

Three-dimensional arrangement of F-actin in the contractile ring of fission yeast

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The contractile ring, which is required for cytokinesis in animal and yeast cells, consists mainly of actin filaments. Here, we investigate the directionality of the filaments in fission yeast using myosin S1 decoration and electron microscopy. The contractile ring is composed of around 1,000 to 2,000 filaments each around 0.6 μm in length. During the early stages of cytokinesis, the ring

consists of two semicircular populations of parallel filaments of opposite directionality. At later stages, before contraction, the ring filaments show mixed directionality. We consider that the ring is initially assembled from a single site in the division plane and that filaments subsequently rearrange before contraction initiates.

Introduction

Cytokinesis in animal and primitive eukaryotic cells is executed by contraction of the contractile ring formed underneath the plasma membrane at the division site (Mabuchi, 1986; Salmon, 1989; Narumiya and Mabuchi, 2002), which is composed mainly of actin filaments (F-actins) and myosin-II. It has been shown by decoration with heavy meromyosin or myosin S1 that the contractile ring F-actin consists of two populations with opposite directionalities, respectively (Sanger and Sanger, 1980; Mabuchi et al., 1988), which supports the idea that the contractile ring contracts by sliding of F-actins over each other via myosin filaments (Mabuchi and Okuno, 1977; Mabuchi, 1986). How myosin and actin assemble into the ring has frequently been studied with the fission yeast *Schizosaccharomyces pombe* because many mutant strains that show defects in ring formation have been obtained (Feierbach and Chang, 2001; Rajagopalan et al., 2003).

S. pombe cells are cylindrical, and grow during interphase by elongation at cell ends where F-actin forms patch structures (Marks and Hyams, 1985) and longitudinal F-actin cables originate (Marks and Hyams, 1985; Arai et al., 1998; Arai and Mabuchi, 2002; Kamasaki et al., 2005). These F-actin structures are considered to function in polarized growth of the cell (Kamasaki et al., 2005). During early mitosis, the novel aster-like structure of F-actin cables is formed near duplicated spindle pole bodies through reorganization of the interphase F-actin structures. From the aster the leading F-actin cables that encircle the cell at the

equator elongate, which have been considered to represent the primary contractile ring, and the contractile ring is established during anaphase from these structures (Arai and Mabuchi, 2002). Cytokinesis progresses by constriction of the ring followed by septum formation (Gould and Simanis, 1997; Rajagopalan et al., 2003). Participation of myosin-II (McCollum et al., 1995; Naqvi et al., 1999; Motegi et al., 2000), the formin Cdc12 (Chang et al., 1997), and the actin-depolymerizing factor Adf1 (Nakano and Mabuchi, 2006) is requisite for assembly of the contractile ring. This suggests that polymerization of actin may be a crucial step in assembly of the ring because all of these proteins from this or other organisms can induce or accelerate actin polymerization in vitro (Hayashi et al., 1977; Mabuchi, 1983; Kovar et al., 2003), and are localized at the division site at very early stage of mitosis (Chang et al., 1997; Chang, 1999; Motegi et al., 2000; Wu et al., 2003). However, it has not been known how these proteins actually function in the course of the ring assembly including the timing and precise site of function. The main reason for this is that all of the localization studies of these and other relevant proteins have so far been performed with fluorescence microscopy. Ultrastructural analyses of the process of ring assembly are now required in order to elucidate spatial organization of the assembly at a molecular level. Here, we investigated arrangements of F-actin in the ring by electron microscopy in order to understand basic structure of the ring and how actin is assembled into the ring structure.

