Genetic and morphological evidence for two parallel pathways of cell-cycle-coupled cytokinesis in *Dictyostelium*

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Summary

Myosin-II-null cells of *Dictyostelium discoideum* cannot divide in suspension, consistent with the dogma that myosin II drives constriction of the cleavage furrow and, consequently, cytokinesis (cytokinesis A). Nonetheless, when grown on substrates, these cells exhibit efficient, cell-cycle-coupled division, suggesting that they possess a novel, myosin-II-independent, adhesion-dependent method of cytokinesis (cytokinesis B). Here we show that double mutants lacking myosin II and either AmiA or coronin, both of which are implicated in cytokinesis B, are incapable of cell-cycle-coupled cytokinesis. These double mutants multiplied mainly by cytokinesis C, a third, inefficient, method of cell division, which requires substrate adhesion and is independent of cell cycle progression. In contrast, double mutants lacking AmiA and coronin were no sicker than each of the single mutants, indicating that the severe defects of myosin II$^-$/AmiA$^-$ or myosin II$^-$/coronin$^-$ mutants are not simple additive effects of two mutations. We take this as genetic evidence for two parallel pathways both of which lead to cell-cycle-coupled cytokinesis. This conclusion is supported by differences in morphological changes during cytokinesis in the mutant cell lines.

Movies available on-line

Key words: Cellular slime mold, Myosin II, AmiA, Coronin, GFP-histone H1

Introduction

In animal cells, mitotic cell division involves a highly coordinated series of events that lead to the formation of two daughter cells (for reviews, see Glotzer, 1997; Robinson and Spudich, 2000; Wolf et al., 1999). Initially, a mitotic spindle forms, and the chromosomes become aligned along the equator of the cell. Next, during anaphase, the chromosomes move toward either pole, and contractile ring components, including non-muscle myosin II and actin, assemble the cleavage furrow around the equator. Then, during telophase, following constriction of the furrow, a midbody forms at the intercellular bridge that connects the two daughter cells. Finally, the midbody is severed, resulting in complete separation of the two daughter cells. The detailed structure of the contractile ring is still not fully resolved, but it is generally believed to consist primarily of actin filaments interspersed with bipolar myosin II filaments. Powered by the motor activity of myosin II, the actin filaments are believed to slide over one another, drawing the ring tighter. The hypothesis that myosin II carries out the essential motor function in this process is supported by an early cytological study in which microinjection of anti-myosin antibodies inhibited cytokinesis in starfish blastomeres (Mabuchi and Okuno, 1977). This notion was further strengthened by later molecular genetic studies using the cellular slime mold *Dictyostelium discoideum*, which divides in a manner that is very similar to cultured animal cells. In these studies inactivation of the myosin II heavy chain gene (*mhcA*) by antisense RNA expression (Knecht and Loomis, 1987) or by gene disruption (De Lozanne and Spudich, 1987; Manstein et al., 1989) blocked cytokinesis in cells maintained in suspension. Reintroduction of functional *mhcA* (Egelhoff et al., 1990), but not a mutant form that lacked motor activity (Yumura and Uyeda, 1997), fully restored the ability of myosin-II-null (*mhcA*$^-$) cells to divide.

Interestingly, when maintained on a solid surface, *mhcA*$^-$ cells were capable of efficiently dividing, despite the absence of functional myosin II. Division of *mhcA*$^-$ cells on solid surfaces was originally attributed to a cell-cycle-independent process termed ‘traction-meditated 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. (Neujahr et al., 1997b; Neujahr et al.,1998) revealed that, although 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 minutes at 22°C, which is only two-fold 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. It is further speculated that this myosin-II-independent cytokinesis emerged earlier in eukaryotic evolution than the myosin-II-dependent method, although the molecular mechanism by which the equatorial cleavage furrow is formed in this case remains unknown (Gerisch and Weber, 2000; Uyeda et al., 2000). Unfortunately, the terms that have been used to describe the various methods of cell division in *Dictyostelium* are rather confusing. In this paper, we use the terms cytokinesis A, B and C. Cytokinesis A refers to the myosin-II-dependent and adhesion-independent division method, which wild-type cells in suspension use for proliferation. Cytokinesis B is the myosin-II-independent and adhesion-dependent division method, by which *mhcA*$^-$ cells on substrates divide. Both cytokinesis A and B are coupled to the progression of the cell cycle. In contrast, cytokinesis C is an adhesion-dependent division method that is not coupled with the cell cycle. As will be described later, certain cell lines of *Dictyostelium* appear to depend on this method of division for proliferation, and for that reason, we call it a form of cytokinesis, even though it is not coupled to the cell cycle.

In parallel with the apparent functional differences between cytokinesis A and B, these two modes of cytokinesis require different protein factors. As discussed above, cytokinesis A depends on the motor activity of myosin II, whereas cytokinesis B does not. It is notable that cell division is partially disrupted in cells lacking *amiA* (Nagasaki et al., 1998) or *corA* (de Hostos et al., 1993), the gene encoding coronin. These mutants are moderately multinucleate when grown on solid surfaces, although they divide with efficiencies comparable to those of wild-type cells in suspension, suggesting these two genes play important roles in cytokinesis B in *Dictyostelium*. Coronin contains WD repeats with sequence similarities to the β-subunit of trimeric G proteins (de Hostos et al., 1991). Analysis of a GFP fusion protein (Fukui et al., 1999; Maniak et al., 1995; Rauchenberger et al., 1997) and a knockout mutant (de Hostos et al., 1993) showed that coronin participates in the remodeling of the cortical actin cytoskeleton, which is required for efficient phagocytosis and macropinocytosis. The *amiA* gene (Nagasaki et al., 1998), also known as *piaA* (Chen et al., 1997), was originally cloned as a gene required for chemotaxis by insertional mutagenesis of *Dictyostelium*, but homologous genes were later identified in the genome databases of yeast and humans (Nagasaki et al., 1998) (A.N. and T.Q.P.U., unpublished). Genetic analyses have suggested that AmiA is involved in communication between cyclic AMP receptor and adenylyl kinase (Nagasaki et al., 1998; Chen et al., 1997). However, little is known about how AmiA functions in vivo, since this protein has not been purified biochemically, and its predicted amino acid sequence does not have significant homology with known motifs or functional domains except for a leucine zipper domain.

The findings summarized above raise the intriguing possibility that *Dictyostelium* has two mechanistically distinct methods of cell-cycle-coupled cytokinesis. To test this hypothesis, we constructed double mutant cell lines in which both cytokinesis A and B were anticipated to be defective. Analysis of these cells fully supported the hypothesis, providing genetic evidence for the presence of two parallel pathways via which cell-cycle-coupled division is achieved in *Dictyostelium*. Furthermore, microscopic observation of the division process of the mutant cell lines suggested that the two cell-cycle-coupled methods of cytokinesis are mechanistically rather different. The definitions of cytokinesis A, B and C described above are functional and do not necessarily imply mechanistic differences among the three methods. On the basis of these results, however, we will propose a re-definition of these terms from mechanistic viewpoints. The implications of these results are discussed in terms of cytokinesis in higher animal cells.

Materials and Methods

Cell culture

Parental *Dictyostelium discoideum* wild-type AX2 cells were grown axenically in HL-5 medium (Sussman, 1987) supplemented with penicillin and streptomycin at 22°C. *CorA*$^-$, *amiA*$^-$ and *corA*$^-$/*amiA*$^-$ cells were cultured in HL-5 in the presence of penicillin, streptomycin and 10 μg/ml blasticidin-S. Cells carrying the *Dictyostelium* expression vector, pBIG (Ruppel et al., 1994), were grown in medium containing penicillin, streptomycin and 10 μg/ml G418. The cells were usually maintained on 9 cm plain plastic Petri dishes. In some experiments, however, cells were grown in suspension, in Teflon conical flasks on a shaker rotating at ~140 rpm.

