Genetic Approaches to Dissect the Mechanisms of Two Distinct Pathways of Cell Cycle-coupled Cytokinesis in Dictyostelium

Akira Nagasaki¹, Makoto Hibi¹,², Yukako Asano¹, and Taro Q.P. Uyeda¹*

¹Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology Tsukuba, Ibaraki 305-8562, Japan and ²Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

**ABSTRACT.** *Dictyostelium discoideum* is a unique experimental organism which allows genetic analysis of the mechanism of cytokinesis of the animal type, and a number of mutations which affect cytokinesis in one way or other have been identified. Myosin II filaments accumulate in the equatorial region, and myosin II-null cells cannot divide in suspension, indicating that active, myosin II-dependent constriction of the cleavage furrow contributes to bisection of the cell. We refer to this method of cytokinesis as cytokinesis A. On substrates, however, myosin II-null cells divide efficiently in a cell cycle-coupled manner. This adhesion-dependent but myosin II-independent division method, which we termed cytokinesis B, is carried out by a pathway that is genetically distinct from that of cytokinesis A. Morphological analyses suggested that cytokinesis B is driven by radial traction forces generated along polar peripheries, which indirectly cause furrow ingression. Identification of two redundant pathways have allowed us to search genes involved in either pathway by mutagenizing cells which are already defective in one of the pathways. This approach enabled us to identify a number of novel cytokinesis-related genes, as well as to reclassify known genes as cytokinesis-related.

**Key words:** cellular slime mold/myosin II/amIA/coronin

Cytokinesis of animal cells involves a highly coordinated series of events that leads to the formation of two daughter cells (for review, see Glotzer, 1997). Initially, a mitotic spindle forms, and the chromosomes become aligned along the equator of the cell. During anaphase, the chromosomes move toward either pole and contractile ring components, including non-muscle myosin II and actin, accumulate around the equator. Accompanying furrowing of the equatorial region two daughter cells are formed and finally separated by scission of the cytoplasmic bridge connecting the two.

Cell biological experiments have indicated that the mitotic spindle determines the position of the cleavage furrow (reviewed in Oegema and Mitchison, 1997; Rappaport, 1986), but it is still unknown what are the signals that are released from the spindle to stimulate the equatorial cortex in a temporally and spatially regulated manner. Regarding the physical mechanism of cell bisection, classic immunofluorescence studies as well as genetic experiments to eliminate myosin II expression have demonstrated that, at least in certain cases, myosin II-dependent constriction of the equatorial region provides the division force. However, it is still unclear how the contractile rings are assembled, and whether this method of cytokinesis is universally employed in all types of cytokinesis of the animal cells. Furthermore, constriction of the equatorial region is not sufficient to sever the cytoplasmic strand at the final stage of cytokinesis, and there appears to be a whole new set of machinery to carry out this task, although the actual mechanism of this process is still controversial (Zeitlin and Sullivan, 2001). Thus, there are still many fundamental questions left unresolved in the mechanism of cytokinesis.

One thing which was missing in the past studies of the cytokinesis mechanisms is a genetically tractable simple organism which carries out cytokinesis of the animal type. The fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* are well established genetically tractable animals, but because they are multicellular organisms, defects in cytokinesis are lethal and manifested...
as developmental defects, making further mechanistic investigations and detailed cytological analyses complicated. On the other hand, the cellular slime mold *Dictyostelium discoideum* is a unicellular ameba with a small haploid genome. *Dictyostelium* ameba divides in a manner very similar to animal cells in culture, and is recently emerging as a unique experimental system to dissect the mechanism of cytokinesis of the animal type. A number of novel, as well as known, cytokinesis-related genes have been identified in *Dictyostelium* (Robinson and Spudich, 2000; Uyeda and Yumura, 2000).

We are using the *Dictyostelium* system to investigate whether active constriction of the equatorial region is the sole means to divide in a cell cycle-coupled manner. The results reviewed here suggest that *Dictyostelium* has a novel, myosin II-independent but adhesion-dependent method of cell cycle-coupled cytokinesis, which is driven by traction forces generated along the polar peripheries. Furthermore, the results in the literature suggest that similar, myosin II-independent method of cytokinesis is preserved in higher animal cells. Our ongoing genetic approach to reveal novel genes involved in the myosin II-dependent and independent pathways is also described.