Results and discussion

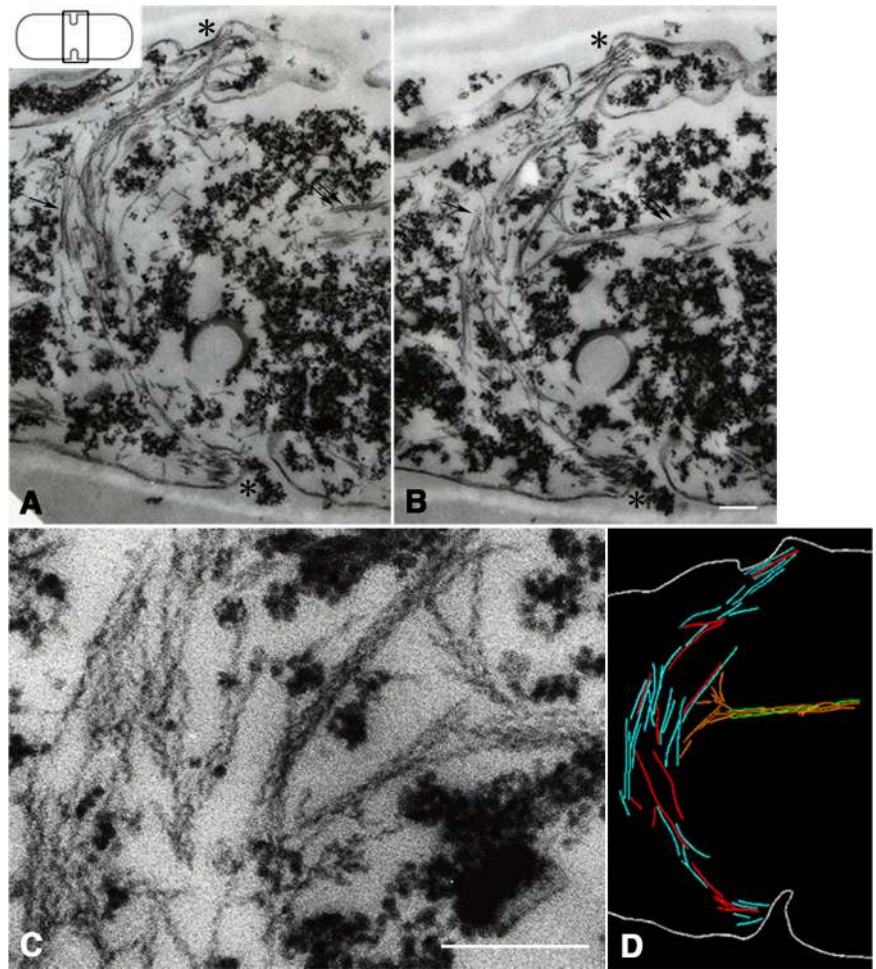
We used both wild-type cells and *cdc25-22* mutant (Russell and Nurse, 1986) cells synchronized at M phase. Cell wall materials

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Figure 1. Images of an S1-decorated contractile ring in longitudinal grazing sections of a dividing *cdc25* cell. An illustration of the observed region in the cell is shown in the top left. (A and B) Serially sectioned images of the ring. These two sections adjoin each other, (A) being close to the surface of the cell. Asterisks indicate position of the furrow. Single arrows, the contractile ring. Double arrows, an F-actin cable. (C) Magnified image of a central part of B. (D) Tracings of F-actin shown in A. F-actins whose pointed end faced the top asterisk in A are colored red, whereas those having the opposite directionality are colored blue. For the longitudinal cable, F-actins whose pointed end pointed to the left are colored orange, while those having the opposite directionality are colored green. White line, cell membrane. Bars, 0.2 μm .



were enzymatically digested and the cells were permeabilized with Triton X-100. Myosin S1 was added to the cells to decorate F-actin and the cells were processed for examination by transmission electron microscopy. It has been confirmed that the structure of actin cytoskeleton in these cells is preserved through this procedure (Kamasaki et al., 2005; Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200612018/DC1>).

