Animal cell division: a fellowship of the double ring?

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Summary

Despite a century of research into the nature of animal cell division, a molecular explanation for the positioning of the actomyosin contractile ring has remained elusive. The discovery of a novel interaction between regulators of Rho family small GTPases has revealed a link between the mitotic microtubules and the contractile ring during the later stages of mitosis. The properties of the interacting Rho regulators suggest a molecular model for the positioning and initiation of contractile ring furrowing in animal cells. In this 'double ring' model, centralspindlin complexes, localized by the action of their kinesin-like protein component, position and activate a cortical equatorial ring of Rho GTPase exchange factors. The resulting ring of activated Rho would then trigger a cascade of events leading to formation and constriction of the contractile ring.

Key words: Cytokinesis, Rho GTPase, PBL/Ect2 RhoGEF, Contractile ring, PAV-KLP/MKLP1, Central spindle, Microtubules

Introduction

There is much that is remarkable about cytokinesis, the process that physically divides one cell into two. For example, a single lipid bilayer must be separated into two without destroying the integrity of the membrane. In addition, the ring of membrane furrowing responsible for division must be positioned accurately to partition the contents so that each daughter cell is provided with the genetic and biochemical materials required for life.

Appropriate partitioning is achieved by positioning the plane of cleavage between the recently separated chromosomes during anaphase. The regulation of this positioning, as well as many other aspects of cytokinesis, has been the subject of speculation since the late 19th century (Rappaport, 1971). More than a century later, we still have no clear molecular understanding of many of the vital steps involved, including the mechanism that positions the plane of cleavage. Here, we discuss recent studies of regulators of the Rho family of GTPases that have provided a long-sought-after, if speculative, molecular model for the positioning of the contractile ring in animal cells.

Cytokinesis: more than a pinch of actomyosin

Animal cytokinesis requires the assembly of an actomyosin contractile ring that constricts during cytokinesis (for a review, see Glotzer, 2001). The ring assembles during anaphase, and myosin activity appears to drive membrane furrowing by applying a contractile force to F-actin filaments (Karess et al., 1991; Mabuchi and Okuno, 1977). Assembly of the actomyosin network is stimulated locally. In *Xenopus* embryos, for example, actomyosin assembly and initiation of furrowing occurs initially in one region and proceeds around the perimeter until the complete ring is formed (Noguchi and Mabuchi, 2001). Partial furrowing can be induced by experimental manipulation (Rappaport and Ebstein, 1965) or occur in mutant cells (Giansanti et al., 1998) (Fig. 1). It is also clear that there is no special pre-existing cortical structure at the site of contractile ring formation prior to the onset of anaphase. Rappaport and Ebstein showed that shifting the mitotic spindle to new positions rapidly induces furrowing activity in a new region of the plasma membrane (Rappaport and Ebstein, 1965).

Rho GTPases and actin regulation

The molecular events that lead to formation and activation of the actomyosin contractile ring are poorly understood. A body of inhibitor and genetic data points to the central importance of the Rho GTPase in this process (Hirose et al., 2001; Imai et al., 2002; Jantsch-Plunger et al., 2000; Larochelle et al., 1996; Mabuchi et al., 1993; Prokopenko et al., 1999; Tatsumoto et al., 1999). Rho family GTPases play important roles in the regulation of actin dynamics (for a review, see Etienne-Manneville and Hall, 2002). Initial characterization of the Rho, Rac and Cdc42 members of this family, by expression in mammalian cultured cells, indicated that active Rho induces the formation of actin stress fibers, Rac induces the formation of lamellipodia and Cdc42 induces the formation of philopodia.

It is now well established that Rho-family GTPases induce changes in actin organization in response to intracellular and extracellular signals. These small G proteins act through a range of effectors, such as the kinases Pak (p21-activated kinase) (reviewed by Bokoch, 2003) and ROCK (Rho kinase) (reviewed by Riento and Ridley, 2003), and actin-modulating proteins such as the Diaphanous-related formin-homology proteins (reviewed by Evangelista et al., 2003) and WASp

