluted from a control BSA column (Fig. 1B, lane 9), it seemed to be specifically associated with the F-actin column.

Immunoblotting experiments involving anti-EF-1α or anti-skeletal muscle actin antiserum indicated that the 49 and 42 kDa proteins were *Tetrahymena* EF-1α and rabbit skeletal muscle actin, respectively (data not shown). To ascertain the isoelectric point of the 60 kDa protein, the 0.5 M KCl fraction was subjected to two-dimensional gel electrophoresis. The isoelectric point of the 60 kDa protein was 7.5, while those of the 49 and 42 kDa proteins corresponded to those of *Tetrahymena* EF-1α (pI 9.0) and rabbit skeletal muscle actin (pI 5.3), respectively (data not shown).

N-Terminal Amino Acid Sequences of Proteolytic Fragments of the Tetrahymena Actin-Binding Protein—The amino-terminal amino acid sequence of the 60 kDa protein was analyzed. However, on several cycles of automated Edman degradation no amino acid was released at any step, indicating that the amino-terminal of the 60 kDa protein was blocked. So, a partial amino acid sequence of the 60 kDa protein was determined using several fragments obtained on CNBr digestion. A homology search of the NBRF database showed that the amino acid sequence of one fragment obtained on CNBr digestion exibited similarity to a region near the actin-binding domain of yeast fimbrin (Fig. 2A).

Specificity of the Antibody against the 21-Mer Oligopeptide of the Tetrahymena Actin-Binding Protein—To investigate the intracellular localization of the 60 kDa protein, we prepared an antibody against a synthetic 21-mer oligopeptide that exibited the sequence similarity to a region near the actin-binding domain of yeast fimbrin. The specificity of the antibody, which had been affinity purified

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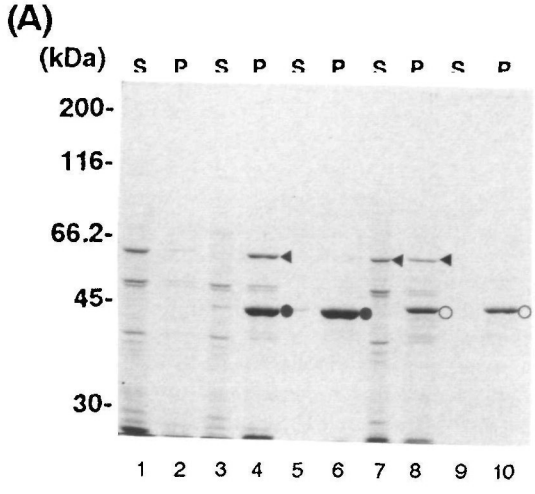
using the 60 kDa protein, was analyzed by immunoblotting. As shown in Fig. 2, the antibody reacted specifically with the 60 kDa protein in the 0.5 M KCl fraction, while it reacted with a 71 kDa protein in *T. pyriformis* and *T. thermophila* cell extracts. These results suggest that the 60 kDa protein was a digestion product of the 71 kDa protein, and that the digestion occurred in the carboxyl terminal region of the 71 kDa protein during the extraction and F-actin affinity column chromatography. The antibody against the 21-mer oligopeptide was designated as the anti-71 kDa protein antibody.

Characterization of the 60 kDa Fragment—To examine direct binding between the 60 kDa fragment and rabbit skeletal muscle actin, the 0.5 M KCl fraction and rabbit skeletal muscle actin were mixed and incubated in buffer A containing 100 mM KCl, and then the mixture was centrifuged. In the case of the 0.5 M KCl fraction alone, most of the 60 kDa fragment was not precipitated on the high speed centrifugation (Fig. 3A, lane 2). In the presence of rabbit skeletal muscle actin, the 60 kDa fragment was co-precipitated with F-actin (Fig. 3A, lane 4).

