Actin-depolymerizing Protein Adf1 Is Required for Formation and Maintenance of the Contractile Ring during Cytokinesis in Fission Yeast[□] [□]

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The role of the actin-depolymerizing factor (ADF)/cofilin-family protein Adf1 in cytokinesis of fission yeast cells was studied. Adf1 was required for accumulation of actin at the division site by depolymerizing actin at the cell ends, assembly of the contractile ring through severing actin filaments, and maintenance of the contractile ring once formed. Genetic and cytological analyses suggested that it collaborates with profilin and capping protein in the mitotic reorganization of the actin cytoskeleton. Furthermore, it was unexpectedly found that Adf1 and myosin-II also collaborate in assembling the contractile ring. Tropomyosin was shown to antagonize the function of Adf1 in the contractile ring. We propose that formation and maintenance of the contractile ring are achieved by a balanced collaboration of these proteins.

INTRODUCTION

Regulation of the actin cytoskeleton is important for various cellular activities, including cytokinesis. In animal cells, the actomyosin contractile ring is formed in the cleavage furrow cortex, and cytokinesis progresses through its contraction. The function of myosin-II is essential for both formation and contraction of the ring. Several actin-modulating proteins are also considered to be involved in these processes.

Studies using the fission yeast *Schizosaccharomyces pombe* have been contributing to understanding of the components required for cytokinesis (reviewed by Balasubramanian *et al.*, 2004). Fission yeast cells are cylindrical and enveloped with a cell wall. In interphase cells, actin is organized as cortical F-actin patches that are localized at the growing ends of the cell where the cell wall is newly synthesized, and as F-actin cables running along the long axis of the cell (Marks and Hyams, 1985; Arai *et al.*, 1998). During cell division, the patches disappear from the ends, the asterlike structure made of cables is formed in the middle cortex of the cell, and it seems to be packed to form the F-actin contractile ring (Arai and Mabuchi, 2002). Myosin-II heavy chain, Myo2, and light chain, Cdc4, are essential for formation of the contractile ring (McCollum *et al.*, 1995; Kitayama *et al.*, 1997; May *et al.*, 1997; Motegi *et al.*, 2000). Myo2 accumulates at the midregion, being controlled temporally by dephosphorylation of its tail, where Mid1/Dmf1 (Chang *et al.*, 1996; Sohrmann *et al.*, 1996) has previously been

deposited, before accumulation of F-actin (Motegi *et al.*, 2004). Septum is formed after the contraction of the contractile ring and separates the daughter cells completely.

Several genes that control actin dynamics also have been identified to be essential for formation of the contractile ring. The products of all of these genes are colocalized with the contractile ring. *cdc3⁺* encodes a G-actin binding protein, profilin (Balasubramanian *et al.*, 1994), which may induce the directed polymerization of actin in the presence of a formin/diaphanous family protein, Cdc12 (Chang *et al.*, 1997; Kovar *et al.*, 2003). The PSTPIP/PCH-family protein Cdc15 (Fankhauser *et al.*, 1995) has recently been reported to directly bind to Cdc12 and control its localization (Carnahan and Gould, 2003). *cdc8⁺* encodes tropomyosin, which localizes at both the contractile ring and F-actin cables and is considered to be required for stabilization of F-actin in these structures (Balasubramanian *et al.*, 1992; Arai *et al.*, 1998). *rng2⁺* encodes an F-actin cross-linking protein, IQGAP (Eng *et al.*, 1998). IQGAP may be involved in actin assembly in the cleavage furrow of sea urchin eggs together with the Rho-family small GTPase Cdc42 (Nishimura and Mabuchi, 2003).

The actin-depolymerizing factor (ADF)/cofilin-family proteins are conserved low-molecular-weight actin-modulating proteins in eukaryotic cells (reviewed by Chen *et al.*, 2000). These proteins bind to actin in both polymerized and monomeric forms and sever F-actin into fragments and promote dissociation of monomers from the pointed end of the filament (Mabuchi, 1983; Nishida *et al.*, 1984; Maciver *et al.*, 1991). ADF/cofilin has been localized to the regions where active movements occur, such as lamellipodia of migrating cells (Dawe *et al.*, 2003) and cleavage furrow of dividing cells (Nagaoka *et al.*, 1995; Abe *et al.*, 1996; Ono *et al.*, 2003). Suppression of ADF/cofilin activities prevents completion of cytokinesis in some animal cells but does not prevent its formation (Gunsalus *et al.*, 1995; Somma *et al.*, 2002; Kaji *et al.*, 2003; Ono *et al.*, 2003). Conversely, the *Xenopus* ADF/cofilin, XAC, is localized to the cleavage furrow from an early stage in cytokinesis and required for formation of the furrow (Abe *et al.*, 1996). Here, we investigated the role of an ADF/cofilin-family protein, Adf1,

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Table 1. List of strains used in this study

Strain	Genotype	Source
JY1	<i>h</i> [−]	Laboratory stock
<i>leu1-32</i>	<i>h</i> [−] <i>leu1-32</i>	Laboratory stock
JY333	<i>h</i> [−] <i>ade6-M216 leu1-32</i>	Laboratory stock
JY741	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18</i>	Laboratory stock
JY746	<i>h</i> ⁺ <i>ade6-M210 leu1-32 ura4-D18</i>	Laboratory stock
KAD1	<i>h</i> [−] / <i>h</i> ⁺ <i>ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18 adf1⁺/adf1::ura4⁺</i>	Laboratory stock
KAD4	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18 adf1::ura4⁺ pREP41HA-adf1</i>	This study
KAD8	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18 adf1::[adh-GFP-adf1-ura4⁺]</i>	This study
KAD11	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18 adf1KMC-ura4⁺</i>	Lab. stock
KAD31	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18 adf1-1</i>	This study
KAD35	<i>h</i> [−] <i>ade6-M216 leu1-32 ura4-D18 adf1-1-ura4⁺</i>	This study
KAD37	<i>h</i> ⁺ <i>ade6-M216 leu1-32 ura4-D18 adf1-1-ura4</i>	This study
<i>cdc3 adf1-1</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 adf1::ura4⁺ cdc3-124 pREP41-adf1</i>	This study
<i>cdc4 adf1-1</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 adf1::ura4⁺ cdc4-8 pREP41cdc4</i>	This study
<i>nda3 adf1-1</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 adf1-1-ura4⁺ nda3-KM311</i>	This study
<i>nda3 cdc8 adf1-1</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 adf1-1-ura4⁺ cdc8-110 nda3-KM311</i>	This study
<i>nda3 cdc8</i>	<i>h</i> [−] <i>leu1-32 cdc8-110 nda3-KM311</i>	This study
<i>cdc25</i>	<i>h</i> [−] <i>cdc25-22</i>	Laboratory stock
<i>cdc3</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 cdc3-124</i>	Laboratory stock
<i>cdc4</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 cdc4-8</i>	Laboratory stock
<i>cdc8</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 cdc8-110</i>	Laboratory stock
<i>cdc12</i>	<i>h</i> [−] <i>ura4-D18 cdc12-112</i>	Laboratory stock
<i>cdc15</i>	<i>h</i> [−] <i>ura4-D18 cdc15-140</i>	Laboratory stock
Δ <i>myo1</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 myo1::Km^r</i>	Laboratory stock
<i>myo2</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 myo2-E1</i>	Laboratory stock
<i>arp3</i>	<i>h</i> [−] <i>leu1-32 ura4-D18 arp3-cs1</i>	Laboratory stock
Δ <i>for3</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 for3::ura4⁺</i>	Laboratory stock
Δ <i>acp1</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 acp1::ura4⁺</i>	Laboratory stock
Δ <i>acp2</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 acp2::ura4⁺</i>	Laboratory stock
Δ <i>fim1</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 fim1::ura4⁺</i>	Laboratory stock
<i>rng2</i>	<i>h</i> [−] <i>ade6-M210 leu1-32 ura4-D18 rng2-D5</i>	Laboratory stock
<i>cdc7</i>	<i>h</i> [−] <i>ura4-D18 cdc7</i>	Laboratory stock
<i>cdc14</i>	<i>h</i> [−] <i>cdc14-118</i>	Laboratory stock
<i>nda3</i>	<i>h</i> [−] <i>leu1-32 nda3-KM311</i>	Laboratory stock

