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On the Role of Myosin-II in Cytokinesis: Division of *Dictyostelium* Cells under Adhesive and Nonadhesive Conditions

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We have investigated the role of myosin in cytokinesis in *Dictyostelium* cells by examining cells under both adhesive and nonadhesive conditions. On an adhesive surface, both wild-type and myosin-null cells undergo the normal processes of mitotic rounding, cell elongation, polar ruffling, furrow ingression, and separation of daughter cells. When cells are denied adhesion through culturing in suspension or on a hydrophobic surface, wild-type cells undergo these same processes. However, cells lacking myosin round up and polar ruffle, but fail to elongate, furrow, or divide. These differences show that cell division can be driven by two mechanisms that we term Cytokinesis A, which requires myosin, and Cytokinesis B, which is cell adhesion dependent. We have used these approaches to examine cells expressing a myosin whose two light chain-binding sites were deleted (ΔBLCBS-myosin). Although this myosin is a slower motor than wild-type myosin and has constitutively high activity due to the abolition of regulation by light-chain phosphorylation, cells expressing ΔBLCBS-myosin were previously shown to divide in suspension (Uyeda *et al.*, 1996). However, we suspected their behavior during cytokinesis to be different from wild-type cells given the large alteration in their myosin. Surprisingly, ΔBLCBS-myosin undergoes relatively normal spatial and temporal changes in localization during mitosis. Furthermore, the rate of furrow progression in cells expressing a ΔBLCBS-myosin is similar to that in wild-type cells.

INTRODUCTION

Conventional myosin II (referred to as myosin throughout this text) is thought to be the motor responsible for the constriction of the cleavage furrow during cytokinesis. It has been shown to be localized in the furrow both by immunofluorescence (Yumura and Fukui, 1985) and by using fluorescently labeled myosins (Sanger *et al.*, 1989; Debiasio *et al.*, 1996; Moores *et al.*, 1996). More importantly, disruption of myosin function disrupts cell division. Antimyosin antibodies injected into starfish blastomeres inhibit furrow formation (Mabuchi and Okuno, 1977). Genetic proof that myosin is required for cytokinesis in suspension first came from studies with *Dictyostelium*. Cells not expressing functional myosin fail to divide in suspension (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein *et al.*, 1989), and reintroduction of myosin gene on a plasmid into the myosin-null cells rescues the cytokinesis defect (Egelhoff *et al.*, 1990). Such rescued cells express normal amounts of myosin and behave identically to wild-type cells (Egelhoff *et al.*, 1990).

Very little is known about the molecular basis for the establishment of the cleavage furrow or about the details of the cell division events. It is hypothesized that actin filaments are anchored to the membrane, and myosin bipolar thick filaments motor the actin filaments to generate the force and the movement needed to constrict the furrow (Schroeder, 1973; Maupin and Pollard, 1986). By ultrastructural studies, actin filament bundles are found beneath the cortex of the furrow region, with their barbed ends anchored to

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the membrane (Schroeder, 1973; Maupin and Pollard, 1986). Drugs that act to destabilize or stabilize actin filaments, such as cytochalasin or phalloidin, disrupt furrowing (Hamaguchi and Mabuchi, 1982; Inoue, 1990). Actin-modulating proteins are also found to play a role in cytokinesis. Deletion of coronin decreases the efficiency of division on a surface in *Dictyostelium* (de Hostos *et al.*, 1993). Other proteins, such as profilin and α-actinin, may play an important role as well (for a review, see Fishkind and Wang, 1995).