**Discovery of a novel, mhcA-independent, adhesion-dependent and cell cycle-coupled method of cytokinesis in Dictyostelium**

*Dictyostelium* has a single heavy chain gene for myosin II (*mhcA*), and gene knock-out and antisense RNA expression experiments demonstrated that myosin II is essential for cytokinesis in suspension (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). Interestingly, when maintained on a solid surface, *mhcA-* cells were capable of efficiently dividing, despite the absence of functional myosin II. Division by *mhcA-* cells on solid surfaces was originally attributed to a cell cycle-independent process termed “traction mediated cytofission”, in which different parts of a large multinucleate cell move in different directions, producing smaller cell fragments with reduced numbers of nuclei (Spudich, 1989). However, more recent detailed microscopic analyses by Neujahr et al. (1997b, 1998) revealed that, while attached to solid surfaces, *mhcA-* cells are able to divide in a cell cycle-coupled fashion using a process termed “attachment-assisted mitotic cleavage”. This process is extremely efficient, with more than 90% of cells dividing successfully following nuclear division, and the morphological changes during the division process, including formation of an equatorial cleavage furrow, are similar to those seen in wild type cells grown on substrates. Moreover, this process is fairly rapid, taking approximately 3–4 min at 22°C, which is only twofold slower than that of wild-type cells under similar conditions. It is thus evident that cell cycle-coupled cytokinesis can proceed in *Dictyostelium* grown on solid substrates in the absence of myosin II. These observations further suggested the intriguing possibility that *Dictyostelium* has two parallel pathways that both lead to cell cycle-coupled cytokinesis, one of which is dependent on myosin II and independent of substrate adhesion, and the other of which is dependent on adhesion and does not require myosin II.

**AmiA and coronin are involved in attachment-assisted mitotic cleavage**

One approach to examine whether *Dictyostelium* has two parallel pathways for cell cycle-coupled cytokinesis involves use of mutations that specifically affect one of the two pathways, prepare double mutants in various combinations, and ask what phenotype these mutants show. The classic, adhesion-independent pathway requires myosin II, and therefore, mutations that specifically affect attachment-assisted mitotic cleavage were needed for this genetic approach.

*AmiA*, also known as *piaA*, was originally isolated as a gene required for chemotaxis (Chen et al., 1997; Nagasaki et al., 1998), and has been implicated in intracellular signal transduction between the cAMP receptor and adenyl cyclase, but its exact molecular function has not been elucidated. Coronin, coded by the *corA* gene, is an actin binding protein which is implicated in macropinocytosis (deHostos et al., 1991, 1993), but again its mechanism of action is not well understood. Both of these genes were first discovered in *Dictyostelium*, but were later identified in various organisms ranging from yeasts to human (coronin by Heil-Chapdelaine et al., 1997, and Nagasaki et al., 2000, and amiA/piaA by Chen et al., 1997, and Nagasaki et al., 1998). Interestingly, *amiA-* and *corA-* cells are reported to show partial defect in cytokinesis and the defects are severer on substrates than in suspension (deHostos et al., 1993; Nagasaki, et al., 1998), in sharp contrast with the case of *mhcA-* cells. These data suggested the important roles for AmiA and coronin in attachment-assisted mitotic cleavage, and prompted Nagasaki et al. (manuscript submitted) to construct mutant cell lines of *Dictyostelium* that lacked either *mhcA, amiA*, or *corA*, and those which lacked two of the three genes in all three combinations.

Phenotypic analyses of these cell lines indicated that mutants lacking *mhcA* and either *amiA* or *corA* were severely defective in cytokinesis on substrates as well as in suspension, while those which lacked *amiA* and *corA* were no worse than those which lacked *amiA* or *corA* singly (Fig. 1). These genetic data led Nagasaki et al. (submitted) to conclude that *Dictyostelium* has two parallel pathways that both lead to cell cycle-coupled cytokinesis. It was interpreted that AmiA and CorA are involved in the same pathway, attachment-assisted mitotic cleavage, which is why double mutants lacking both *amiA* and *corA* were no sicker than the *amiA*–*corA* single mutants.