and its cooperation with other actin-modulating proteins in cytokinesis in *S. pombe*.

MATERIALS AND METHODS

Genetic Techniques and DNA Manipulation

S. pombe strains used in this study are listed in Table 1. The media used have been described previously (Moreno *et al.*, 1991). Complete medium YE and minimum medium EMM were used for growing *S. pombe* strains. MEA was used for induction of conjugation and sporulation. All plates contained 2% agar. Standard procedures for *S. pombe* genetics were followed according to Alfa *et al.* (1993) and Moreno *et al.* (1991).

Gene Expression in Fission Yeast

Fission yeast expression vectors pREP1 and pREP41 (Maundrell, 1993) were used in this study. These vectors contain *Saccharomyces cerevisiae* LEU2, the expression of which complements the nutrient defect of the *leu1* mutant in *S. pombe*. pREP1 has a stronger *mtt1* promoter than pREP41. The repression of exogenous genes is induced by adding 5 μ M thiamine to the medium. pREP41HA-*adf1* contains nucleotides encoding a hemagglutinin (HA)-epitope tag-fused full-length cDNA of *adf1*⁺ (1~414 base pairs) at the NdeI- and SalI-cloning site in pREP41. pREP41cdc4 contains a full-length cDNA of *cdc4*⁺ (1~426 base pairs) at the NdeI- and BamHI-cloning site in pREP41. pREP1cdc8 contains a full-length cDNA of *cdc8*⁺ (1~486 base pairs) at the NdeI- and BamHI-cloning site in pREP1.

A His-tag fused cDNA for porcine cofilin, Cof1, Cof1F82, and Cof1A120 (gifts from Dr. K. Moriyama, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; Moriyama and Yahara, 1999), was expressed under the control of the promoter region of *adf1*⁺ (−849~−1) in pUC119 containing the autonomous replicating sequence of *S. pombe* and *S. cerevisiae* ADE2 whose expression complements the nutrient defect of the *ade6* mutant in *S. pombe*.

Observation of the Phenotype of *adf1* Null Cells

To observe the phenotype of *adf1* null cells, the *adf1*⁺/*adf1::ura4*⁺ diploid strain (KAD1) was transformed with pREP41HA-*adf1* and sporulated. The

spores were spread on EMM in the absence of uracil and leucine and incubated at 25°C for 5 d. Several colonies were removed, and it was confirmed by Southern blotting that the chromosomal *adf1*⁺ was disrupted. This *adf1::ura4*⁺ strain containing pREP41HA-*adf1* (KAD4) could not grow on an EMM plate containing thiamine. To determine the phenotype for the depletion of Adf1, thiamine was added to an exponentially growing culture of KAD4 in EMM at 30°C, and after 0, 8, 10, 12, 15, and 19 h, the cells were fixed and processed for immunoblotting and fluorescence microscopic observation.

Generation of *adf1* Mutant Strains

The pUC18-derived vector pcaf1 contains *EcoRI* (−1981-base pair) and *KpnI* (1510-base pair) fragments of *adf1*⁺ which lacks a second intron and has SalI and BglII sites downstream of the stop codon (627 base pairs). To introduce random mutations into *adf1*⁺, we amplified the region between the XbaI (−416 base pairs) and SalI sites (645 base pairs) using *adA*f (5′-tgattgagtagct-ggaacattc-3′) and *adS* (5′-gggtcgacttactacgagtaaccttc-3′) by PCR in the presence of 0.05 mM MnCl₂. The PCR product was digested with XbaI and SalI, and the 1.1-kb fragment was ligated to XbaI- and SalI-treated pcaf1. *Escherichia coli* XL1blue was transformed with the ligation product. About 10,000 clones of the transformants were collected and stored at −80°C. KAD4 was transformed with *EcoRI*- and *KpnI*-treated plasmids isolated from this library, and the transformant was spread on YE plates containing 5-fluoroorotic acid (Sigma-Aldrich, St. Louis, MO), which inhibits the growth of cells expressing *ura4*⁺. After incubation at 25°C for 5 d, clones that were unable to grow at 37°C were selected. One of these clones was called *adf1-1* (KAD31). Genomic DNA derived from KAD31 was amplified using *adA*f and *adS*, and the PCR product was cloned in XbaI- and SalI-sites in pUC18. After the sequence analysis of this vector, pUCad1-1, it was revealed that Leu57 in Adf1 was replaced by Ser in the mutant protein. To confirm whether this mutation is responsible for *adf1-1*, we replaced chromosomal *adf1*⁺ with *adf1-1* as follows. pUCad1-1 was digested with SacI and SalI, and the 0.4-kb fragment was cloned into SacI- and SalI-treated pcaf1 containing *ura4*⁺ at the BglII site (pcaf1::ura4⁺). A diploid strain constructed by mating JY741 and JY746 was transformed with this construct, pcaf1-1::ura4⁺, which had been digested with *EcoRI* and *KpnI*. The transformant was sporulated, and the spores were spread on EMM containing adenine and leucine, and incubated

at 25°C for 5 d. Several colonies were examined, and it was confirmed by Southern blotting that the chromosomal *adf1*⁺ was replaced with *adf1-1::ura4*⁺. This strain, *KAD35*, showed a temperature-sensitive growth defect similar to *KAD31*.

To introduce random mutations into the C-terminal region of Adf1, *adf1*⁺ cDNA was amplified using the oligonucleotides adEN (5'-ccgaattccatgctcttttccaggtg-3') and adCmut (5'-gggggtcgtactactacnannnnnnnnnnaagaactgtc-3'). The PCR product was digested with SacI and SalI, and the 0.4-kb fragment was ligated to SacI- and SalI-treated p*cadf1::ura4*⁺. *XL1blue* was transformed with the ligation product. About 10,000 clones of the transformants were removed and stored at -80°C. *JY746* was transformed with EcoRI- and KpnI-treated plasmids isolated from this library and plated on EMM containing adenine and leucine. After incubation at 25°C for 5 d, clones that were unable to grow at 37°C were selected. One of these clones was named *adf1KMC*. For determination of the sites of mutation in *adf1KMC*, the genomic DNA was amplified by PCR. After sequence analysis of the PCR product, it was revealed that E132, K133, and R136 in Adf1 were changed to K, M, and C, respectively, in *adf1KMC*.