Actin is a highly conserved protein in eukaryotes, but *Tetrahymena* actin has some unusual properties. It lacks binding ability as to phalloidin, muscle α-actinin and tropomyosin, and does not inhibit DNase I activity (*20, 28*). On the other hand, it shares many essential properties with skeletal muscle actin, such as K^+ or Mg^{2+}-dependent polymerization into filaments, binding with muscle heavy meromyosin to form arrowhead structures, and activation of myosin Mg^{2+}-ATPase activity (*20*). Thus, it was very important to determine whether or not the 60 kDa fragment interacts with *Tetrahymena* actin. The interaction



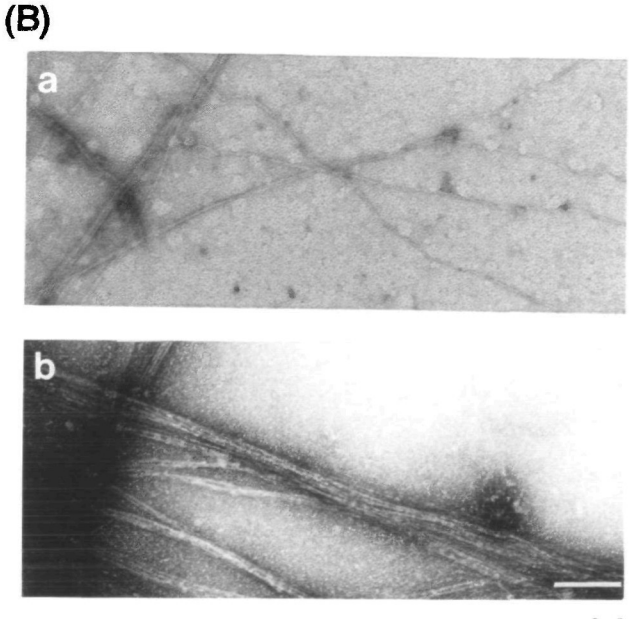


Fig. 3. **Binding of the *Tetrahymena* 60 kDa fragment to skeletal muscle F-actin or *Tetrahymena* F-actin, and *Tetrahymena* F-actin bundles induced by the 60 kDa fragment.** A: The 0.5 M KCl fraction, rabbit skeletal muscle actin and/or *Tetrahymena* actin were mixed and incubated for 30 min at room temperature in buffer S. The mixtures were ultracentrifuged at 100,000 × *g* for 30 min. The supernatants and pellets obtained on ultracentrifugation were analyzed by SDS-PAGE. The supernatants (S) and pellets (P) of the samples containing the 0.5 M KCl fraction alone (lanes 1 and 2), the 0.5 M KCl fraction and skeletal muscle actin (lanes 3 and 4), skeletal muscle actin alone (lanes 5 and 6), the 0.5 M KCl fraction and *Tetrahymena* actin (lanes 7 and 8), and *Tetrahymena* actin alone (lanes 9 and 10) are shown. The bands corresponding to the 60 kDa fragment, rabbit skeletal muscle actin and *Tetrahymena* actin are denoted by ◂, ●, and ○, respectively. B: 200 μg/ml *Tetrahymena* F-actin alone (a) or a mixture of *Tetrahymena* F-actin and the 0.5 M KCl fraction (b) was incubated for 30 min at room temperature, and then each mixture was analyzed by electron microscopy after negative staining. The bar represents 100 nm.