Construction of GFP-histone

Dictyostelium histone H1 cDNA was amplified by RT-PCR using a pair of oligonucleotides, 5′-GGATCCAATGGGTCCAAAAGCAC-CAAC-3′ and 5′-GAGCTCCTATTTTTTGGCAGCGACTT-3′. These oligonucleotides add *Bam*HI and *Sac*I recognition sites at either end of the PCR product, enabling it to be subcloned into GFP/pBig downstream of the GFP coding sequence. Subsequent expression of GFP-H1 was driven by the promoter of actin 15.

Generation of knockout mutants

Each cell line was generated by homologous recombination in wild-type, *mhcA*$^-$ (Ruppel et al., 1994) or *corA*$^-$ (Fukui et al., 1999) cells. Disruption of *amiA* in wild-type or *mhcA*$^-$ cells was achieved using pKO amiA(Bsr) (Fig. 2A), which resulted in the blasticidin resistance gene being inserted into the coding region of *amiA*. *AmiA*$^-$/*corA*$^-$ and *amiA*$^-$/*mhcA*$^-$ double knockout cells were generated using the targeting vector pKO amiA(neo) in *corA*$^-$ or *mhcA*$^-$ cells. This gene disruption construct consisted of an *amiA* gene with its promoter and part of the coding sequence being replaced by a cassette conferring neomycin resistance. The insertion position of the neomycin resistance gene was the same as that of the original REMI mutant (Nagasaki et al., 1998). To create an *mhcA*$^-$/*corA*$^-$ double knockout strain, the Neo selectable marker cassette was inserted between the two *Eco*RV restriction sites within the motor domain of *mhcA*; the resultant plasmid was then used to knockout *mhcA* in *corA*$^-$ cells.

Fluorescence microscopy

Cells were transfected with GFP-H1/pBig by electroporation, after which the transfectants were incubated on a plastic Petri dish with a thin glass bottom (IWAKI, Japan). The modified HL-5 culture medium, which minimized background fluorescence, contained 3.85 g/l of glucose, 1.78 g/l of Proteose Peptone (Difco), 0.45 g/l of yeast extract (Difco), 0.485 g/l of KH2PO4 and 1.2 g/l of $Na_2HPO_4{\cdot}12H_2O$ and was sterilized by filtration (pore size, 0.25 μm). Cells expressing GFP-H1 were observed under a fluorescence microscope (IX50; Olympus, Japan) equipped with a UPlan Apo 40X oil immersion objective lens (Olympus). Time-lapse images were acquired with a CCD camera (C5985; Hamamatsu Photonics, Japan) for 10 hours with intervals of 30-120 seconds between frames using a time-lapse recording system (ARGAS-20, Hamamatsu Photonics). For montage

sequences, video images were digitized using NIH image software version 1.61.

Results

Three methods of cytokinesis in *Dictyostelium*

Consistent with the aforementioned reports, *Dictyostelium* was observed to make use of three apparently distinct modes of cytokinesis (Fig. 1). Wild-type cells were able to carry out cytokinesis when embedded and cultured in low melting temperature agarose gel, a condition mimicking suspension culture. Under this condition, mitotic wild-type cells organized contractile machinery around the equator to form a cleavage furrow (Fig. 1A), and the two daughter cells moved away from each other after separation. *MhcA*$^-$ cells were only capable of attachment-assisted mitotic cleavage on solid surfaces. The morphological changes during this process resembled those of normal cytokinesis in wild-type cells grown on solid substrates (Fig. 1B); however, detailed microscopic observation revealed subtle differences between the two processes, which will be described later. The third method of cytokinesis, which is cell-cycle-independent, was most prominently observed when large, multinucleate *mhcA*$^-$ cells, prepared by culture in suspension for a few days, were replaced onto solid substrates. These multinucleate cells were rapidly torn into several pieces, each containing nuclei, apparently driven by uncoordinated ameboid movement of different parts of the large cells, regardless of the cells' stage in the cell cycle (Fig. 1C).

Hereafter we use the terms 'cytokinesis A' and 'cytokinesis B' to describe the two cell-cycle-coupled methods of cytokinesis. Cytokinesis A refers to the myosin-II-dependent and adhesion-independent division method, which wild-type cells in suspension rely on for proliferation. Cytokinesis B is the myosin-II-independent and adhesion-dependent division method, by which *mhcA*$^-$ cells on substrates divide. Wild-type cells on substrates probably utilize both cytokinesis A and B, although the relative contributions made by each is not known and possibly variable, as will be discussed later. We refer to the cell-cycle-independent division as 'cytokinesis C' (Fig. 1C). Because the division process of cytokinesis A or B of mononucleate cells is typically completed within 3-4 minutes under our experimental conditions, we operationally judge division that occurred more than 30 minutes after the nuclear division to be cell cycle independent, and hence, cytokinesis C. Cytokinesis C, like cytokinesis B, depends on adhesion to solid substrates and does not require myosin II.

Disruption of *mhcA*, *amiA* and *corA* in *Dictyostelium*

Inactivation of the *mhcA* or *amiA* gene in wild-type, *mhcA*$^-$ or *corA*$^-$ cells was achieved by homologous recombination (Table 1). The gene-disruption constructs consisted of the coding region and/or promoter of *mhcA* or *amiA*, part of which was replaced by a gene conferring resistance to the antibiotic blasticidin S or G418 (Fig. 2A). Selective disruption of targeted genes was confirmed by genomic PCR (Fig. 2B); the primers used are indicated by arrows in Fig. 2A. The generation of *corA*$^-$ cells was described previously (Fukui et al., 1999).

Table 1. Strains used in this study

	Genotype	Parental strain	Source
AX2		–	Watts and Ashworth, 1970
HTU1	*amiA (Bsr)*	AX2	Nagasaki et al., 1998
HS1	*mhcA (Thy)*	AX3	Ruppel et al., 1994
coronin$^-$	*corA (Bsr)*	AX2	Fukui et al., 1999
HTU2	*amiA (Neo)*	AX2	This study
HTU3	*mhcA (Neo)*	AX2	This study
HTU4	*amiA (Bsr), mhcA (Thy)*	AX3	This study
HTU5	*amiA (Bsr), mhcA (Neo)*	AX2	This study
HTU6	*amiA (Neo), mhcA (Thy)*	AX3	This study
HTU7	*corA (Bsr), mhcA (Neo)*	AX2	This study
HTU8	*amiA (Neo), corA (Bsr)*	AX2	This study

Two strains of *mhcA*$^-$ and three strains of *amiA*$^-$/*mhcA*$^-$ cells were created to assess possible phenotypic differences derived from differences in the parent strains. These redundant strains showed identical phenotypes in our assays, however, and results from HTU3, HTU5 and HTU6 are not presented. The HS1 *mhcA*$^-$ strain was originally constructed by Ruppel et al. (Ruppel et al., 1994) using the *THY1* gene (Dynes and Firtel, 1989) as a selection marker in JH10, a thymidine auxotroph derivative of AX3 (Hadwiger and Firtel, 1992).

