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Interactions among a Fimbrin, a Capping Protein, and an Actin-depolymerizing Factor in Organization of the Fission Yeast Actin Cytoskeleton

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We report studies of the fission yeast fimbrin-like protein Fim1, which contains two EF-hand domains and two actin-binding domains (ABD1 and ABD2). Fim1 is a component of both F-actin patches and the F-actin ring, but not of F-actin cables. Fim1 cross-links F-actin *in vitro,* but a Fim1 protein lacking either EF-hand domains (Fim1A12) or both the EF-hand domains and ABD1 (Fim1A2) has no actin cross-linking activity. Overexpression of Fim1 induced the formation of F-actin patches throughout the cell cortex, whereas the F-actin patches disappear in cells overexpressing Fim1A12 or Fim1A2. Thus, the actin cross-linking activity of Fim1 is probably important for the formation of F-actin patches. The overexpression of Fim1 also excluded the actin-depolymerizing factor Adf1 from the F-actin patches and inhibited the turnover of actin in these structures. Thus, Fim1 may function in stabilizing the F-actin patches. We also isolated the gene encoding Acp1, a subunit of the heterodimeric F-actin capping protein. *fim1 acp1* double null cells showed more severe defects in the organization of the actin cytoskeleton than those seen in each single mutant. Thus, Fim1 and Acp1 may function in a similar manner in the organization of the actin cytoskeleton. Finally, genetic studies suggested that Fim1 may function in cytokinesis in cooperation with Cdc15 (PSTPIP) and Rng2 (IQGAP), respectively.

INTRODUCTION

In eukaryotes, the actin cytoskeleton plays important roles in various cellular events such as migration, organelle transport, morphogenesis, and cytokinesis. Its reorganization in the cell is believed to be regulated by a variety of actin-modulating proteins (Pollard and Cooper, 1986; Ayscough, 1998; Kreis and Vale, 1999). Among these proteins, the actin cross-linking proteins are considered to contribute to the three-dimensional (3-D) organization of the actin cytoskeleton (Matsudaira, 1991; Puius *et al.*, 1998). These proteins function through two or more F-actin–binding domains in the molecule. The distance between the domains is an important determinant of the type of F-actin structure that forms: cross-linkers in which the two binding domains are in close proximity tend to form tightly packed bundles, whereas those in which the two domains are distant tend to form loose aggregates.

A number of actin cross-linking proteins, such as α-actinin, spectrin, ABP-280, dystrophin, and ABP-120 share a homologous F-actin–binding domain (ABD) that has been conserved during evolution (Matsudaira, 1991; Puius *et al.*, 1998). Most of these proteins have a single ABD and function as multimers. In contrast, fimbrin has two ABDs tandemly arranged in a single polypeptide chain. Fimbrin was first identified in chicken intestinal microvilli (Matsudaira and Burgess, 1979; Bretscher and Weber, 1980) and is now known to be associated with actin bundles in various structures in vertebrate cells, including membrane ruffles, microspikes, and focal contacts. Fimbrin is widely conserved from yeast to human (Puius *et al.*, 1998). Sac6p, the homologue of fimbrin in the budding yeast *Saccharomyces cerevisiae*, has been identified both by F-actin affinity column (Drubin *et al.*, 1988) and through dominant suppression of a temperature-

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Table 1. Fission yeast strains used in this study

Strain	Genotype	Source
JY1	h^-	Lab. stock
leu1	h^- *leu1-32*	Lab. stock
JY336	h^+ *ade6-M216 leu1-32*	Lab. stock
JY741	h^- *ade6-M216 leu1-32 ura4-D18*	Lab. stock
JY746	h^+ *ade6-M210 leu1-32 ura4-D18*	Lab. stock
cdc3	h^- *ade6-M210 leu1-32 ura4-D18 cdc3-124*	Lab. stock
cdc4	h^- *leu1-32 ura4-D18 cdc4-8*	Lab. stock
cdc7	h^- *ura4-D18 cdc7-24*	Lab. stock
cdc8	h^- *ade6-M210 leu1-32 ura4-D18 cdc8-110*	Lab. stock
cdc11	h^- *ura4-D18 cdc11-136*	Lab. stock
cdc12	h^- *ura4-D18 cdc12-112*	Lab. stock
cdc14	h^- *ura4-D18 cdc14-118*	Lab. stock
cdc15	h^- *ura4-D18 cdc15-140*	Lab. stock
cdc25	h^- *cdc25-22*	Lab. stock
rng2	h^- *ade6-M210 leu1-32 ura4-D18 rng2-D5*	M. K. Balasubramanian
nda3	h^- *nda3-KM311*	Lab. stock
cps8	h^- *leu1-32 cps8*	J. Ishiguro
KFM101	h^+ *ade6-M216 leu1-32 ura4-D18 fim1*$^+$::*ura4*$^+$	This study
KFM102	h^- *ade6-M210 leu1-32 ura4-D18 fim1*$^+$::*ura4*$^+$	This study
KAC101	h^+ *ade6-M216 leu1-32 ura4-D18 fim1*$^+$::*ura4*$^+$	This study

sensitive actin mutation (Adams *et al.*, 1989). It has been shown that Sac6p localizes to actin patches and actin cables in *S. cerevisiae* (Drubin *et al.*, 1988); moreover, it has been reported that loss-of-function mutants of Sac6p display abnormal organization of the actin cytoskeleton (Adams *et al.*, 1991). In addition, two human homologues of fimbrin, T- and L-plastin, can replace the function of Sac6p in *sac6* mutant cells (Adams *et al.*, 1995).

In the fission yeast *Schizosaccharomyces pombe*, actin is organized during interphase as cortical F-actin patches that are localized at the growing ends of the cell and F-actin cables running along the long axis of the cell (Marks and Hyams, 1985; Arai *et al.*, 1998). The functions of these structures are thought to be important for cell morphogenesis (Kobori *et al.*, 1989; Nurse, 1994; Ishiguro and Kobayashi, 1996; Ishiguro, 1998). During mitosis, the F-actin ring forms in the middle cortex of the cell, where it contracts during cytokinesis as does the contractile ring in animal cells (Marks and Hyams, 1985; Kanbe *et al.*, 1989; LeGoff *et al.*, 1999). However, the mechanisms of actin cytoskeleton regulation are not well known in fission yeast. Only a few actin-modulating proteins such as Cdc3 (profilin; Balasubramanian *et al.*, 1994), Cdc8 (tropomyosin; Balasubramanian *et al.*, 1992; Arai *et al.*, 1998), component proteins of the Arp2/Arp3 complex (Lees-Miller *et al.*, 1992; Balasubramanian *et al.*, 1996; McCollum *et al.*, 1996; Arai *et al.*, 1998; Morrell *et al.*, 1999), and Adf1 (actin depolymerizing factor/cofilin; K. Nakano, M. Kawamukai, and I. Mabuchi, unpublished results) have been characterized. Therefore, the identification of additional actin-binding proteins should be an important step in elucidating the mechanisms of actin regulation.

In this article, we report studies of the function of an *S. pombe* fimbrin-like actin cross-linking protein, Fim1, which is a component of both the F-actin patches and the F-actin ring. We also report the identification and initial characterization of an actin-capping protein, Acp1. Our studies suggest that regulation of the actin cytoskeleton involves interactions among Fim1, Adf1, and Acp1.