If the two parallel pathways identified by the *mhcA-* and
amiA\textsuperscript{−} or corA\textsuperscript{−} mutations are the only two major cell cycle-coupled methods of cytokinesis, it is predicted that the cells lacking mhcA and either amiA or corA would divide only through cell cycle-uncoupled routes. Examination of cells expressing GFP-histone, which enabled visualization of mitotic events in each cell under the fluorescence microscope, demonstrated that mitosis and cytokinesis were mostly uncoupled in these mutant strains that are unable to carry out both classic cytokinesis and attachment-assisted mitotic cleavage (Nagasaki \textit{et al}., submitted).

\textbf{Classic cytokinesis and attachment-assisted mitotic cleavage achieve bisection of the cells in physically distinct manners}

The genetic evidence does indicate that the classic cytokinesis and attachment-assisted mitotic cleavage are distinctive pathways, but does not tell how different these two are. In one extreme case, the AmiA/coronin pathway might complement the loss of myosin II by providing contractile forces in the equatorial region and achieve attachment-assisted mitotic cleavage. In this scenario, the classic cytokinesis and attachment-assisted mitotic cleavage are similar and differ only in terms of force-generating elements in the equatorial region. In the other extreme case, attachment-assisted mitotic cleavage might cleave a cell in a manner physically distinct from that of classic cytokinesis. In support of the first case, Robinson and Spudich (2000) and Gerisch and Weber (2000) proposed that dynamic changes in the actin cytoskeleton which occur in the equatorial region somehow cause myosin II-independent active equatorial furrowing. They argued that the contractile forces generated by the dynamics of the actin cytoskeleton are insufficient to complete the division when not supported by solid substrates. In disagreement with these views, Nagasaki \textit{et al}.
(submitted) proposed that the latter possibility is more likely, based on the following four reasons.

First, the morphological changes of cells during classic cytokinesis and attachment-assisted mitotic cleavage are rather different (Nagasaki \textit{et al}., submitted). AmiA\textsuperscript{−} or corA\textsuperscript{−} cells on substrates always detach from the substrate, and become more or less spherical, when they perform classic cytokinesis. In contrast, mhcA\textsuperscript{−} cells on substrates remain adherent over the entire ventral surfaces throughout the attachment-assisted mitotic cleavage process. These observations suggest that these two cytokinesis processes mechanistically different. Second, AmiA and coronin are not motor proteins, and are not known to activate other motor proteins (deHostos \textit{et al}., 1991; Chen \textit{et al}., 1997; Nagasaki \textit{et al}., 1998). Third, intracellular localization of AmiA and coronin are either pan-cortical (A. Nagasaki and K. Sutoh, unpublished) or mostly polar (deHostos \textit{et al}., 1991; Fukui \textit{et al}., 1999), and do not support their primary roles in equatorial regions. Finally, if the AmiA/coronin pathway somehow drives active equatorial furrowing, one would expect to see some ingression even in abortive division of mhcA\textsuperscript{−} cells in suspension. In contrary to this prediction, Zang and Spudich (1997) failed to detect any sign of furrowing in these cells, even though the cells elongated axially following mitosis.
What then is the physical mechanism of cell bisection in attachment-assisted mitotic cleavage? Uyeda et al. (2000) proposed that \( mhcA^- \) cells on substrates probably divide, depending on radial polar extension forces generated against the substrates. These forces would draw cytoplasm out of the equatorial region, and cause passive ingestion of the equatorial region. This scenario is consistent with the fact that attachment-assisted mitotic cleavage is absolutely dependent on substrate adhesion. Furthermore, after separation, the two daughter cells migrate away from each other, in a manner morphologically similar to locomoting keratocytes. In keratocytes, it is generally accepted that the forward movement is driven by radial extension forces generated along the front arciform lamellipodia (Lee et al., 1993), which is dependent on the Arp2/3-regulated actin polymerization (Borisy and Svitkina, 2000). Because cytokinesis of \( mhcA^- \) cells and migration of the two daughter cells from each other is one smoothly continuous process, it seems reasonable to assume that the radial extension forces are generated during the division process as well.

