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Tetrahymena Fimbrin Localized in the Division Furrow Bundles Actin Filaments in a Calcium-Independent Manner

Shin Shirayama and Osamu Numata*
Institute of Biological Sciences, University of Tsukuba, Ibaraki 305-8572

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In cytokinesis, the contractile ring constricts the cleavage furrow. However, the formation and properties of the contractile ring are poorly understood. Fimbrin has two actin-binding domains and two EF-hand Ca^{2+}-binding motifs. Ca^{2+} binding to the EF-hand motifs inhibits actin-binding activity. In *Tetrahymena*, fimbrin is localized in the cleavage furrow during cytokinesis. In a previous study, *Tetrahymena* fimbrin was purified with an F-actin affinity column. However, the purified *Tetrahymena* fimbrin was broken in to a 60 kDa fragment of a 70 kDa full length fimbrin. In this study, we investigated the properties of recombinant *Tetrahymena* fimbrin. In an F-actin cosedimentation assay, *Tetrahymena* fimbrin bound to F-actin and bundled it in a Ca^{2+}-independent manner, with a K_d of 0.3 μM and a stoichiometry at saturation of 1: 1.4 (*Tetrahymena* fimbrin: actin). In the presence of 1 molecule of *Tetrahymena* fimbrin to 7 molecules of actin, F-actin was bundled. Immunofluorecence microscopy showed that a dotted line of *Tetrahymena* fimbrin along the cleavage furrow formed a ring structure. The properties and localization of *Tetrahymena* fimbrin suggest that it bundles actin filaments in the cleavage furrow and plays an important role in contractile ring formation during cytokinesis.

Key words: actin, Ca^{2+}, cytokinesis, fimbrin, *Tetrahymena*.

Abbreviations: AtFim1, *Arabidopsis* fimbrin; CaM, calumodulin; EF-1α, elongation factor-1α; Fim1p, *Schizosaccharomyces pombe* fimbrin.

The actin cytoskeleton plays important roles in cell migration, organelle transport, and cytokinesis. Cytokinesis in animal cells takes place through constriction of the cleavage furrow. The force for constriction is generated by the contractile ring, which is composed of actin, myosin, and a number of actin-modulating proteins (*1*). Many studies on these proteins have provided useful information on the structure and dynamic organization of the contractile ring. However, the molecular mechanisms of formation and contraction of the contractile ring are unknown.

The ciliate *Tetrahymena* is a good experimental system for studying cytokinesis. Heat treatment causes *Tetrahymena* cells to divide synchronously. The contractile ring structure of *Tetrahymena*, which is similar to those in animal cells, is composed of several division-associated structures (*2*). Some proteins involved in cytokinesis are localized in the *Tetrahymena* cleavage furrow, such as actin (*3*), p85 as a division plane determination factor (*4*), profilin as an actin polymerizing factor (*5*), and elongation factor-1α (EF-1α) (*6*) and fimbrin (*7*) as actin filament bundling factors. Ca^{2+} is thought to be an important signal as to the organization of cytokinesis. The localization of cytosolic calcium in the cleavage furrow has been observed (*8*). Additionally, Ca^{2+}/calmodulin (CaM) signaling is related to regulation of cytokinesis (*9*). In *Tetrahymena*, CaM is localized in the cleavage furrow, and Ca^{2+}/CaM regulates the division plane determination by p85 (*4*) and inhibits the actin bundling activity of EF-1α (*10*).

In our previous study, *Tetrahymena* fimbrin, which was isolated with an F-actin affinity column, was found to be localized in the cleavage furrow during cytokinesis (*7*). That was the first report showing localization of a fimbrin/plastin family protein to the cleavage furrow. Recently, Wu *et al*. (*11*) reported that *Schizosaccharomyces pombe* fimbrin (Fim1p) is also localized in the cleavage furrow.

Fimbrin, which is conserved from yeast to man (*12*), was first identified in chicken intestinal microvilli, and forms tight actin bundles in microvilli (*13*). Fimbrin is required for cell morphogenesis (*14*) and endocytosis (*15*). Fimbrin/plastin family proteins are monomeric actin-bundling proteins, because they have two tandem α-actinin type actin-binding domains (*16*, *17*). Most fimbrins have two EF-hand Ca^{2+}-binding motifs at their NH_2 terminal ends. In *Dictyostelium* plastin (*18*) and human L-plastin (*19*), Ca^{2+} binds to these EF-hand motifs and inhibits their F-actin binding activity. Although Fim1p and *Arabidopsis* fimbrin (AtFim1) have two and one EF-hand motif, respectively, the actin-binding activities of Fim1p and AtFim1 are Ca^{2+}-independent (*20*, *21*). *Tetrahymena* fimbrin has no EF-hand motif but has two tandem α-actinin type actin-binding domains (*22*), suggesting that *Tetrahymena* fimbrin should also be Ca^{2+}-independent.

Proteases in *Tetrahymena* are so strong that we cannot purify the full length *Tetrahymena* fimbrin, and cannot accurately and quantitatively determine the properties of

*To whom correspondence should be addressed. Tel/Fax: +81-29-853-6648, E-mail: numata@sakura.cc.tsukuba.ac.jp

Tetrahymena fimbrin *in vitro*. Therefore, the relationships between fimbrin and other actin-bundling proteins in the contractile ring, such as EF-1α, are not fully understood.