MATERIALS AND METHODS

Genetic Techniques and DNA Manipulation

The *S. pombe* strains used in this study are listed in Table 1. The growth media have been described previously (Moreno *et al.*, 1991). Complete medium YEA and minimum medium EMM were used to grow *S. pombe* strains. MEA was used to induce conjugation and sporulation. Standard procedures for *S. pombe* genetics were followed according to Alfa *et al.* (1993) and Moreno *et al.* (1991). Standard methods were used for DNA manipulations (Sambrook *et al.*, 1989). Amino-acid sequence alignments and calculation of protein similarities were performed with the use of the DNA Star software (DNASTAR Inc., Madison, WI).

Identification of Fim1 by F-actin Affinity Column Chromatography

Extracts of the wild-type *S. pombe* strain *JY1* were prepared as described by Arai *et al.* (1998). F-actin columns for affinity chromatography were prepared according to Miller and Alberts (1989) and Terasaki *et al.* (1997). The column was equilibrated with A-buffer (50 mM HEPES, 2 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA, 1.1 M glycerol, 0.05% NP-40, 2 mM TAME, 0.1 mM PMSF, 10 μg/ml leupeptin, pH 7.5). After the cell extract was applied, the column was washed with three column volumes of A-buffer. Bound proteins were then eluted with A-buffer containing 0.2 M KCl, 1 mM ATP, and 3 mM $MgCl_2$.

Cloning of fim1$^+$ *and Gene Expression in Fission Yeast*

cDNA clones encoding full-length Fim1 (amino acids 1–614), Fim1A12 (amino acids 108–614), and Fim1A2 (amino acids 369–614) were amplified by PCR with the use of an *S. pombe* cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) as the template and were cloned into the *Eco*RI and *Sal*I sites of the pBluescriptSK$^-$ (pSK) vector. To construct the vectors pSKfim1, pSKfim1A12, and pSKfim1A2, we used the after PCR primers: 5′-cccgaattccatatgttagctcttaaacttcaaaag-3′ and 5′-ccggtcgacgtactcacttccgaagttg-3′ for Fim1; 5′-gaattccatatgtcttcctcaagtgtttctcatacc-3′ and 5′-ccggtcgacgtactcacttccgaagttg-3′ for Fim1A12; and 5′-cccgaattccatatggagccttta-

aatgaagaggaaaagc-3′ and 5′-ccggtcgacgtactcacttccgaagttg-3′ for Fim1A2. pSKfim1EA1 (amino acids 1–429) and pSKfim1A1 (amino acids 108–429) were constructed by deletion of the *Hpa*I and *Sal*I regions in pSKfim1 and pSKfim1A12, respectively. These constructs were digested with *Nde*I and *Xho*I, and the inserts were ligated into *Nde*I and *Sal*I sites of the fission yeast expression vectors, pREP1 or pREP81 (Maundrell, 1993), respectively. pREP1 has a promoter stronger than pREP81 (Forsburg, 1993). Expression of exogenous genes from these plasmids was repressed by 5 μM thiamin in medium.

To express the fusion protein FimA2-YFP (a yellow-green variant of the *Aequorea victoria* green fluorescent protein) in *cdc8* cells, pEYFP (Clontech) was used as a template for PCR with the use of the primers 5′-ggggatccgtcgacgcatatgcagatctcaatggtgagcaagggc-3′ and 5′-ggggagctctagctcgagaattccttgtacagctcgtccatgccg-3′. After the PCR, the 0.7-kb product was digested with *Nde*I and *Eco*RI and subcloned together with the 0.8-kb fragment derived from digesting pSKfim1A2 with *Eco*RI and *Xho*I into the *Nde*I and *Sal*I sites of pREP1. The pREP1YFP-Fim1A2 vector was then transformed into *cdc8* cells.

Protein Expression and Purification

After digestion with *Eco*RI and *Xho*I, the DNA fragment encoding Fim1 or each of the truncated Fim1 proteins was inserted into the *Eco*RI and *Sal*I sites of the GST protein vector pGEX4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). The GST fusion proteins were purified according to the manufacturer's protocol. For use in cosedimentation assays with F-actin, the GST fusion proteins were cleaved with 1 U/μl human thrombin (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) in thrombin buffer (0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT) at 25°C for 1 h or 4°C overnight. Thrombin was then removed by incubation with *p*-aminobenzamidine-agarose beads (Sigma Chemical Co., St. Louis, MO) at 4°C for 30 min. In addition, the 1.8-kb *Eco*RI- and *Sal*I-digested fragment encoding Fim1 was cloned into *Eco*RI and *Sal*I sites of pMAL2c (New England Biolabs, Inc., Beverly, MA) for production of a maltose-binding protein fused with Fim1 (MAL-Fim1). MAL-Fim1 was purified with amylose resin according to the manufacturer's protocol.

Production of Antisera

GST-Fim1 was emulsified with Freund's complete adjuvant (Difco, Detroit, MI), and 0.1 mg protein was injected into a female white rabbit. After the first injection, 0.2 mg protein each emulsified with Freund's incomplete adjuvant (Difco) was injected into the rabbit at 2-week intervals. After the fifth injection, serum was harvested. The antibodies were purified from the antiserum by membrane affinity adsorption with the use of MAL-Fim1 according to the method of Smith and Fisher (1984). Fim1 was detected by Western blotting as described previously (Nakano *et al.* 1997).

F-actin Cosedimentation Assay

Forty-five microliters of 2 μM rabbit skeletal muscle F-actin in F-buffer (0.1 M KCl, 2 mM $MgCl_2$, 0.7 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM DTT, and 10 mM Tris-HCl, pH 7.5) and 5 μl of 4 μM wild-type or mutant Fim1 were mixed and incubated at room temperature for 2 h. The actin filament bundles were sedimented by centrifugation at 10,000 × *g* for 20 min, whereas the actin filaments were sedimented at 100,000 × *g* for 30 min. Equal portions of the pellets and the supernatants were loaded onto SDS-10% polyacrylamide gels.

Microscopy

The cells were fixed and processed for fluorescence microscopy as described previously (Arai *et al.*, 1998). For immunolocalization of Fim1, actin, or Arp2, the affinity-purified anti-Fim1 antibodies, anti-actin antibodies (Arai *et al.*, 1998), or anti-Arp2 antibodies (Morrell *et al.*, 1999), respectively, were used. The cells were stained with rhodamine-phalloidin or bodipy-phallacidin as described (Alfa *et al.*, 1993; Arai *et al.*, 1998) and were visualized by two approaches. Conventional images were obtained with the use of a Zeiss Axioskop fluorescence microscope equipped with a Plan Apochromat 63× lens (Carl Zeiss, Oberkochen, Jena, Germany) and photographed on Kodak T-MAX ASA 400 films (Eastman Kodak, Rochester, NY). 3-D images were obtained with the use of a Delta Vision system (Applied Precision, Issaquah, WA) attached to an Olympus IX-70-SIF fluorescence microscope equipped with a UplanApo 100× oil lens (Olympus, Tokyo, Japan) as described previously (Motegi *et al.*, 2000).

Gene Disruption

To disrupt the *fim1*$^+$ gene, we prepared a construct containing the 4.5-kb *Bgl*II and *Sac*I genomic DNA fragment in which the *fim1*$^+$ open reading frame (ORF) lacking a region coding for the six C-terminal amino acid residues was replaced by *ura4*$^+$. In detail, *S. pombe* genomic DNA was amplified by PCR with the use of the after two sets of primers: 5′-attgcctgtacaaaaagaacacac-3′ and 5′-gccggtaccacacgatgtgtgctttggg-3′, and 5′-gccctgcagtttaatggccgtataatttg-3′ and 5′-gcgaagcttacatgttgcctgctaaagccgc-3′. The PCR products corresponded to upstream and downstream regions of *fim1*$^+$, respectively. The PCR product derived from the first set of primers was digested with *Sac*I and *Kpn*I, and the second product was digested with *Hin*dIII and *Pst*I. These DNA fragments were inserted into the *Sac*I and *Kpn*I sites, and *Hin*dIII and *Pst*I sites, respectively, in the pUC18 vector containing the *ura4*$^+$ marker gene in the *Hin*cII site. This construct, pfim1::ura4$^+$, was digested with *Bgl*II and *Sac*I and used to transform a diploid produced by mating *JY741* and *JY746*. The correct integration was verified by Southern blotting.