To understand this complex molecular process, we are using the cellular slime mold *Dictyostelium discoideum* as a model system. The efficiency of homologous recombination in this organism is very high, making molecular genetic manipulations possible. Thus, the single-copy myosin heavy chain gene was deleted, and the phenotypes of the resulting myosin-null cells were characterized (Manstein *et al.*, 1989; Spudich, 1989). Furthermore, mutated forms of the myosin gene can be introduced into these myosin-null cells and expressed. These mutant myosins can then be tested for their ability to restore wild-type function in vivo. In addition, they can be purified and assayed biochemically for their ATPase activity and their ability to move actin filaments in vitro (Kron and Spudich, 1986). Importantly, attachment of green fluorescent protein (GFP) to the N terminus of myosin allows the myosin dynamics in living cells to be followed in real time (Moores *et al.*, 1996; Sabry *et al.*, 1997). This GFP-myosin was shown to function like wild-type myosin by both in vitro and in vivo assays (Moores *et al.*, 1996).

A detailed characterization of the role of myosin in cytokinesis has been hampered by the complication that cells on an adhesive surface undergo a process we have called "traction-mediated cytofission" (Spudich, 1989), which is not necessarily coupled to mitosis but can occur in interphase cells as well. To characterize cytokinesis, cells must be able to be examined in the absence of such adhesion forces. Furthermore, in the case of the myosin-null cells, which fail to divide in suspension, it is important to be able to score for cells in mitosis. Here we describe approaches that have allowed us to characterize both wild-type and myosin-null cells as they proceed through mitosis, both in the absence and in the presence of an adhesive surface.

Furthermore, we illustrate the use of these approaches to characterize cells carrying a mutant form of myosin, one which lacks the light chain-binding domain of the myosin molecule. Detailed examination of this mutant was of special interest because phosphorylation of the myosin regulatory light chain (RLC) has been implicated in the control of timing for cytokinesis. Satterwhite *et al.* (1992) proposed that the signal for the onset of cytokinesis is the phosphorylation regulation of myosin light chain by cyclin-p34^{cdc2}. In their model, active cyclin–p34^{cdc2} complexes phosphorylate myosin light chains at serine-1, serine-2, or threonine-9 during prophase and metaphase. The phosphorylation of these sites inhibits myosin activity. At the metaphase–anaphase transition; however, the activity of cyclin-p34^{cdc2} drops drastically. Therefore, myosin is released from inhibition and acts to drive cytokinesis. In vivo phosphorylation data (Yamakita *et al.*, 1994) support this hypothesis. RLC from mitotic cells is phosphorylated at the serine-1 and serine-2 and, much less extensively, at serine-19, a site that is known to activate myosin. At the start of cytokinesis, phosphorylation is increased 20 times at serine-19, whereas phosphorylation at serine-1 and -2 is decreased by half. In another study, using biosensors to detect the phosphorylation state of the myosin light chain, Debiasio *et al.* (1996) reported global phosphorylation of myosin at the onset of anaphase.

These studies in mammalian cells indicate that changes in RLC phosphorylation accompany mitosis, but do not address whether it actually controls the timing of cytokinesis. The molecular genetic tools available in *Dictyostelium* make it ideal for addressing these questions. RLC phosphorylation is carried out by multiple myosin light chain kinases (MLCK) in this organism. Cells in which the gene for MLCK-A is disrupted are able to divide in suspension, yet cultures show an increased number of multinucleate cells, suggesting that MLCK-A contributes to, but is not essential for, cytokinesis (Smith *et al.*, 1996). However, in a related study, Ostrow *et al.* (1994) found that cells expressing a mutant RLC whose activating phosphorylation site has been changed to an alanine are able to divide in suspension. This finding suggests that the lower activity of unphosphorylated myosin is sufficient for cytokinesis. Constitutively active myosin has been engineered by internally truncating the heavy chain to remove the RLC-binding site (ΔRLCBS-myosin) (Uyeda and Spudich, 1993) or both the RLC and the essential light chain-binding sites (ΔBLCBS-myosin) (Uyeda *et al.*, 1996). Cells in which wild-type myosin has been replaced by one of these constitutively active mutants are able to grow in suspension. These findings complicate the issue of the role of light-chain phosphorylation in cytokinesis. To determine whether the light chain-binding region of the myosin is essential for normal spatial and temporal control of assembly of the contractile ring and for normal constriction of the cell, we used the approaches described here to investigate the behavior of cells expressing a ΔBLCBS myosin during cytokinesis.