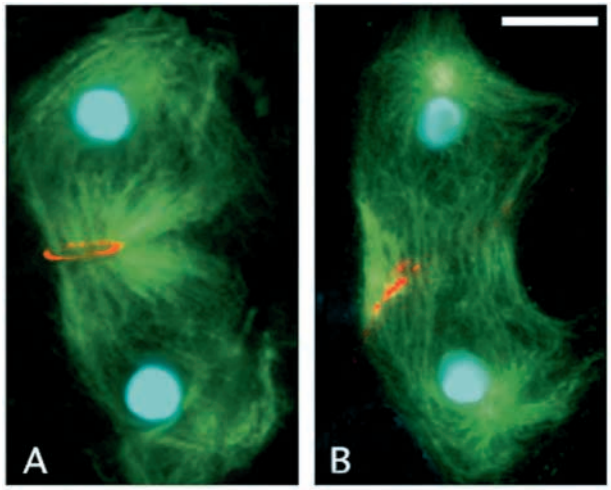

Fig. 1. Formation of a partial contractile ring during the first meiotic division of a *D. melanogaster* spermatocyte carrying a mutation in the *chickadee* (profilin) gene. A wild-type spermatocyte is shown in A and the *chickadee* mutant spermatocyte shown in B. Microtubules are stained green, DNA blue and F-actin red. Reproduced, with permission, from Giansanti et al. (Giansanti et al., 1998).

family proteins (reviewed by Badour et al., 2003). During cytokinesis, Rho GTPase appears to be acting through some of these effectors. The Diaphanous formin-homology protein (Tatsumoto et al., 1999) and members of the Rho-dependent kinases (Kosako et al., 2000; Madaule et al., 1998) have been implicated as targets of activated Rho.

The role of the mitotic apparatus

The plane of cytokinesis is established between the separated chromosomes and lies perpendicular to the axis of chromosome separation. The cleavage plane could be specified by the position of the chromosomes at metaphase, by the overlap in the bipolar mitotic spindle, or by an inhibitory signal emitted at the spindle poles. Elegant experiments on marine invertebrate embryonic cells by Rappaport (Rappaport, 1961) showed that furrows can form between spindle poles independently of chromosome position but only where astral microtubules from each pole are close to the cortex of the cell (Fig. 2). These observations argued against a role either for chromosomes or for spindle pole inhibition and led to a model in which an organizing factor is directed along astral microtubules to concentrate at the point at which the plus ends of the microtubules meet or interdigitate (Devore et al., 1989).

Although astral microtubules appear to be sufficient to induce furrowing in some cases, microtubule arrangements in cells of different sizes and structures can vary considerably during anaphase. One common feature of anaphase cells is the formation of bundled microtubules in the midzone between the separated chromosomes. These midzone microtubules are collectively referred to as the central spindle. In some cells, such as *Drosophila melanogaster* spermatocytes, the central spindle contacts the cell cortex (Cenci et al., 1994). A role for the midzone microtubules in generating the cytokinetic stimulus has been suggested previously (Wheatley and Wang, 1996; Bonaccorsi et al., 1998; Gatti et al., 2000; Bucciarelli et al., 2003). Wheatley and Wang showed that in normal rat kidney (NRK) cells with multiple spindles, furrowing is induced when the midzone microtubules lie close to the cortex and is independent of the position of the spindle poles or astral microtubules. Bonaccorsi et al., have shown that *D. melanogaster asterless* mutant male meiotic cells, which lack astral microtubules but develop normal midzone microtubules, are capable of undergoing cytokinesis. These and other studies have led Gatti and his colleagues to conclude that formation of the central spindle and the contractile ring apparatus are interdependent (Cao and Wang, 1996; Gatti et al., 2000; Giansanti et al., 1998; Somma et al., 2002). As noted above, furrowing does not require formation of a complete ring and a local association between microtubules and the cortex is enough to induce local furrowing (Wheatley and Wang, 1996).

Connecting the contractile ring to microtubules

All the evidence, therefore, points to a key relationship between microtubules and the establishment of the cytokinetic furrow, but how do the microtubules position the contractile ring? This question is difficult to answer. Cytokinesis occurs too quickly for easy biochemical analysis. *D. melanogaster* tissue culture cells, for example, spend less than 0.5% of their time in cytokinesis; so the contractile apparatus is assembled for a relatively short time and is present in a small proportion of the cells. A proven approach to the dissection of complex spatially or temporally limited biological processes is the application of genetic analysis, allowing identification of key genes on the basis of mutants that disrupt the process. Over the past decade or so, numerous mutations that affect cytokinesis have been described in *D. melanogaster* and *Caenorhabditis*