To disrupt the *acp1*$^+$ gene, we prepared a construct containing the 2.8-kb *Spe*I and *Afl*II genomic DNA fragment in which the *Acc*I and *Spe*I region including the *acp1*$^+$ ORF was replaced by *ura4*$^+$. The diploid strain was transformed with this construct, pacp1::ura4$^+$, after digestion with *Spe*I and *Afl*II.

RESULTS

Identification and Localization of Fim1 in Fission Yeast

We used F-actin affinity column chromatography to look for novel actin-binding proteins in fission yeast. When an *S. pombe* cell lysate was applied to the F-actin affinity column, several proteins including a major 67-kDa protein were obtained by elution with 0.2 M KCl. Digestion of the 67-kDa protein by lysyl endopeptidase gave rise to two internal amino acid sequences: YRLLEEDETLDQFLRLP and KAVENCNYAVDLG. The sequences of these peptides corresponded closely to those of peptides from Sac6p (Adams *et al.*, 1991) and also matched sequences in the recently reported sequence of *S. pombe* Fim1 (Accession No. 97208; Wu *et al.*, 2001). Fim1 belongs to the fimbrin family of actin cross-linking proteins, which contain 2 EF-hand domains and a tandem duplication of actin-binding domains (ABD1 and ABD2, from the N-terminal to the C-terminal).

In whole-cell homogenates of wild-type cells, affinity-purified anti-Fim1 antibodies recognized specifically a single band corresponding to the molecular weight of Fim1, whereas in *fim1*-null cells no band was detected (Figure 1A). By immunofluorescence microscopy, we found that Fim1 localized as patch-like structures mainly at the ends of interphase cells, most of which corresponded with F-actin patches (Figure 1, B and C). By contrast, Fim1 was not

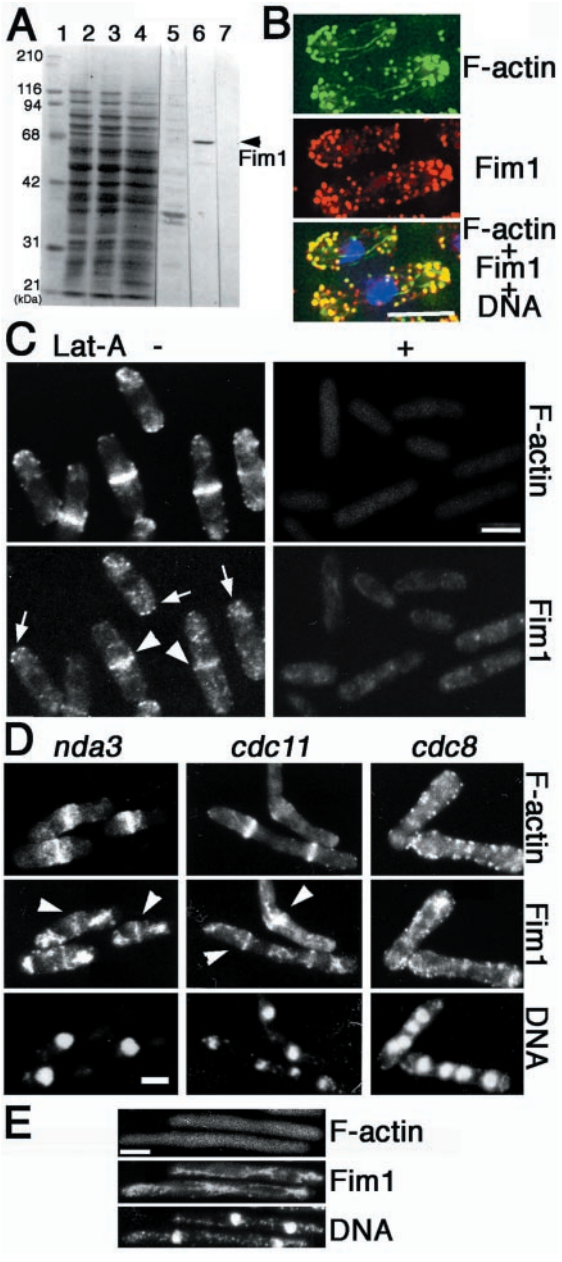

Figure 1. Fim1 is a component of F-actin patches and the F-actin ring. (A) Anti-Fim1 antibodies specifically recognize Fim1. Molecular marker proteins (lane 1), extracts of wild-type cells (lanes 2, 3, 5, and 6), and *fim1*-null cells (lanes 4 and 7) were subjected to SDS-PAGE. Lanes 1–4 show CBB staining; lanes 5–7 show immunoblots with the use of preimmune serum (1/100 dilution; lane 5) or affinity-purified anti-Fim1 antibodies (lanes 6 and 7). Arrowhead indicates the Fim1 band. (B) Three-dimensional localization of F-actin (green), Fim1 (red), and DNA (blue) in wild-type cells. (C) Effect of Lat-A on the localization of Fim1. Cells were treatment with 1 μM Lat-A for 10 min. Arrowheads and arrows indicate localization of Fim1 in the F-actin ring and F-actin patches, respectively. (D) Localization of F-actin, Fim1, and DNA in *nda3* cells incubated at 20°C for 8 h or in *cdc11* or *cdc8* cells incubated at 37°C for 6 h. Arrowheads indicate Fim1 localization to the F-actin ring. (E) Localization of F-actin, Fim1, and DNA in *cdc25* cells released from G2/M arrest for 40 min in the presence of 1 μM Lat-A. Bars, 5 μm.

localized to F-actin cables. In mitotic cells, Fim1 was observed to accumulate in the middle region of the cell (Figure 1C, arrowheads) where the F-actin ring is formed. We also confirmed these localizations of Fim1 by observing expression of the YFP-Fim1 fusion protein in live cells (our unpublished result). These localizations of Fim1 were almost entirely lost in cells incubated with latrunculin A (Lat-A), an actin-monomer sequestering substance (Cove et al., 1987) (Figure 1C). Thus, Fim1 is likely to be associated with F-actin at the ends of interphase cells and in the middle region of mitotic cells.

We next studied Fim1 localization in the cell division mutant cells (Figure 1D). The *nda3* mutant strain has a defect in mitotic spindle formation and arrests at prophase (Hiraoka *et al.*, 1984). The arrested cells have an F-actin ring, but it does not contract (Chang *et al.*, 1996). The *cdc11, cdc7*, and *cdc14* cells form an F-actin ring but not a septum (Fankhauser and Simanis, 1993, 1994). We found that in these cells, Fim1 was colocalized with F-actin at the middle region (Figure 1D, arrowheads; our unpublished result). Thus, nuclear division, contraction of the F-actin ring, and septation are probably not required for the accumulation of Fim1 at the division site.