In this paper, we show that *Tetrahymena* fimbrin bound and bundled F-actin in a Ca^{2+}-independent manner. An immunofluorescence study showed that *Tetrahymena* fimbrin was localized in the cleavage furrow as a ring structure during cytokinesis.

MATERIALS AND METHODS

Cell Culture—Tetrahymena thermophila was axenically cultivated in SPP medium (*23*) containing 1% proteose peptone, 0.1% yeast extract, 0.2% dextrose, and 0.003% Fe-EDTA at 26°C until the early stationary phase.

Cloning of Tetrahymena Fimbrin Full Length cDNA—Based on the *Tetrahymena* fimbrin cDNA sequence, primers S-1 and AS-1 for cloning the 5′end of cDNA, and primers S-2 and AS-2 for cloning the 3′end of cDNA were synthesized. Primer S-1 containing a *Bam*HI site comprised 5′-GCGGATCCATGAATAAGCAAGAAAGAATATC-3′. Primer AS-1 was 5′-GCTTTATTTAGCTTCGAC-3′. Primer S-2 was 5′-ATGGATACCTTAAAGACTGCTGTAAAG-3′. Primer AS-2 containing a *Bam*HI site comprised 5′-GCGGATCCAGTAATCTGTAAGAAAGGAAGC-3′. PCR was performed for 30 cycles using primers S-1 and AS-1, or primers S-2 and AS-2 with ExTaq DNA polymerase at 94°C for 30 s, at 50°C for 1 min, and at 72°C for 1 min, with first strand cDNA as the template. The amplified DNA fragments were digested with *Taq*, and then ligated with Ligation High (TOYOBO, Japan). The ligated DNA fragments were subcloned into the pT7blue vector (NOVAGEN, USA) digested with *Bam*HI.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out using a Mutan-Super Express Km site-directed mutagenesis system (TAKARA, Japan), according to the manufacturer's instructions. *Tetrahymena* fimbrin cDNA was subcloned into the *Bam*HI site of pKF18k. The following oligonucleotides were used to change TAA and TAG to CAA and CAG, respectively: TmNo.1, 5′-ATACAGCGCAATAATATAAAAAATAATATATATTTACA-3′; TmNo.2, 5′-AGCAATCAAAAACAGATTAAGTCTG-3′; TmNo.3, 5′-GCTAAACAAAGCGACACAAATGCTATTCACTCATTTACTCAAGATG-3′; TmNo.4, 5′-AATTGATCCTCAAAGCAATAAG-3′; TmNo.5, 5′-TCTTGTTTGGCAGATTATCAAGGCTCAAATGTTG-3′; TmNo.6, 5′-CTACGCAGTACAATTAGCTAAGGATTTACATTTACAAATTGTTGG-3′; TmNo.7, 5′-TATTGTTTGGCAACTCATGAGAAAGCAATCATTAC-3′; TmNo.8, 5′-AGCTATCAATCAGGATTTG-3′; and TmNo.9, 5′-GCGGATCCAACCTGTAAGAAAGGAAGC-3′. The altered nucleotides are underlined. The results of site-direct mutagenesis were confirmed by the dideoxy chain termination method with an ABI Prism DNA sequencer 377.

Expression and Purification of Tetrahymena Fimbrin—*Tetrahymena* fimbrin cDNA with eleven CAA and four CAG codons changed from TAA and TAG codons, respectively, was amplified by PCR using Primer S-1 and TmNo.9. The resulting PCR products were cloned in-frame into expression vector pQE60 (QIAGEN, Germany) using the *Bam*HI site. Expression and purification of the recombinant protein were performed essentially as described in the manufacturer's manuals. The recombinant *Tetrahymena* fimbrin was expressed in *Escherichia coli*, strain M15 [prep4], and purified with Ni-NTA agarose (QIAGEN, Germany). The purified recombinant *Tetrahymena* fimbrin contained a ten-residue C-terminal extension including a 6′His tag.

Gel electrophoresis and Immunoblotting—SDS-PAGE was performed on a 10% running gel with a 3% stacking gel according to the method of Laemmli (*24*). Gels were stained with Coomassie Brilliant Blue R-250. Immunoblotting was carried out according to the method of Towbin *et al.* (*25*), using alkaline phosphatase-conjugated goat anti-rabbit IgG (Biosource International Inc, USA). Proteins were detected with a BCIP/NBT phosphatase substrate system (KPL, USA).

Preparation of Rabbit Skeletal Muscle Actin—Rabbit skeletal muscle actin was purified according to the procedure of Pardee and Spudich (*26*).

F-actin Cosedimentation Assay—The recombinant *Tetrahymena* fimbrin and rabbit skeletal muscle actin were diluted with F-buffer (10 mM imidazole, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 5 μg/ml leupeptin, pH 7.5). After ultracentrifugation at 200,000 ×*g* for 20 min, fimbrin (2 μM) was mixed with F-actin (3 μM) in 200 μl of F-buffer containing 5 mM EGTA, 10 nM, and 10 μM or 1 mM $CaCl_2$. The mixture was incubated for 30 min at 26°C, and then ultracentrifuged at 200,000 ×*g* for 30 min. The supernatant and pellet obtained on ultracentrifugation were analyzed by SDS-PAGE. The gels were scanned and the intensity of bands was determined with Image Gauge (FUJI FILM, Japan).