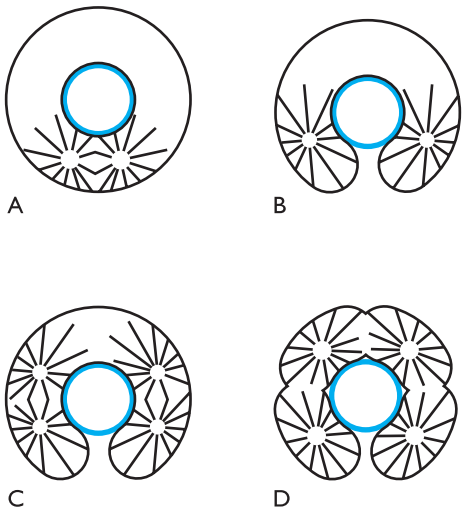

Fig. 2. Manipulation of sea urchin embryos to generate ectopic astral spindles in the absence of chromosomes revealed the significance of microtubules, rather than chromosomes, in positioning the contractile apparatus. The blue ring represents a solid cylinder pushed through the center of an embryo to create a torus. The first mitotic division induces furrowing at the spindle midzone, but no cleavages are observed at positions distant from the spindle. The second mitotic divisions induce furrowing at the two spindle midzones, but, in addition, furrowing appears between the two spindle poles in the absence of metaphase chromosomes or anaphase movements. Adapted, with permission, from Rappaport (Rappaport, 1971).

elegans, and recent studies using these model organisms have broken new ground in our efforts to understand the molecular events of animal cytokinesis.

Three genes in particular have been important to these new developments: those encoding the *D. melanogaster* Rho GTP exchange factor (GEF); Pebble (PBL) (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 1999); the plus-end-directed kinesin-like motor proteins known as ZEN-4 in *C. elegans* (Raich et al., 1998) and Pavarotti (PAV-KLP) in *D. melanogaster* (Adams et al., 1998); and the Rho-family GTPase-activating protein (GAP) known as CYK-4 in *C. elegans* (Jantsch-Plunger et al., 2000) and RacGAP50C in *D. melanogaster* (Somers and Saint, 2003). Each of these has orthologs in mammals. The mouse ortholog of *pbl*, *Ect2*, was originally identified as a proto-oncogene and then shown to be required for cytokinesis (Tatsumoto et al., 1999). MgcRacGAP, the mammalian ortholog of CYK-4/RacGAP50C, and the CHO1/MKLP1 vertebrate orthologs of ZEN-4/PAV-KLP are also required for cytokinesis (Chen et al., 2002; Kuriyama et al., 2002). Thus, all three encoded proteins are evolutionarily conserved cytokinesis factors.

pbl appears to be required for the earliest steps in cytokinesis, because embryonic *D. melanogaster* cells lacking PBL fail to show evidence of contractile ring function (Lehner, 1992; Prokopenko et al., 1998; Somma et al., 2002). Genetic interactions and yeast two-hybrid studies indicated that Rho1/RhoA is the target of PBL RhoGEF activity (O'Keefe et al., 2001; Prokopenko et al., 1999). PBL localizes to the contractile ring during cytokinesis (Prokopenko et al., 1999), where it appears to activate Rho locally and trigger the cascade of events that results in contractile ring function.

An important recent development is the discovery that CYK-4 and ZEN-4 associate to form a complex, known as centralspindlin, that can bundle microtubules (Mishima et al., 2002). Microtubule bundling is a feature of the anaphase/telophase microtubule network and appears to play an important role in generating the midzone microtubule network. The centralspindlin complex also appears to be a conserved feature of animal cytokinesis. The human and *D. melanogaster* CYK-4 and ZEN-4 orthologs form a complex (Mishima et al., 2002; Somers and Saint, 2003), and embryonic cells lacking the *Drosophila zen-4* ortholog, *pav*, exhibit defects in microtubule bundling (Adams et al., 1998).

A double ring to bind them?

Recently a connection between the contractile ring and the anaphase microtubule network was made with the discovery that the PBL RhoGEF can bind to RacGAP50C (Somers and Saint, 2003). The proteins bind strongly in a yeast two-hybrid system and co-immunoprecipitate from embryonic extracts. How does this interaction between the microtubule-associated centralspindlin complex and contractile ring-associated PBL manifest itself in the cell? Three-dimensional imaging of PBL, RacGAP50C and PAV-KLP in *D. melanogaster* embryonic epithelial cells revealed that a cortical, microtubule-associated ring of centralspindlin complexes is juxtaposed to a ring of PBL RhoGEF (Somers and Saint, 2003). This double ring arrangement is present in cells at the onset of constriction and, as constriction proceeds, in rings of ever decreasing size until the centralspindlin complexes become concentrated in the midbody.