In contrast, Fim1 was not localized to the middle of *cdc3*, *cdc4*, *cdc8*, and *cdc12* cells at the restrictive temperatures at which these cells cannot form the F-actin ring (Figure 1D; our unpublished result). This suggests that Fim1 accumulation at the division site probably depends on the F-actin ring. To confirm this hypothesis, *cdc25* cells that had been arrested at G2/M were released from the arrest in the presence of Lat-A (Figure 1E). Although nuclear division took place in these cells, the F-actin ring was not formed and Fim1 did not accumulate at the middle of the cell. Therefore, localization of Fim1 to the division site is dependent on F-actin.

F-actin Binding of Fim1

To study the function of Fim1 in vitro, we produced full-length and truncated Fim1 proteins with the use of a bacterial expression system and estimated the F-actin–binding ability of these proteins by both an F-actin cosedimentation assay and an F-actin–bundling assay (Figure 2, A and B). F-actin alone was mostly sedimented by high-speed centrifugation but not by the low-speed centrifugation (Figure 2C, a), whereas in the presence of full-length Fim1, most of the F-actin was sedimented by low-speed centrifugation. We also observed that Fim1 induced aggregation of rhodamine–phalloidin-labeled F-actin (Figure 2C, b). These results suggest that Fim1 has an F-actin cross-linking ability in vitro. In addition, Ca^{2+} did not seem to affect the interaction between Fim1 and F-actin, because almost all the actin sedimented with Fim1 by the low-speed centrifugation either in the presence or absence of Ca^{2+} (Figure 2D).

Next, we studied the activity of the truncated proteins in vitro. Fim1A12, Fim1A2, and Fim1EA1 cosedimented with F-actin at the high speed, but not at the low speed, whereas Fim1A1 and Fim1E did not cosediment with F-actin (Figure 2C, a). The F-actin–bundling activity was not seen in any of the truncated proteins (Figure 2C, b).

Overexpression of Fim1 or Truncated Proteins

We examined the effect of overexpressing Fim1 or its truncated proteins in wild-type cells (Figure 2A). Overexpression of Fim1, Fim1A12, or Fim1A2 inhibited cell growth, and

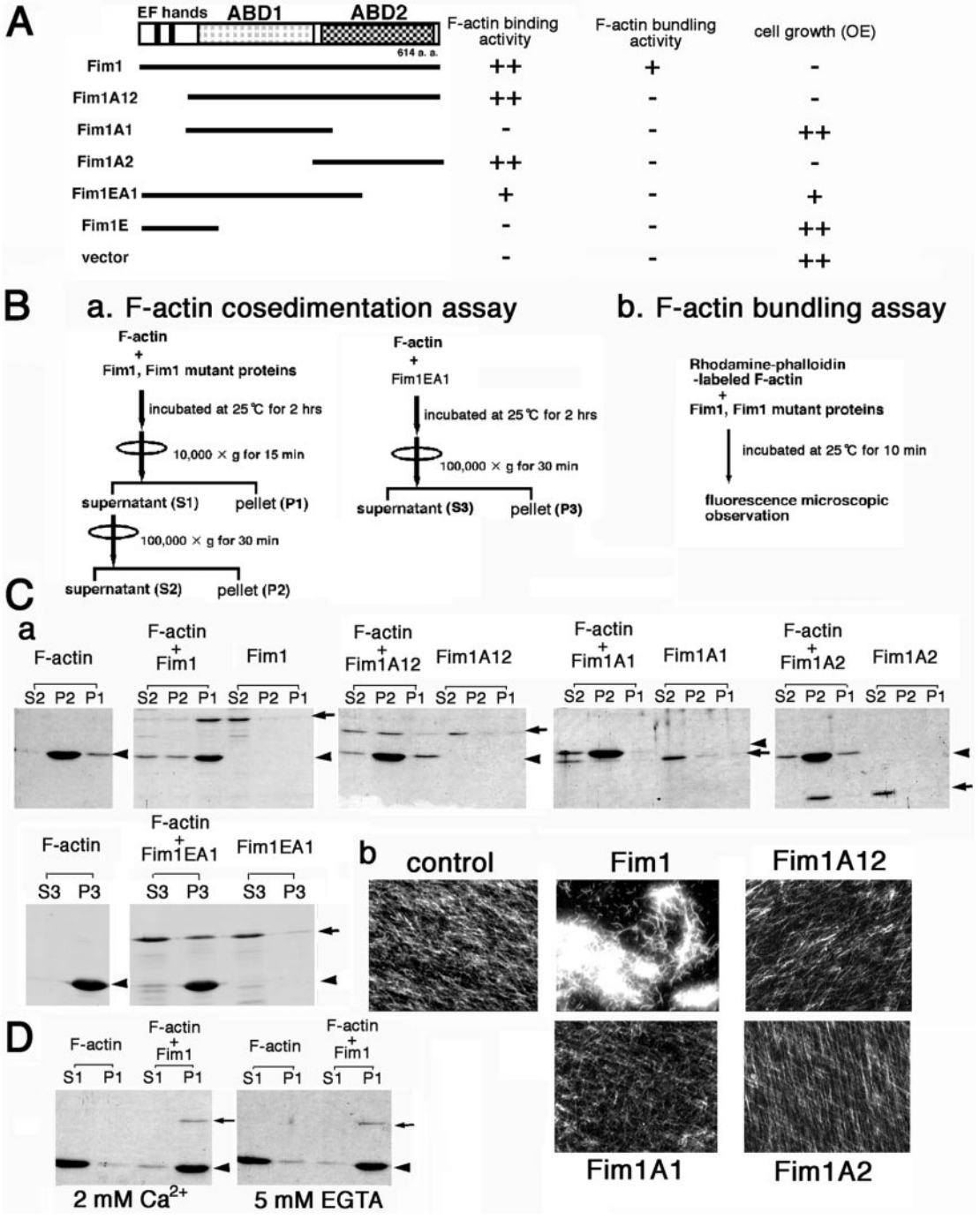

Figure 2. Fim1 is an F-actin cross-linking protein. (A) Functional analysis of Fim1 and its truncated proteins. Action of Fim1 or its truncated proteins on F-actin in vitro and effect of overexpression of Fim1 or its truncated proteins are shown. (B) Experimental schemes. The F-actin–binding abilities of Fim1 and its truncated proteins were estimated by F-actin cosedimentation assay (a), and F-actin-bundling ability was determined by fluorescence microscopy (b). (C) Full-length Fim1 is required for the F-actin cross-linking activity. (D) Ca^{2+} does not affect the F-actin cross-linking activity of Fim1. Arrowheads and arrows indicate the bands of actin and Fim1 or its truncated proteins, respectively.

that of Fim1EA1 partially inhibited it, whereas that of Fim1A1 did not show any effect. These effects correlated well with the in vitro actin-binding abilities of these proteins (see above).

