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Progress towards understanding the mechanism of cytokinesis in fission yeast

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Abstract

We use fission yeast to study the molecular mechanism of cytokinesis. We benefit from a long history in genetic analysis of the cell cycle in fission yeast, which provided the most complete inventory of cytokinesis proteins. We used fluorescence microscopy of proteins tagged with fluorescent proteins to establish the temporal and spatial pathway for the assembly and constriction of the contractile ring. We combined biochemical analysis of purified proteins (myosin-II, profilin, formin Cdc12p and cofilin), observations of fluorescent fusion proteins in live cells and mathematical modelling to formulate and test a simple hypothesis for the assembly of the contractile ring. This model involves the formation of 65 nodes containing myosin-II and formin Cdc12p around the equator of the cell. As a cell enters anaphase, actin filaments grow from formin Cdc12p in these nodes. Myosin captures actin filaments from adjacent nodes and pulls intermittently to condense the nodes into a contractile ring.

Keywords

actin; contractile ring; cytokinesis; fission yeast; fluorescent fusion protein; formin; myosin

Introduction

Understanding the mechanism of cytokinesis has proved to be more challenging than expected in the 1970s, when pioneering experiments showed that a contractile ring of actin filaments [1] and myosin-II [2,3] constricts the cleavage furrow in animal cells. At that point next to nothing was known about the genetics of cytokinesis, so no one knew the full inventory of cytokinesis proteins. Biochemists lacked assays for reconstituting any of the steps in cytokinesis except for the enzymatic interaction of myosin-II with actin filaments. Light microscopy was limited to DIC (differential interference contrast) or phase contrast of live cells and fluorescent antibody staining of fixed cells. Electron microscopy showed that the contractile ring disassembles as it constricts [4], but provided little information regarding the mechanisms of contractile ring assembly, constriction or disassembly.

Now, 30 years later, the technology for studying cytokinesis has improved dramatically, but much is yet to be done, since we now appreciate that cytokinesis is extraordinarily complex at the molecular level. Cytokinesis in fission yeast depends on more than 60 gene products [5], but the exact number is not yet known. Fruitflies use a similar group of proteins for cytokinesis [6], so the core machinery seems to have been conserved. Thus contemporary

cells have inherited from their common eukaryotic ancestor a core set of cytokinesis proteins with common functions in spite of the fact that animals and fungi diverged 800 million years ago. This is good news, because insights from diverse experimental organisms can be combined to appreciate general principles about the hardware and logic of cytokinesis. Some cells use this hardware in novel and interesting ways, but that should not divert the field from the main challenge of understanding the common features of cytokinesis.

Advantages of studying cytokinesis in fission yeast

Over the last decade our laboratory has moved from using *Acanthamoeba* and vertebrate tissue culture cells to using the fission yeast *Schizosaccharomyces pombe* for most of our work on the actin cytoskeleton and cytokinesis. Historical and technical advantages attracted us to fission yeast. Pioneering classical genetics by Nurse et al. [7] established the most complete genetic inventory of cytokinesis proteins in any organism (reviewed in [5]). The haploid genome of vegetative fission yeast allows the phenotypes of recessive mutations to reveal themselves. Since many cytokinesis genes are essential for viability, the ability to make conditional mutations (usually temperature-sensitive) was essential in the screens for cytokinesis mutations. A diploid stage allows for genetic crosses. A key advantage has been facile methods using homologous recombination to manipulate the genome. One can delete, replace, modify or tag any gene in the organism in days.

The ability to integrate GFP (green fluorescent protein) or its cousins [CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein)] anywhere in the genome has been particularly valuable for microscopic studies of cytokinesis in live cells (Figure 1). The field has generated hundreds of strains expressing proteins with a fluorescent tag. These strains can be constructed with the fluorescent protein integrated into the genome at the N- or C-terminus of a protein with the gene under the control of its native promoter. Therefore all copies of particular protein can be tagged and quantified, a vast advantage over expressing a tagged protein from a plasmid with the native protein in the background. Replacing a wild-type gene with a tagged gene is the first test of the physiological function of the tagged protein. Even if the tagged protein supports normal growth and morphology, subtle defects may escape detection. However, genetic crosses allow for an additional test of the tagged protein. Combining the tagged protein with mutations in genes known to interact with the tagged gene can bring out synthetic phenotypes. With a modest amount of experimentation, it is possible to find a way to tag most proteins in a way that retains full function. We have been frustrated a few times and have been unable to tag actin, tropomyosin and several subunits of the Arp2/3 complex (actin-related protein 2/3 complex) without compromising function [8].