High-Speed and Low-Speed Cosedimentation Assays—The recombinant *Tetrahymena* fimbrin and rabbit skeletal muscle actin were diluted with F-buffer. After ultracentrifugation at 200,000 ×g for 20 min, fimbrin (2 μM) was mixed with F-actin (3 μM) in 200 μl of F-buffer containing 5 mM EGTA, and 10 nM, 10 μM or 1 mM $CaCl_2$. The mixture was incubated for 30 min at 26°C, and then centrifuged at low-speed (12,000 ×*g* for 15 min). After the centrifugation, supernatant was ultracentrifuged at high-speed (200,000 ×*g* for 30 min). The supernatants and pellets obtained on low-speed and high-speed centrifugation were analyzed by SDS-PAGE. The gels were scanned and the intensity of bands was determined with Image Gauge (FUJI FILM, Japan).

Negative Staining Electron Microscopy—The recombinant *Tetrahymena* fimbrin and rabbit skeletal muscle actin were diluted with MES buffer (10 mM MES, 60 mM KCl, 2 mM $MgCl_2$, 5% glycerol, 0.75 mM 2-mercaptoethanol, 5 μg/ml leupeptin, pH 6.5). After ultracentrifugation of fimbrin at 200,000 ×g for 20 min, it (0.3 μM) was mixed with F-actin (2 μM) in the 200 μl of F-buffer containing 5 mM EGTA or 5 mM $CaCl_2$, and then incubated for 30 min at 26°C. The mixtures were mounted on carbon-coated grids and negatively stained with 3% uranyl acetate. The grids were observed under a JEOL 100CXII electron microscope at an accelerating voltage of 80 kV.

Antibodies and Immunofluorescence—An anti-*Tetrahymena* fimbrin antiserum was prepared in the previous study (*7*). The antibody was affinity-purified with the recombinant *Tetrahymena* fimbrin by the method of Talian *et al.* (*27*). *Tetrahymena* cells were fixed with 0.2%

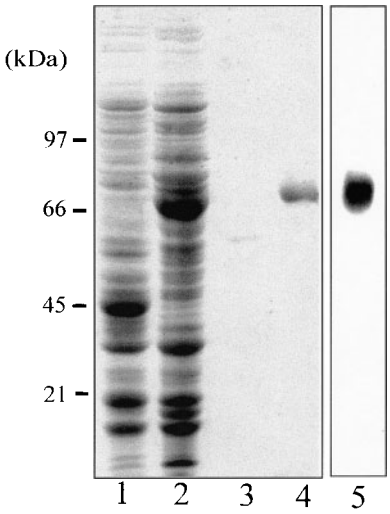

Fig. 1. **Expression and purification of the recombinant *Tetrahymena* fimbrin.** *E. coli* cells transformed with pQE60- *Tetrahymena* fimbrin were solubilized by sonication before (lane 1) and after 1 mM IPTG induction (lane 2). The soluble fraction obtained on sonication was incubated with Ni-NTA agarose resin. The Ni-NTA agarose resin was washed with 50 mM imidazole (lane 3). The bound proteins were eluted with 250 mM imidazole (lane 4). Proteins were resolved by 15% SDS-PAGE and stained with CBB. The fraction eluted with 250 mM imidazole was subjected to immunoblotting with anti-*Tetrahymena* fimbrin antiserum (lane 5). Molecular mass markers are on the left.

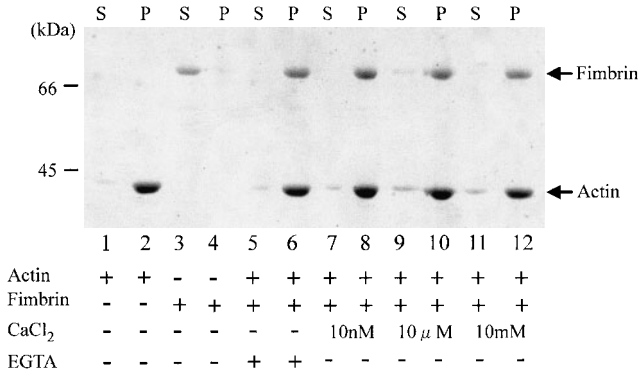

Fig. 2. **Binding of *Tetrahymena* fimbrin to skeletal muscle F-actin.** Fimbrin and rabbit skeletal muscle F-actin in F-buffer were incubated and ultracentrifuged; the supernatants (S) and pellets (P) were analyzed by 15% SDS-PAGE. S and P of the samples containing actin alone (lanes 1 and 2), fimbrin alone (lanes 3 and 4), actin and fimbrin with 5 mM EGTA (lanes 5 and 6), actin and fimbrin with 10 nM $CaCl_2$ (lanes 7 and 8), actin and fimbrin with 10 μM $CaCl_2$ (lanes 9 and 10), and actin and fimbrin with 1 mM $CaCl_2$ (lanes 11 and 12) are indicated. The bands corresponding to fimbrin and actin are indicated by arrows. Molecular mass markers are on the left.

formaldehyde in methanol at –20°C. Fixed cells were incubated with the affinity-purified rabbit anti-*Tetrahymena* fimbrin antibody (diluted 1:15 with 5% nonfat skimmed milk in PBS) for 12 h at room temperature. The specimens were incubated with FITC-conjugated goat anti-rabbit IgG antibody (KPL, USA; diluted 1:100 with 5% nonfat skimmed milk in PBS) for 1 h at room temperature. The specimens were mounted with PBS containing 50% glycerol and 0.1 mg/ml of anti-fading reagent ρ-phenylenediamine. The specimens were observed under a confocal microscope, LSM510 (Carl Zeiss, Germany).