The Fim1A1-overexpressing cells showed normal F-actin structures (Figure 3A). In contrast, severe defects in the organization of the actin cytoskeleton were observed in Fim1-overexpressing cells; F-actin patches were formed

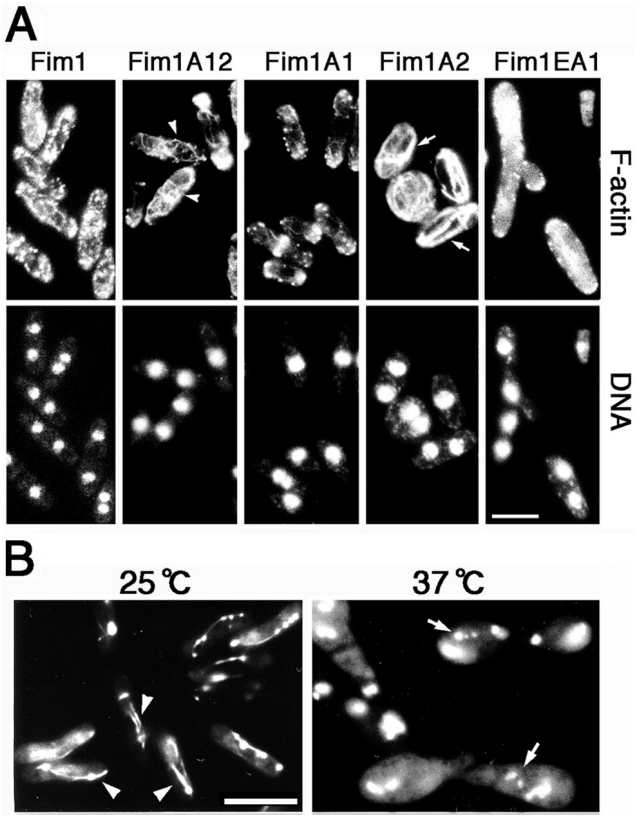

Figure 3. (A) Phenotype of cells overexpressing Fim1 or its truncated proteins. Wild-type cells carrying pREP1fim1, pREP1fim1A12, pREP1fim1A1, pREP1fim1A2, or pREP1fim1EA1 were fixed and stained with bodipy-phallacidin and DAPI after incubation at 30°C for 18 h in EMM. Arrowheads indicate the cells containing abundant F-actin cables. Arrows indicate unusually thick F-actin cables. (B) Formation of thick F-actin cables involves Cdc8 (tropomyosin). *cdc8* cells containing pREP1YFP-fim1A2 grown at 25°C in EMM without thiamine for 12 h were incubated at 25°C (left) or 37°C (right) for 6 h, and the distribution of YFP-Fim1ABD2 was observed. Arrowheads and arrows indicate YFP-Fim1A2-cables and -balls, respectively. Bars, 10 μm.

abundantly all over the cell cortex, whereas F-actin rings and F-actin cables were scarcely seen. It could be that a major part of the actin in the cell may be used in forming the F-actin patches in the Fim1-overexpressing cells and that there was only an insufficient amount of actin left to form the F-actin ring and F-actin cables. In these cells, the percentage of binucleate cells was increased (76% compared with 18% in the control cells), suggesting that cytokinesis was impaired in these cells. Further nuclear divisions did not occur in most of these binucleate cells. On the other hand, F-actin cables were formed abundantly in cells overexpressing Fim1A12 or Fim1A2. In particular, unusually thick cables were formed and the F-actin patches disappeared in the Fim1A2-overexpressing cells. Moreover, F-actin ring formation was inhibited in these cells: 48% of the Fim1A12-overexpressing cells and 55% of the Fim1A2-overexpressing cells were binucleate. In cells overexpressing Fim1EA1, F-actin patches were scattered all over the cell cortex, and the F-actin ring often had an irregular shape and direction. In these cells, 65% were multinucleate. Because the phenotype of these cells was different from that of the Fim1A2-overexpressing cells, ABD1 and ABD2 may have different functions in the cells.

Cdc8 (tropomyosin) appears to be an essential component of the F-actin cable (Arai *et al.*, 1998), and it was localized to the unusually thick F-actin cables formed in the Fim1A2-overexpressing cells (our unpublished result). To ask if the thick F-actin cables were derived from the normal F-actin cables, we examined *cdc8* cells overexpressing YFP-Fim1A2 (Figure 3B). At the permissive temperature, overexpression of YFP-Fim1A2 resulted in formation of thick cables of YFP-Fim1A2, which were similar to the thick F-actin cables seen in Fim1A2-overexpressing cells. In addition, ball-like structures of YFP-Fim1A2 were also seen that were not seen when YFP-Fim1A2 was expressed in wild-type cells. At the restrictive temperature, the F-actin cables disappeared, and instead the number of the balls seemed to increase. Thus, Cdc8 functions in the formation of the thick F-actin cables. It is tempting to speculate that the F-actin cables are normally dynamic and that Fim1A2 may stabilize the cables and thereby induce the formation of the thicker cables.

Fim1 Inhibits the Depolymerization of Actin

We have recently found that the actin-depolymerizing protein Adf1 is an essential component of the F-actin patch and the F-actin ring in *S. pombe* and that loss of function of Adf1 causes the formation of abundant F-actin patches all over the cell cortex (our unpublished results). Because this phenotype is similar to that of Fim1-overexpressing cells, we examined the localization of Adf1 in Fim1-overexpressing cells. Adf1 was localized normally when Fim1 was not overexpressed but was mostly diffused into the cytoplasm in Fim1-overexpressing cells (Figure 4A). This result suggests that Fim1 excludes Adf1 from the F-actin patches. We also observed that overexpression of Fim1A2 or Fim1EA1 was sufficient to exclude Adf1 and that the effect of Fim1A2 was stronger than that of Fim1EA1 (our unpublished result).

We next examined the rate of actin turnover in Fim1-overexpressing cells by with the use of Lat-A (Figure 4B). No F-actin structures were seen in wild-type cells 10 min after the addition of Lat-A. In contrast, most of the Fim1-overexpressing cells maintained F-actin structures. Therefore, turnover of actin is probably inhibited in the presence of Fim1. Fim1 may play an important role in the organization of the actin cytoskeleton by stabilizing F-actin.

Fim1 and the Capping Protein Acp1 Control Organization of the F-actin Patch

Although *fim1*$^+$ was not essential for cell viability, some *fim1*-null cells had an irregular shape (Figure 5), as reported also by Wu *et al.* (2001). In addition, we found a strong genetic interaction between Fim1 and actin: a double mutant made from the *fim1*-null strain and an actin mutant strain, *cps8*, showed synthetic lethality. This defect was suppressed by the expression of Fim1 but not by Fim1A12 or Fim1A2. To observe the phenotype of the *fim1cps8* double mutant, we transformed the cell with pREP81fim1. The *fim cps8* cells containing pREP81fim1 grew normally in the absence of thiamin, but the growth rate was reduced and the cells