Cytokinesis defects in single and double mutants affecting cytokinesis A or B

When *amiA*$^-$ and *corA*$^-$ cells were cultured on solid surfaces,

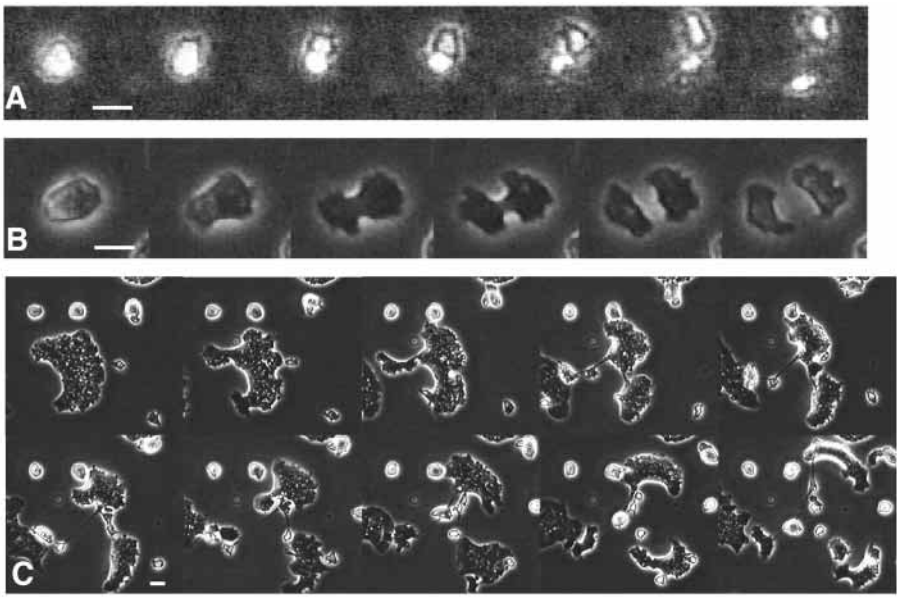

Fig. 1. Three modes of cytokinesis in *Dictyostelium*. A series of time-lapse phase contrast images obtained at 30 second intervals are shown. (A) Wild-type AX2 cells were embedded in low melting temperature agarose and cultured without solid surfaces. In this condition, wild-type cells divide using cytokinesis A. (B) *mhcA*$^-$ cells were cultured on a plastic dish to allow adhesion to a solid surface. Division of *mhcA*$^-$ cells is driven by cytokinesis B. (C) Multinucleate *mhcA*$^-$ cells grown in suspension for 3 days were then placed on a plastic dish. These giant cells divide by cytokinesis C. Bars, 10 μm.

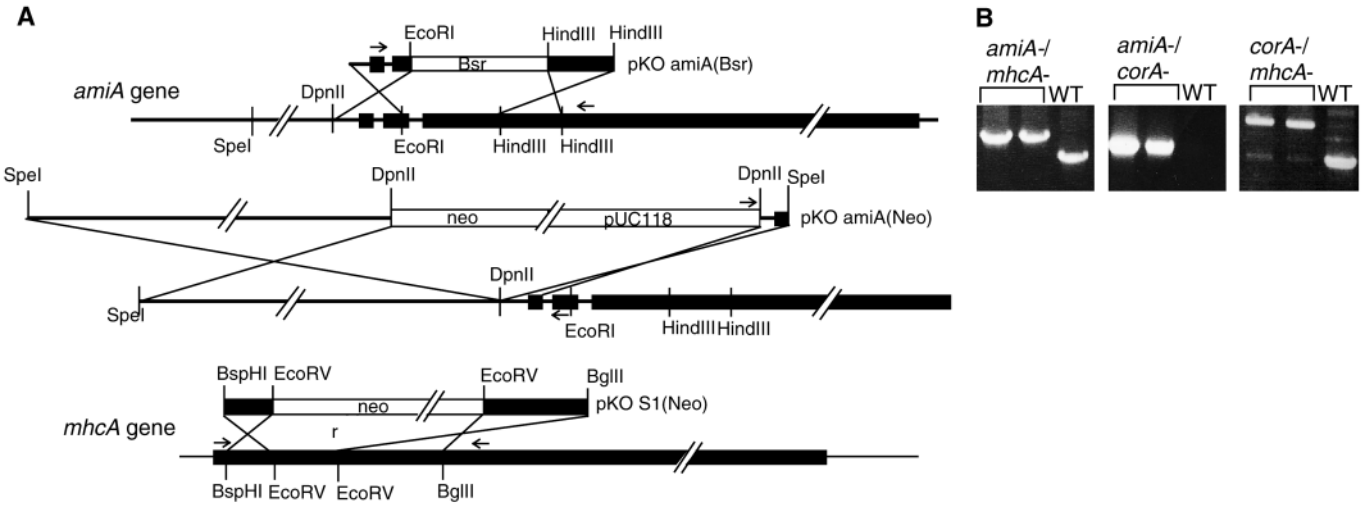

Fig. 2. Disruption constructs targeting *mhcA* and *amiA*, and confirmation of disruption using genomic PCR. (A) The targeting vector used to knockout *amiA* was constructed by replacing part of its coding region and promoter with the blasticidin S [pKO amiA(Bsr)] or G418 [pKO amiA(Neo)] resistance gene. In the targeting vector for *mhcA*, a portion of the motor domain was replaced with the G418 resistance cassette. Expression of all drug resistance genes was driven by the actin 15 promoter. Thick lines indicate coding sequences of *amiA* and *mhcA*. (B) Mutant cells were identified by a shift in size of the PCR products. (Left) Knockout of *amiA* in *mhcA*$^-$ cells (HS1), yielding HTU1; (middle) knockout of *amiA* in *corA*$^-$ cells, yielding HTU8; (right) knockout of *corA* in *mhcA*$^-$ cells (HS1), yielding HTU7. Arrows in A show the positions of the primers used for genomic PCR.

they tended to become larger and flatter than wild-type or *mhcA*$^-$ cells (Fig. 3C-E,G). Most notably, staining of the nuclei with 4,6-diamidino-2-phenylindole (DAPI) revealed that some of these mutants had become moderately multinucleate. When assayed three days after dilution to new plate cultures, 63% and 68% of *amiA*$^-$ and *corA*$^-$ cells had more than two nuclei, respectively (Fig. 3A). Less than 4% of wild-type cells maintained on substrates were multinucleate. In contrast, when cultured in suspension for three days, 44% of the wild-type cells became multinucleate, indicating the limited efficiency of cytokinesis A. Suspension culture of *amiA*$^-$ and *corA*$^-$ mutant cells yielded multinucleate cells. The fractions of multinucleate cells (52% and 41%, respectively) were somewhat smaller than but similar to those on substrates (Fig. 3B). These values are also comparable to that of wild-type cells grown in suspension. In summary, wild-type cells on substrates divide with nearly 100% efficiency, whereas *amiA*$^-$ and *corA*$^-$ cells either in suspension or on substrates and wild-type cells in suspension divide at similarly compromised efficiencies (30~60%). In other words, *amiA*$^-$ and *corA*$^-$ cells are more severely disrupted in cytokinesis than wild-type cells on substrates, but they divide with efficiencies similar to that of wild-type cells in suspension.

That *amiA*$^-$ and *corA*$^-$ cells exhibit more severely disrupted cytokinesis than wild-type cells only when cultured on substrates. This is in sharp contrast to *mhcA*$^-$ cells, which exhibit severely defective cytokinesis only in suspension. These results suggest that AmiA and coronin might play important roles in cytokinesis B and that we now have mutations that selectively affect cytokinesis A (*mhcA*$^-$) and cytokinesis B (*amiA*$^-$ and *corA*$^-$). On the basis of that premise, we set out to dissect genetically the mechanism of cytokinesis in *Dictyostelium*. When cultured on solid substrates, cytokinesis in cells lacking both AmiA and myosin II (*amiA*$^-$/*mhcA*$^-$) was much more disrupted than that in cells lacking only AmiA (Fig. 3F,J) or double mutants lacking coronin and myosin II (*corA*$^-$/*mhcA*$^-$) (Fig. 3H,J). The efficient suppression of cytokinesis in *amiA*$^-$/*mhcA*$^-$ and *corA*$^-$/*mhcA*$^-$ cells appeared to reflect a synergistic effect of the loss of myosin II and AmiA or coronin rather than being merely an additive effect of two gene disruptions; indeed *amiA*$^-$/*corA*$^-$ double mutants were no more severely affected than cells carrying a single mutation (Fig. 3I,J).