RESULTS

Expression and Purification of Tetrahymena Fimbrin—For the cloning of *Tetrahymena* fimbrin full length cDNA, four primers were designed. Fragments of *Tetrahymena* fimbrin cDNA of 170 and 1,570 bp length were amplified by PCR with primers S-1 and AS-1, and primers S-2 and AS-2, respectively. These fragments were digested with *Taq* I and then ligated. The ligated cDNA was used in this study as the *Tetrahymena* fimbrin full-length cDNA.

To obtain recombinant *Tetrahymena* fimbrin, we attempted to express the *Tetrahymena* fimbrin cDNA in *E. coli*. Since *Tetrahymena* transcribes universal stop codons, TAA and TAG as glutamine codons, *Tetrahymena* genes with TAA and TAG in their open reading frames are not expressed in *E. coli*. To express the *Tetrahymena* fimbrin cDNA in *E. coli*, we changed eleven TAA and four TAG to "universal" glutamine codons, CAA and CAG, by site-directed mutagenesis. The changed *Tetrahymena* fimbrin cDNA was amplified by PCR with primer S-1 and TmNo.9. The PCR product was subcloned into expression vector pQE60, in which *Tetrahymena* fimbrin cDNA was fused to six consecutive histidine codons to facilitate purification of the recombinant *Tetrahymena* fimbrin. *Tetrahymena* fimbrin was expressed in *E. coli* and then purified by Ni^{2+} chelating column chromatography. Expression of the recombinant *Tetrahymena* fimbrin in *E. coli* was induced by the addition of 1 mM IPTG (Fig. 1, lane 2). The recombinant *Tetrahymena* fimbrin was found in the soluble fraction of the total bacterial lysate and was bound to Ni-NTA agarose resin. As expected, the recombinant *Tetrahymena* fimbrin was purified as a single protein of 70 kDa (Fig. 1, lane 4) and specifically reacted with the anti-*Tetrahymena* fimbrin antiserum (Fig. 1, lane 5).

F-actin Binding Activity of Tetrahymena Fimbrin—To examine the binding activity of the recombinant *Tetrahymena* fimbrin as to F-actin, we performed a cosedimentation assay with the recombinant *Tetrahymena* fimbrin and F-actin. In the case of F-actin alone, most F-actin was precipitated on high-speed centrifugation (Fig. 2, lane 2). On the other hand, in the case of fimbrin alone, most fimbrin was not precipitated (Fig. 2, lane 3). In the presence of F-actin and fimbrin, fimbrin was coprecipitated with F-actin (Fig. 2, lane 6).

Tetrahymena fimbrin has no EF-hand motifs at its N terminal end. We examined the Ca^{2+} sensitivity of its actin-binding activity. Fimbrin was coprecipitated with F-actin whether $CaCl_2$ existed in the F-buffer or not (Fig. 2, lanes 8, 10, and 12). Therefore, *Tetrahymena* fimbrin bound to F-actin in a Ca^{2+}-independent manner.

The molar ratio of the *Tetrahymena* fimbrin to F-actin was examined by means of a cosedimentation assay. Fimbrin coprecipitation with F-actin increased with the fimbrin concentration (Fig. 3A). The binding of fimbrin to F-actin was saturated at about 0.7 fimbrin molecules per actin molecule. Scatchard analysis of the binding of fim-

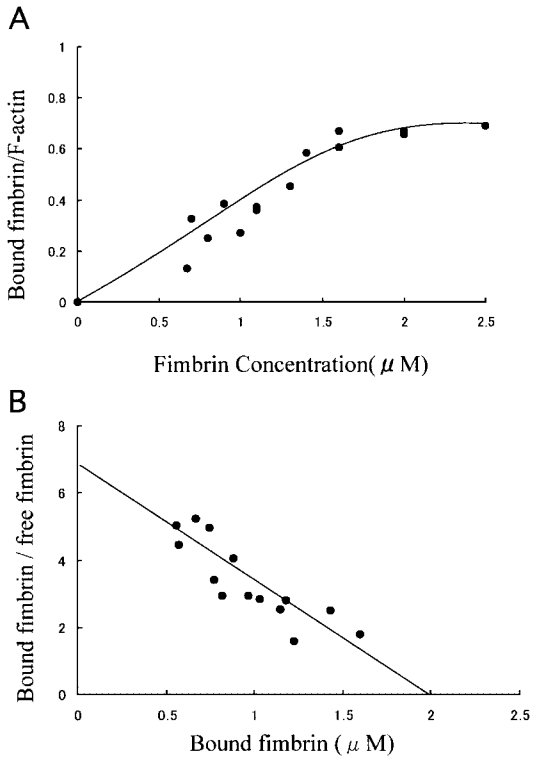

Fig. 3. **The binding ratio of *Tetrahymena* fimbrin to F-actin with various fimbrin concentrations.** (A) Various concentrations of fimbrin (0.67–2.5 μM) and 1.5 μM rabbit skeletal muscle F-actin were mixed in F-buffer and then analyzed by means of a cosedimentation assay. (B) Scatchard analysis of the data in (A).

brin to F-actin indicated that K_d = 0.3 μM and the binding ratio of fimbrin : actin was 1:1.4 (Fig. 3B).