Both the wild-type cells and the *cdc25* cells at M phase showed a bundle of microfilaments at the division site often associated with ingressions of plasma membrane in longitudinal grazing sections. S1 decoration to form arrowhead structures showed that these filaments were composed of F-actin (Fig. 1, A–C; Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200612018/DC1>). In Fig. 1 D, the F-actins whose pointed ends faced the top asterisk in Fig. 1 B are shown in red, whereas those showing the opposite directionality are shown in blue. It is apparent that the ring was composed of F-actins of opposite orientations from each other. A longitudinal F-actin cable attached to the ring is seen in Fig. 1. Such cables have been found by fluorescence microscopy both before and after establishment of the ring (Arai et al., 1998; Arai and Mabuchi, 2002). The S1 decoration showed that these cables consisted of F-actin of opposite orientations. Another feature of these cables was that they were fragmented at the end close to the ring and then seemed to be incorporated into the ring structure.

The F-actin ring of a *cdc25* cell sectioned parallel to the division plane around the equator was observed as loose bundles of F-actin lying underneath the cell membrane and encircling the cytoplasm (Fig. 2 A). We examined the entire structure of six contractile rings by serial sectioning of the rings; these rings were confined in 14–33 serial sections (55 nm/section; Table I). Stages of the rings in the process of cytokinesis were determined as follows. Dividing nuclei were seen adjacent to edges of rings #1 and #2, but they were not seen in the vicinity of rings #3 to #6 (unpublished data). Therefore, it was considered that rings #1 and #2 were in cells at early anaphase B. Diameters of the median circles of rings #1 to #4 were similar and they were 2.3–2.5 μm , while those of rings #5 and #6 were significantly smaller (Table I). Thus, rings #1 to #4 were not yet contracting, while rings #5 and #6 were contracting when the cells were permeabilized. This suggests that the cells possessing rings #3 and #4 were at late anaphase B.

The total number of F-actin in the rings was between 1,100 and 2,100 (Table I). F-actins whose pointed ends were clockwise on the photographs are shown in red, and those whose pointed ends were counterclockwise are shown in blue. F-actins of unclear directionality or those oriented perpendicular to the plasma membrane are shown in yellow (Fig. 2, B and D). The ratio of clockwise-oriented F-actins to counterclockwise-oriented ones in the contractile ring was roughly 1:1 for all the rings analyzed (Table I).