Morphological changes during the mitotic phase in mutants and wild-type cells

Detailed analyses of the morphological changes that occur during cytokinesis revealed subtle but reproducible differences among the wild-type and mutant cell lines. For example, *amiA*$^-$ and *corA*$^-$ cells grown on substrates always rounded up and became detached from the substrate when they carried out cytokinesis successfully – as judged by their refractile appearance in phase contrast micrographs (Fig. 4C,D) - and in most cases they remained so throughout the cleavage process, until the two daughter cells were completely separated. Furthermore, dividing *amiA*$^-$ and *corA*$^-$ cells often drifted over the substrate, suggesting that substrate adhesion is greatly decreased during this division process. This manner of cell division is similar to that of wild-type cells cultured in suspension, in agarose (Fig. 1A), or on hydrophobic surfaces (Zang et al., 1997). Under these conditions, cytokinesis A powered by myosin-II-dependent active furrowing of the contractile rings is the sole means of division, which is consistent with our premise that AmiA and coronin are required for cytokinesis B.

MhcA$^-$ cells also first retracted pseudopods and temporarily rounded up slightly when entering mitosis, but in contrast to *amiA*$^-$ or *corA*$^-$ cells, they remained attached to the substrate during the entire division process. Moreover, during anaphase

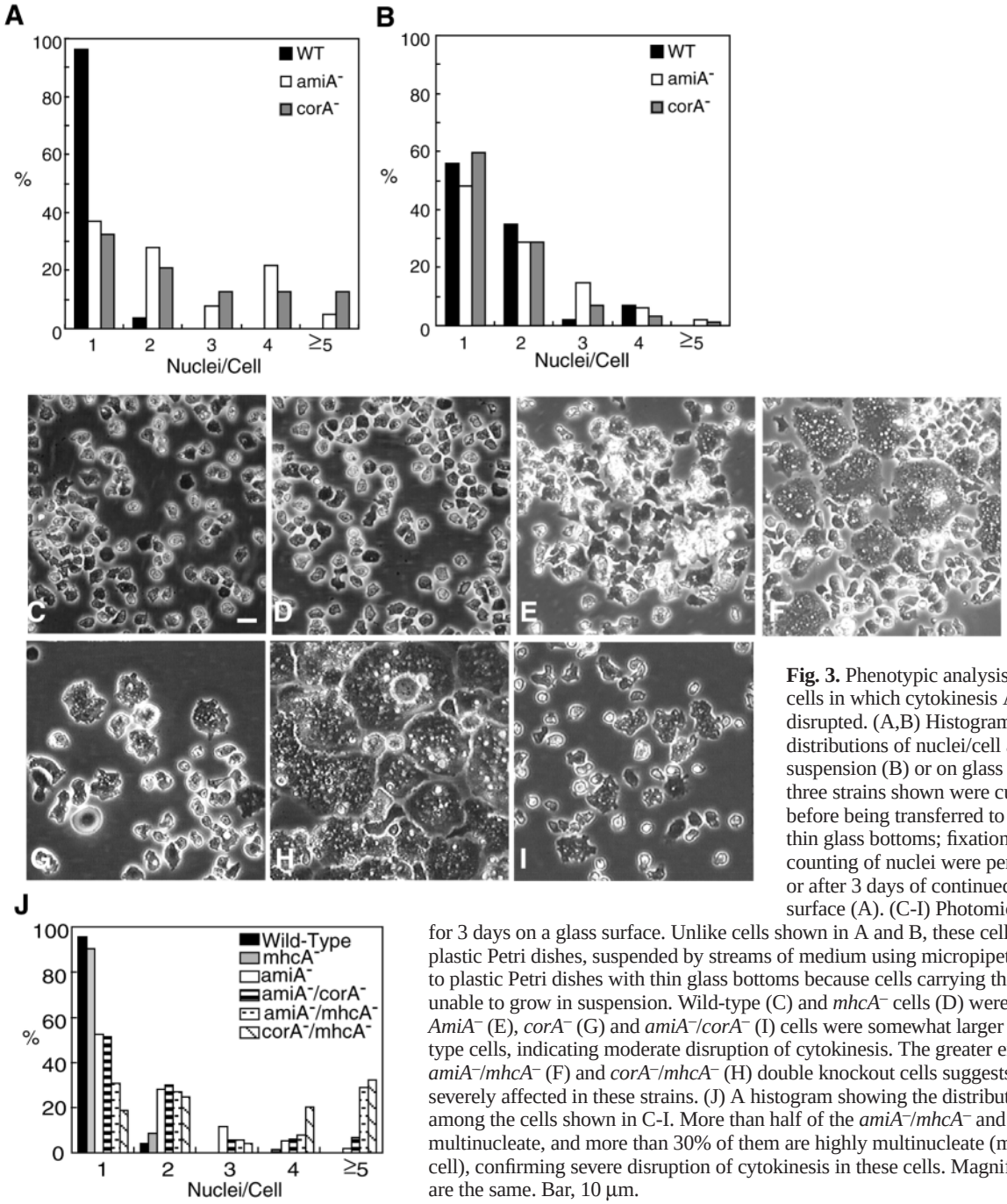

Fig. 3. Phenotypic analysis of wild-type and mutant cells in which cytokinesis A and/or B has been disrupted. (A,B) Histograms showing the distributions of nuclei/cell among cells grown in suspension (B) or on glass surfaces (A). Cells of all three strains shown were cultured in suspension before being transferred to plastic Petri dishes with thin glass bottoms; fixation, DAPI staining and counting of nuclei were performed immediately (B) or after 3 days of continued growth on the glass surface (A). (C-I) Photomicrographs of cells grown for 3 days on a glass surface. Unlike cells shown in A and B, these cells were precultured on plastic Petri dishes, suspended by streams of medium using micropipettes, diluted, and replated to plastic Petri dishes with thin glass bottoms because cells carrying the *mhcA*– mutation were unable to grow in suspension. Wild-type (C) and *mhcA*– cells (D) were mostly mononucleate. *AmiA*– (E), *corA*– (G) and *amiA*–/*corA*– (I) cells were somewhat larger and flatter than wild-type cells, indicating moderate disruption of cytokinesis. The greater enlargement of *amiA*–/*mhcA*– (F) and *corA*–/*mhcA*– (H) double knockout cells suggests cytokinesis is more severely affected in these strains. (J) A histogram showing the distributions of nuclei/cell among the cells shown in C-I. More than half of the *amiA*–/*mhcA*– and *corA*–/*mhcA*– cells are multinucleate, and more than 30% of them are highly multinucleate (more than five nuclei per cell), confirming severe disruption of cytokinesis in these cells. Magnifications in panels C-I are the same. Bar, 10 μm.

or telophase, *mhcA*– cells would resume their flat appearance, adhering to the substrate over their entire ventral surfaces (Fig. 1B, Fig. 4B). Mitotic wild-type cells grown on substrates were initially adherent, much like mitotic *mhcA*– cells, but like *amiA*– or *corA*– cells, they rounded up by the time cleavage furrows formed and remained detached until division was complete. Thus the morphological changes seen during cytokinesis in wild-type cells grown on substrates are intermediate between those of *amiA*– and *corA*– cells and those of *mhcA*– cells, which suggests that, on substrates, wild-type cells may employ both cytokinesis A and B to guarantee efficient and faithful bisection.