F-Actin Bundling Activity of Tetrahymena Fimbrin—To examine the F-actin bundling activity of *Tetrahymena* fimbrin, we performed high-speed and low-speed cosedimentation assays. *Tetrahymena* fimbrin and rabbit skeletal muscle F-actin were mixed, incubated at 26°C for 30 min in F-buffer, and then centrifuged at 12,000 ×*g* (low-speed). The supernatant obtained on low speed centrifugation was ultracentrifuged again at 200,000 ×*g* (high-speed). The actin filament bundles were precipitated on low-speed centrifugation and the single actin filaments were precipitated on high-speed centrifugation (Fig. 4). F-actin alone was not precipitated on low-speed centrifugation (Fig. 4, lane 1), but mostly precipitated on high-speed centrifugation (Fig. 4, lane 4). Fimbrin was precipitated on neither low- nor high-speed centrifugation (Fig. 4, lanes 6 and 8). In the presence of F-actin and fimbrin, fimbrin was coprecipitated with F-actin on low-speed centrifugation (Fig. 4, lane 10).

Next, we examined the Ca^{2+} sensitivity of the F-actin bundling activity of *Tetrahymena* fimbrin. In the presence of 10 nM, 10 μM, or 1 mM $CaCl_2$, most fimbrin was coprecipitated with F-actin on low-speed centrifugation (Fig. 4, lanes 14, 18, and 22). These results show that *Tetrahymena* fimbrin has no Ca^{2+} sensitivity.

We examined the molar ratio of *Tetrahymena* fimbrin and actin required for F-actin bundling. As the amount of fimbrin increased, the amount of F-actin precipitated on

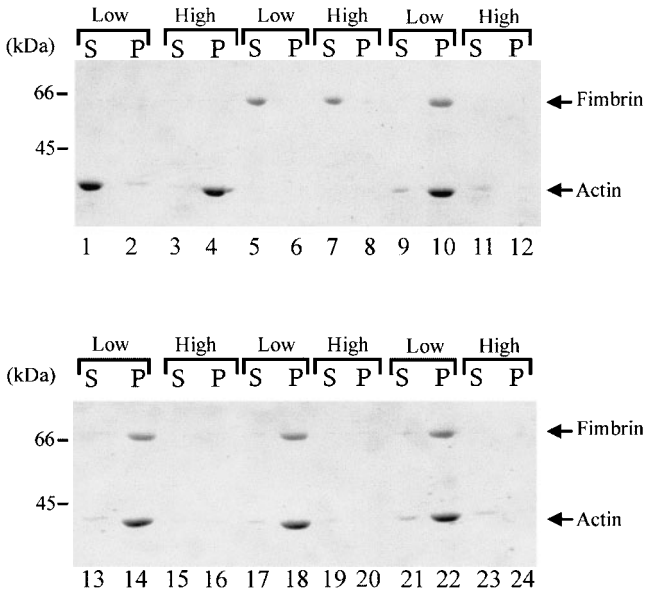

Fig. 4. **Bundling of rabbit skeletal muscle F-actin by *Tetrahymena* fimbrin.** Low-speed centrifuged samples (Low) and high-speed centrifuged samples (High) were analyzed by 15% SDS-PAGE. The supernatants (S) and pellets (P) of the samples containing F-actin alone (lanes 1–4), fimbrin alone (lanes 5–8), F-actin and fimbrin (lanes 9–12), F-actin and fimbrin with 10 nM $CaCl_2$ (lanes 13–16), F-actin and fimbrin with 10 μM $CaCl_2$ (lanes 17–20), and F-actin and fimbrin with 1 mM $CaCl_2$ (lanes 21–24) are indicated. The bands corresponding to fimbrin and F-actin are indicated by arrows. Molecular mass markers are on the left.