The link between the PBL RhoGEF and the centralspindlin complexes suggested a model for the positioning of the contractile ring (Somers and Saint, 2003). This 'double ring' model postulates that the kinesin-like protein component (PAV-KLP/ZEN-4//CHO1/MKLP1) moves centralspindlin complexes to the interdigitated or juxtaposed plus ends of microtubules located at the midzone, where they form a microtubule-associated ring underneath the future cleavage site (Fig. 3). The interaction between the RacGAP50C/CYK-4/MgcRacGAP and the PBL/Ect2 is proposed to result in the positioning of the cortical RhoGEF at this site. This, in turn, would result in formation of a ring of activated Rho and, consequently, organization of the actomyosin contractile ring (Fig. 3).

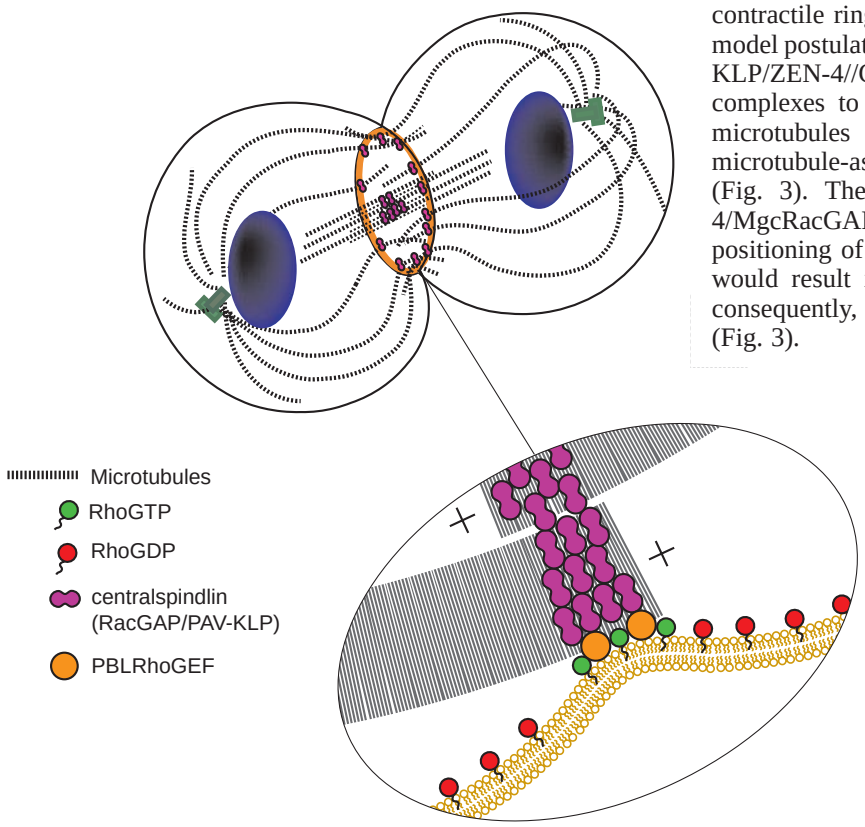

Fig. 3. The double ring model of cytokinesis positioning. Centralspindlin complexes move to the midzone along microtubules where they concentrate and activate the RhoGEF through a direct protein-protein interaction. This leads to a cortical ring of activated Rho1 between the separated chromosomes. The activated Rho1 leads to formation and activation of the actomyosin contractile ring (not shown).

This model accounts for conflicting observations (discussed above) concerning the roles of the astral microtubules and the midzone/central spindle microtubules. The arrangement and type of microtubule network may, in fact, not be important except in being able to deliver the centralspindlin complex to the midzone cortex. The way this is achieved may depend on the cell type. In large cells, such as those involved in the initial divisions of the sea urchin embryo, where the central spindle is far from the cortex, astral microtubules may play this role. Conversely, in the case of *D. melanogaster* spermatocytes or NRK cells, it may be the central spindle that delivers the centralspindlin complexes to create the cortical double ring with PBL and induce furrowing.

A general mechanism for animal cytokinesis?