In this paper, we examine in detail the division of *Dictyostelium* cells expressing wild-type myosin, no myosin, or the fusion protein GFP-ΔBLCBS-myosin under adhesive and nonadhesive conditions to understand myosin's role in cytokinesis. Under nonadhesive conditions, cells expressing wild-type myosin and

GFP-ΔBLCBS-myosin are able to round up, stretch out, furrow, polar ruffle, and divide successfully, whereas myosin-null cells retain their ability to round up and polar ruffle, but fail to form a furrow and divide. All three types of cells are able to divide successfully with similar changes in morphology on an adhesive glass surface. However, analysis reveals that myosin-null cells, unlike wild-type cells and GFP-ΔBLCBS-myosin cells, do not have a constant rate of cleavage furrow constriction. In light of these results, we discuss myosin's role in cytokinesis and propose that cytokinesis can occur by two mechanisms: Cytokinesis A and Cytokinesis B.

MATERIALS AND METHODS

Plasmid Construction

A GFP-ΔBLCBS-myosin (deletion of Both Light Chain Binding Sites) expression plasmid was constructed as follows. pBIG-GFPmyo (Moores *et al.*, 1996) was digested with *Xba*I and *Bst*XI. The resulting 2.7-kilobase (kb) fragment containing the actin 15 promoter, GFP, and a part of myosin motor domain was then ligated to the 8-kb fragment of pMyD-ΔBLCBS-myosin (Uyeda *et al.*, 1996), similarly digested with *Xba*I and *Bst*XI. A 0.8-kb *Sac*I–*Sac*I fragment containing the blasticidin resistance gene from pBsr2 (Sutoh, 1993) was then dropped into the resulting plasmid, also digested with *Sac*I. A clone with the correct orientation was selected, resulting in pGFP-ΔBLCBS-Bsr.

A nuclear localization signal (NLS)-GFP expression plasmid p66 was constructed as follows. The plasmid pRSET (a kind gift of Dr. Roger Tsien, University of California, San Diego) containing the cDNA for S65T GFP was used as a template for polymerase chain reaction. Primers were designed to fuse the minimal NLS from SV40 T antigen (Kalderon *et al.*, 1984) PKKKRKV to the N terminus of GFP, while adding a *Kpn*I site to the 5′ end and a *Xba*I site to the 3′ end. The sequence for the 5′ end primer was 5′-GAGGGTACCCC-AAAAAAGAAACGTAAAGTTTCAAAAGGTGAAGAACTTTTC-ACTGG-3′, and the sequence of the 3′ end primer was 5′-CACTC-TAGAAGCTTATTTGTATAGTTCATCCATGC-3′. The resulting polymerase chain reaction product was digested with *Kpn*I and *Xba*I and inserted into an expression vector pDXA-3C containing G418 resistance (Manstein *et al.*, 1995), also digested with *Kpn*I and *Xba*I.

Manipulation of Dictyostelium *Cells*

HS1, a myosin null cell line (Ruppel *et al.*, 1994), was transformed with pGFP-ΔBLCBS-Bsr. Individual clones were grown at 21°C in HL5 media (Sussman, 1987), supplemented with Pen-Strep (60 U/ml of penicillin; 60 μg/ml of streptomycin), and 5 μg/ml of blasticidin (ICN Pharmaceuticals, Costa Mesa, CA). HS1 transformed with p66 were grown at 21°C in HL5 with Pen-Strep and 10 μg/ml of G418 (Geneticin; Life Technologies, Gaithersburg, MD). JH10 cells, the parent strain of the HS1 cells, were cultivated at 21°C in HL5 media supplemented with Pen-Strep and 20 μg/ml of thymidine. Cells expressing GFP-myosin or GFP alone were produced and maintained as previously described (Moores *et al.*, 1996). HS2206, another myosin-null cell line (Manstein *et al.*, 1989) was grown in HL5 media, supplemented with Pen-Strep and 10 μg/ml of G418.