Immunoblotting

Preparation of cell extracts and SDS-gel electrophoresis were performed as described previously (Moreno *et al.*, 1991). For immunoblotting, affinity-purified anti-Adf1 antibodies (our unpublished data), monoclonal anti-actin antibody N350 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), anti-HA antibodies (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-His antibodies (G-18; Santa Cruz Biotechnology) were used. Quantitative analysis was performed by ImageJ software downloaded from <http://rsb.info.nih.gov/ij/>.

Microscopy

The cells were fixed and processed for immunofluorescence microscopy as described previously (Alfa *et al.*, 1993). For immunolocalization of Adf1, actin, tubulin, Cdc8, and Cdc4, affinity-purified anti-Adf1 antibodies, monoclonal anti-tubulin antibody TAT1 (a gift from Dr. K. Gull, University of Manchester, Manchester, United Kingdom), anti-Cdc8 serum (Arai *et al.*, 1998), and anti-Cdc4 serum (a gift from Dr. M. K. Balasubramanian, National University of Singapore, Singapore) were used, respectively. Staining of the cells with Calcofluor (Sigma-Aldrich), rhodamine-phalloidin (Molecular Probes, Carlsbad, CA), and Bodipy-phalloidin (Molecular Probes) was performed as described previously (Alfa *et al.*, 1993; Arai *et al.*, 1998). Latrunculin-A (Lat-A) (Wako Pure Chemicals, Osaka, Japan) was used to sequester G-actin in the cell and to estimate the rate of turnover of actin in vivo.

Conventional fluorescence microscopy was performed using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a Plan Apochromat × 63 lens and photographed on Kodak T-MAX ASA 400 film. Three-dimensional (3-D) reconstruction and time-lapse observations were made using a Delta Vision system (Applied Precision, Issaquah, WA) attached to an Olympus IX-70-SIF fluorescence microscope equipped with a UplanApo × 100 lens (Olympus, Tokyo, Japan) as described previously (Motegi *et al.*, 2000).

Observation of Green Fluorescent Protein (GFP) Fusion Proteins

The pGFP gene (Clontech, Mountain View, CA) was amplified using gfpN (5'-ggcatatgtgagcaaggcgaggag-3') and gfpC (5'-gggaattcctgtacagctctgcatcg-3'), and the PCR product was digested with PstI and EcoRI. *adf1*⁺ was amplified with adEN (5'-ccgaattccatgctcttttccaggtg-3') and adC (5'-gggggatccttactacgagtaaccttc-3'), and the PCR product was digested with EcoRI and BamHI. These fragments were ligated simultaneously to PstI- and BamHI-treated pART1 containing an *adh1* promoter (McLeod *et al.*, 1987). We confirmed that this plasmid, pART1GFP-Adf1, could rescue the *adf1* null cells (our unpublished data). To prepare an integrated strain, pART1GFP-Adf1 was digested with EcoRV and SacI. The 2.2-kb fragments containing the GFP gene fused with *adf1*⁺ under the control of the *adh* promoter were ligated to HpaI- and SacI-treated p*cadf1::ura4*⁺. The construct, padh::GFP-Adf1::ura4⁺, was digested with ApaLI and KpnI, and a diploid strain obtained by mating *JY741* and *JY746* was transformed with it. The transformant was sporulated, and the spores were spread on EMM containing adenine and leucine, and incubated at 25°C for 5 d. Several colonies were picked up, and it was confirmed by Southern blotting that the genomic *adf1*⁺ gene was replaced with *adh::GFP-Adf1::ura4*⁺. This GFP-Adf1-integrated strain (*KAD8*) was used for time-lapse observations using the Delta Vision system as described above.

To observe the behavior of the contraction of the F-actin ring, yellow fluorescent protein (YFP)-fused Cdc4 was expressed from pREP1 in EMM with 5 μM thiamine at 25°C. The expression of YFP-Cdc4 affected neither of growth nor shape of the cell. Measurement of diameter of the F-actin ring was performed using Softworx software (Applied Precision).

RESULTS

Dynamic Behavior of Adf1 in Cytokinesis

We first observed the behavior of Adf1 during cytokinesis by expressing GFP-Adf1. The GFP-Adf1-integrated strain expressed GFP-Adf1 at a level similar to that of Adf1 in the wild-type cells and grew normally (our unpublished data), suggesting that GFP-Adf1 was functional like the wild-type Adf1. The 3-D views of the GFP-Adf1 localization in a mitotic cell are shown in Figure 1A and Supplemental Movie 1. In an early step, some GFP-Adf1 patches occurred in the middle cortex of the cell (1~2 min in Figure 1A). Then, a fibrous structure emerged in this region, which was in contact with the patches (3 min in Figure 1A). This structure did not seem to be stable: it appeared and then disappeared within a minute. During a few minutes, the number of the fibrous structure in this example increased up to three to our eyes. They were then arranged as an equatorial circle (see Supplemental Movie 1). Finally, the fibrous structures were connected with each other to form a ring, and the ring was thickened (9 min in Figure 1A). During cytokinesis, the GFP-Adf1 ring contracted, and the GFP-Adf1 patches reappeared around it during the contraction. Figure 1B shows the process of formation of these patches. GFP-Adf1 often protruded from the ring during the contraction. These protrusions changed into patches within a few seconds. After completion of the contraction of the ring, the GFP-Adf1 patches remained in the region where the septum was formed (180 s in Figure 1B, b). These results suggested that Adf1 was involved in controlling the dynamic organization of the actin cytoskeleton through the course of cytokinesis.

The Adf1 accumulation at the midregion was observed neither in mutants incapable of formation of the contractile ring such as *cdc3* (profilin), *cdc4* (myosin light chain), *cdc8* (tropomyosin), and *cdc12* (formin) nor in the cells supplied with 1 μM Latrunculin-A (Lat-A) to abolish the ring formation (Supplemental Figure 1). Alternatively, it was observed in a SIN mutant *cdc14* or a β-tubulin mutant *nda3*, which formed the ring but the ring did not contract at the restrictive temperature. Therefore, the Adf1 localization at the ring was dependent on F-actin and was independent on contraction of the ring.

Overexpression of Adf1 Abolishes F-Actin-containing Structure and Results in Failed Cytokinesis

To overexpress Adf1 in the wild-type cells, we transformed these cells with pREP1*adf1*, pREP41*adf1*, or empty vector. At 20 h after removal of thiamine, the expression level of Adf1 was markedly higher in the cells containing pREP1*adf1* (18.4-fold of control) or slightly higher in those containing pREP41*adf1* (2.3-fold) than in the control cells, respectively (Figure 2A). The expression of Adf1 from pREP1 completely inhibited the growth of the cell, whereas that from pREP41 partially inhibited (our unpublished data).