Using fully functional fluorescent fusion proteins, we learned that the total fluorescence of a live cell gives a direct measure of the number of copies of the protein [9]. Furthermore, the total fluorescence is the same (Figure 1) whether the protein is dispersed in the cytoplasm (such as myosin-II during interphase) or concentrated in one part of the cell (such as myosin-II in the contractile ring during cytokinesis). This approach allowed us to count the number of proteins in contractile ring precursors, contractile rings, spindle pole bodies and actin patches.

One can also use homologous recombination to replace the native promoter of any gene with an inducible promoter, such as *nmt* promoters, which are repressed by thiamine in the medium. This strategy allows one to dial up or down the concentration of any protein in the cell. If the protein has a fluorescent tag, then total cell fluorescence reveals the concentration of the protein in each cell.

Biochemical methods are less well developed, but improving steadily with time. We routinely purify native myosin-II [10] and Arp2/3 complex [11] directly from yeast cells. We also purified proteins from *Escherichia coli*, such as recombinant fission yeast profilin [12], cofilin [13], capping protein [14] and domains of formins [15,16].

Time course of cytokinesis

Stepping into this well-developed field, our first task was to become oriented. We chose to do so by determining the time that cytokinesis proteins concentrate at the site of cleavage [8]. We used a fluorescent fusion protein associated with spindle pole bodies as an internal clock. We defined the moment of spindle pole body separation as zero time and counted backwards and forwards in time-lapse movies. The behaviour of the cells is highly uniform, allowing us to track the time that fluorescent fusion proteins concentrate at the site of cytokinesis (Figure 2). The whole process takes place at a deliberate pace over approx. 80 min. Each protein arrives at a specific time with only small variations from cell to cell (S.D. of < 3 min).

The order of events along this time-line is consistent with genetic dependencies [5], so we take it to be the biochemical pathway. In the first stage, the cell assembles approx. 65 nodes of protein in a band approx. 3 μm wide around the equator of the cell. These nodes contain approx. 20 copies each of anillin (Mid1p), myosin-II (Myo2p and its light chains) and IQGAP (Rng2p) along with a small number (~2) of formin Cdc12p dimers. The broad band of nodes assembles between time -10 min and zero time. At time +2 min, actin filaments begin to polymerize between the nodes, which condense into a tight contractile ring around the equator of the cell over 10 min. The cell completes anaphase B between time+12 min and +30 min, during which the contractile ring matures by adding tropomyosin, alpha-actinin and an unconventional myosin-II (Myp2p with its light chains). Between +40 min and +70 min, constriction of the ring invaginates the plasma membrane as glucan synthetases lay down an extracellular septum in the furrow [17]. Myo2 concentrates in the ring as it constricts, but the other components disassemble. Fusion of the plasma membrane allows the daughter cells to separate. This outline of events directed us to investigate the molecules and their interactions along the pathway.

Myosin-II

Lord and Pollard [10] purified and characterized Myo2, the fission yeast myosin-II required for both the assembly and constriction of the contractile ring [18–21]. This was, to my knowledge, the first myosin to be purified from a fungus. Myo2 consists of the Myo2p heavy chain with light chains Rlc1p and Cdc4p [10,22]. Myo2 has two heads and a coiled-coil tail (J. Friend, personal communication) and ATPase activity stimulated by actin filaments.

Although crude preparations of Myo2 moves actin filaments in an *in vitro* assay, this activity disappeared as Lord and Pollard [10] purified the protein. Thus it was determined that the purification process separates Myo2 from Rng3p, a protein with a UCS domain that supports myosin function during cytokinesis [23]. Rng3p allowed purified Myo2p to move unloaded actin filaments at 400 nm/s in an *in vitro* assay. Rng3p has two salutary effects on Myo2: it promotes interaction with actin filaments in the presence of ATP; and it prolongs the lifetime of myosins in cells [24].