Protein Purification

Proteins were isolated as described for wild-type myosin (Ruppel *et al.*, 1994) and further purified using an agarose gel filtration column (Bio-Gel A-15m, 100–200 mesh, Bio-Rad, Richmond, CA). The concentration of protein was determined using the Bradford assay (Bradford, 1976), with rabbit skeletal myosin as the standard.

Electrophoresis and Immunoblots

Equal amounts of *Dictyostelium* whole cell lysates were loaded onto two SDS/7.5% polyacrylamide gels. These gels were then either stained with Coomassie brilliant blue or transferred onto nitrocellulose paper. Quantitation of the amount of myosin in cells was done by densitometry on the Coomassie-stained gel. The density in the region of the myosin band was integrated and ratioed over the integration of two other major bands in the lanes to correct for loading. The blots were probed with My6, a monoclonal anti-*Dictyostelium* myosin antibody, or an anti-GFP antibody, followed by appropriate secondary antibodies conjugated to horseradish peroxidase (Bio-Rad). An enhanced chemiluminescence system (Amersham, Arlington Heights, IL) was used to visualize the signals.

To visualize the light chains and the heavy chain, purified myosins were loaded onto an SDS/15% polyacrylamide gel and stained with Coomassie.

Imaging Cells in Suspension

Cells were imaged in suspension as described by Egelhoff *et al.* (1991), with the following modifications on the assembly of the hanging drop chamber. A drop of media (8 μl or 14 μl) containing about 10^4 cells was hung from a clean square 22-mm no. 1 glass coverslip (Corning, Corning, NY). This coverslip was then placed onto a greased O-ring such that the drop was in the central empty space of the O-ring. A 12-mm circular no. 1 glass coverslip (Corning), was then placed on the other side of the O-ring, completing the chamber. To prevent fogging, this coverslip was treated with Photoflo (Eastman Kodak, Rochester, NY) and then rinsed with water. In other preparations, we washed the bottom coverslip with methylene chloride (VWR, South Plainfield, NJ), followed by 1 h of soaking in 0.1% (vol/vol) octadecyltrichlorosilane (Sigma Chemical, St. Louis, MO) dissolved in toluene (Baker, Phillipsburg, NJ), and finally rinsed with fresh toluene and ethanol.

Cells were observed for less than 3 h in the hanging drop. Beyond 3 h, they are able to adhere to the air–media interphase of the droplet.

Preparation of a Hydrophobic Monolayer Substrate

Glass coverslips, 25 × 25 mm^2 (no. 2, Corning), were cleaned with "piranha" solution at 80°C for 30 min. Piranha solution is a mixture of concentrated sulfuric acid and 30% hydrogen peroxide in a 3:1 volume ratio. ***WARNING:*** *Piranha solution should be handled with extreme care; in some circumstances, especially when it comes in contact with significant quantities of an oxidizable organic material, it can detonate spontaneously.* This treatment was followed by extensive rinsing with nanopure (18 MΩ cm resistance) water and finally blown dry with a stream of argon passed through a 0.2-μm filter. Cleaned coverslips then were placed in a solution of 15 μl of octadecyltriethoxysilane (United Chemical Technologies, Bristol, PA) in a mixture of 20 ml wet isooctane (Aldrich, Milwaukee, WI), 5 ml carbon tetrachloride (Aldrich), and 125 μl acetic acid (Baker). This reaction was done in 30-ml Teflon vials, which were baked at 120°C for at least 2 h before use. It was allowed to proceed overnight at 22°C. The next day, coverslips were taken out of solution, rinsed with methylene chloride (Baker), subjected to sonication in methylene chloride for 10 min, rinsed with isopropanol and water, and finally blown dry with a stream of argon. The thickness of the resulting monolayer was determined as 2.4 ± 0.2 nm by ellipsometrical measurement on oxidized Si(111) wafers.