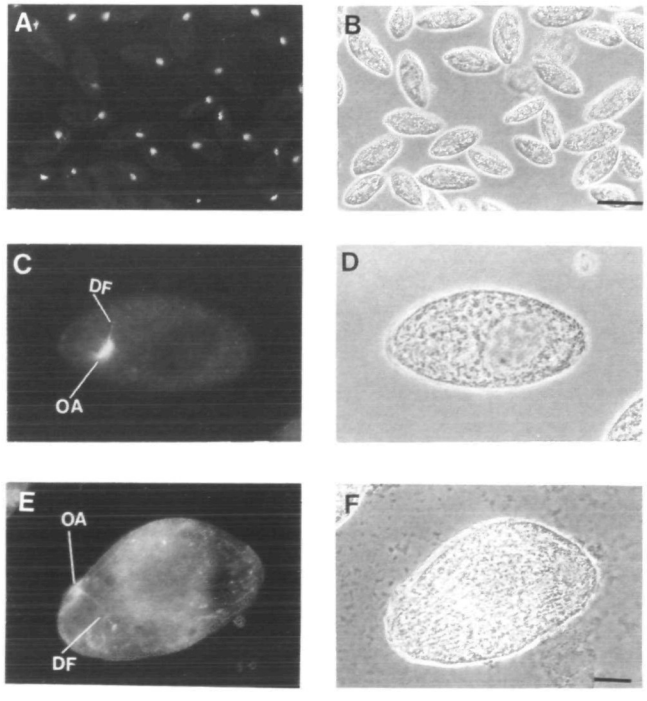


Fig. 4. **Localization of the 71 kDa protein and actin in *T. pyriformis* interphase cells.** Fluorescence photomicrographs of interphase cells stained with the anti-71 kDa protein antibody (A and C) or the anti-*Tetrahymena* actin antiserum (E). (B), (D), and (F) are phase-contrast images of the fields shown in (A), (C), and (E), respectively. (A) and (B) are low magnification images and (C-F) are high magnification images. The bars in (B) and (F) indicate 40 μm and 10 μm, respectively. Note that the 71 kDa protein and actin were colocalized in the oral apparatus (OA) and the deep fiber bundles (DF).

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between the 60 kDa fragment and *Tetrahymena* actin was examined by means of a co-sedimentation experiment. The latter showed that the 60 kDa fragment was co-precipitated with *Tetrahymena* actin under the same conditions as for skeletal muscle actin (Fig. 3A, lane 8).

To investigate the effect of the 60 kDa fragment on the F-actin organization, a mixture of the 0.5 M KCl fraction and *Tetrahymena* F-actin was examined by electron microscopy after negative staining. The mixture contained many F-actin bundles (Fig. 3). Although the 0.5 M KCl fraction contained a very small amount of EF-1α, the contribution of contaminating EF-1α to the actin bundle formation would be negligible, because (i) the F-actin bundles formed by the 0.5 M KCl fraction looked looser than those formed by *Tetrahymena* EF-1α, and (ii) the interaction conditions for this experiment, such as a weak alkaline pH (pH 7.5) and high ionic strength (120 mM KCl), markedly reduce the F-actin bundling activity of *Tetrahymena* EF-1α (*29*). Thus, these results suggest that the 60 kDa fragment contains actin-binding domain(s) and induces the bundling of F-actin.

Localization of the Tetrahymena 71 kDa Protein in Interphase Cells—For immunofluorescence staining, *Tetrahymena* cells were fixed with 0.2% formaldehyde in methanol. The 71 kDa protein was localized in the oral apparatus and the deep fiber bundles in interphase *T. pyriformis* cells (Fig. 4, A and C). This localization corresponds to that of actin (Fig. 4E) (*30*). On the other hand, in *T. thermophila* the 71 kDa protein was localized in the oral apparatus and the contractile vacuole pores (Fig. 5, B and C). These staining patterns were not observed with preimmune serum (data not shown). Since the oral apparatus is involved in phagocytosis, we examined the relationship between food vacuoles and the 71 kDa protein localization. *Tetrahymena* cells were incubated in culture medium containing 2% India Ink, and then the cells that had ingested carbon particles were subjected to immunofluorescence staining with the anti-71 kDa protein antibody. No im-