Although Myo2 is essential for viability, biochemical analysis of purified Myo2 with mutations in either the heavy chain or light chains revealed that fission yeast cells survive and divide with less than 5% of wild-type Myo2 activity. Myo2 is soluble in high

concentrations of salt and insoluble in physiological salt concentrations, but we do not yet know the structure of the insoluble species.

Formins

Kovar et al. [16,25,26] characterized how the cytokinesis formin Cdc12p regulates actin polymerization. Chang et al. had established that Cdc12p is required for fission yeast to assemble actin filaments for the contractile ring [27] and that formin For3p is responsible for the actin filaments in interphase actin cables [28]. Pioneering studies of the budding yeast formin Bni1p revealed that the FH (formin homology) 2 domain of formins stimulates actin polymerization and appeared to be localized at or near the fast-growing barbed ends of the filaments [29,30]. Kovar et al. [26] found that the FH2 domain of Cdc12p nucleates actin filaments but strongly inhibits elongation of the barbed ends of these filaments. Crystal structures of the Bni1p FH2 domains established that FH2 domains form doughnut-shaped head-to-tail dimers [31] that can wrap around actin filaments [32].

Located N-terminal to the FH2 domain, Cdc12p and other formins have a proline-rich FH1 domain that binds profilin, a small actin monomer-binding protein [27]. Profilin regulates actin assembly by Cdc12p constructs with both FH1 and FH2 domains by suppressing nucleation [15] but allowing barbed ends to grow at nearly full speed [26]. Profilin might simply displace the FH2 cap from barbed ends, but observations of filaments growing from formins immobilized on microscope slides [25] showed that FH1FH2 domains remain attached to barbed ends as thousands of actin subunits add to the end.

Kovar et al. [16] learned much more about the mechanism of action of formin FH1FH2 domains by comparing the properties of four formins. Cdc12p is at one extreme; in the absence of profilin, Cdc12p FH1FH2 slows barbed end elongation by approx. 99%, and an optimal concentration of profilin allows elongation at nearly the rate of a free barbed end. Mouse mDia1FH1FH2 is another extreme; it slows barbed end elongation by only 10% and profilin can stimulate elongation at five times the rate of a free barbed end [16,33]. This is remarkable, because actin filament elongation is diffusion-limited.

A mathematical model by Vavylonis et al. [34] and extensive characterization of FH1FH2 constructs made from Bni1p [15] provided hypotheses to explain the variable effects of FH2 domains on elongation and how formins can break the diffusion limit (Figure 3). All formin FH2 domains slow down barbed end elongation. The extent of inhibition depends on the fraction of the time that the formin-actin complex on the barbed end is in a closed state that cannot elongate and an open state that can elongate. For Cdc12pFH2, the rapid equilibrium between these two states strongly favours the closed state. mDia1FH2 is largely in the open state. The ability of FH1 domains to enhance elongation depends on their multiple polyproline tracks to bind profilin-actin. This creates a high local concentration of profilin-actin bound to the FH1 domain. The flexibility of FH1 domains allows any one of these tethered actins to bind rapidly to the barbed end of the filament. Profilin dissociates from the new subunit on the barbed end, allowing addition of more subunits. Although profilin promotes elongation of filaments associated with formin FH1FH2 domains, profilin strongly inhibits the nucleation of actin filaments.

Formin FH2 domains rarely dissociate from growing barbed ends, but the rate of dissociation is directly proportional to the rate of elongation [15]. Our interpretation is that the elongation cycle includes a step with a low probability ($\sim 10^{-4}$) for FH2 dissociation. Consequently, a formin-like Cdc12p is highly processive, allowing it to nucleate, elongate and anchor the barbed end of an actin filament.

Formation of the contractile ring by condensation of nodes

Knowing these properties of Cdc12p and Myo2, we proposed [35] that nodes condense into a ring by a search and capture mechanism (Figure 4). The idea was that a small number of Cdc12p dimers in each node nucleate actin filaments. These filaments grow radially in random directions as they add subunits to their barbed ends, associated with Cdc12p in the nodes. When the pointed end of a growing filament passes close to another node, Myo2 binds the filament and walks toward the formin on its barbed end. This produces a force that pulls on the adjacent node. We imagined that the combined traction of the nodes on each other condenses them into a continuous ring.