4′,6-Diamidino-2-Phenylindole (DAPI) Staining

Cells expressing NLS-GFP were fixed and stained with DAPI, as described previously (De Lozanne and Spudich, 1987).

Imaging Cells on a Surface

Cells were placed in a chambered coverslip (Nunc, Naperville, IL) filled with MES buffer (20 mM 2-[morpholino]ethane-sulfonic acid, pH 6.8/0.2 mM $CaCl_2$/2 mM $MgSO_4$) at 22°C. Imaging was done as described (Sabry *et al*., 1997). Images of cells on the nonadhesive hydrophobic surface were taken within 3 h after the cells had settled to the surface.

Data Analysis

Images of cell division on a surface were analyzed using Image-1 Metamorph (Universal Imaging Corp, West Chester, PA). The cleavage furrow widths, cell lengths, and internuclear distances were measured by drawing subjective lines on the images by hand. The measurement of the line length, obtained by the line length command, was logged into Microsoft Excel files. Images of cell division in suspension were digitized and analyzed using the Optimas software (Optimas, Bothell, WA). Least square regression analysis was carried out using Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

Myosin Null Cells, Unlike Wild-Type Cells, Cannot Form a Cleavage Furrow in the Absence of Adhesion

Cells expressing wild-type myosin are able to divide in shaking culture, while cells lacking functional myosin fail to do so (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein *et al*., 1989). To determine whether myosin-null cells fail to initiate a furrow or simply fail in the completion of furrowing, we imaged cells suspended in the bottom of a droplet hanging from a coverslip. This procedure eliminated complications due to surface adhesion forces.

An example of a cell expressing wild-type myosin from its endogenous copy of the myosin gene (referred to as wild-type cells throughout this text) undergoing cytokinesis in suspension is shown in Figure 1A. During interphase, the cell was very active, constantly sending out pseudopods and showing rapid movements of vesicular elements. However, at the onset of mitosis, it rounded up and the cell surface became very smooth. We define time 0 as the start of this quiescence. After 140 s, the cell elongated into a cylindrical shape and started to form a cleavage furrow. The cleavage furrow was very apparent after 210 s, along with ruffling of the polar edges. After 350 s, the daughter cells pinched off and separated, completing cytokinesis. Change of cell activity from dynamic to quiescent is a good indicator of mitosis, as all cells undergoing this change proceeded to divide during our observations.

Like wild-type cells, myosin-null cells were very active during interphase and became rounded and quiescent during mitosis (for example, see the cell shown in Figure 1B). As with the wild-type cells, time 0 was defined as the onset of quiescence. After 230 s, ruffling started to occur at the poles of the cell, similar in timing to the wild-type cells. However, all 10 myosin- null cells examined remained relatively rounded, and cleavage furrows failed to form. The ruffling continued, yet the cell did not show any sign of cleavage. After 570 s, the cell took on the appearance of an interphase cell, with the reestablishment of vesicular movements and pseudopod dynamics. The frequency of these mitotic events was quite similar to that observed in the wild-type cells. We observed 10 such events in 170 cell-hours for myosin-null cells, whereas nine wild-type cells divided in 350 cell-hours. No cleavage furrow formation was observed in myosin-null cells in fresh suspension droplets. However, *Dictyostelium* cells should not be allowed to remain in the hanging drop for more than 3 h. Over time, they adhere to the media–air interphase of the droplet, possibly because the cells either secrete substances into the medium or because some cells lyse, leaving a film to which cells can adhere. These adherent null cells are flatter in shape than cells freshly placed in a hanging drop, and they are able to divide under these conditions.