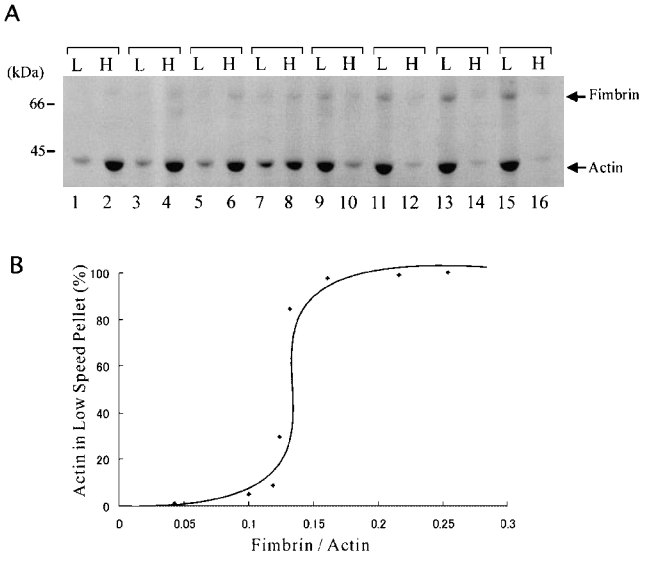

Fig. 5. **Bundling F-actin increased depending on the concentration of *Tetrahymena* fimbrin.** (A) Various concentrations of fimbrin (0.12–0.75 μM) and 3 μM F-actin were analyzed by means of high-speed and low-speed cosedimentation assays. The low- (L) and high-speed centrifugation pellets (H) were analyzed by SDS-PAGE. The concentrations of fimbrin incubated with F-actin were 0.12 μM (lanes 1 and 2), 0.3 μM (lanes 3 and 4), 0.36 μM (lanes 5 and 6), 0.38 μM (lanes 7 and 8), 0.42 μM (lanes 9 and 10), 0.48 μM (lanes 11 and 12), 0.66 μM (lanes 13 and 14), and 0.75 μM (lanes 15 and 16). The bands corresponding to fimbrin and F-actin are indicated by arrows. Molecular mass markers are on the left. (B) The percentage of the amount of actin in the low-speed centrifugation pellet was plotted against the fimbrin/actin concentration ratio and fitted to a sigmoidal curve.

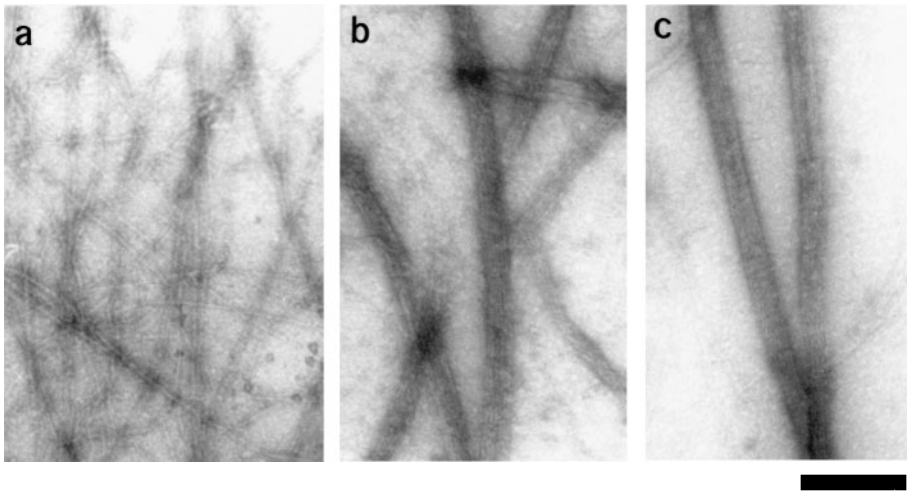

Fig. 6. **F-actin bundles induced by *Tetrahymena* fimbrin.** Electron micrographs show 2 μM F-actin alone (a), a mixture of 2 μM F-actin and 0.5 μM fimbrin (b), and a mixture of 2 μM F-actin and 0.5 μM fimbrin with 5 mM $CaCl_2$ (c). The bar represents 100 nm.

low-speed centrifugation increased (Fig. 5A); we fitted this relationship to a sigmoidal curve (Fig. 5B). When the molar ratio of fimbrin/actin was 0.15, the amount of bundled F-actin was saturated.

F-Actin Bundles Induced by Tetrahymena Fimbrin—We examined the F-actin bundling activity of *Tetrahymena* fimbrin under an electron microscope. In the case of F-actin alone, single actin filaments were observed (Fig. 6a). In the presence of fimbrin, tightly bundled actin filaments were observed (Fig. 6b). Many tightly bundled actin filaments were observed in the presence of 5 mM $CaCl_2$ (Fig. 6c) as well as in the absence of $CaCl_2$.

Localization of Tetrahymena Fimbrin—Our previous study showed that *Tetrahymena* fimbrin is localized in the oral apparatus, deep fibers, and contractile vacuole pores in interphase cells, and in the cleavage furrow in dividing cells (7). In this study, anti-*Tetrahymena* fimbrin antibody was affinity-purified with the recombinant *Tetrahymena* fimbrin and samples were observed under a confocal laser microscope. Among all the proteins in the *Tetrahymena* cell lysate, affinity-purified anti-*Tetrahymena* fimbrin antibody only reacted with *Tetrahymena* fimbrin (Fig. 7A, lane 2). In this study, *Tetrahymena* fimbrin was found to be localized in the apical ring and oral apparatus but not in contractile vacuole pores in interphase cells (Fig. 7B). The localization to the apical ring was a new finding in this study. In the previous study, anti-*Tetrahymena* fimbrin antibody was affinity- purified with the 60 kDa fragment of a 70 kDa full length fimbrin. Therefore, the difference in localization of fimbrin between the previous and present studies is due to the fimbrin used to obtain affinity-purified antibodies.