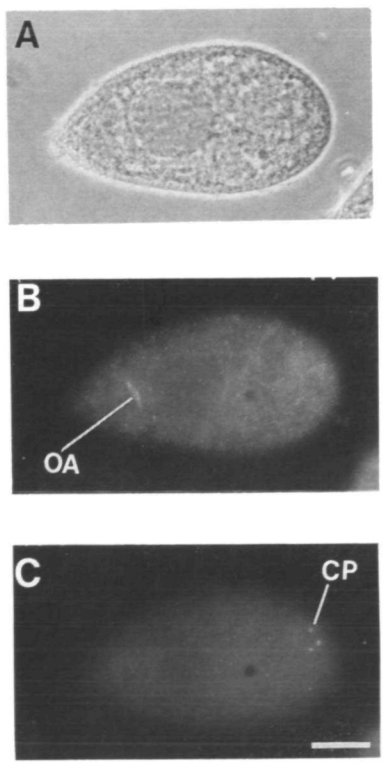

Fig. 5. **Localization of the 71 kDa protein in *T. thermophila* interphase cells.** (A) is a phase-contrast image of the cell shown in (B) and (C). (B) and (C) are fluorescence photomicrographs of an interphase cell stained with the anti-71 kDa protein antibody, focusing on the oral apparatus (B) and the contractile vacuole pores (C), respectively. The bar in (C) indicates 10 μm. The 71 kDa protein is localized in the oral apparatus (OA) and the contractile vacuole pores (CP) in *T. thermophila*.