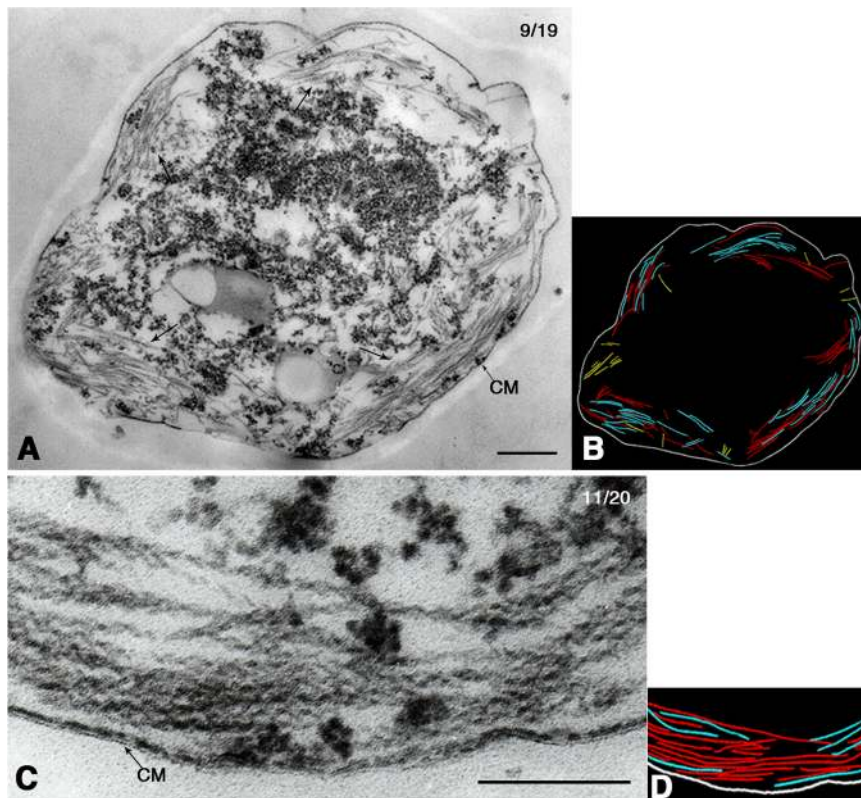


Figure 2. Images of S1-decorated contractile rings in equatorially sectioned *cdc25* cells. (A) An image of section #9 of the contractile ring at late anaphase B (ring #4, the total section number = 19). Arrows, contractile ring F-actins. CM, cell membrane. (B) Tracings of F-actin shown in A. F-actins whose pointed end was oriented clockwise are colored red, while those oriented counterclockwise are colored blue. F-actins of other orientations or unknown orientation are colored yellow. White line, cell membrane. (C) A magnified image of a part of section #11 of an early anaphase B ring (ring #1, the total section number = 20). In this region, F-actins whose pointed ends are oriented counterclockwise form a cluster. (D) Tracings of F-actin in C. Bars, 0.2 μm .

The average length of randomly extracted 100 F-actins in rings #1 to #4 was 0.6 μm , while that in rings #5 and #6 was 0.45 μm (Table I; Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200612018/DC1>). This suggests that F-actins in the ring shortened as it contracted. It was also found that F-actins having opposite directionalities were not mixed homogeneously in the ring, but those of a same directionality were seen to form clusters (Fig. 2, C and D). In addition, branching of F-actin was hardly seen in both longitudinal grazing sections (4 rings) and equatorial sections (rings #1 to #6).

Next, we performed a three-dimensional reconstruction of the contractile rings in which directionality of each F-actin is indicated (Fig. 3 A; Fig. S3, B and C; and Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200612018/DC1>). It is remarkable in both of the early stage rings #1 and #2 that F-actins with the same directionality occupied about a half of the ring on the whole, as if the F-actin ring was composed of two semicircles of uniform, but opposite polarity. On the other hand, F-actins in the rings of later stages seemed to be mixed homogeneously on the whole (Fig. 3 A; Fig. S3, B and C; and Videos 1 and 2). To quantitatively show this feature, we divided all serial images of the 6 contractile rings into 12 equal segments, respectively, counted F-actins in each segment, calculated percentages of F-actins of the major two directionalities, and then obtained absolute value of difference in the percentages for every segment (Fig. 3 B; Fig. S3 C). For rings #1 and #2, the differences were minimum in the bottom (segment 6) and the top (segment 1) segments, indicating that numbers of F-actins of opposite directionalities were close in these segments, while the differences were maximum in the right and left segments,

indicating that F-actins of same directionalities were dominant in these regions. Furthermore, the differences were larger in many of the segments of rings #1 and #2 than in the segments of rings #3 to #6 (Fig. 3 B; Fig. S3 C). This suggests that overlapping of the filaments of opposite directionalities progress from early anaphase B to late anaphase B/cytokinesis.

The fact that the fission yeast contractile ring is composed of antiparallel actin filaments of 1:1 number ratio strongly suggests that this structure is contractile, providing that myosin filaments are present in the ring. Though we did not see myosin filament-like structures in the ring in thin sections, it has been known that the myosin-II heavy chain Myo2 is colocalized to the ring, and is necessary in establishment of the ring (Kitayama et al., 1997; Naqvi et al., 1999; Motegi et al., 2000). The present finding is important because no evidence for actual contraction of the ring in fission yeast cell has been obtained, although it shrinks during cytokinesis.