To be certain that myosin-null cells undergoing quiescence and polar ruffling were truly in mitosis, we developed a method for unambiguously following nuclear division in living cells. We engineered and expressed in myosin-null cells a fusion protein, NLS-GFP. The seven amino acids from SV40 T antigen, PKKKRKV, shown to be sufficient for nuclear localization (Kalderon *et al*., 1984), were fused to the N terminus of S65T GFP, which is a brighter variant of GFP (Heim *et al*., 1995). This NLS-GFP localized to the nuclei of *Dictyostelium* cells during interphase (Figure 2A and B). Fixed cells stained with DAPI, which binds DNA, confirmed the location of the nuclei (Figure 2C). Importantly, we observed that nuclear localization of the NLS-GFP is lost during early stages of mitosis. Figure 2D–F shows a cell during early anaphase, as revealed by DAPI staining (Figure 2F). At this stage, NLS-GFP was diffuse throughout the cell (Figure 2E), which enabled us to identify cells that were about to undergo mitosis-coupled division. As the cells entered telophase (Figure 2, G–I), NLS-GFP relocalized into the nucleus and remained nuclear throughout cytokinesis (Figure 2, J–L). The expression of NLS-GFP does not hamper cell division, since wild-type cells expressing this protein can grow in suspension.

Armed with this tool, we proceeded to examine the behavior of myosin-null cells during and after mitosis in the absence of adhesion. We did not use the hanging drop method due to several limitations. First, since there was a gap between the droplet and the bottom coverslip, a long working distance objective was required. This limited us to lenses with lower numerical aperture and thus lower resolution. Furthermore, this technique was quite labor intensive. Refocusing must be done about every 15 s, since it is very difficult to keep the cells in the same plane of focus. Therefore, in order to easily visualize cells in the absence of adhe-