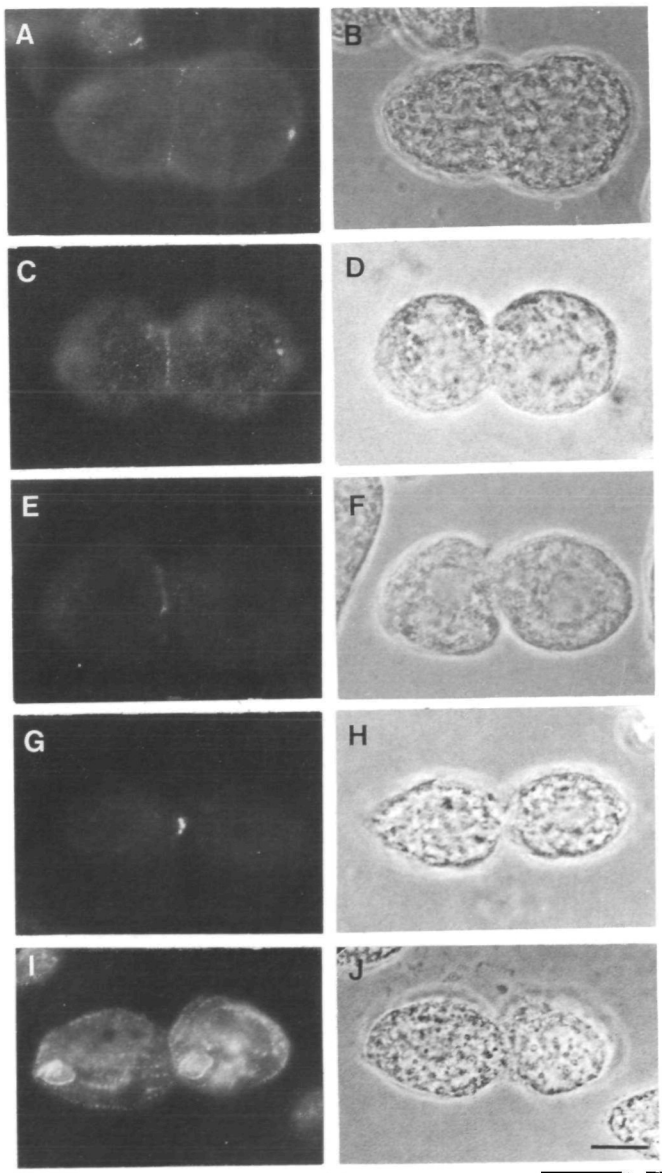

Fig. 6. **Localization of the 71 kDa protein in dividing *T. thermophila* cells.** Dividing cells at various stages of cytokinesis (A–H) were stained with the anti-71 kDa protein antibody. Micrographs of immunofluorescence images (A, C, E, and G) and those of phase-contrast images (B, D, F, and H) are arranged in order: early (A, B), mid- (C, D), late (E, F), and end (G, H) stages of cytokinesis. (I) shows the immunofluorescence localization of *T. thermophila* actin in the mid-stage of cytokinesis in dividing cells. (J) shows a phase-contrast image of (I). The bar in (J) indicates 10 μm. Note that the 71 kDa protein and actin are localized in the division furrow.

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munofluorescence appeared around the food vacuoles (data not shown). These results imply that the 71 kDa protein may have some function in the early stage of endocytosis in the oral apparatus.

Localization of the Tetrahymena 71 kDa Protein in Dividing Cells—When dividing *T. thermophila* cells at various stages were examined, the equatorial furrow regions of cells were found to be stained from the early to the end stage of cytokinesis (Fig. 6, A-D). At an early stage of cytokinesis, a thin dotted line along the fission zone fluoresced with the anti-71 kDa protein antibody (Fig. 6A). At the mid-stage of cytokinesis, small dots of the 71 kDa protein converged on large dots and then a thick dotted line appeared in the division furrow (Fig. 6B). The dots of the 71 kDa protein joined each other, as the furrow constriction proceeded. From the late to the end stage of cytokinesis, the fluorescence appeared as large dots in the division furrow (Fig. 6, C and D). Virtually no fluorescence was observed in the oral apparatuses or contractile vacuole pores of dividing cells, but strong fluorescence in these organelles was observed when cytokinesis had been completed. On the other hand, *T. thermophila* actin was localized in the division furrow, oral apparatus and basal body (Fig. 6, I and J). These results indicate that actin and the 71 kDa protein are colocalized in the division furrow.

Localization of the Tetrahymena 71 kDa Protein in Permeabilized Cells—To determine whether or not the *Tetrahymena* 71 kDa protein exists as an insoluble form in the division furrow and the oral apparatus, soluble proteins in the cytoplasm were first extracted with 0.25% NP-40 and then localization of the 71 kDa protein was examined in the cytoskeletal cell models. It was found that virtually no fluorescence appeared in the division furrow (data not shown). Therefore, the 71 kDa protein may undergo a weak interaction with cytoskeletal actin filaments in the division furrow, the oral apparatus and the contractile vacuole pores.

DISCUSSION

In this study we found a 60 kDa fragment of a 71 kDa F-actin-binding protein in *Tetrahymena* cell lysates, using F-actin affinity column chromatography (Fig. 1). Although the 60 kDa fragment was found to be a digestion product of the 71 kDa protein, co-sedimentation experiments and electron microscopy demonstrated that the 60 kDa fragment was able to bind to both *Tetrahymena* actin and skeletal muscle actin (Fig. 3A), and to induce actin filament bundling (Fig. 3B). *Tetrahymena* actin binds to skeletal muscle myosin and induces myosin Mg^{2+}-ATPase activity, while it lacks binding ability as to phalloidin, muscle α-actinin and tropomyosin, and does not inhibit DNase I activity (*20, 28*). These unusual properties of *Tetrahymena* actin appeared to be due to the presence of six variable regions within protozoan actins (*31*). The fact that the 60 kDa fragment binds to skeletal muscle F-actin as well as to *Tetrahymena* F-actin suggests that it may bind to a region conserved in *Tetrahymena* and rabbit skeletal muscle actins. We tried to isolate the intact 71 kDa protein from *Tetrahymena*, but failed because of proteolytic digestion. To further examine the 71 kDa protein's function *in vitro*, we are attempting cloning of the 71 kDa protein gene.