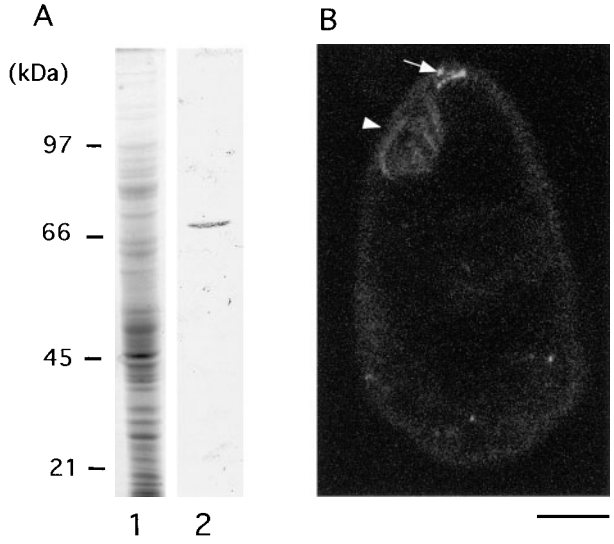

Fig. 7. **Localization of *Tetrahymena* fimbrin in an interphase cell.** (A) The specificity of the affinity-purified anti-*Tetrahymena* fimbrin antibody. A *T. thermophila* cell lysate was separated by SDS-PAGE followed by staining with CBB (lane 1) and then analyzed by immunoblotting with the affinity-purified anti-*Tetrahymena* fimbrin antibody (lane 2). Molecular mass markers are on the left. (B) Immunofluorescence localization of *Tetrahymena* fimbrin in an interphase cell. The arrow indicates the apical ring. The arrowhead indicates the oral apparatus. The scale bar represents 10 μm.

During cytokinesis, *Tetrahymena* fimbrin was localized in the cortical region of the cleavage furrow as a ring structure at the early to late stages of cytokinesis (Fig. 8). At an early stage of cytokinesis, fimbrin was localized in a thin dotted line along the cleavage furrow (Fig. 8a). From the mid to late stages of cytokinesis, fimbrin was localized in a thick dotted line in the division furrow (Fig. 8, b and c). At the final stage of cytokinesis, fluorescence also appeared in an intercellular bridge, *i.e.* on the verge of segregation into daughter cells (Fig. 8d). This clearly shows the ring localization of fimbrin in *Tetrahymena*.

DISCUSSION

The contractile ring is composed of actin, myosin, and many actin-binding proteins. *Tetrahymena* fimbrin is localized in the cleavage furrow. Our previous study was the first to show the localization of fimbrin in the cleavage furrow (7). This localization suggests that *Tetrahymena* fimbrin plays a role in the formation of the contractile ring. Although fimbrin tightly bundles actin filaments, the contractile ring is thought to comprise flexible actin bundles. Therefore, the biochemical properties of *Tetrahymena* fimbrin are very interesting. However, its properties were poorly revealed in the previous study,

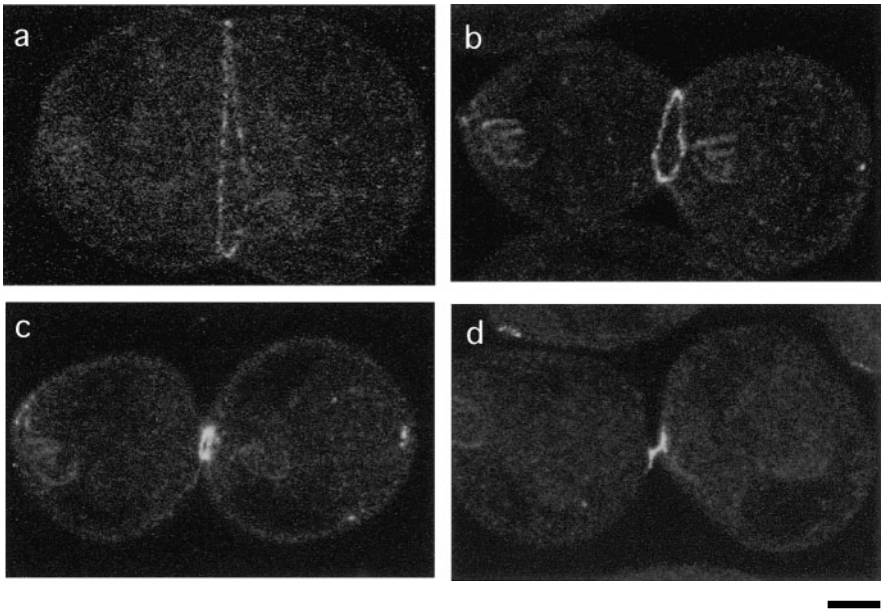

Fig. 8. **Localization of *Tetrahymena* fimbrin in dividing cells.** Dividing cells at various stages of cytokinesis were stained with the affinity-purified anti-*Tetrahymena* fimbrin antibody. Confocal laser micrographs of immunofluorescence images are shown, arranged in the order of early (a), mid (b), late (c), and end (d) stages of cytokinesis. The scale bar represents 10 μm.

because *Tetrahymena* fimbrin was eluted as a 60 kDa fragment from an F-actin affinity column; the purification of full length *Tetrahymena* fimbrin was impossible. In this study, we succeeded in elucidation of the biochemical properties of *Tetrahymena* fimbrin *in vitro* using the recombinant protein and found its localization along the contractile ring on confocal laser microscopy.

Ca^{2+} is thought to be an important signal for the organization of cytokinesis in *Tetrahymena*. Ca^{2+}/CaM regulates the division plane determination by p85 (*4*) and inhibits the actin bundling activity of EF-1α (*10*). The F-actin binding activity of human L-plastin is reduced by 10 μM $CaCl_2$ (*19*). Similarly, the actin-binding activity of *Dictyostelium* plastin and chicken fimbrin is Ca^{2+}-sensitive (*18*, *28*). However, *Tetrahymena* fimbrin lacks EF-hand motifs. A cosedimentation assay and electron microscopy showed that *Tetrahymena* fimbrin bound to actin filaments, and bundled them in the presence of 10 nM, 10 μM, or 1 mM $CaCl_2$ (Figs. 2 and 4). Therefore, the actin filament-binding activity of *Tetrahymena* fimbrin is Ca^{2+}-insensitive.