Although features of this model may be correct, Monte Carlo simulations of the model showed that the hypothesis is incomplete [36]. Simulations with only search, capture and traction reactions (Figures 5a–5c) result in condensation of the nodes into clumps around the equator rather than a ring with only small gaps between nodes as observed in cells.

Careful examination of live cells expressing the Myo2 regulatory light chain with a triple GFP-tag revealed the missing reactions. Each node forms in about 1 min and then remains relatively stationary on the inside of the plasma membrane. The small motions are consistent with two-dimensional diffusion with $D \sim 20 \text{ nm}^2 \cdot \text{s}^{-1}$, several orders of magnitude smaller than diffusion of a protein with single transmembrane helix. Diffusion is also limited in cells treated with Latrunculin A, so the nodes are not anchored by actin filaments. The large mass of nodes ($> 22\,000 \text{ kDa}$, based on our incomplete inventory of the proteins) may account for their restricted motion.

At time +1 min, Cdc12p joins the nodes, and at time +2 min, actin filaments begin to grow from the nodes. Over the next 10 min, each node undergoes a succession of short discontinuous motions. The directions of motion are nearly random. The mean velocity is $30 \text{ nm} \cdot \text{s}^{-1}$ and the mean duration of movements is about 20 s. We postulated that this behaviour arises from random breaks of the connections between nodes, arising either from severing filaments linking nodes or Myo2 dissociating from the filaments linking nodes. We have not yet been able to observe single actin filaments directly in live cells, because actin fused with GFP or YFP does not incorporate into contractile ring filaments (most likely due to incompatibility with Cdc12p). GFP-fused to the calponin homology domains of Rng2p (GFP-CHD, from F. Chang) appears to mark all of the actin filaments in live fission yeast. Dual labelling of nodes with Rlc1p and actin filaments with GFP-CHD revealed linear elements containing actin filaments growing between nodes. Some of these linear elements dissociated from nodes and others broke in the middle.

Simulations with connections between nodes breaking randomly not only reproduce the start and stop motions of the nodes in live cells, but also assemble the nodes into rings in 10 min as observed in cells. The numerical parameters used in these simulations influence the time to assemble a ring and the distribution of nodes in the ring, so we have tried to measure as many parameters as possible (Table 1). The outcome of the simulations is more sensitive to the values of some of these parameters than others, but the model tolerates a range of parameter values around the observed values.

This combination of experiments and theoretical calculations provides evidence that fission yeast assemble contractile rings by an undirected random process involving polymerization of actin filaments, which explore the neighbouring space. Myo2 captures these filaments and applies tension between the nodes. The diffusion coefficient of nodes and the velocity of their motions gives the force required, approx. 6 pN, in the range of force produced by a few myosin molecules. Frequent breaks in the connections allows for many rounds of

experimentation and correction of errors. The arrangement of nodes in a band on the inner surface of a cylinder constrains their motions and results in condensation into a ring.

Open questions

Many questions remain. How is anillin Mid1p targeted to the plasma membrane in a broad band around the equator? Previous work established that Polo kinase releases Mid1p from the nucleus [37] and the nodes form near the position of the nucleus [38]. Local diffusion from the nucleus and negative signals from the poles of the cell both contribute to concentrating nodes near the nucleus [37,39,40]. However, we do not know the receptor for Mid1p on the inner surface of the plasma membrane or the organization of anillin Mid1p, myosin-II Myo2, IQGAP Rng2p, PCH/F-BAR protein Cdc15p and formin Cdc12p in nodes. Nor do we understand what activates Cdc12p to nucleate actin filaments only after it joins the nodes. Events during the long interval between completion of the ring and the onset of constriction are a mystery, as is the mechanism by which the SIN (septation initiation network) co-ordinates ring constriction with septum formation [41]. The organization of the mature ring and its mechanism of disassembly during constriction remain to be determined.

Animal cells also depend on anillin, myosin-II, formins and actin to assemble contractile rings [42,43]. Myosin-II can be found in clusters that move somewhat like the nodes in fission yeast [44], so other cells may also use a search, capture, pull and release strategy to assemble contractile rings.

Our progress on contractile ring assembly suggests that a combination of quantitative live cell observations, detailed biochemical and biophysical characterization of the components and mathematical modelling will all be essential to answer these complicated questions and to reach the goal of a understanding the molecular mechanism of cytokinesis.