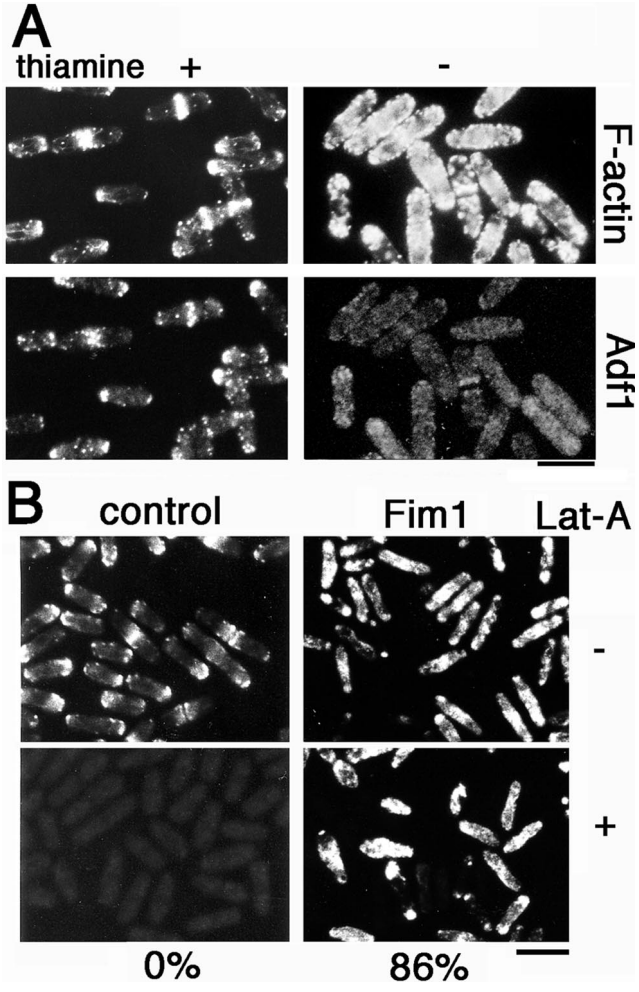

Figure 4. Fim1 affects the turnover of F-actin in vivo. (A) Fim1 excludes the actin-depolymerizing protein Adf1 from F-actin structures. Wild-type cells containing pREP1fim1 were grown at 30°C in EMM with (+) or without (−) thiamine for 18 h, and processed for visualization of F-actin (top) and Adf1 (bottom). (B) Fim1 inhibits the turnover of F-actin. Wild-type cells containing pREP1 or pREP1fim1 grown at 30°C in EMM for 18 h were fixed and stained with bodipy-phallacidin before or 10 min after addition of 1 μM Lat-A. The numbers at the bottom represent the percentage of cells with F-actin structures remaining. Bars, 10 μm.

showed an aberrant shape in the presence of thiamin; these cells were swollen and finally lysed (Figure 5).

We then examined the actin cytoskeleton in these cells by staining with antibodies against actin or Arp2 as a marker of the F-actin patch; it has been demonstrated that Arp2 localizes to the F-actin patches but neither to the F-actin ring nor to the F-actin cables (Morrell *et al.*, 1999). It was found that the F-actin patches tended to be scattered in the cortex of the *fim1*-null cells compared with those of the wild-type cells or the *cps8* cells. Thus, Fim1 probably functions in cell morphogenesis by controlling the localization of the F-actin patches.

The *S. cerevisiae* fimbrin Sac6p has a close relationship with the CapZ/β-actinin family actin-capping protein, which is a heterodimer of α- and β-subunits, Cap1p and

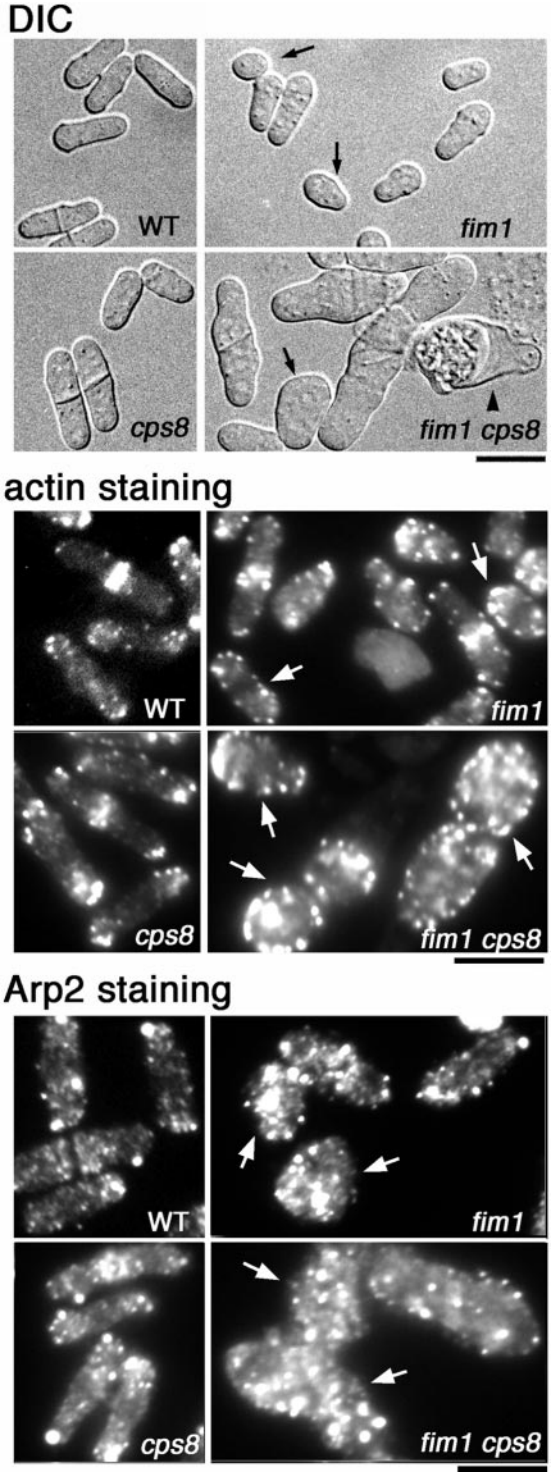

Figure 5. Fim1 functions together with actin in cell morphogenesis. Wild-type cells (WT), *fim1*-null cells (*fim1*), *cps8* cells (*cps8*), and *fim1 cps8* double-mutant cells containing pREP81fim1 (*fim1 cps8*) grown in YEA at 25°C were observed by DIC microscopy or immunofluorescence microscopy with anti-actin antibodies or anti-Arp2 antibodies. Arrows and arrowhead indicates depolarized cells and a lysed cell, respectively. Bars, 10 μm.