When *amiA*–/*mhcA*– cells were cultured on solid surfaces for three days, most became multinucleated, with some becoming extremely large and highly multinucleate (Fig. 3J). Moreover, among the mononucleate *amiA*–/*mhcA*– cells, most did not divide during a time-lapse observation period of 8 hours (not shown). Given that their doubling time is normally about 8 hours (Zada-Hames and Ashworth, 1978), these observations suggest that the majority of *amiA*–/*mhcA*– cells do not divide in a cell-cycle-coupled manner. Time-lapse observation of large, multinucleate *amiA*–/*mhcA*– cells showed that a portion of these cells often moved in different directions by assembling multiple leading edges, which sometimes tore the large cell

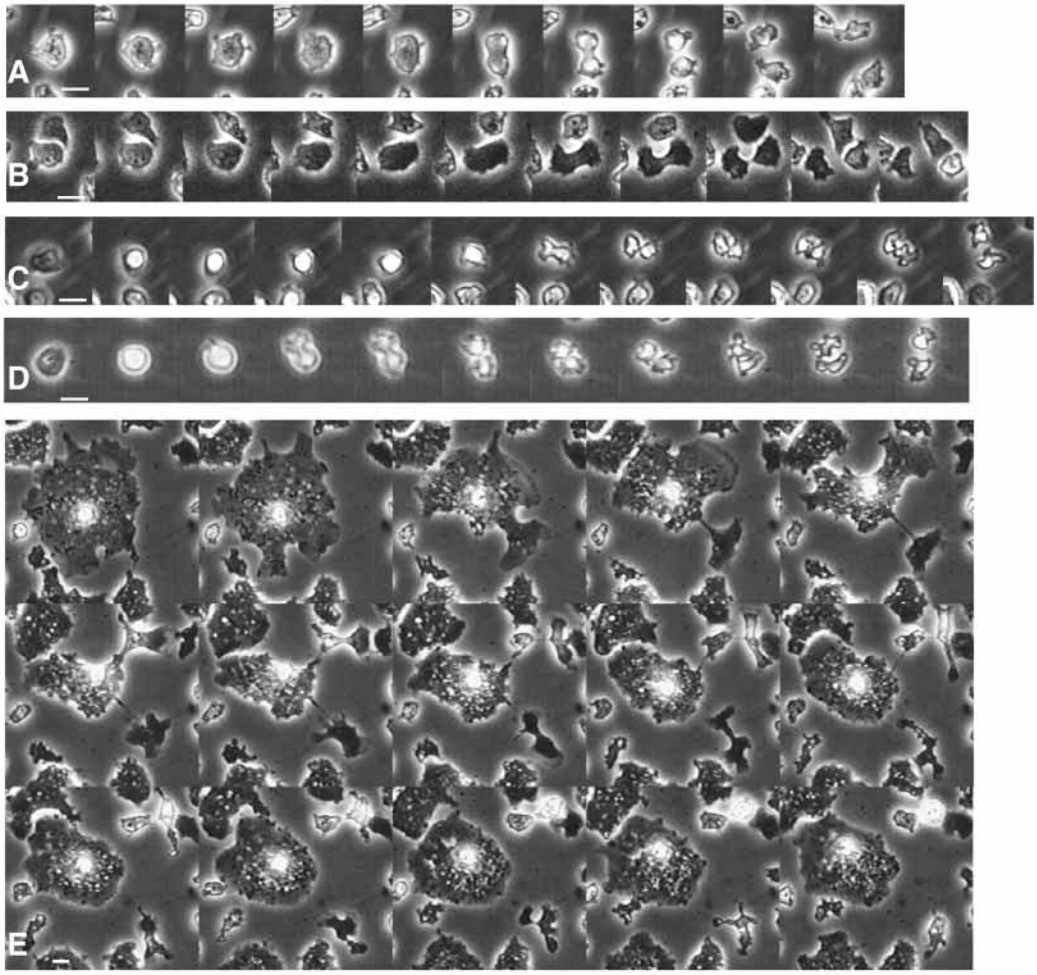

Fig. 4. Sequences of morphological changes during division on a solid substrate. Cells were attached to plastic dishes for 2 days before observation. Each panel shows a series of phase contrast images recorded with intervals of 60 seconds between frames. (A-E) Mitotic wild-type (A), mitotic *mhcA*$^-$ (B), mitotic *amiA*$^-$ (C), mitotic *corA*$^-$ (D) and *amiA*$^-$/*mhcA*$^-$ (E) cells. The cell cycle stage of the cell in E was not determined, but is most probably interphase. Bars, 10 μm. Magnifications of A-D are the same.

into several fragments (Fig. 4E). This method of cytokinesis resembles the cytokinesis C observed when large, multinucleate *mhcA*$^-$ cells produced by culture in suspension were placed on a solid surface (Fig. 1C). That type of division is apparently uncoupled from the cell cycle, as most of the large cells initiated this process immediately following adhesion to the substrate (data not shown). We suggest that the morphologically similar division of large *amiA*$^-$/*mhcA*$^-$ cells is also uncoupled from the cell cycle.

Visualization of cell cycle progression using GFP-histone

To further investigate the relationship between cell cycle progression and division events, we visualized progression of the cell cycle as a function of the expression of histone H1 fused with the green fluorescent protein (GFP-H1; Fig. 5A). Using time-lapse observation of GFP-H1 fluorescence, we determined that during interphase GFP-H1 was distributed uniformly within nuclei (Fig. 5B). When cells entered the mitotic phase, however, the pattern of GFP-H1 fluorescence varied in a characteristic fashion that enabled it to be used to track the cell within the cell cycle. First the contour of the nucleus became obscure and the chromosomes condensed (Fig. 5C); after which the two sets of chromosomes separated, and two daughter nuclei were formed (Fig. 5D). These changes in patterns of GFP fluorescence (see Movie 1 at http://jcs.biologists.org/supplemental), and direct observation of nuclear division in particular, allowed us to determine the onset of anaphase unambiguously within each cell.

Of the cells entering mitosis, more than 90% of the wild-type and *mhcA*$^-$ (Fig. 5D) cells on substrates successfully divided into two daughter cells (n=20 and 40, respectively). Of 17 *amiA*$^-$ cells that we were able to follow throughout the mitosis process, as judged by GFP-H1 localization, 10 successfully divided within 30 minutes (59% success). This number may not be accurate, however, since we were unable to follow the majority of the mitosis events of *amiA*$^-$ cells in video sequences, owing to detachment of the mitotic cells from the surface and the resultant drifting. Also, detachment of the mitotic cells carried nuclei out of the focal plane, which obscured the fluorescence images of GFP-H1.