**Cytokinesis A, B and C**

Unfortunately, the terms that have been used to describe the various methods of cell division in *Dictyostelium*, (classic) cytokinesis, attachment-assisted mitotic cleavage, and traction-mediated cytofission, are rather confusing. For that reason, we recently extended a proposal by Zang et al. (1997) and redefined functionally the three apparently distinct methods of cell division as cytokinesis A, B, and C (Uyeda et al., 2000). In that view, cytokinesis A was defined as the cell cycle-coupled, adhesion-independent and myosin II-dependent method of division, cytokinesis B as the cell cycle-coupled, adhesion-dependent, and myosin II-independent method of division, and cytokinesis C as the cell cycle-uncoupled, adhesion-dependent and myosin II-independent method of division. Cytokinesis A is the same as the classic, textbook cytokinesis, and cytokinesis B is the same as attachment-assisted mitotic cleavage (Neujahr et al., 1997b). Cytokinesis C is analogous to traction-mediated cytofission (Spudich, 1989), though the relationship between the latter and the cell cycle has not been defined.

Based on the more recent morphological and other observations (Nagasaki et al., submitted; Uyeda et al., 2000) which are summarized above, however, Nagasaki et al. (submitted) proposed mechanistic definitions of cytokinesis A, B and C. In this view, cytokinesis A is the cell cycle-coupled division method which is driven by active equatorial constriction, and cytokinesis B is the cell cycle-coupled division method which is driven by radial polar extension which indirectly causes equatorial ingression (Fig. 2). Clearly more mechanistic studies are needed to test this proposal.

**Searching for more cytokinesis-related genes**

A number of studies have already been conducted to identify genes involved in cytokinesis in *Dictyostelium*, by randomly mutagenizing wild type cells and isolating clones that show cytokinetic defects (Adachi et al., 1994; Larochelle et al., 1996; Robinson and Spudich, 1998). Also, a large number of disruption mutants lacking known genes have been tested for their ability to divide and grow under laboratory conditions. These studies have identified a number of novel genes involved in the later stages of cytokinesis, i.e., completion of the furrowing process and severing of the thin cytoplasmic strands connecting the two daughter cells (Adachi et al., 1997; Larochelle et al., 1997; Gerald et al., 1998, 2001; Kwak et al., 1999). Also, a large number of genes are now known to affect the efficiency of cytokinesis, as one of their pleiotropic effects. However, surprisingly few mutations are known to affect specifically earlier steps of cytokinesis, including determination of the cleavage position, assembly of the contractile ring, and initiation of constriction (DeLozanne and Spudich, 1987; Faix et al., 1996). We reasoned that this is largely due to the fact that there are two parallel, redundant pathways that lead to cytokinesis of cells grown on substrates. Except for one study by Larochelle et al. (1996), all screening thus far conducted to search clones defective in cytokinesis out of randomly mutagenized pools of cells were performed on cells maintained on plastic plates, a condition which allows both cytokinesis A and B to function. In fact, \( mhcA^- \), \( amiA^- \), and \( corA^- \) single mutations exhibit only subtle phenotype on substrates, and should not show up as cytokinesis mutants if screened this way. Selection of cells defective in cytokinesis in suspension cultures should enable searching mutants with impaired cytokinesis A in the absence of contributions from cytokinesis B, but it is extremely laborious since this is a negative selection (i.e., desired mutants would become large and multinucleate and eventually lyse, while the background wild type cells continue to grow).