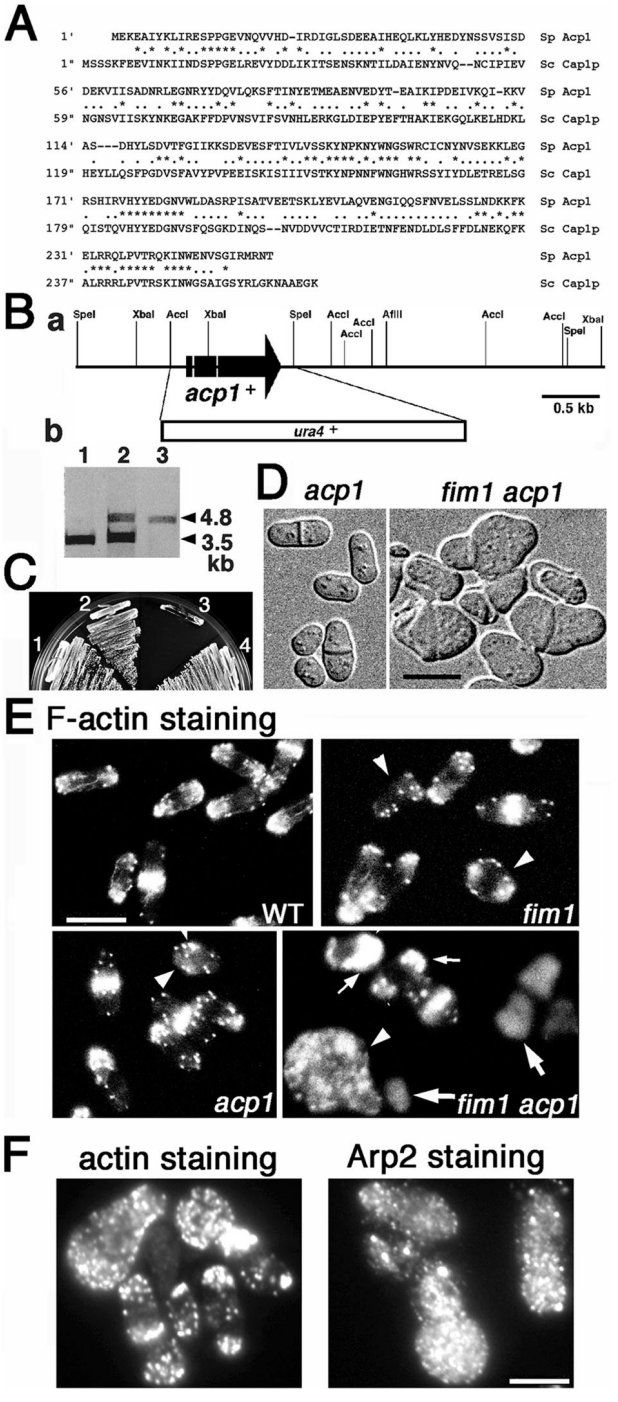

Figure 6. Characterization of the actin-capping-protein gene *acp1*$^+$. (A) Amino-acid sequence alignments of *S. pombe* Acp1 and *S. cerevisiae* Cap1p. Identical and conserved residues between the 2 proteins are denoted by asterisks and dots, respectively. (B) (a) Strategy of *acp1*$^+$ gene disruption. (b) Confirmation of the *acp1*$^+$ gene disruption. Genomic DNA derived from the parental diploid cells (lane 1), the *acp1* disrupted diploid cells (lane 2), or the *acp1* disrupted haploid cells (lane 3) was digested with *Xba*I, and subjected to Southern hybridization with the use of the 3′ UTR region of *acp1*$^+$ as a probe. (C) Genetic interaction between *fim1*$^+$ and *acp1*$^+$. Wild-type cells (1), *acp1*-null (2) and *fim1*-null (4) cells, and *fim1 acp1* null cells (3) were streaked on YEA plates and incubated at 25°C for 5 d. (D–F) Phenotype of the *fim1 acp1* null cells. Wild-type cells, *fim1*- and *acp1*-null cells, and *fim1 acp1* null cells grown in YEA at 25°C were observed by DIC microscopy (D) or fixed and stained with bodipy-phallacidin (E). The *fim1 acp1* null cells were also stained with antibodies against actin or Arp2 (F). Arrowheads, cells in which the F-actin patches were scattered in the cell cortex; small arrows, abnormal accumulation of F-actin; large arrows, cells lacking the F-actin structures. Bars, 10 μm.

Cap2p (Adams *et al.*, 1993; Karpova *et al.*, 1993). A gene encoding a protein similar to Cap1p has recently been revealed by the fission yeast genome project (Figure 6A); we named it *acp1*$^+$ and investigated its function in relation to that of Fim1. Gene disruption (Figure 6B) revealed that *acp1*$^+$ is not essential for cell viability (Figure 6C). However, severe defects in cell growth and cell shape were seen in *fim1acp1* null cells (Figure 6, C and D). Staining of F-actin revealed that its organization was moderately defective in the single mutants: F-actin patches were widely distributed in the cell cortex (33% of the *fim1*-null cells and 17% of the *acp1*-null cells). However, in the *fim1 acp1* null cells, severe defects in the organization of the actin cytoskeleton were observed: 40% of the double null cells had no discrete F-actin structure, 31% had depolarized F-actin patches, and 15% had F-actin clusters. This was confirmed by immunofluorescence microscopy with the use of antibodies against actin or Arp2 (Figure 6F). These defects were suppressed by the expression of Fim1 but not by Fim1A12 or Fim1A2. Thus, Fim1 and Acp1 may function in a similar manner in organizing the actin cytoskeleton in fission yeast.

A Possible Role of Fim1 in Cytokinesis

As described above, Fim1 is likely to be a component of the F-actin ring. To investigate further the possible role of Fim1 in cytokinesis, we made double mutants of *fim1*-null with cell-division mutations such as *cdc3* (Balasubramanian *et al.*, 1994), *cdc4* (McCollum *et al.*, 1995), *cdc8* (Balasubramanian *et al.*, 1992), *cdc12* (Chang *et al.*, 1997), *rng2* (Eng *et al.*, 1998), *cdc15* (Fankhauser *et al.*, 1995), *cdc7* (Fankhauser and Simanis, 1994), *cdc11* (Nurse *et al.*, 1976), and *cdc14* (Fankhauser and Simanis, 1993). We detected synergistic effects between *fim1*$^+$ and *rng2*$^+$ and between *fim1*$^+$ and *cdc15*$^+$: the double mutants did not form colonies at 30°C, whereas each single mutant did (Figure 7A). Moreover, cytokinesis was impaired in these double mutant cells at 27°C, a semipermissive temperature (Figure 7B). Fim1 may therefore play a role in cytokinesis that is linked with Rng2 and Cdc15 functions.

DISCUSSION

The actin cytoskeleton is considered to play important roles in cell morphogenesis and cytokinesis. To clarify the mechanisms by which the organization of the actin cytoskeleton is controlled in fission yeast, we searched for novel actin-modulating proteins with the use of F-actin affinity column chromatography. In this study, we characterized the function of Fim1, which belongs to the fimbrin family of actin cross-linking proteins. We found that Fim1 is involved in controlling cell shape through regulation of the actin cytoskeleton cooperatively with the actin-capping protein

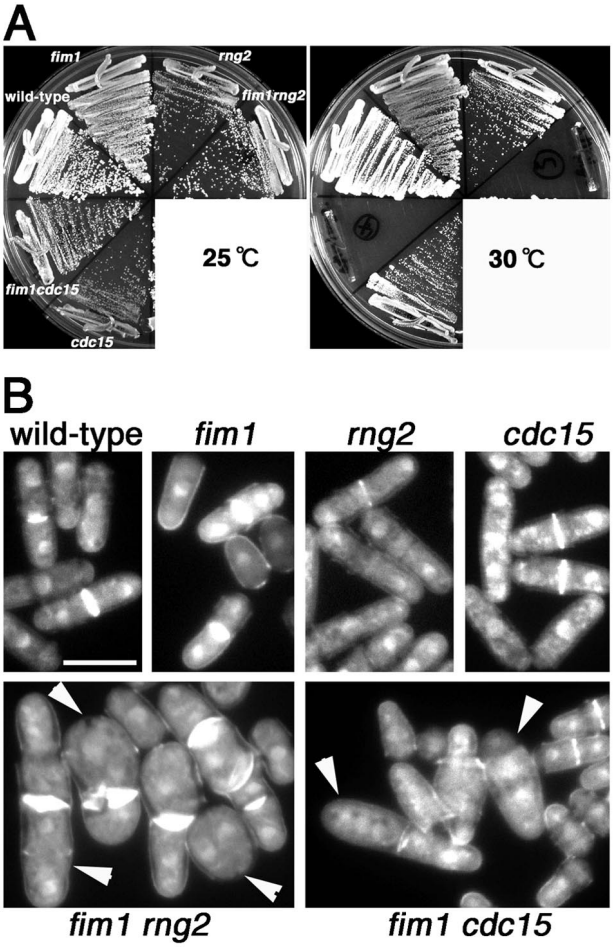

Figure 7. Genetic interactions among *fim1*$^+$, *rng2*$^+$, and *cdc15*$^+$. (A) Wild-type cells, the three single mutants, and the *fim1 rng2* and *fim1 cdc15* double mutants were streaked on YEA plates and incubated at 25°C or 30°C for 4 d. (B) The cells grown at 27°C were fixed and stained with Calcofluor and DAPI. Arrowheads indicate multinucleate cells. Bar, 10 μm.

Acp1. Moreover, Fim1 may function in cytokinesis as a component of the F-actin ring.