We estimated the average length and the number of actin filaments in the contractile ring to be around 0.6 μm and 1,100–2,100, respectively. Taking the filament number as 2,000, total actin monomers comprising one ring would be 444,000. The actin concentration in the cytoplasm including all the organelles in the wild-type fission yeast cells has recently been estimated to be 8.7 μM (Takaine and Mabuchi, 2007), which is significantly smaller than that reported previously (31.3 [in a minimal medium] or 63.2 [in a rich medium] μM ; Wu and Pollard, 2005). Supposing that actin concentration in the *cdc25* cell is same as that in the wild-type cell, and the length and diameter of the cytoplasmic compartment of a mitotic *cdc25* cell are 28.6 and 2.84 μm , respectively (Kamasaki et al., 2005; this paper), number of actin

Table 1. Structure of F-actin contractile rings obtained by analyses of equatorially sectioned images

	Ring #1	Ring #2	Ring #3	Ring #4	Ring #5	Ring #6
Cell cycle stage	early anaphase B	early anaphase B	late anaphase B	late anaphase B	cytokinesis	cytokinesis
Number of serial sections	14	20	32	19	33	26
Number of total filaments in each F-actin ring (%)	1,119 (100)	1,571 (100)	1,460 (100)	2,000 (100)	2,112 (100)	1,066 (100)
Number of clockwise filaments (%)	468 (41.8)	710 (45.2)	509 (34.9)	899 (45)	731 (34.6)	356 (33.3)
Number of counterclockwise filaments (%)	581 (51.9)	645 (41.1)	676 (46.3)	873 (43.7)	886 (42.0)	427 (40)
Number of other filaments (%)	70 (6.3)	216 (13.7)	275 (18.8)	228 (11)	495 (23.4)	283 (26.5)
Length of F-actin (average \pm SD μ m)	0.61 \pm 0.16	0.59 \pm 0.16	0.59 \pm 0.15	0.58 \pm 0.17	0.45 \pm 0.11	0.45 \pm 0.13
Estimated diameter of the ring (μ m)	2.31	2.38	2.33	2.54	1.43	1.89

The average length of F-actin was obtained for randomly extracted 100 F-actins. Some of these F-actins spanned two serial sections. Diameter of the ring was estimated as follows. First, the area enclosed by a median circle of a ring, which passes through midpoints of the width of the ring was estimated using NIH Image. The diameter was then calculated supposing that the median circle was a complete circle.

molecules in the mitotic cell is roughly 920,000. Therefore, \sim 50% of the actin molecules are used to form the contractile ring. This agrees with the observation by fluorescence microscopy that a majority of actin filaments in the mitotic cell seems to form the contractile ring (Arai and Mabuchi, 2002).

We found that the actin filaments in the contracting ring were shorter than those in the ring before contraction. It has been considered that the filaments must be disassembled during contraction because the contractile ring retains its width, depth from the plasma membrane, and density of filaments during contraction and finally disappears when contraction is complete (Schroeder, 1972). Our observation demonstrated for the first time that this is the case. The shortening of the filaments is likely to be due to depolymerization of actin (Mabuchi, 1986) especially induced by Adf1, which is localized to the ring throughout the course of cytokinesis and necessary for disassembly of the ring (Nakano and Mabuchi, 2006).

The overall structure of the F-actin contractile ring revealed here leads us to predict important features of formation and establishment of the contractile ring in fission yeast cells. Polymerization of actin seems to be a crucial step in the ring assembly. It has been believed that actin polymerizes in the cell preferably from the barbed end (Pollard and Borisy, 2003). Therefore, the fact that the ring at early anaphase B seemed to roughly comprise two semicircles of unidirectional F-actins, the directionalities of which were opposite from each other, suggests that formation of the ring contains a step where polymerization of actin may take place mainly at one site in the medial cortex of the cell and elongation occurs in both directions in the equatorial plane (Fig. 3 C). A strong candidate for such a site is the center of the aster-like structure of F-actin cables, which is formed near spindle pole bodies during prophase and from which the leading cable(s) elongate (Arai and Mabuchi, 2002). An intriguing possibility is that the formin Cdc12 may localize to the center of this structure. This formin is necessary for elongation of the leading cable(s) from this region (Arai and Mabuchi, 2002). It functions in vitro to promote nucleation of actin polymerization at barbed ends (Kovar et al., 2003). It is localized