The control cells showed normal F-actin structures: patches at the cell ends and longitudinal cables were seen in interphase cells, and the contractile ring was seen in dividing cells. However, no F-actin structure was seen in the cells containing pREP1*adf1*, or F-actin structures were somewhat diminished in the cells containing pREP41*adf1* (Figure 2B). These results suggested that Adf1 functions to disintegrate F-actin structures in vivo. As much as 70% of the cells containing pREP1*adf1* were binucleate at 20 h after the induction, whereas 20% of the control cells were binucleate. Calcofluor staining revealed that septum materials formed a number of dots in the middle of the cells containing pREP1*adf1* or abnormal clusters in the cells containing pREP41*adf1* (Figure 2B). These results suggested

Figure 1. Behavior of Adf1 during cytokinesis. (A) Time lapse 3-D images of a dividing cell expressing GFP-Adf1 obtained every minute. Insets, images of the midregion of the cell as seen from an end. Because this cell was placed obliquely to the optical plane, the midregion was obliquely cut to make these images. Therefore, some cortical patches are projected inside of the cell. At an early stage, a few GFP-Adf1 patches occurred in the middle cortex of the cell (1 and 2 min). A GFP-Adf1 filament occurred in the equatorial cortex at 3 min (arrowhead), elongated at 4 min, and partially disappeared at 5 min. A complete GFP-Adf1 ring was formed at 9 min. Bar, 5 μ m. (B) Contraction of the GFP-Adf1 ring during cytokinesis. A single focal plane observed every second in the early (a) or late (b) stages of cytokinesis is shown. Arrows, the GFP-Adf1 ring. Arrowheads, protrusions formed from the ring, which then became patches. Bars, 3 μ m.

that the proper expression level of Adf1 is important for cytokinesis and after normal septation.

In addition, it was found that the overexpression of Adf1 lead to formation of bars in the cytoplasm, which contained Adf1 (Figure 2C). Immunofluorescence study using anti-actin antibodies revealed that those structures contained actin, but they were not stained with rhodamine-phalloidin (our unpublished data). Similar bars containing Cofilp has been reported in budding yeast mutants defective in actin, profilin, or tropomyosin (Moon *et al.*, 1993).

Adf1 Is Required for Reorganization of the Actin Cytoskeleton in Cell Division

It has been found that *adf1*⁺ is essential for cell viability (our unpublished data). To investigate the role of Adf1 in vegetative growth of the cell, we prepared an *adf1* null strain containing

pREP41HA-*adf1* (*KAD4*) to control expression level of *adf1*⁺. The expression level of HA-Adf1 in the *KAD4* cells was similar to that of Adf1 in the wild-type cells (Figure 3A). In contrast, the growth rate of these cells was reduced 12~15 h after the addition of thiamine (Figure 3B). The expression level of HA-Adf1 in this period was much lower than that before the addition of thiamine (Figure 3C). Moreover, the proportion of binucleate cells in the *KAD4* strain increased strikingly after the addition of thiamine (Figure 3D), suggesting that cytokinesis was inhibited by depletion of Adf1.

We examined the distribution of F-actin in the *KAD4* cells (Figure 3E). Before the addition of thiamine, F-actin was organized in a manner similar to the wild-type cells. F-actin patches were localized at the cell ends during interphase and gradually disappeared from the ends during metaphase to anaphase. In contrast, most of the interphase *KAD4* cells

Figure 2. Effect of overexpression of Adf1 on the F-actin structures. (A) Expression levels of Adf1 in Adf1-overexpressing cells. Extracts of wild-type cells carrying pREP1, pREP1*adf1*, or pREP41*adf1*, respectively, cultured at 30°C for 20 h after removal of thiamine were subjected to immunoblot analysis using antibodies against actin as a loading marker (top) and Adf1 (bottom). Equal amounts of the cell extracts were loaded. (B) Overexpression of Adf1 diminishes F-actin structures. The wild-type cells carrying pREP1, pREP1*adf1*, or pREP41*adf1* were fixed and stained with bodipy-phalloidin (top) and 4,6-diamidino-2-phenylindole (DAPI) (middle) or Calcofluor (bottom) after incubation at 30°C for 20 h in EMM. Arrows, Calcofluor-stained dots in the middle of the cells. Arrowheads, abnormally formed septa. (C) Overexpression of Adf1 leads to formation of bar containing Adf1 in cytoplasm. The wild-type cells carrying pREP1*adf1* were fixed and stained with anti-Adf1 antibodies after incubation at 30°C for 20 h in EMM. Arrowheads, Adf1-containing bars. Bars, 10 μ m.

rowheads, abnormally formed septa. (C) Overexpression of Adf1 leads to formation of bar containing Adf1 in cytoplasm. The wild-type cells carrying pREP1*adf1* were fixed and stained with anti-Adf1 antibodies after incubation at 30°C for 20 h in EMM. Arrowheads, Adf1-containing bars. Bars, 10 μ m.

Figure 3. Adf1 is essential for cytokinesis. (A) Expression levels of Adf1 in wild-type (*JY1*) cells and HA-Adf1 in *adf1* null cells expressing pREP41HA-*adf1* (*KAD4*). Extracts of those cells cultured at 30°C without thiamine were subjected to immunoblot analysis using antibodies against actin (top) and Adf1 (bottom). Equal amounts of cell extracts were loaded. (B) Growth of *KAD4* cells in EMM with (open circles) or without (closed circles) thiamine at 30°C. (C) Immunoblots obtained using antibodies against actin as a loading marker (top) and Adf1 (bottom) in *KAD4* cells after the addition of thiamine. Equal amounts of cell extracts were loaded. (D) Percentages of mono- (open bars), bi- (hatched bars), or multi (closed bars)-nucleate *KAD4* cells after addition of thiamine. (E) Fluorescence microscopy of Adf1-depleted cells during the cell cycle. *KAD4* cells were incubated for 12 h with (+) or without (−) thiamine. The 3-D images of the cells stained with rhodamine-phalloidin (left and red in the merged images at the right), TAT1 (middle and green in the merged images), and DAPI (blue in the merged images) are shown. Small arrow, no accumulation of F-actin at the midregion of the cell. Arrowheads, abnormal accumulation of F-actin bundles at the division site of anaphase cells. Large arrows, F-actin accumulated at the septum invaginating from one side of the cell. (F) Localization of Cdc4 (myosin-II light chain) in Adf1-depleted cells (3-D images). *KAD4* cells cultured for 12 h with or without thiamine were fixed and stained with rhodamine-phalloidin (top and red in the merged images), anti-Cdc4 serum (middle and green in the merged images) and DAPI (blue in the merged images). Arrowheads, abnormal accumulation of Cdc4 at the division sites of mitotic cells. (G) Depletion of Adf1 causes a septation defect. After addition of thiamine, *KAD4* cells were fixed and stained with Calcofluor at the time points indicated. Arrows, accumulation of septum materials at one side of the cell near the division site. Arrowheads, septum materials accumulated as dots. (H) Terminal phenotype of Adf1-depleted cells. *KAD4* cells were incubated for 15 h with thiamine. The 3-D images of the cells stained with rhodamine-phalloidin (left and red in the merged images), TAT1 (middle and green in the merged images), and DAPI (blue) are shown. Bars, 10 μ m.