In contrast, the majority of the mononucleate *amiA*$^-$/*mhcA*$^-$ cells failed to complete cell division, even though nuclear division proceeded normally and each of the two new nuclei temporarily migrated to the respective lobes of an elongated cell (Fig. 5E). Of 30 mononucleate *amiA*$^-$/*mhcA*$^-$ cells that went through nuclear division, only seven successfully divided within 30 minutes (23% success) (see also Movies 2 and 3 at http://jcs.biologists.org/supplemental for failed cytokinesis of mononucleate and multinucleate *amiA*$^-$/*mhcA*$^-$ cells). When mononucleate or binucleate *amiA*$^-$/*mhcA*$^-$ cells successfully

divided following nuclear division, two or four mononucleate daughter cells were generated, respectively. This indicates that cleavage furrows were formed between every nascent nucleus. On the other hand, when large highly multinucleate *amiA*$^-$/*mhcA*$^-$ cells successfully divided following nuclear division (three out of 12 cases), only a small number of large, unilateral furrows were formed, resulting in generation of two or three multinucleate cell fragments (Fig. 5F) (Movie 4 at http://jcs.biologists.org/supplemental) (see also Neujahr et al., 1998). Conversely, when large multinucleate *amiA*$^-$/*mhcA*$^-$ cells on substrates divided, retrospective examination of the time-lapse images demonstrated that in most cases there were no preceding nuclear division within 30 minutes (data not shown), and these division processes were judged to be cytokinesis C (Fig. 5F).

Discussion

There is ample experimental evidence to suggest that the contractile force produced by myosin II is essential for cytokinesis in a variety of eukaryotic cells lacking cell walls (Glotzer, 1997; Robinson and Spudich, 2000; Wolf et al., 1999). Consistent with that evidence, *Dictyostelium* cells, which lack functional myosin II, become multinucleated and eventually lyse when cultured in suspension (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). When cultured on solid surfaces, however, these cells are able to divide normally, in a cell-cycle-coupled manner, even without myosin II (Neujahr et al., 1997b), suggesting that a myosin-II-independent mechanism of cell division exists in *Dictyostelium*. In addition, microscopic observation indicates that myosin-II-independent cytokinesis includes two apparently distinct processes (Uyeda et al., 2000; Zang et al., 1997) (see also Fig. 1B,C), resulting in a total of three methods of cytokinesis in *Dictyostelium*: cytokinesis A, a cell-cycle-coupled, myosin-II-dependent and adhesion-independent division method, which appears to be driven by active constriction of the contractile rings (classic cytokinesis); cytokinesis B, which is a cell-cycle-coupled division method employed by *mhcA*$^-$ cells on substrates [attachment-assisted mitotic cleavage (Neujahr et al., 1997b)]; and cytokinesis C, a division method that is not coupled to the cell cycle. Cytokinesis C requires substrate adhesion but does not depend on myosin II [traction-mediated cytofission (Spudich, 1989)]. The present study was carried out to genetically dissect the mechanisms responsible for these three division processes in *Dictyostelium*.

To achieve that goal, we used gene disruption to generate cell lines that were unable to carry out cytokinesis A and/or B. The *mhcA*$^-$ mutation selectively inhibited cytokinesis in cells grown in suspension, which is indicative of cytokinesis A impairment, whereas the *amiA*$^-$ (Nagasaki et al., 1998) and *corA*$^-$ (de Hostos et al., 1993) mutations more severely affected cells cultured on substrates, which is indicative of cytokinesis B impairment. Both *amiA*$^-$ and *corA*$^-$ were originally cloned in *Dictyostelium*, but homologs were subsequently identified in the yeast and human genomes [coronin (Heil-Chapdelaine et al., 1998; Iizaka et al., 2000; Parente et al., 1999; de Hostos, 1999), *amiA*/*piaA* (Chen et al., 1997; Nagasaki et al., 1998)]. Coronin was originally purified from precipitated actin-myosin complexes as an actin-binding protein (de Hostos et al., 1991) and was later implicated in phagocytosis on the basis of *corA* gene knockout experiments (de Hostos et al., 1993). *AmiA*, also known as *piaA*, was cloned

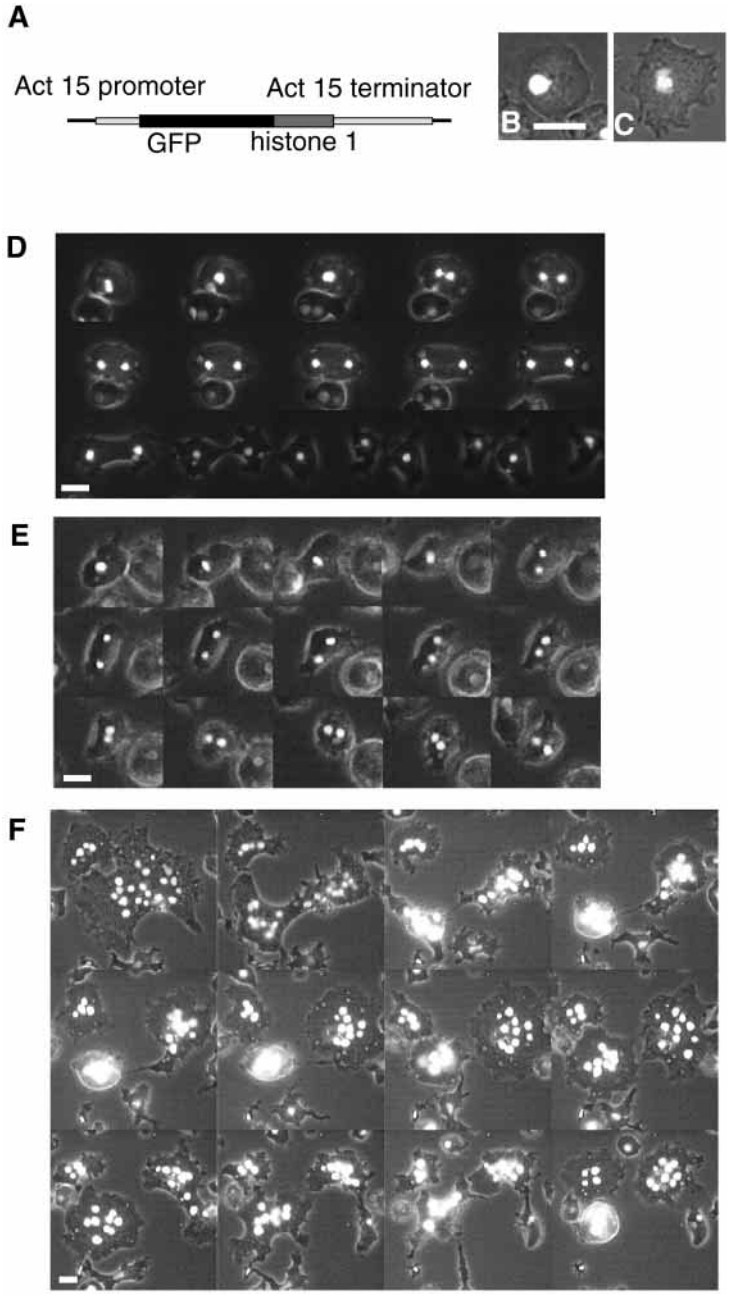

Fig. 5. Visualization of the cell cycle as a function of GFP-histone expression. (A) Structure of the GFP-tagged histone H1 chimeric gene. (B,C) Confocal images of the nucleus were identified in interphase (B) and mitotic (C) cells by GFP-H1 fluorescence. (D-F) A series of time-lapse micrographs of GFP fluorescence images superimposed on phase contrast images. They show differing cytokinesis processes in three strains of GFP-H1-expressing cells grown on solid substrates: *mhcA*$^-$ cells undergoing cytokinesis B (D); *amiA*$^-$ cells failing to divide (E); and *amiA*$^-$/*mhcA*$^-$ cells undergoing cytokinesis C (F). Bars, 10 μm.

as a chemotaxis-related gene (Chen et al., 1997; Nagasaki et al., 1998). Although their functions in vivo are not yet fully understood, earlier phenotypic analyses clearly indicated that the process we have termed cytokinesis B was impaired in knockout mutants lacking either *amiA* or *corA* and that these genes can be used to dissect the mechanism of cytokinesis in *Dictyostelium*. In addition, we engineered and expressed GFP-H1, which allowed easy visual identification of mitotic cells among a population of cells using fluorescence microscopy (Fig. 5). Armed with these tools, we set out to examine cytokinesis defects caused by disruption of *mhcA*, *amiA* and *corA*, alone or in combination.