The binding ratio of *Tetrahymena* fimbrin to actin was 1 to 1.4 (Fig. 3). *Tetrahymena* fimbrin has two actin-binding domains. If these actin-binding domains had the same affinity to actin, the molecular ratio of fimbrin to actin would be 1 to 2. The ratio of 1 to 1.4 thus suggests that these actin-binding domains have different binding affinities to F-actin. *Dictyostelium* plastin and AtFim1 have been reported to have two different K_d values for high and low concentrations of fimbrin (*18*, *20*). In addition, genetic analysis of *S. cerevisiae* fimbrin (sac6p) showed that its two actin-binding domains bind to the same region of actin but have different affinities to it (*29*). Human L-plastin (*19*), *Dictyostelium* plastin (*18*), chicken fimbrin (*30*), sac6p (*28*), and AtFim1 (*20*) bind to actin at ratios from 1:2 to 1:8. The molecular ratio of *Tetrahymena* fimbrin to actin is higher than those of other fimbrins. Since the actin-binding domains in *Tetrahymena* fimbrin are thought to have different K_d values and one of them exhibits higher affinity to actin, the average dissociation constant (K_d) of fimbrin as to actin was 0.3 μM in this experiment. The K_d value of *Tetrahymena* fimbrin is similar to those (0.35–0.5 μM) of *Dictyostelium* plastin and AtFim1 at a low fimbrin concentration. These data suggest that most *Tetrahymena* fimbrin binds to actin, and that *Tetrahymena* fimbrin exhibits higher affinity to actin than do other fimbrins.

At the molar ratio of 1 fimbrin to 7 actins, fimbrin caused complete actin filament bundling (Fig. 5). This ratio was in good agreement with the ratio of human L-plastin to actin in actin filament bundling (*19*). In addition, 2-dimensional and 3-dimensional analysis of actin filament bundles induced by fimbrin revealed that one fimbrin molecule binds to eight actin molecules, and then forms hexagonal latticed actin bundles (*31*). Our data suggest that *Tetrahymena* fimbrin and other fimbrins form similar hexagonal actin bundles.

In interphase cells, strong immunofluorescence for *Tetrahymena* fimbrin appeared in the apical ring and faint immunofluorescence appeared in the oral apparatus (Fig. 7B). The apical ring contains microfilaments, individually approximately 5 to 7 nm in diameter, and is situated directly underneath the apical crown of basal-body couplets in *Tetrahymena* (*32*). Its chemistry is unknown, but an anti-actin immunofluorescence study showed that it does not contain actin (*3*). Therefore, the apical ring is distinct from the contractile ring associated with the division furrow in *Tetrahymena*. In interphase cells, *Tetrahymena* actin was localized in the oral apparatus and deep fibers, and the proximity of the cytoproct, which are organelles involved in endocytosis and exocytosis, respectively (*3*). In addition, *Tetrahymena* actin was localized at the periphery of food vacuoles, suggesting that actin was involved in endocytosis and exocytosis (*3*). Although yeast fimbrin associates functionally with actin structures involved in the internalization step of endocytosis *in vivo* (*33*), *Tetrahymena* fimbrin does not associate with actin filaments around food vacuoles or the cytoproct (Fig. 7B), and has no function in endocytosis and exocytosis.

During cytokinesis, *Tetrahymena* fimbrin was localized in the cleavage furrow as a ring structure and the immunofluorescence for fimbrin in the cleavage furrow intensified as cytokinesis progressed (Fig. 8). In addition, *Tetrahymena* fimbrin was localized in the intercellular bridge at the end stage of cytokinesis (Fig. 8d). These results suggest that *Tetrahymena* fimbrin was localized along the contractile ring and should be involved in the dynamic organization of the contractile ring. Thus, we proposed that *Tetrahymena* fimbrin associates especially with actin filaments in the contractile ring and plays an important role in contractile ring organization.

Another actin-binding protein, EF-1α, is localized in the *Tetrahymena* cleavage furrow and its actin-bundling activity is regulated by Ca^{2+}/CaM. The K_d value of EF-1α as to F-actin is 3.4 μM (*34*), suggesting that the binding activity of *Tetrahymena* fimbrin should be higher than that of EF-1α. In *Tetrahymena*, two actin-bundling proteins, which exhibit different Ca^{2+} sensitivities and different binding activities as to F-actin, may regulate the organization of actin filaments in the contractile ring.

This study clearly showed that *Tetrahymena* fimbrin, which is localized in the cleavage furrow, is a Ca^{2+}-insensitive actin bundling protein. It exhibits high affinity to F-actin and induces F-actin bundles *in vitro*. The biochemical properties of *Tetrahymena* fimbrin are similar to those of other fimbrin/plastin family proteins except for its high affinity to F-actin. Therefore, *Tetrahymena* fimbrin possibly plays a key role in the formation of the contractile ring during cytokinesis. Further studies involving gene transformation through gene knockout techniques on the role of fimbrin in contractile ring formation may shed light on the complete mechanism of cytokinesis in *Tetrahymena*.

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