showed abundant F-actin patches localized at the end(s) after 12 h in the presence of thiamine, and these patches largely stayed there even in anaphase. Moreover, accumulation of F-actin at the division site was not seen in 67% of

the metaphase cells (Figure 3E, small arrows) containing the short spindle and in 21% of the anaphase cells in which a spindle had elongated. During anaphase, 52% of the cells had a few thick F-actin bundles, which were randomly ori-

Figure 4. Phenotype of *adf1-1* mutant. (A) Mutation in Adf1 causes a severe decrease in the rate of actin depolymerization in vivo. Cultures of the wild-type, *adf1-1*, and *adf1KMC* cells grown at 25°C were divided equally and incubated further at 25 or 37°C for 10 min. The cells were fixed and stained with bodipy-phalloidin at the indicated time points after addition of 1 μ M Lat-A. The percentage of cells containing F-actin is indicated on the vertical axis. (B) Adf1 disappears rapidly from the actin cytoskeleton in *adf1-1* cells after a shift to 37°C. Wild-type or *adf1-1* cells grown at 25°C were fixed after additional incubation at 25 or 37°C for 10 min. The 3-D images of the cells stained with bodipy-phalloidin (top and green in the merged images at the right), anti-Adf1 antibodies (middle and red in the merged images), and DAPI (blue) are shown. Arrow, deformed contractile ring. Bar, 10 μ m.

ented in the medial region of the cell (Figure 3E, arrowheads), whereas only 27% of the cells were able to form the contractile ring. Myosin light chain Cdc4 has been localized specifically to the contractile ring (McCollum *et al.*, 1995). By immunofluorescence microscopy, we confirmed that Cdc4 (myosin-II light chain) was localized specifically to a part of the medial F-actin bundles in the mid-anaphase cells (Figure 3F). Therefore, at least a part of these structures is likely to be abnormally formed contractile ring. The accumulation of F-actin in the medial region increased after the spindle breakdown, that is, the onset of cytokinesis in the control cells. However, the F-actin was frequently accumulated at one side of the *KDA4* cells, where unilateral septation occurred (Figure 3E, large arrows). Most of these cells were not able to divide. We also examined the *KAD4* cells by staining with Calcofluor (Figure 3G). The septum materials were often accumulated at one side of the middle cortex of these cells. Thus, the abnormally formed contractile ring in the Adf1-depleted cells seemed to retain activity to induce formation of the septum although it was irregularly shaped. These observations indicate that Adf1 functions in mitosis to timely disassemble F-actin patches at the ends of the cell, which may be the source of actin to form the contractile ring. It also seemed to be required to assemble functional contractile ring having a proper configuration.

At 15 h after the addition of thiamine, F-actin patches were randomly distributed all over the surface of *KAD4* cells, which contained two nuclei, interphase microtubules, and several dots of septum materials accumulated around

the midregion (Figure 3, G and H). As shown in Figure 3D, the progression of the nuclear division was arrested at 19 h in the *KAD4* cells. This may be because cell elongation was inhibited by the depletion of Adf1.

Adf1 Functions to Maintain the Structure of the Contractile Ring

We generated two temperature-sensitive *adf1* mutant strains, *adf1-1* and *adf1KMC*. *adf1-1* showed a stronger growth defect than *adf1KMC* at a restrictive temperature 37°C (Supplemental Figure 2). We compared the rates of actin depolymerization in vivo between these mutant cells and wild-type cells using Lat-A (Lappalainen and Drubin, 1997). The rate of disappearance of the actin cytoskeleton after treatment with Lat-A is expected to reflect the rate of subunit dissociation from F-actin in vivo, because the drug sequesters G-actin but does not disassemble F-actin directly (Coué *et al.*, 1987). In the wild-type cells, all the F-actin disappeared within 5 min in the presence of 1 μ M Lat-A at 25°C or at 37°C (Figure 4A). In contrast, the rate was reduced to some extent even at 25°C and to a great extent at 37°C in both of the mutants. The expression level of each mutant protein was constant between 25 and 37°C (our unpublished data). Because the inhibitory effect was prominent at 37°C in *adf1-1* cells compared with that in *adf1KMC* cells, we used the *adf1-1* strain for further analysis.

The organization of F-actin and localization of Adf1 in *adf1-1* cells were investigated. At 25°C, both the actin cytoskeleton and localization of Adf1 were normal (Figure 4B). The structure of the contractile ring looked normal during

Figure 5. Adf1 is an essential component of the contractile ring. After *nda3* cells or *nda3 adf1-1* cells were incubated at 19°C for 6 h, they were released from the mitotic checkpoint by shifting the temperature to 37°C and fixed at the indicated time points for observation. The 3-D images of the cells stained with rhodamine-phalloidin (top and red in the merged images), anti-Cdc4 (myosin-II light chain) antibodies (middle and green in the merged images), and DAPI (blue) are shown. Arrowheads indicate septated sites of the cells. Bar, 10 μ m.

contraction in *adf1-1* cells and also in *adf1KMC* cells, although the rate of contraction of the rings in these cells was slightly slower than that of wild-type cells (Supplemental Figure 3). At 37°C, however, F-actin patches tended to be clustered together at the cell ends, F-actin cables became curly, and the contractile ring was deformed (Figure 4B). Adf1 was diffused in the cytoplasm in *adf1-1* cells within 10 min after the shift to 37°C in contrast to the wild-type cells. Thus, the association of Adf1 with F-actin may be critical for organizing the F-actin structures through the cell cycle.

To explore the function of Adf1 as a component of the contractile ring in detail, we used cold-sensitive *nda3* mutant cells, which are arrested at metaphase at the restrictive temperature of 19°C (Figure 5). Contractile ring containing Cdc4 was organized but did not contract under this condition. The contraction of the ring was induced after completion of nuclear division by incubating these cells for 20 min at the permissive temperature of 37°C. In the *nda3 adf1-1* double mutant cells, the ring was disintegrated and curly irregular

F-actin cables were developed within 10 min after the shift to 37°C, although the ring was normally organized in mitotic-arrested cells at 19°C because Adf1 activity was maintained. Moreover, the ring structure of Cdc4 was also disintegrated within 20 min. We confirmed that Adf1 disappeared from the actin filaments in these cells (our unpublished data). This result suggested that the activity of Adf1 was required for maintaining the structure of the contractile ring. Septation was initiated in these cells after the progression of mitosis, even though the contractile ring was absent. However, most of the cells were unable to separate into two daughter cells (40 min in Figure 5).

Interaction of Adf1 with F-Actin Is Controlled by Tropomyosin in the Contractile Ring

When Adf1 was overexpressed in wild-type cells, all the F-actin structures, including the contractile ring, disappeared (Figure 2), despite that Adf1 is an essential component of the ring. Therefore, there may be a mechanism by which Adf1

Figure 6. Cdc8 (tropomyosin) antagonizes the function of Adf1 in the contractile ring. (A) Cdc8 interferes with the localization of Adf1 in the contractile ring. Wild-type cells carrying pREP1 (top) or pREP1cdc8 (bottom) were grown at 30°C without thiamine for 20 h and processed for 3-D imaging for F-actin (left and green in the merged images), Adf1 (middle and red), and DNA (blue). Arrowheads, position of the contractile ring. (B) Cdc8 protects F-actin from depolymerization induced by Adf1 in the contractile ring. After *nda3 cdc8* double mutant cells or *nda3 cdc8 adf1-1* triple mutant cells were incubated at 19°C for 6 h, the cells were released from the mitotic checkpoint by shifting the temperature to 37°C and fixed after 10 min. The 3-D images of the cells stained with bodipy-phalloidin (left and green in the merged images), anti-Cdc8 serum (middle and red in the merged images), and DAPI (blue) are shown. Bars, 10 μ m.

activity is controlled in the contractile ring. It has been reported that vertebrate ADF/cofilin-family proteins are controlled via phosphorylation of their N-terminal serine residues. However, no defect was observed in a strain expressing mutated Adf1 in which the N-terminal Ser4 was substituted with Ala (our unpublished data). Thus, the association of Adf1 with F-actin is likely to be regulated in another way.