to a single spot in the mid-region of the cell during early mitosis (Chang et al., 1997; Chang, 1999; Yonetani, A., and F. Chang, personal communication). Furthermore, it is localized at the center of an aster-like structure formed in *cdc4* mutant cells at a restrictive temperature (Chang et al., 1997). The *cdc4⁺* gene encodes the myosin-II essential light chain (McCollum et al., 1995) and its mutation affects myosin-II functions, one of which is likely to accomplish ring formation together with F-actins after formation of the aster-like structure (Motegi et al., 2000). Therefore, ring formation would not proceed beyond the aster-like structure in the *cdc4* mutant cells.

In addition, presence of clusters of F-actin of a same directionality in the contractile ring may also reflect a process of formation of the ring. It has been reported that the F-actin cables accumulate as rays of the aster-like structure are somehow fused with the leading cable to form the contractile ring during anaphase (Arai and Mabuchi, 2002). The ring thus may contain remnants of the cables, each of which probably consists of unidirectional F-actins.

It was also shown that in late anaphase B contractile ring, which was not contracting yet, F-actins of opposite directionalities were mixed homogeneously on the whole. Therefore, there must be mechanism(s) by which F-actins are rearranged in the ring during anaphase before contraction initiates. There may be two possible mechanisms for this rearrangement: First, sites for polymerization of actin may be scattered in the division plane at the later stage of contractile ring formation, although polymerization may occur mainly at one site at the beginning as mentioned above. This idea seems to be supported by localization of Cdc12. Although Cdc12 mainly localizes to a single spot during early mitosis, it then spreads in the division plane (Chang, 1999; Yonetani, A., and F. Chang, personal communication). Thus, this protein would be able to polymerize actin at various spots in the division plane during anaphase. Actually, we have observed that Cdc12 forms a ring containing 10–20 strong spots during anaphase (unpublished data). It has recently been published that Cdc12 is scattered as a broad band of nodes at the division site during early mitosis (Wu et al., 2006). We do not know