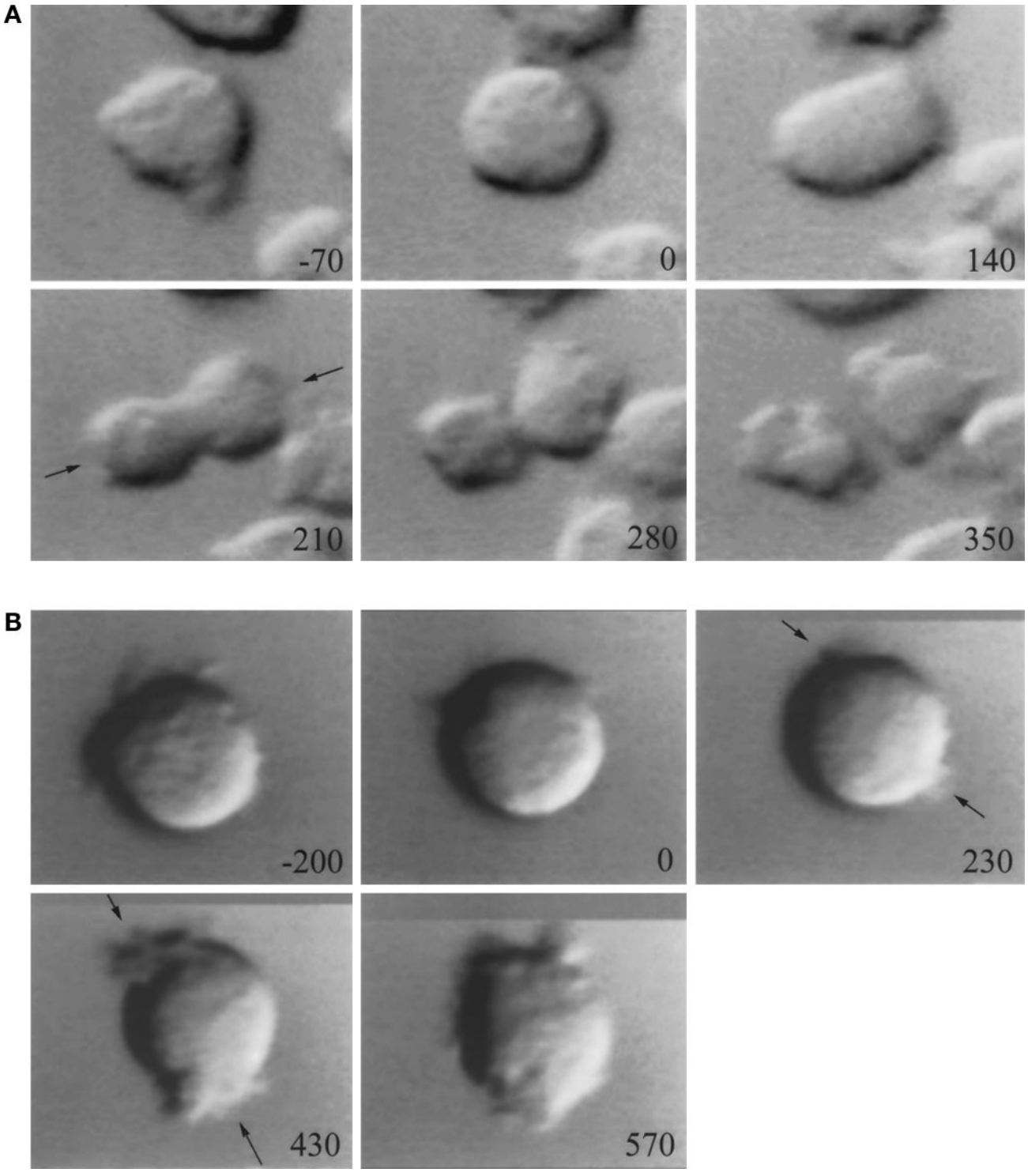

Figure 1. *Dicytostelium* myosin-null cells fail to form furrows during cytokinesis in suspension as shown by sequential differential interference contrast images. In these images, time 0 indicates the start of rounding and quiescence. (A) Cells expressing wild-type myosin can round up, elongate, furrow, and separate. Times of the panels are −70, 0, 140, 210, 280, and 350 s. Ruffling of the polar regions, as indicated by black arrows, coincided with the furrowing (210 s). (B) Myosin-null cells cannot divide in suspension. Times of the panels are −200, 0, 230, 430, and 570 s. They can round up, but they fail to elongate and furrow. However, like wild-type cells, they are able to polar ruffle, as indicated by arrows, at about the same time as wild-type cells (230 s).