Fim1 has an F-actin, cross-linking activity in vitro. This activity derives from the two actin-binding domains, ABD1 and ABD2. A computer analysis of the amino acid sequences of fimbrins showed that ABD1 and ABD2, respectively, are highly conserved among the corresponding regions of these proteins including Fim1, Sac6p (Adams *et al.*, 1989) and *Dictyostelium* fimbrin (Prassler *et al.*, 1997; Table 2). However, the similarity between ABD1 and ABD2 in the same fimbrin molecules is lower. We observed that Fim1A2 bound more strongly to F-actin than did Fim1EA1 in vitro. Moreover, Fim1A1 did not bind to F-actin. Interestingly, it has recently been reported that ABD1 and ABD2 of *Arabidopsis thaliana* fimbrin may have different affinities for F-actin (Kovar *et al.*, 2000). Brower *et al.* (1995) have reported that a mutation in the ABD2 in Sac6p gives rise to a phenotype different from that of a mutation in the ABD1. In addition, the phenotype of Fim1A2-overexpressing cells was quite different from that of Fim1EA1-overexpressing cells. Thus, the two ABDs seem to act on actin differently both in vitro and in vivo. In fission yeast, ABD2 may bind tightly to F-actin, whereas the cross-linking activity of Fim1 may be controlled by ABD1: Fim1 may contribute to formation of the 3-D actin cytoskeleton when ABD1 is somehow activated to bind to F-actin. It may also be possible that the binding of ABD2 to F-actin changes conformation of the Fim1 molecule so that ABD1 binds more tightly to F-actin. However, we cannot exclude the possibility that Fim1A1 and Fim1EA1, especially the former, may have been misfolded or unstable.

It has been demonstrated that the actin-binding activity of *Dictyostelium* fimbrin is suppressed in the presence of Ca^{2+} (Prassler *et al.*, 1997). However, we found that the activity of Fim1 was independent of Ca^{2+}. Similarly, chick fimbrin has been shown to cross-link F-actin even in the presence of Ca^{2+} (Bretscher, 1981). Thus, the effect of Ca^{2+} on fimbrin seems to vary among the species. Recently, Watanabe *et al.* (2000) reported the isolation of a fimbrin-like protein from *Tetrahymena pyriformis* that lacks EF-hand domains. Therefore, there could be a mechanism other than Ca^{2+} control that regulates the F-actin cross-linking activity of fimbrin.

Immunofluorescence microscopy showed that Fim1 is a component of both the F-actin patches and the F-actin ring. Because the overexpression of Fim1 markedly induced formation of the F-actin patches all over the cell cortex, Fim1 may have a positive function in the formation of the F-actin patches in vivo, although it is not essential for the formation. The function of Fim1 in this process seems to require the F-actin cross-linking activity of Fim1, because overexpression of Fim1A12 or Fim1A2, neither of which has F-actin cross-linking ability, did not induce the F-actin patch formation.

It was unexpected that Fim1 is not a component of the F-actin cable, because Fim1 cross-links F-actin to form bundles and the F-actin cable seems to be a bundle of F-actin filaments cross-linked with each other. It was also unexpected that Fim1A12 and Fim1A2, which have no F-actin cross-linking activity, induced thick F-actin cables when overexpressed in the cell. Moreover, we observed that YFP-Fim1A2 was colocalized with Cdc8 (tropomyosin) in the F-actin cables (our unpublished result). This result implies that Fim1 may be able to bind F-actin cable through its ABD2. At this moment, we do not know the reason why

Table 2. Similarity of ABDs in fimbrins

	Sc ABD1	Dd ABD1	Sp ABD2	Sc ABD2	Dd ABD2
Sp ABD1	76[a]	56[a]	23	23	21
Sc ABD1	—	55[a]	21	22	21
Dd ABD1	—	—	21	20	21
Sp ABD2	—	—	—	62[a]	47[a]
Sc ABD2	—	—	—	—	44[a]

Values are expressed as percentage. ABD, actin-binding domain.
[a] N-terminal ABD and C-terminal ABD in *S. pombe* Fim1, *S. cerevisiae* Sac6p, and *D. discoideum* fimbrin are shown as Sp ABD1 and Sp ABD2, Sc ABD1 and Sc ABD2, and Dd ABD1 and Dd ABD2, respectively.

Fim1 is not localized to the F-actin cable in the cell. It is important to elucidate the molecular mechanism of the F-actin cable assembly.

Another function of Fim1 appears to be to stabilize the F-actin structure. This idea is supported by the following evidence. First, overexpression of Fim1 reduced the rate of actin depolymerization induced by Lat-A. Second, F-actin structures decreased in *fim1 acp1* null cells, and the shape of the cells became aberrant. This phenotype was suppressed by the expression of Fim1 but not by that of truncated Fim1 proteins lacking the F-actin cross-linking activity. Acp1 probably contribute to stabilization of the F-actin structures by capping the barbed ends. In *S. cerevisiae*, Karpova *et al.* (1995) have also reported that F-actin is unstable in the absence of capping protein or fimbrin. Third, the morphological defect of *fim1*-null cells was enhanced in a *fim1 cps8* double mutant, and this was suppressed by the expression of Fim1 but not by that of the truncated Fim1 proteins. The *cps8* mutation is caused by an amino acid substitution in the hydrophobic loop of actin, which is considered to affect the stabilization of F-actin (Ishiguro and Kobayashi, 1996). Fourth, we found that overexpression of Fim1 excludes Adf1 from the F-actin structures. Adf1 functions to facilitate depolymerization of F-actin (K. Nakano, I. Mabuchi, unpublished results). Thus, the exclusion of Adf1 from F-actin would lead to its stabilization. Fim1 and Adf1 are likely to function in a competitive manner to control the stability of the F-actin structure in the cell.

During cytokinesis, Fim1 accumulates in the F-actin ring; this accumulation is dependent on F-actin but not on nuclear division or septation. Recently, it has been shown that a fimbrin-like protein is localized in the division furrow in *T. pyriformis* (Watanabe *et al.*, 1998). Thus, fimbrin-family proteins may be involved in cytokinesis in eukaryotic cells. To explore this possibility, we made several double mutants from the *fim1*-null strain and cytokinesis mutants and found that Fim1 shows a genetic interaction with the IQGAP-family protein Rng2 and the PSTPIP-family protein Cdc15. Eng *et al.* (1998) have reported that the temperature-sensitive *rng2* mutant accumulates F-actin cables in the medial region of mitotic cells but fails to organize these cables into the F-actin ring under the restrictive condition. It has been shown that bovine IQGAP1 cross-links actin filaments in vitro (Bashour *et al.*, 1997). Thus, the cells lacking the function of Rng2 may be defective in the F-actin cross-linking activity. In addition, very recently, the α-actinin-like actin cross-linking protein Ain1 has been characterized in fission yeast; it appears to functions cooperatively with Fim1 in the formation of the F-actin ring (Wu *et al.*, 2001). Therefore, the cross-linking of F-actin by these proteins may be required for the formation of the F-actin ring. PSTPIP-family proteins have been reported to be involved in cytokinesis in fission yeast cells (Fankhauser *et al.*, 1995; Demeter *et al.*, 1998), budding yeast cells (Kamei *et al.*, 1998; Lippincott and Li, 1998), and mammalian cells (Spencer *et al.*, 1997). In fission yeast, the predominant defect in *cdc15* cells is that F-actin patches are not formed around the division site after formation of the F-actin ring (Balasubramanian *et al.*, 1998). The F-actin patches are considered to induce septum formation after contraction of the F-actin ring. Fim1 may be involved in the formation of the F-actin patches cooperatively with Cdc15 after ring formation.

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