Based on this reasoning, we started screening cytokinesis mutants beginning with mutant strains already defective in either cytokinesis A or B. If, for example, \( mhcA^- \) cells are mutagenized randomly and allowed to form colonies on plates and colonies with large and multinucleate cells are screened visually, we should be able to identify mutants defective in cytokinesis B. This strategy has been so far very successful, and we have already identified seven cytokinesis defective clones starting from \( mhcA^- \) cells, and two cytokinesis defective clones starting from \( amiA^- \) or \( corA^- \) cells. Two of the seven mutations derived from \( mhcA^- \) cells were subjected to further analysis, and were demonstrated to exhibit severe cytokinetic defects only when combined with the \( mhcA^- \) mutation (M. Hibi, Y. Asano, A. Nagasaki, M. Takahashi, A. Yamagishi, and T. Q. P. Uyeda, unpublished data). We thus believe that this is a promising new approach to identify more cytokinesis-related mutations, and ulti-
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Cytokinesis in Dictyostelium discoideum ultimately, to understand the mechanisms of cytokinesis more fully.

**Cytokinesis B appears to be conserved in higher animal cells**

O’Connell et al. (1999) reported that microinjection of the C3 enzyme, which inhibits endogenous rho, into adherent, mitotic cells (normal rat kidney cells and 3T3 fibroblasts) did not inhibit equatorial furrow formation and even induced additional ectopic furrows. The equatorial and ectopic furrows formed in C3-injected cells did not contain higher levels of actin and myosin II filaments, properties reminiscent of cleavage furrows in mhcA–Dictyostelium cells undergoing cytokinesis B. On the other hand, microinjection of C3 into poorly adherent HeLa cells failed to induce ectopic furrows, and inhibited cytokinesis. Thus when rho is inactivated, mitotic vertebrate cells in culture are able to carry out adhesion-dependent, cell cycle-coupled cytokinesis, apparently independent of myosin II. More recently, O’Connell et al. (2001) discovered that local application of cytochalasin D to the equatorial region of dividing normal rat kidney cells accelerated the furrowing process, rather than inhibiting it, while its application to the polar region inhibited the furrowing to occur. This observation again supports the idea that contractile activities in the equatorial region is not essential for the equatorial furrowing and cytokinesis of these adherent cells in culture. In addition, Zurek et al. (1990) observed that injection of anti-myosin antibodies into epitheloid kidney cells, which diminished levels of myosin II in the equatorial region, only delayed cytokinesis, and all of the injected cells eventually divided successfully. This finding was interpreted by those investigators to mean that there was sufficient residual myosin II in the equatorial region to drive the cleavage slowly, but we propose an alternative interpretation: that adherent epitheloid cells are able


References


to divide in the absence of myosin II, albeit more slowly than in its presence, just as mhclA+ Dictyostelium cells take twice as long to divide than the wild-type cells.

This is not to say that animal cells do not in general require myosin II-dependent constriction of the contractile ring for successful cytokinesis. For example, microinjection of anti-myosin antibodies into starfish eggs inhibited their division, confirming the dependence of this process on contractile forces produced by myosin II (Mabuchi and Okuno, 1977). This would be analogous to the failure of mhcA- Dictyostelium cells to divide in suspension, a condition under which cytokinesis A is the only mechanism of cell division.

Another situation in which myosin II was shown to be essential for cytokinesis in animal cells is during embryogenesis of Drosophila melanogaster (Young et al., 1993) and Caenorhabditis elegans (Guo and Kemphues, 1996). Cells in developing animal tissues are surrounded by other cells and extracellular matrix, and in that sense are adherent to substrates, a condition which allows cytokinesis B in Dictyostelium. One possible reason why these cells may require functional myosin II for cytokinesis despite the presence of a substrate is that they are physically confined within small spaces so that the daughter cells cannot tear themselves apart by moving away from each other. This scenario is reminiscent of the observation that mhcA- Dictyostelium cells are unable to carry out cytokinesis B when sandwiched between a glass surface and a sheet of agarose (Neujahr et al., 1997a; Yumura and Uyeda, 1997). Alternatively, cells in developing tissues may be inherently less motile than cells cultured in vitro, and therefore cannot generate sufficient traction forces.

More studies are warranted to clarify the roles myosin II plays during cytokinesis of higher animal cells.
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