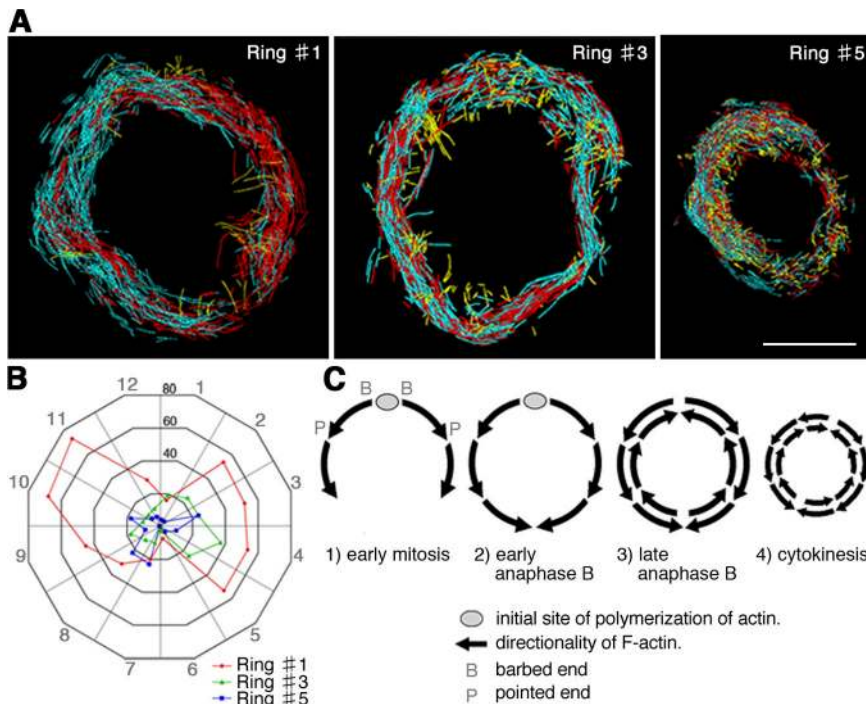


Figure 3. Three-dimensionally reconstructed images of the F-actin contractile ring in *cdc25* cells. (A) Three-dimensional images of rings #1 (early anaphase B), #3 (late anaphase B), and #5 (cytokinesis). Bar, 1 μm . (B) The degrees of unidirectionality of the F-actins in segments of the contractile rings #1, #3, and #5. All the images of serial sections of these rings were divided into 12 segments as shown here. Actin filaments having the "blue" and the "red" directionalities were counted for each segment. Percentages of the blue F-actin and the red F-actin were obtained for each segment and the absolute value of difference between the percentages was plotted. The rings in A and the star diagrams in B are set so that the segment in which the difference was minimum (segment 6) is placed at the bottom. The scale numbers indicate the absolute value of difference between the percentages. (C) Possible processes of contractile ring formation and contraction in fission yeast. (1) A primary ring is initiated to form from a site for polymerization of actin through function of Cdc12 or other unknown proteins during early mitosis. (2) In early anaphase B, F-actins having a same directionality occupy about a half of the ring on the whole, as if the F-actin ring is roughly composed of two semicircles. (3) In late anaphase B, F-actins having opposite directionalities from each other are mixed homogeneously. Cdc12 and/or Myo2 may be involved in this rearrangement process. (4) Contraction of the ring occurs by sliding between the F-actins of opposite directionalities. This is accompanied by shortening of the F-actins (Mabuchi, 1986).

what the reasons for the discrepancy are, but this observation might have been done after its spreading. Myosin-II (Myo2) that accumulates as multiple spots (Motegi et al., 2000) at the division site through binding to Mid1 (Motegi et al., 2004) is also capable of organizing actin because mutant Myo2s, which prematurely accumulate at the presumptive division site even during G2 phase, induce F-actin accumulation at the same site during G2 (Motegi et al., 2004). These proteins could produce antiparallel F-actins in each semicircle during anaphase. If this type of rearrangement takes place, actin in the original semicircles would be turning over. It has been suggested that the contractile ring actin turns over even after establishment of the ring in *Xenopus* eggs (Noguchi and Mabuchi, 2001), and demonstrated in cultured cells (Guha et al., 2005). A FRAP study on GFP-Cdc8 (tropomyosin) also suggests that actin turns over in the contractile ring in fission yeast cells (Pelham and Chang, 2002). The fact that the actin-depolymerizing factor, Adf1, which promotes actin turnover, is necessary for maintenance of the contractile ring structure in addition to its formation in fission yeast (Nakano and Mabuchi, 2006), supports the idea of turnover of actin in the ring.

Second, F-actins could slide over each other being mediated by Myo2 without accompanying contraction of the ring, and therefore the mixed directionality is produced during late anaphase B. It may also be responsible for redistribution of Cdc12 nucleation sites in the division plane. This sliding without contraction would occur if the F-actins are only weakly or not attached to the cell membrane. Unfortunately, it has not been known when the contractile ring F-actin firmly attaches to the cell membrane during formation of the ring. Actin dynamics

and actin–membrane interaction in the contractile ring must be investigated in order to clarify mechanism of contractile ring formation.

Mitoses in fission yeast cells and animal cells are different in that the nuclear membrane persists and the mitotic spindle is formed inside the nucleus in fission yeast, while the mitotic apparatus is formed after breakdown of the nuclear membrane in animal cells, which has a symmetric microtubular structure. In fission yeast, a signal for contractile ring assembly is thought to be transferred from the nucleus to the cell cortex (Chang and Nurse, 1996) through the spindle pole bodies (Arai and Mabuchi, 2002); a strong candidate for a division signal molecule, Plo1, which is a fission yeast orthologue of the Polo kinase that is first localized to the spindle pole bodies and then transferred to the mid-ring structure during early mitosis (Bahler et al., 1998). This agrees well with the present observation that the contractile ring actin assembly initiates at one site in fission yeast.