Because Adf1 is not localized to the F-actin cables although it binds to F-actin in vitro (our unpublished data), there may be a mechanism that excludes Adf1 from the F-actin cables. Cdc8 (tropomyosin) is a component of F-actin cables, which are considered to be precursors of the ring (Arai *et al.*, 1998; Arai and Mabuchi, 2002). It is essential for the accumulation of F-actin in the midregion (Balasubramanian *et al.*, 1992; Chang *et al.*, 1996). In addition, tropomyosin stabilizes F-actin from depolymerization caused by ADF (Bernstein and Bamburg, 1982). Indeed, Cdc8 interferes with the association of Adf1 with F-actin in vitro (our unpublished data). Thus, we examined the effect of overexpression of Cdc8 in the wild-type cells. Both F-actin cables and contractile rings were curly and often very thick in the cells overex-

pressing Cdc8 (Figure 6A). These F-actin patterns were similar to those in *nda3 adf1-1* cells (Figure 5). Twenty-six percent of the mitotic Cdc8-overexpressing cells formed an aberrant contractile ring, 84% of which showed no Adf1 signal in the ring. The rate of actin depolymerization in the ring induced by Lat-A was reduced in these cells: 19% of the contractile ring remained after 5-min incubation with 1 μ M Lat-A, whereas no ring was observed in the control cells under the same conditions. The expression level of Adf1 was not affected by the overexpression of Cdc8 (our unpublished data). Therefore, it is likely that excess Cdc8 interfered with the interaction between Adf1 and F-actin in these cells. We examined whether elevation of the expression level of Adf1 neutralize the phenotype of the Cdc8-overexpressing cells. However, the overexpression of Adf1 did not suppress the abnormal formation of the contractile ring in the Cdc8-overexpressing cells (our unpublished data).

Next, we investigated the role of Cdc8 in stability of the contractile ring once formed. We examined *nda3 cdc8* double mutant cells released from mitotic arrest at 19°C, which is

Figure 7. F-actin-severing activity of ADF/cofilin may be involved in assembly of the contractile ring. (A) Growth of *KAD4* cells expressing porcine Cof1, Cof1F82, or Cof1A120. Both actin-depolymerizing and -severing activities decrease in Cof1A120, whereas only actin-depolymerizing activity decreases in Cof1F82. The cells incubated on EMM plates with or without thiamine at 25°C for 3 d. The expression of either Cof1 or Cof1F82 rescued the growth defect of *KAD4* cells with thiamine. In contrast, Cof1A120 was not able to substitute effectively for the function of *S. pombe* Adf1. Each porcine cofilin protein was tagged with 6×His at the N terminus. Mock indicates the cells containing empty vector. (B) Immunoblots of *KAD4* cells expressing Cof1, Cof1F82, or Cof1A120 incubated for 16 h at 25°C with or without thiamine using antibodies against actin, Adf1, or 6×His. Equal amounts of cell extracts were loaded. (C) Fluorescence micrographs of Cof1-expressing cells. Cells incubated with thiamine in B were fixed and processed for 3-D imaging with bodipy-phalloidin (top and green in the merged images), TAT1 (red), and DAPI (blue). An arrow, an anaphase cell without contractile ring. Small arrowheads, contractile rings formed by the function of Cof1 or Cof1F82. Large arrowhead, an abnormal accumulation of F-actin bundles at the division site of an anaphase cell expressing Cof1A120. Asterisk (*) indicates that this cell could not accomplish cytokinesis. Bar, 10 μ m.

the restrictive temperature for the *nda3* mutation, by raising the temperature to 37°C, which is, in contrast, the restrictive-temperature for the *cdc8* mutation. Cdc8 was localized to the contractile ring during the mitotic arrest. However, it disappeared from the midregion and the contractile ring concomitantly disappeared soon after the shift to 37°C (Figure 6B). This result indicated that the Cdc8 activity is required for maintaining the contractile ring structure. Next, we examined *nda3 cdc8 adf1-1* triple mutant cells. In these cells, twisted F-actin cables were rapidly formed at the midregion after the function of both Adf1 and Cdc8 was lost. These cables were confirmed to be derived from the contractile ring, because Cdc4 (myosin-II light chain) was localized there (our unpublished data). These results indicated that Adf1 disassembled the contractile ring completely without the function of Cdc8 and that both Adf1 and Cdc8 were necessary to maintain the proper dynamic structure of the contractile ring.

Actin-severing Activity of ADF/Cofilin Is Likely Required for Assembling the Contractile Ring

ADF/cofilin-family proteins are supposed to control the organization of the actin cytoskeleton by both severing F-actin and accelerating dissociation of G-actin from the pointed end of the filaments. Thus, we investigated whether these activities are involved in contractile ring formation. The substitution of Ser120 of porcine cofilin Cof1 with Ala decreases both the actin-severing and -depolymerizing activities, whereas that of Tyr82 with Phe decreases only the actin-depolymerizing activity (Moriyama and Yahara, 1999). First, we investigated the effects of expression of wild-type Cof1 or each mutant protein on the growth of the Adf1-depleted cells (Figure 7A). The expression of Cof1F82 enabled the cells to grow as well as that of the wild-type Cof1. In contrast, the growth of the Adf1-depleted cells expressing Cof1A120 was significantly slow. There was no difference in

expression level between Cof1A120 and Cof1F82 after the depletion of Adf1, although the expression levels was higher than that of wild-type Cof1 (Figure 7B). Next, we examined the organization of the actin cytoskeleton in these cells. The expressions of Cof1 and Cof1F82 restored formation of the contractile ring in the Adf1-depleted cells (Figure 7C and Table 2). In contrast, the Adf1-depleted cells expressing Cof1A120 could not assemble a proper contractile ring similar to the cells having empty vectors and often failed to divide. Therefore, it was likely that the actin-severing activity of Cof1 functioned in assembling the contractile ring in the Adf1-depleted cells.

Genetic Interaction between Adf1 and Actin-modulating Proteins

We investigated the genetic interaction between *adf1*⁺ and the genes encoding actin-modulating proteins as shown in Table 3. Double mutant of *adf1* and Δ *acp1*, Δ *acp2*, *cdc3*, *cdc4*, or *myo2* was lethal. Although spores of each double mutant

Table 2. Frequency (%) of F-actin ring formation in *KAD4* cells expressing Cof1 or its mutant

Protein expressed	Normal ring	Abnormal ring	No accumulation of F-actin
Mock	20.2	47.7	32.1
Cof1	59.4	27.4	13.2
Cof1F82	66.1	21.1	12.8
Cof1A120	27.0	57.7	15.3

More than 100 cells containing a long spindle were examined for each strain.