Cytokinesis in double mutants lacking myosin II and either AmiA or coronin was severely disrupted in cells grown on substrates (Fig. 3). In contrast, the phenotype of cells lacking both AmiA and coronin was no worse than those of cells lacking one or the other (Fig. 3J). The severe defects observed in *mhcA*$^{-}$/*amiA*$^{-}$ and *mhcA*$^{-}$/*corA*$^{-}$ cells were thus synergistic rather than additive, which strongly suggests the presence of two independent pathways leading to mitotic cytokinesis in *Dictyostelium*. Furthermore, the fact that *mhcA*$^{-}$/*amiA*$^{-}$ and *mhcA*$^{-}$/*corA*$^{-}$ cells only infrequently undergo cell-cycle-coupled division under the present experimental conditions (23% in the case of *mhcA*$^{-}$/*amiA*$^{-}$) indicates that the inhibition of cytokinesis B by either the *amiA*$^{-}$ or *corA*$^{-}$ mutation is fairly strong, just as the *mhcA*$^{-}$ mutation strongly inhibits cytokinesis A. That *amiA*$^{-}$ or *corA*$^{-}$ cells divide as efficiently as wild-type cells in suspension indicates that these proteins play only unessential roles, if any, in cytokinesis A.

The very low rate of cell-cycle-coupled cytokinesis in cells defective in both cytokinesis A and B further suggests that these two are the only two major pathways of cell-cycle-coupled division in *Dictyostelium*. When these cultures become dense, however, the success rates of cell-cycle-coupled cytokinesis increase, and consequently, the fraction of multinucleate cells decreases. Time-lapse observation showed that when these double mutants in dense cultures go into mitotic phase, a neighbor cell often crawls towards the equatorial region of the mitotic cell and apparently helps the mitotic cell to divide by walking over the equatorial region (A.N. and T.Q.P.U., unpublished). A similar phenomenon was recently reported for another amoeba, *Entamoeba invadens* (Biron et al., 2001). The authors suggested that the *Entamoeba* cells chemotax towards equatorial region of mitotic cells. We speculate that perhaps a similar, third, cell-cycle-coupled process of cytokinesis exists in *Dictyostelium*, and we are currently investigating this point.

Because cleavage furrows are formed in the equatorial regions during telophase in both cytokinesis A and B, the positioning and timing signals of both mechanisms must derive from the mitotic apparatus. This suggests the possibility that cytokinesis A and B may share the same upstream regulatory mechanism. The final stage of cytokinesis in both cases involves scission of the cytoplasmic strand connecting the two daughter cells, another process that may be shared by the two cytokinesis mechanisms. Most probably, the two mechanisms diverge from one another in the middle stages of cytokinesis, during furrow formation in particular (Fig. 6C). The present genetic evidence does not address how extensive is the divergence between the two pathways, however. In one extreme case, AmiA and coronin, factors essential for cytokinesis B, might complement

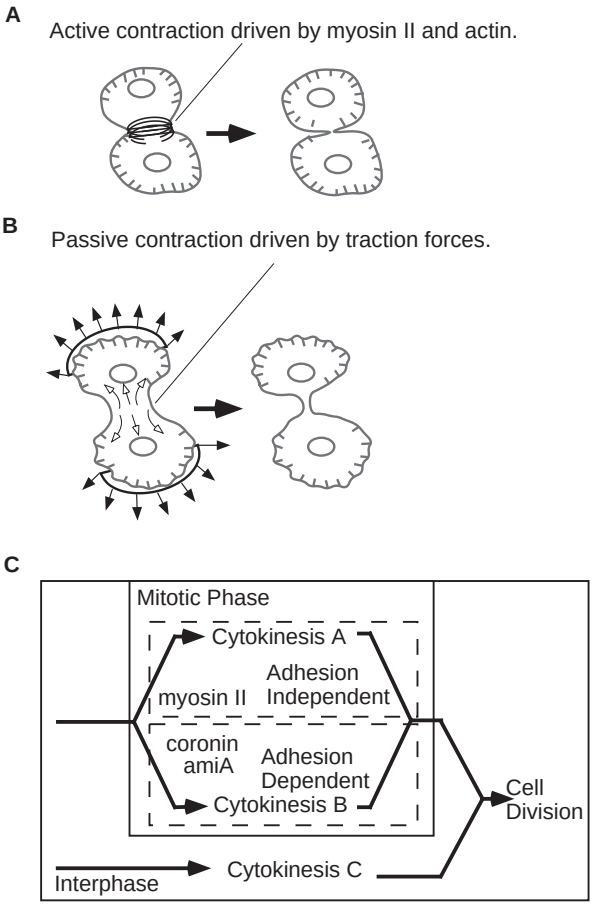

Fig. 6. A schematic diagram depicting two pathways leading to cell-cycle-coupled cell division in *Dictyostelium* cells. (A) Cytokinesis A. Mitotic AmiA^{-} or coronin^{-} cell carries out cytokinesis by active contraction of the cleavage furrow which depends on actin and myosin II. (B) Cytokinesis B. A mitotic myosin II-null cell divides by passive contraction of the cleavage furrow. In this case, cytoplasm in equatorial region is withdrawn indirectly (white arrows inside the cell) by traction forces generated along polar peripheries (black arrows). (C) Summary of three methods of cytokinesis in *Dictyostelium*. Cytokinesis A requires myosin II expression, but adhesion to a substrate is not necessary. Cytokinesis B is not dependent on myosin II but adhesion is indispensable. These two mechanisms of cell division occur immediately following nuclear division and are somehow coordinated in wild-type cells. The third pathway, cytokinesis C, is cell cycle independent and occurs during interphase.

the loss of myosin II by providing contractile force in the equatorial region. Robinson and Spudich (Robinson and Spudich, 2000) and Gerisch and Weber (Gerisch and Weber, 2000) proposed that the equatorial region of mitotic *mhcA*$^{-}$ cells may contract actively without myosin II. According to these hypotheses, cytokinesis A and B are mechanistically similar, differing only in terms of the force-generating element that constricts the furrow. Alternatively, cytokinesis A and B might achieve furrow formation via physically distinct mechanisms.

To further investigate the difference between cytokinesis A and B, we carried out detailed morphological analyses of each mutant cell line during cytokinesis. Mitotic *mhcA*$^{-}$ cells were flat and adhered to the surface of the culture dish during the

furrowing and separation processes. After separation, the two daughter cells were elongated laterally rather than longitudinally. These observations led us to speculate that division of these cells (cytokinesis B) is achieved by passive contraction of the equatorial region driven by radial traction forces produced along the polar peripheries against the surface (Uyeda et al., 2000). A similar model has been proposed by Neujahr et al. (Neujahr et al., 1997b) on the basis of their observation of morphological changes in large *mhcA*$^-$ cells during cytokinesis with respect to the behavior of mitotic apparatus. In contrast, division of *amiA*$^-$ or *corA*$^-$ cells, which are unable to carry out cytokinesis B, is primarily dependent on active constriction of the contractile ring powered by myosin II (Fig. 6A; cytokinesis A). When *amiA*$^-$ or *corA*$^-$ cells cultured on substrates divide successfully, they usually detached from the surface and rounded up before forming cleavage furrows (Fig. 4C,D); the two daughter cells remained detached and more or less spherical until they had completely separated. These differences in the sequence of morphological changes during cytokinesis A and B favor the possibility that the two processes are mechanistically rather different.