The 71 kDa protein is colocalized with actin in the oral apparatus and the deep fiber bundles in interphase cells (Figs. 4 and 5), while this protein is localized in the division furrow from the early to the end stage of cytokinesis (Fig. 6). These observations lead to the inference that the 71 kDa protein binds to actin filaments and then induces the bundling of actin filaments *in vivo*. The immunofluorescence staining and immunoblotting experiments involving permeabilized cells showed that the 71 kDa protein may undergo a weak interaction with cytoskeletal actin filaments (data not shown). Therefore, the 71 kDa protein may function as a sensitive regulator of the actin filament organization. On the other hand, the 60 kDa fragment of the 71 kDa protein strongly bound to actin in an F-actin affinity column. The difference in the actin-binding activity between the 71 kDa protein and the 60 kDa fragment may depend on the existence of the 11 kDa portion which was removed from the 71 kDa protein during the purification steps. The 11 kDa portion may regulate the actin-binding activity of the 71 kDa protein.

The partial amino acid sequence of the 60 kDa fragment was similar to on near the actin-binding domain of yeast fimbrin (Fig. 3). Yeast fimbrin identified using an F-actin affinity column binds to actin and forms actin bundles *in vitro*, and it associates functionally with actin structures involved in the development and maintenance of cell polarity, morphogenesis, and the internalization step of endocytosis *in vivo* (*15, 25, 32, 33*). The predicted relative molecular mass of yeast fimbrin is 71.7 kDa (*15*). The F-actin bundles formed by the 60 kDa fragment were similar to those formed by yeast fimbrin. In *Tetrahymena* cells, the 71 kDa protein and actin are colocalized in the oral apparatus, which is involved in endocytosis. In yeast, actin and fimbrin are required for the internalization step of endocytosis (*33*). Thus, we suggest that the 71 kDa protein may be a homologue of fimbrin in *Tetrahymena*, in terms of the isolation method, partial amino acid sequence, apparent molecular weight, interaction with actin, and localization. Although fimbrin and actin are colocalized in surface microvilli, the membrane ruffle and substratum attachment points in several cultured cells, there has been no report of the coexistence of fimbrin with actin filaments in the division furrow. If the 71 kDa protein is a *Tetrahymena* fimbrin homologue, this is the first report of the occurrence of fimbrin in the division furrow.

In *Tetrahymena*, p85 (*34, 35*) and profilin (*9*) have been reported to be present in the division furrow region. p85 was found to be localized in the equatorial basal bodies just before the formation of the fission zone, which coresponds to the position of the fission plane (*34*). On the other hand, at a restrictive temperature, *Tetrahymena* cell division-arrest *cdaA1* mutant cells fail to form a fission zone and undergo a subsequent furrowing, and do not show equatorial localization of p85 (*34*). These observations suggest that p85 is a crucial factor as to the cell division plane. The localization of *Tetrahymena* profilin in the division furrow coincided with that of contractile ring microfilaments in terms of both position and timing, and the deposition of profilin started just before the onset of division furrow constriction (*9*). Since profilin is known, in general, to interact only with G-actin, *i.e.* not with F-actin, it is likely that the colocalization of actin and profilin in the division furrow is due to an indirect interaction. In the presence of excess ATP, profilin promotes actin polymerization by

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increasing the rate of nucleotide exchange on the actin monomer (*36*), and lowering the critical concentration of ATP-actin (*37*). Therefore, profilin localized in the prospective division furrow of *Tetrahymena* cells may play a role in the rapid formation of contractile ring microfilaments.

Immunofluorescence for the 71 kDa protein appeared as small dots along the equatorial plane, and deposition of the 71 kDa protein in the division furrow started just after the onset of division furrow constriction. As the division furrow became constricted along the equatorial plane, small equatorial dots seemed to gather gradually and became a thick dotted line. We found that the 60 kDa fragment binds directly to F-actin and induces the bundling of F-actin *in vitro*, suggesting that the 71 kDa protein in the division furrow may bind to the contractile ring microfilaments and play a role in the bundling of these microfilaments. Thus, we speculate that in *Tetrahymena*, p85, profilin and the 71 kDa protein, in that order, appear in the division furrow and function in determination of the division plane, formation of contractile ring microfilaments and bundling of microfilaments, respectively.