Table 3. Genetic interaction between *adf1*⁺ and genes controlling the actin cytoskeleton or cytokinesis

Strain	Gene product	Phenotype of double mutant with	
		<i>adf1-1</i>	<i>adf1KMC</i>
<i>cdc3-124</i>	Profilin	SL	SL
<i>cdc4-8</i>	Myosin-II essential light chain	SL	SL
<i>cdc8-110</i>	Tropomyosin	NE	NE
<i>cdc12-112</i>	Diaphanous/formin-family protein	NE	NE
<i>cdc15-140</i>	PSTPIP/PCH-family protein	NE	NE
<i>Δmyo1</i>	Myosin-I heavy chain	NE	NE
<i>myo2-E1</i>	Myosin-II heavy chain	SL	SL
<i>arp3-cs1</i>	Essential component of Arp2/3 complex	NE	NE
<i>Δfor3</i>	Diaphanous/formin-family protein	NE	NE
<i>Δacp1</i>	α-Subunit of F-actin-capping protein	SL	SL
<i>Δacp2</i>	β-subunit of F-actin-capping protein	SL	SL
<i>Δfim1</i>	Fimbrin	NE	NE
<i>rng2-D5</i>	IQGAP	NE	NE
<i>cdc7-24</i>	Ser/Thr-protein kinase in the SIN pathway	SG	SG

Growth of each double mutant cells was compared with those of corresponding parent cells at 25°C, a permissive temperature for the parent mutants. Double mutant cells between *adf1* mutants and *cdc3-124*, *cdc4-8*, *myo2-E1*, *Δacp1*, or *Δacp2* did not grow from spores from parent strains (n ≥ 20). SL, synthetic lethal; SG, slow growth; and NE, no effect.

could germinate, the germs were elongated and sometimes branched with abnormal septa (our unpublished data).

We then examined the terminal phenotype of the double mutants by using a thiamine-repressible promoter. *cdc3 adf1-1* cells containing pREP41*adf1* grew as well as *cdc3* cells without thiamine at the permissive temperature of 25°C and formed the contractile ring normally (our unpublished data). In contrast, the accumulation of F-actin in the midregion was delayed in 53% of the double mutant cells at 25°C in the presence of thiamine: no accumulation was observed during metaphase to early anaphase in these cells (Figure 8A). Moreover, formation of the contractile ring was impaired in 85% of the *cdc3 adf1-1* cells even at late anaphase or telophase. This phenotype was

very similar to that of *Adf1*-depleted cells as mentioned above. In addition, we observed a similar terminal phenotype in a double mutant *adf1-1 Δacp1* or *adf1-1 Δacp2* strain (our unpublished data). Thus, we concluded that *Adf1* functions cooperatively with *Cdc3* (profilin) and actin-capping protein to form the F-actin ring.

We then examined the relationship between *Adf1* and myosin-II. The *myo2 adf1-1* double mutant strain was inappropriate for this purpose because cytokinesis was often impaired in these cells even when *Adf1* or *Myo2* was expressed from pREP41. Thus, we observed the phenotype of *cdc4 adf1-1* cells containing pREP41*cdc4*. Formation of the contractile ring progressed normally in this strain without thiamine at 25°C (our unpublished data). Repression of *Cdc4* (myosin-II light chain) expression did not affect the accumulation of F-actin in the midregion during metaphase or early anaphase (Figure 8B). However, during late anaphase, contractile ring formation was disturbed in the *cdc4 adf1-1* double mutant cells: 74% of the cells still formed an asterlike structure of F-actin cables, a precursor of the contractile ring (Arai and Mabuchi, 2002), in the midregion, and only 24% developed a normal contractile ring. Under the same conditions, all of the *cdc4* and *adf1-1* single mutant cells were able to form the contractile ring. It has been postulated that myosin-II functions in assembling the contractile ring by interacting with F-actin in the midregion (Motege *et al.*, 2000). Thus, we concluded that *Adf1* induces assembly of the ring from F-actin accumulated at the division site together with myosin-II and that this role of *Adf1* is different from the one collaborative with profilin and capping protein.

In addition, a weak genetic interaction was detected between *adf1*⁺ and *cdc7*⁺, which encodes a protein kinase in a SIN signaling pathway (Fankhauser and Simanis, 1994) and monitors the cytokinesis checkpoint (Liu *et al.*, 2000) (Table 3). The two double mutant strains, *adf1-1 cdc7* and *adf1KMC cdc7*, did not form colonies at 30°C, whereas each single mutant did. Moreover, the double mutant cells were often abnormally elongated at 25°C, whereas the single mutant cells showed a normal shape (our unpublished data). Therefore, *Adf1* may also be involved in formation of the septum.

DISCUSSION

Adf1 Generates G-Actin Necessary for Formation of the Contractile Ring

We first demonstrated that most of the F-actin patches remained at the ends of mitotic cells when the expression of *Adf1* is suppressed. This may be a reason why accumulation of F-actin at the division site was delayed in these cells. *Adf1*

Figure 8. Genetic interaction of *Adf1* with *Cdc3* (profilin) (A) and with *Cdc4* (myosin-II light chain) (B) in assembling the contractile ring. *cdc3* cells, *cdc3 adf1-1* cells containing pREP41HA-*adf1*, *cdc4* cells, and *cdc4 adf1-1* cells containing pREP41*Cdc4* were incubated with thiamine for 18 h at 25°C. F-actin is accumulated as an asterlike structure in the *cdc4 adf1-1* cells at metaphase (middle cell) and even at late anaphase (bottom cell) (asterisk). The 3-D images of the cells stained with bodipy-phalloidin (top and green), TAT1 (red), and DAPI (blue) are shown. Arrowheads and arrows indicate midregions of metaphase cells and anaphase cells, respectively. Bars, 10 μm.

colocalized with the F-actin patch and is involved in turnover of actin in the patch (our unpublished data). We presented lines of evidence that Adf1 functions in depolymerizing actin in the patch: overexpression of Adf1 lead to disappearance of F-actin patches, patches became clustered in *adf1-1* cells, and patches in *adf1-1* cells or *adf1KMC* cells were less sensitive to Lat-A than those in wild-type cells. Therefore, it is plausible that Adf1 disassembles F-actin in the patch during early mitosis by an unknown signal to supply a sufficient amount of actin for formation of the ring. The depolymerized actin, probably in the form of Adf1-G-actin complex, may diffuse in the cytoplasm or actively be transported through some structure such as the longitudinal F-actin cable to the midregion of the cell and repolymerized there as the cables composing the asterlike structure.