This view is further substantiated by three other pieces of information. First, AmiA and coronin are not motor proteins and are not known to activate other motor proteins. Second, the intracellular localization of GFP-AmiA (A.N. and K. Sutoh, unpublished) and native coronin (de Hostos et al., 1991) is not consistent with their primary function in the furrow region, although a more recent analysis of cells overexpressing GFP-coronin demonstrated its presence in the region of the equatorial furrow as well as in the polar regions (Fukui et al., 1999). Third, if AmiA and coronin support active constriction of the contractile ring, *mhcA*$^-$ cells would be expected to show some equatorial furrowing even in the absence of supporting substrates. However, analysis of *mhcA*$^-$ cells on non-adherent, hydrophobic surfaces clearly demonstrated that these cells elongate axially in telophase but show no sign of furrowing (Zang et al., 1997). We therefore propose that *Dictyostelium* cells possess two parallel and mechanistically distinct pathways leading to cell-cycle-coupled division. One mechanism involves active, myosin-II-dependent constriction of the contractile ring, while the other depends on radial traction forces generated along the polar peripheries to indirectly cause passive constriction of the equatorial region. This leads to re-definition of cytokinesis A and B from a general and mechanistic viewpoint: cytokinesis A as the cell-cycle-coupled division method that is driven by active equatorial constriction, and cytokinesis B as the cell-cycle-coupled division method that is driven by opposing traction forces generated along the polar peripheries. More work is needed to further examine this proposal.

Wild-type cells cultured on solid substrates have the potential to carry out both cytokinesis A and B, which raises a question about how they coordinate these apparently distinct processes. Neujahr et al. (Neujahr et al., 1997b) reported that the majority of the wild-type NC4 cells cultured on solid surfaces rounded up and detached from the substrate during most phases of mitosis and cytokinesis. This sequence of shape changes is very similar to that of mitotic *amiA*$^-$ and *corA*$^-$ cells undergoing cytokinesis A. For a minority of cells, however, division on substrates was indistinguishable from that of *mhcA*$^-$ cells, suggesting that each mitotic NC4 cell undergoes cell division either by cytokinesis A or B. Fig. 4A shows a mitotic cell from the AX2 cell line, which was established as an axenic strain from NC4 cells subjected to mutagenesis (Watts and Ashworth, 1970) but which are nonetheless widely used as a 'wild-type' cell line. It appears that the morphological changes in mitotic AX2 cells grown on substrates contain features of both cytokinesis A and B and that these cells probably use both mechanisms. Interestingly, Neuhjahr et al. (Neuhjahr et al., 1997a) discovered that levels of myosin II in the cleavage furrows of AX2 cells flattened by being sandwiched between a glass surface and a sheet of agarose are higher than in the furrows of cells cultured otherwise, suggesting that these cells are likely to employ more of the cytokinesis A pathway under physically demanding conditions. In contrast, the fact that practically all wild-type cells on substrates are mononucleate whereas ~40% of those grown in suspension are multinucleate suggests that cytokinesis B contributes significantly to efficient cytokinesis of wild-type cells on substrates. This is most likely why *amiA*$^-$ and *corA*$^-$ cells on substrates show moderate cytokinetic defects. However, it is not clear why the fraction of multinucleate *amiA*$^-$ or *corA*$^-$ cells is consistently somewhat larger on substrates than in suspension.

Cells that cannot carry out cytokinesis A or B, that is, *amiA*$^-$/*mhcA*$^-$ and *corA*$^-$/*mhcA*$^-$ cells, are still viable on solid surfaces. How then do they multiply? For the first few days, mononucleate *amiA*$^-$/*mhcA*$^-$ cells inoculated onto plastic dishes grew in size with a very low rate of cytokinesis. The resultant large, multinucleate cells then occasionally divided through apparently two different routes, depending on the density of cells. In dense cultures, they often divided in a cell-cycle-coupled manner with the help of neighboring cells in a manner similar to *Entamoeba invadens* (Biron et al., 2001). In contrast, when the cell densities were low, division of large, multinucleate *amiA*$^-$/*mhcA*$^-$ cells was not coupled with cell cycle progression (Fig. 5F). If removed from the support of a solid surface, *mhcA*$^-$ cells became extremely large and highly multinucleate. When returned to a solid surface, many of these multinucleate *mhcA*$^-$ cells immediately begin to divide by forming several leading edges. Originally called 'traction-mediated cytofission' (Spudich, 1989), we renamed this process cytokinesis C by redefining it as an attachment-dependent, cell-cycle-independent division process (Uyeda et al., 2000). The division sequences of multinucleate *amiA*$^-$/*mhcA*$^-$ or *corA*$^-$/*mhcA*$^-$ cells are very similar to those of giant *mhcA*$^-$ cells prepared by culture in suspension (Fig. 1C, Fig. 4E, Fig. 5F), which suggests to us that, on substrates, these cells employ a common mechanism of division, namely cytokinesis C. Cytokinesis C appears mechanistically similar to cytokinesis B. However, cytokinesis B is distinct from cytokinesis C in that it requires a number of gene products that are dispensable for cytokinesis C (reviewed by Uyeda et al., 2000). Furthermore, cytoplasmic bridges connecting separating cell fragments during cytokinesis C are often extremely long (Fig. 1C, Fig. 4E), whereas those connecting daughter cells during cytokinesis B are severed before the two cells move away from each other, suggesting that cytokinesis B has an additional mechanism to regulate the cortical stiffness of the cytoplasmic bridges.

We do not believe cytokinesis B is specific to *Dictyostelium*; it is probably conserved in higher animal cells as well.

O'Connell et al. (O'Connell et al., 1999) reported that microinjection of C3 toxin, which inhibits endogenous rho in adherent, mitotic cells (normal rat kidney cells and 3T3 fibroblasts), did not inhibit equatorial furrow formation and even induced additional ectopic furrows, often resulting in multiple anucleate cell fragments. The equatorial and ectopic furrows formed in C3-injected cells were wider than normal cleavage furrows and did not contain higher levels of actin and myosin II filaments, properties reminiscent of cleavage furrows in *mhcA*$^{-}$ *Dictyostelium* cells undergoing cytokinesis B. In contrast, furrows were not formed when C3 was injected into interphase cells; similarly microinjection of C3 into poorly adherent HeLa cells failed to induce ectopic furrows and, rather, inhibited cytokinesis. Thus when rho is inactivated, mitotic cells in culture are able to carry out adhesion-dependent, cell-cycle-coupled cytokinesis without concentrating myosin II to the furrow regions. More recently, O'Connell et al. (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 instead of inhibiting it, although its application to the polar region inhibited the furrowing. This observation again supports the idea that contractile activities in the equatorial region are not essential for the equatorial furrowing and cytokinesis of these adherent cells in culture. In addition, Zurek et al. (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 to divide in the absence of myosin II, albeit more slowly than in its presence, just as *mhcA*$^{-}$ *Dictyostelium* cells take two-fold longer 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 (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 embryogenesis of *Drosophila melanogastor* (Young et al., 1993) and *Caenorhabditis elegans* (Guo and Kemphues, 1996). Cells in developing animal tissues are surrounded by other cells and the extracellular matrix and in that sense are adherent to substrates, a condition which allows cytokinesis B in *Dictyostelium*. One 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. Future investigation aimed at clarifying the roles of myosin II during cytokinesis in higher animal cells should address these and other questions.

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