The double ring model described here accounts for many observations that have implicated microtubules in the positioning of the contractile ring, The PBL-centralspindlin double ring, and the *pbl*, *pav* and *RacGAP50C* mutant and RNAi phenotypes all support this model (Adams et al., 1998; Lehner, 1992; Prokopenko et al., 1999; Somers and Saint, 2003). In addition, ZEN-4 has been observed to be associated with the furrow in *C. elegans* embryos (Jantsch-Plunger and Glotzer, 1999).

Other evidence, however, is inconsistent with this model. Some of the inconsistencies appear to reflect differences in cytoskeletal organization within different types of cells. For example, whereas studies described above revealed correlations between midzone or astral microtubule concentrations and furrow positioning (Wheatley and Wang, 1996; Giansanti et al., 1998), a recent report shows that *C. elegans* furrowing occurs at a local minimum in the microtubule concentration (Dechant and Glotzer, 2003). In addition, the phenotypes of the *cyk-4* and *zen-4* mutants suggest that they are required only after the onset of cytokinesis, when the advancing furrow reaches the central spindle (Raich et al., 1998; Jantsch-Plunger et al., 2000). The recent observation that Aurora-B-mediated phosphorylation converts MgcRacGAP (the mammalian CYK-4 ortholog) into a RhoGAP (Minoshima et al., 2003) supports the proposal that this factor inactivates Rho as the advancing furrow meets the midbody (Jantsch-Plunger et al., 2000).

Other conflicting evidence has come from studies of mammalian cultured cells. In contrast to the behaviour of RacGAP50C RNAi-treated *D. melanogaster* tissue culture cells (Somers and Saint, 2003), mammalian cells subjected to RNAi directed against CHO1 exhibit later cytokinetic phenotypes (Matuliene and Kurimaya, 2002). In addition, dominant-negative constructs of both Ect2 and CHO1 also block cytokinesis after the onset of furrowing. These studies should be treated with some caution, however, because it is possible that residual levels of the proteins remain at sufficient levels to induce initial furrowing. Indeed, Echard and O'Farrell (Echard and O'Farrell, 2003) have observed that the persistence of maternal PBL correlates with incomplete furrowing in cycle 14 *pbl* mutant cells. Alternatively, it is possible that the PBL-RACGAP50C-PAV-KLP double-ring is not involved in the initial cytokinetic events, but positioned in preparation for a later role at the time of midbody formation, or that it plays a role in relaxing the contacts between epithelial cells so that they can round up for mitosis.

Conclusion and perspectives: upstream and downstream of Rho activation

The double ring model of contractile ring positioning postulates that complexes of a kinesin-like protein (PAV-KLP/ZEN-4/CHO1/MKLP1) and a Rho-family GAP (RacGAP50C/CYK-4/MgcRacGAP) become localized to the overlapping or juxtaposed plus ends of astral and/or midzone microtubules between anaphase chromosomes. There, through a direct interaction between the Rho family GAP and a RhoGEF (PBL/Ect2), the GEF becomes concentrated, generating a cortical ring of activated Rho, and inducing organization and activation of the actomyosin contractile mechanism.

Even if the double ring model turns out to be true, it explains only a small fraction of the events required for successful cytokinesis. For example, it does not explain how microtubules are established so that the plus ends are juxtaposed or interdigitated for the centralspindlin complexes to accumulate and create the plane of division. It also does not explain how the double ring mechanism becomes active during anaphase B. Activation may involve regulators of late mitotic processes, such as polo-like kinase (Carmena et al., 1998), the aurora kinases (Giet and Glover, 2001; Schumacher et al., 1998; Severson et al., 2000) and INCENP (Kaitna et al., 2000; Wheatley et al., 2001), or other factors associated with the centralspindlin-PBL complex, such as the CeCDC-14 phosphatase, which is required for ZEN-4 localization (Gruneberg et al., 2002).

We also know little about the molecular events that occur between the activation of Rho and the formation and activation of the actomyosin contractile ring. In *D. melanogaster*, Anillin accumulates before the appearance of the actomyosin network (Field and Alberts, 1995) and in *X. laevis*, myosin appears before F-actin (Noguchi and Mabuchi, 2001). Septins are also recruited to the ring, perhaps by anillin-actin complexes (Kinoshita et al., 2002). The process of furrowing must involve an exquisite molecular motor that allows the membrane-attached ring to contract.

Although many questions associated with animal cytokinesis remain, it is nonetheless clear that the combined experimental resources of key model organisms such as *C. elegans*, *X. laevis* and *D. melanogaster* will provide the answers.

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