On the other hand, microtubules emanating from the separated centrosomes converge at the equatorial cortex in anaphase animal cells, and are thought to transmit cleavage signals to the cortex. Thus, contractile ring formation is expected to initiate simultaneously at multiple sites in the mid-cortex. It has recently been shown that cleavage signaling molecules are localized to tips of astral microtubules at the equatorial cortex in HeLa cells (Nishimura and Yonemura, 2006). The signal may be transmitted to the cortex through these molecules at multiple sites, each of which may correspond to the single site in fission yeast cells, and basically similar assembly and rearrangement of actin may occur at these sites.

Materials and methods

Yeast strains and growth conditions

Yeast strains and growth conditions were described previously (Kamasaki et al., 2005). *cdc25-22* temperature-sensitive mutant cells were synchronized at M phase.

Electron microscopy

Preparation of cells and electron microscopy were performed as described previously (Kamasaki et al., 2005). In brief, cells were treated for 30 min with 0.5 mg/ml Zymolyase 100T (Seikagaku Corp.) at 30°C to produce spheroplasts, then with 0.5% Triton X-100 dissolved in 20 mM Pipes, 20 mM MgCl₂, 10 mM EGTA, pH 7.0, protease inhibitors, and 1 M sorbitol for permeabilization in the presence of 4.2 μM phalloidin (Sigma-Aldrich) for 1 min, and incubated at 4°C overnight with 1 mg/ml myosin S1. They were fixed with 2% glutaraldehyde (Electron Microscopy Sciences) and 0.2% tannic acid for 1 h at 4°C, postfixed in 2% OsO₄ for 1 h at 4°C, and embedded in Quetol812 (Nissin EM).

Sections were cut with an ultramicrotome (ULTRACUT-S; Reichert-Nissei), stained with 4% uranyl acetate and 0.4% lead citrate, and examined with an electron microscope (JEM 1200EX; JEOL) at 80–100 kV.

Three-dimensional reconstruction

Three-dimensional reconstructions of the F-actin contractile rings decorated with S1 were performed using serially sectioned images. Micrographs originally taken at 25,000× were enlarged to 100,000×. After determining the directionality of each F-actin in the ring, images of the F-actin were traced from the individual electron micrographs. The tracings of F-actin were aligned and superimposed to produce reconstructed images using the TRI/3D SRF II program (Ratoc System Engineering) and Windows NT.

Fluorescence microscopy

Staining of fixed cells with Bodipy-phalloidin and permeabilization of unfixed cells in the presence of rhodamine-phalloidin were performed as described previously (Arai and Mabuchi, 2002; Kamasaki et al., 2005). Both of these cells were finally washed with a solution of 20 mM Pipes, 20 mM MgCl₂, 10 mM EGTA, pH 7.0, and protease inhibitors, and observed in the same solution with an Axioskop fluorescence microscope (Carl Zeiss Microimaging, Inc.) equipped with a Plan Aplanachromat 63×/1.40 oil objective lens and appropriate filters. Images were acquired using a CCD camera (SPOT; Diagnostic Instruments).

Online supplemental material

Fig. S1 shows fluorescent microscopic evidence that no artificial F-actin structure was formed after permeabilization followed by S1 decoration in wild-type and *cdc25* cells. Fig. S2 shows an electron microscopic image of a contractile ring of an S1-decorated wild-type cell. Fig. S3 shows that the ring F-actin became short in contracting contractile ring (A), three-dimensional reconstructed images of the F-actin contractile rings #2, #4, and #6 in *cdc25* cells (B), and degree of unidirectionality of F-actins in segments of these rings (C). This figure suggests that overlapping of F-actins of opposite directionalities progresses during cytokinesis. Video 1 shows that F-actins with the same directionality occupied about a half of the ring on the whole in the early stage ring #1. Video 2 shows that F-actins of opposite directionalities are mixed on the whole in the later stage ring #5. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200612018/DC1>.

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