Cdc3 (profilin) is essential for contractile ring formation and localized around the division site (Balasubramanian *et al.*, 1994). We found that *adf1*⁺ genetically interacted with *cdc3*⁺: both of *adf1-1* and *adf1KMC* were synthetic lethal with *cdc3*, and accumulation of F-actin at the midregion during mitosis and after formation of the contractile ring was impaired in *cdc3 adf1-1* cells. This phenotype indicated that the cooperative function of Adf1 and Cdc3 is involved in these processes. Profilin may be able to control actin dynamics by increasing the rate of nucleotide exchange on G-actin, and promoting the polymerization of actin by lowering the critical concentration (reviewed by Theriot and Mitchison, 1993). It may be able to replace ADF in the ADF-G-actin complex by competing binding to actin (reviewed by Pollard and Borisy, 2003). Thus, it is possible that Cdc3 promotes polymerization of actin into cables at the division site, which is generated by the disassembly of F-actin patches by Adf1. It is also possible that Cdc3 together with Cdc12 (Chang *et al.*, 1997; Kovar *et al.*, 2003) promotes polymerization of the leading F-actin cable (Arai and Mabuchi, 2002) from disassembled actin. However, this is not clear at present because no genetic interaction was seen between *adf1*⁺ and *cdc12*⁺. Alternatively, Adf1-G-actin may directly polymerize into F-actin by the action of myosin-II because myosin-II has been shown to polymerize actin in vitro from the depactin-actin complex (Mabuchi, 1982), and myosin-II may be previously accumulated at the division site (Motegi *et al.*, 2000). How the G-actin for assembling of the ring is generated is illustrated in Figure 9.

In addition, the F-actin-capping protein, the Acp1/Acp2 complex, was also suggested to contribute to the mitotic actin reorganization in cooperation with Adf1. The capping protein may facilitate depolymerization of actin in the patches by preventing polymerization at the barbed end or by limiting the length of F-actin in the contractile ring to secure the number of actin filaments to ensure effective contraction of the ring.

Adf1 Functions in Assembling the F-Actin Ring

GFP-Adf1 occurred as a transient fibrous structure around the division site before the complete formation of the GFP-Adf1 ring. This suggested that Adf1 functions in the early step of formation of the contractile ring. Actually, assembly of the contractile ring was impaired in Adf1-depleted cells, even though F-actin had accumulated at the division site. However, the localization of Adf1 at the division site was dependent on F-actin. Therefore, Adf1 is not a factor such as myosin-II that comes to the division site earlier than F-actin and assembles actin there.

Adf1 showed strong genetic interactions with the myosin-II heavy chain Myo2 and the light chain Cdc4: contractile ring assembly was disturbed in *cdc4 adf1-1* cells, although

Figure 9. Model of actin dynamics induced by Adf1 during cytokinesis.

the asterlike structure was formed. This fact together with the result indicating that the F-actin-severing activity of Adf1 is necessary for the contractile ring assembly leads us to important inferences on cooperation of myosin-II and Adf1. One inference is that both of these proteins function in actin polymerization. We have evidence that *myo2* mutants *myo2S1444A* (Motegi *et al.*, 2004) or *myo2A1409T* (Motegi and Mabuchi, unpublished data) prematurely accumulate at the midregion of the cell during G₂ phase and induce contractile ring formation in this phase. Thus, Myo2 has an ability to induce F-actin assembly at the midregion of the cell. Similarly in frog egg, myosin-II accumulates as dots at the cleavage site earlier than F-actin, and actin assembles on the myosin dots (Noguchi and Mabuchi, 2001). As for Adf1, ADF/cofilin increases the number of nuclei for polymerization by severing F-actin. Thus, explosive polymerization of actin occurs after a short lag time, when ADF/cofilin is present as shown by in vitro experiments with depactin (Mabuchi, 1983), cofilin (Nishida *et al.*, 1984), and actophorin (Maciver *et al.*, 1991). Therefore, it is possible that Myo2 induces polymerization of actin at the division site at an early step of mitosis, and then Adf1 severs these F-actin to induce further polymerization. The second possibility is that the F-actin cables accumulated around the division site could be too long to be included quickly into the ring structure. Thus, Adf1 would sever these F-actin into short filaments, which may easily be incorporated into the ring. Myosin-II, probably in a form of filaments, would rearrange these F-actin pieces into the ring by its cross-linking activity (Mabuchi, 1990). These inferences are depicted in Figure 9.

Maintenance of the Contractile Ring Structure by Functions of Adf1 and Cdc8 (Tropomyosin)

In *adf1-1* cells, the contractile ring was disintegrated, and abnormal cables were formed soon after the loss of Adf1 activity. The disintegration of the ring occurred even in anaphase cells in which the ring did not begin to contract. Thus, function of Adf1 is probably required for maintenance

of the proper contractile ring structure irrespective of its contraction. We further demonstrated that abnormal thick F-actin cables were formed around the division site instead of the contractile ring in the cells overexpressing Cdc8. Adf1 was excluded from the F-actin structures in these cells. In contrast, the contractile ring disappeared in cells overexpressing Adf1. Similarly, the contractile ring disappeared rapidly soon after the loss of Cdc8 activity. Thus, it is likely that Cdc8 functions to protect F-actin from depolymerization by Adf1 in normal cells. Although both Adf1 and Cdc8 are required for the contractile ring to form, the former provides the ring with its dynamic properties, whereas the latter stabilizes the ring. This balanced interaction of these proteins with F-actin is likely important for maintaining the contractile ring.

Depolymerization of F-Actin Occurs during Cytokinesis

During contraction of the F-actin ring, the thickness of the F-actin ring did not seem to change (Supplemental Figure 3). Therefore, actin may be lost from the ring during this process as in dividing animal cells (Schroeder, 1972; Mabuchi, 1986). We found that the GFP-Adf1 ring contracted as division proceeded, indicating that GFP-Adf1 colocalized with the contractile ring during its contraction. Therefore, it is possible that Adf1 disassembles actin in the ring as division proceeds. Similarly, it has been suggested that ADF/cofilin is required for disassembling the contractile ring at a late stage of cytokinesis in animal cells (Gunsalus *et al.*, 1995; Somma *et al.*, 2002; Kaji *et al.*, 2003; Ono *et al.*, 2003). In sand dollar eggs, injection of phalloidin quickly stabilizes the contractile ring and inhibits its contraction (Hamaguchi and Mabuchi, 1982), although phalloidin does not interfere with actin-myosin sliding (Yanagida *et al.*, 1984). This suggests that the depolymerization of actin may be required for contraction of the contractile ring. Here, we found that contraction of the contractile ring is a little slower in the *adf1* mutant cells than in the wild-type cells. Therefore, Adf1 may be involved in controlling contraction of the contractile ring by depolymerizing F-actin. However, no significant difference in velocity of the ring contraction was observed between *adf1-1* and *adf1KMC* strains, whereas the rate of actin turnover was more reduced in the latter than the former cells at 25°C. Thus, it may be that the rate of turnover of actin is not directly related to the rate of ring contraction.

It has been considered that F-actin patches that newly occur around the contractile ring as cytokinesis progresses may somehow function in septation (Balasubramanian *et al.*, 1998). It was observed that GFP-Adf1 patches frequently emerged from the ring during its contraction. Thus, actin depolymerized by Adf1 during contraction of the ring may be reorganized into the patches. We showed a genetic interaction between *adf1*⁺ and *cdc7*⁺, which encodes a protein kinase essential for septation (Fankhauser and Simanis, 1994). Therefore, Adf1 may be involved in septation by inducing a dynamic reorganization of F-actin around the division site after disassembly of the contractile ring.

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