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REFERENCES

1. Mabuchi, I. (1986) Biochemical aspects of cytokinesis. *Int. Rev. Cytol.* **101**, 175-213
2. Salmon, E.D. (1989) Cytokinesis in animal cells. *Curr. Opin. Cell Biol.* **1**, 541-547
3. Satterwhite, L.L. and Pollard, T.D. (1992) Cytokinesis. *Curr. Opin. Cell Biol.* **4**, 43-52
4. Fishkind, D.J. and Wang, Y.L. (1995) New horizons for cytokinesis. *Curr. Opin. Cell Biol.* **7**, 23-31
5. Fujiwara, K., Porter, M.E., and Pollard, T.D. (1978) Alpha-actinin localization in the cleavage furrow during the cytokinesis. *J. Cell Biol.* **79**, 268-275
6. Wallraffe, E., Schleicher, M., Modersitzki, M., Rieger, D., Isenberg, G., and Gerisch, G. (1986) Selection of *Dyctyostelium* mutants defective in cytoskeletal proteins: use of an antibody that binds to the ends of alpha-actinin rods. *EMBO J.* **5**, 61-67
7. Mabuchi, I., Hamaguchi, Y., Kobayashi, T., Hosoya, H., Tsukita, S., and Tsukita, S. (1985) Alpha actinin from sea urchin eggs biochemical properties, interaction with actin and distribution in the cell during fertilization and cleavage. *J. Cell Biol.* **100**, 375-383
8. Ishimoda-Takagi, T. (1979) Localization of tropomyosin in sea urchin egg. *Exp. Cell. Res.* **119**, 423-428
9. Edamatsu, M., Hirono, M., and Watanabe, Y. (1992) *Tetrahymena* profilin is localized in the division furrow. *J. Biochem.* **112**, 637-642
10. Zeuthen, E. (1964) *Synchrony in Cell Division and Growth* (Zeuthen, E., ed.) pp. 99-156, John Wiley, New York
11. Scherbaum, O.H. and Zeuthen, E. (1954) Induction of synchronous cell division in mass culture of *Tetrahymena pyriformis*. *Exp. Cell Res.* **6**, 221-227
12. Yasuda, T., Numata, O., Ohnishi, K., and Watanabe, Y. (1980) A contractile ring and cortical changes found in the dividing *Tetrahymena pyriformis*. *Exp. Cell Res.* **128**, 407-418
13. Yasuda, T., Tamura, R., and Watanabe, Y. (1984) Molecular mechanism of cell division in *Tetrahymena thermophila*. Ultrastructure changes found in a division-arrest ts-mutant. *Zool. Sci.* **1**, 62-73
14. Watanabe, Y., Ohba, H., Hirono, M., and Yasuda, T. (1990) Analysis of furrow formation and furrowing during cell division in *Tetrahymena* using cell-division-arrest mutants. *Ann. N.Y. Acad. Sci.* **582**, 166-178
15. Adams, A.E., Botstein, D., and Drubin, D.G. (1991) Requirement of yeast fimbrin for actin organization and morphogenesis *in vivo*. *Nature* **354**, 404-408
16. Watanabe, Y., Numata, O., Kurasawa, Y., and Katoh, M. (1994) Cultivation of *Tetrahymena* cells in *Cell Biology* (Celis, J.E., ed.) Vol. 1, pp. 398-404, Academic Press, California
17. Laemmli, U.K. (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
18. Towbin, H.T., Stahelin, H.M., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
19. Pardee, J.D. and Spudich, J.A. (1982) Purification of muscle actin. *Methods Enzymol.* **85**, 164-181
20. Hirono, M., Kumagai, Y., Numata, O., and Watanabe, Y. (1989) Purification of *Tetrahymena* actin reveals some unusual properties. *Proc. Natl. Acad. Sci. USA* **86**, 75-79
21. Miller, K.G. and Alberts, B.M. (1989) F-actin affinity chromatography: Technique for isolating previously unidentified actin binding proteins. *Proc. Natl. Acad. Sci. USA* **13**, 4808-4812