Abbreviations used

Arp2/3 complex	actin-related protein 2/3 complex
FH	formin homology
GFP	green fluorescent protein
SIN	septation initiation network
YFP	yellow fluorescent protein

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Figure 1. Fluorescence micrograph of five fission yeast cells in the same field

These cells were expressing the myosin-II regulatory light chain fused to three tandem copies of GFP (Rlc1p-3GFP) from the native promoter in the genome. A Z-series of confocal sections were combined into a maximum intensity projection. Top, interphase cell with diffuse cytoplasmic fluorescence. Second, an early mitotic cell with about half of the total fluorescent myosin concentrated into a broad band of about 65 nodes in the centre of the cell. The nucleus determines the position of the broad band. Third, between zero time and time +10 min, the nodes condense into a compact contractile ring. Bottom, two daughter cells after cytokinesis. The cells are ~3.5 μm wide. Based on the work of Vavylonis et al. [36].

Figure 2. Time course of cytokinesis in fission yeast

Time (min) relative to spindle pole body separation is given on the left. Sketches of cells show steps in division of the nucleus and formation and constriction of the contractile ring. The pathway on the right is based on the times that each of these proteins appears around the equator of the cell and on other information. Anillin Mid1p exits the nucleus and organizes nodes (dots) in a broad band on the inside of the plasma membrane adjacent to the nucleus. Nodes contain anillin Mid1p, myosin-II Myo2, IQGAP Rng2p, formin Cdc12p and PCH/F-BAR protein Cdc15p. At time +2 min, Cdc12p nucleates actin filaments and profilin promotes their elongation. Nodes condense into a contractile ring between time +2 min and +12 min. After a delay of approx. 30 min, signals from the SIN stimulate ring constriction and formation of a septum between the daughter cells. Based on the work of Wu et al. [8].

Figure 3. Hypothesis for the transfer of actin from a formin FH1 domain to the barbed end of an actin filament

The formin FH2 dimer associates with the actin subunits at the barbed end of the filament (large grey circles). Profilin (small dark grey circles) tethers an actin monomer to a polyproline track in the FH1 domain of the formin. FH1 domains are flexible, allowing any actin associated with the FH1 domain to bind to the barbed end of the filament. Subsequently profilin dissociates from the end of the filament and the FH2 domain translocates onto the new terminal subunit. Based on the work of Kovar et al. [16], Vavylonis et al. [34] and Paul and Pollard [15].

Figure 4. Hypothesis for the mechanism that condenses nodes into a contractile ring

These scale drawings show the number of copies of proteins known to be associated with nodes. Myosin-II is shown assembled into minifilaments, although they have not been observed experimentally. Each node is shown with two formin Cdc12p dimers, which are associated with the growing barbed end of an actin filament. Myosin-II captures and pulls on the pointed ends of filaments from adjacent nodes. Reproduced from Wu, J.Q., Sirotkin, V., Kovar, D.R., Lord, M., Beltzner, C.C., Kuhn, J.R. and Pollard, T.D. (2006) Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. *J. Cell Biol.* **174**, 391–402. ©2006, JCB Online by Rockefeller University Press; <http://www.jcb.org>.

Figure 5. Elements of the mechanism that assembles contractile rings

(a) Nodes containing formin Cdc12p and Myo2 grow actin filaments in random directions. Cdc12p is postulated to anchor the growing barbed ends of the filaments in the node. (b) If a filament approaches within distance r_c of a neighbouring node, Myo2 captures the pointed end of the filament. (c) Myo2 pulls on the actin filaments with force F . The net effect of these forces produces velocity V . (d) With a characteristic time, τ_{break} , connections between nodes are broken. (e) Nodes produce new filaments that grow in random directions. Based on the work of Vavylonis et al. [36].

Table 1

The second column lists the limits on these simulations. Parameter values outside these limits produce faulty simulations. For example, if the number of nodes is <50 or the filament growth rate is <40 subunits/s, the nodes form large clumps rather than rings with small gaps between nodes, as observed in cells. The third column lists our best estimates of the parameter values in live cells. Based on the work of Vavylonis et al. [36].

Parameter	Model	Cell
Number of nodes	> 50	~65
Broad band width	<3 μm	2.8 μm
Formins per node	>1	~2
Filament growth rate	>40 subunits/s	80 subunits/s
Connection lifetime	<45 s	20 s