22. Jahnen, W., Ward, L.D., Reid, G.E., Moritz, R.L., and Simpson, R.J. (1990) Internal amino acid sequencing of proteins by in situ cyanogen bromide cleavage in polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **166**, 139-145
23. Liu, F.T., Zinnecker, M., Hamaoka, T., and Katz, D.H. (1979) New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* **18**, 690-693
24. Talian, J.C., Olmsted, J.B., and Goldman, R.D. (1983) A rapid procedure for preparing fluorescein-labeled specific antibodies from whole antiserum: its use in analyzing cytoskeletal architecture. *J. Cell Biol.* **97**, 1277-1282
25. Drubin, D.G., Miller, K.G., and Botstein, D. (1988) Yeast actin binding proteins: Evidence for a role in morphogenesis. *J. Cell Biol.* **107**, 2551-2561
26. Miller, K.G., Field, C.M., and Alberts, B.M. (1989) Actin binding protein from *Drosophila* embryos: A complex network of interacting proteins detected by F-actin chromatography. *J. Cell Biol.* **109**, 2963-2975
27. Terasaki, G.A., Ohnuma, M., and Mabuchi, I. (1997) Identification of actin-binding proteins from sea urchin eggs by F-actin affinity column chromatography. *J. Biochem.* **122**, 226-236
28. Hirono, M., Tanaka, R., and Watanabe, Y. (1990) *Tetrahymena* actin: Copolymerization with skeletal muscle actin and interaction with muscle actin binding proteins. *J. Biochem.* **107**, 32-36
29. Kurasawa, Y., Watanabe, Y., and Numata, O. (1996) Characterization of F-actin bundling activity of *Tetrahymena* elongation factor 1 alpha. *Zool. Sci.* **13**, 371-375
30. Hirono, M., Nakamura, M., Tsunemoto, M., Yasuda, T., Ohba, H., Numata, O., and Watanabe, Y. (1987) Localization and possible biological roles of actin in *Tetrahymena* cells. *J. Biochem.* **102**, 537-545
31. Hirono, M., Sutoh, K., Watanabe, Y., and Ohno, T. (1992) A chemical actin carrying N-terminal portion of *Tetrahymena* actin does not bind to DNase I. *Biochem. Biophys. Res. Commun.* **184**, 1511-1516
32. Adams, A.E., Botstein, D., and Drubin, D.G. (1989) A yeast actin-binding protein is encoded by *SAC6*, a gene found by suppression of an actin mutation. *Science* **243**, 231-233
33. Kubler, E. and Riezman, H. (1993) Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J.* **12**, 2855-2862
34. Ohba, H., Ohmori, I., Numata, O., and Watanabe, Y. (1986) Purification and immunofluorescence localization of the mutant gene product of a *Tetrahymena cdaA1* mutant affecting cell division found in division arrest mutant. *J. Biochem.* **100**, 797-808
35. Numata, O., Suzuki, H., Ohba, H., and Watanabe, Y. (1995) The mutant gene product of a *Tetrahymena* cell-division-arrest mutant *cdaA* is localized in the accessory structure of specialized basal body close to the division furrow. *Zool. Sci.* **12**, 133-135
36. Goldschmidt-Clermont, P.J., Machesky, L.M., Doberstein, S.K., and Pollard, T.D. (1991) Mechanism of the interaction of human platelet profilin with actin. *J. Cell Biol.* **113**, 1081-1089
37. Pantaloni, D. and Carlier, M.-F. (1993) How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* **75**, 1007-1014

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