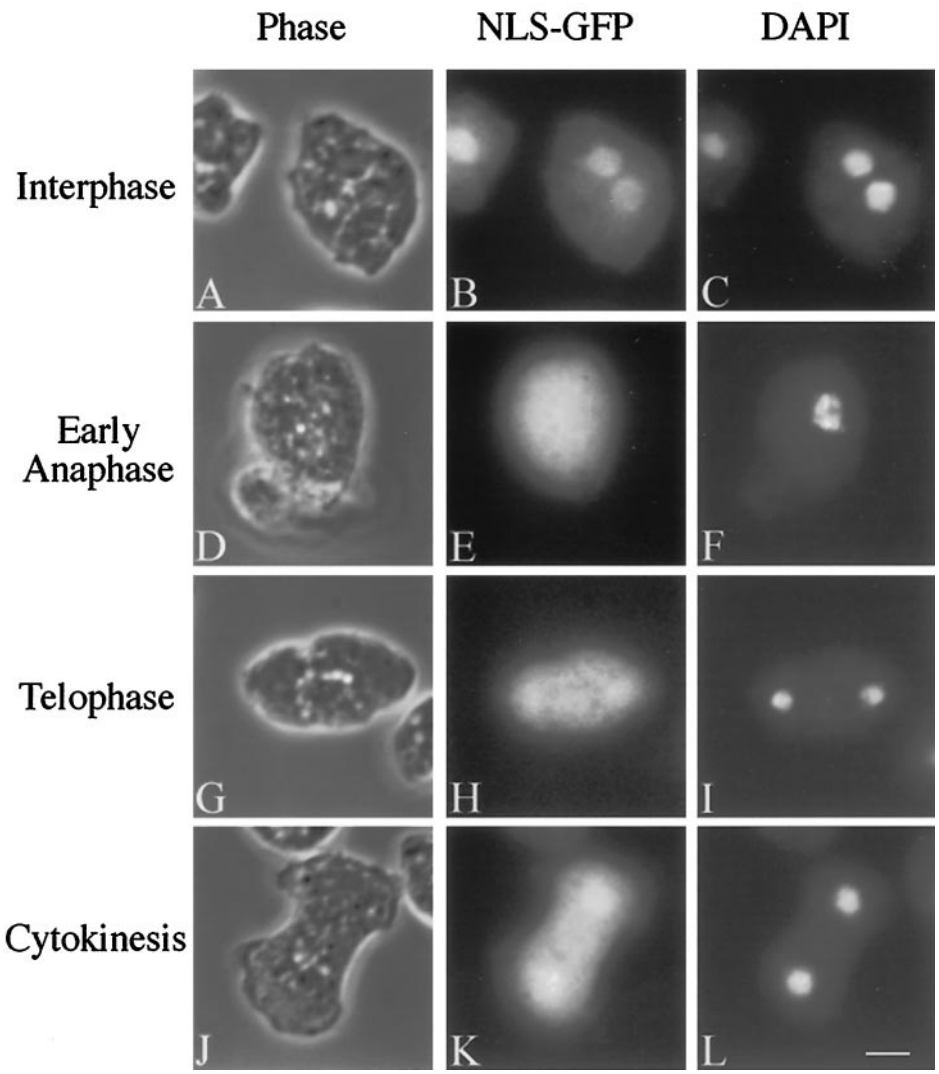

Figure 2. NLS-GFP is localized to the nucleus, except during early mitosis. Myosin-null cells expressing NLS-GFP were fixed and stained with DAPI. The left column shows phase-contrast images, the center column shows NLS-GFP localization, and the right column shows DAPI staining. (A–C) Interphase cells. (D–F) An early anaphase cell. (G–I) A telophase cell. (J–L) A cell undergoing cytokinesis. Bar, 5 μm.

sion, we developed a hydrophobic surface that *Dictyostelium* cells fail to adhere to.

A well-ordered, 2.4 nm thin monolayer with methyl termination was chemically linked to a clean glass coverslip. Myosin-null cells behaved similarly on this nonadhesive, hydrophobic surface as they did in suspension (Figure 3A). At the onset of mitosis, myosin-null cells expressing NLS-GFP rounded up and became quiescent (Figure 3A, 0 s). At this stage, NLS-GFP is not localized in the nucleus. Later in mitosis, NLS-GFP relocalized into the two daughter nuclei, which were at the polar ends of the cell. The polar membrane close to the nuclei ruffled, although the overall cell shape remained relatively round (Figure 3A, 190 s and 485 s). The ruffling continued for about 10 min. Then polar ruffling ended as one of the nuclei left its polar position (Figure 3A, 745 s and 1010 s). All seven myosin-null cells examined behaved this way, although the duration of polar ruffling varied from cell to cell.

In contrast, cells expressing a wild-type myosin fused to GFP (GFP-myosin) were able to divide on the nonadhesive surface (Figure 3B). As in suspension, these cells on the nonadhesive surface rounded up, elongated, furrowed, polar ruffled, and then separated. During mitosis, *Dictyostelium* cells do not break down their nuclear envelopes (Moens, 1976). The nuclei appeared as circles of reduced fluorescence because GFP-myosin is excluded from the nuclei, thus confirming our observations of mitotic divisions.

Myosin-Null Cells Can Divide on an Adhesive Surface

Myosin-null cells grown in suspension become large and multinucleated, as a result of their inability to

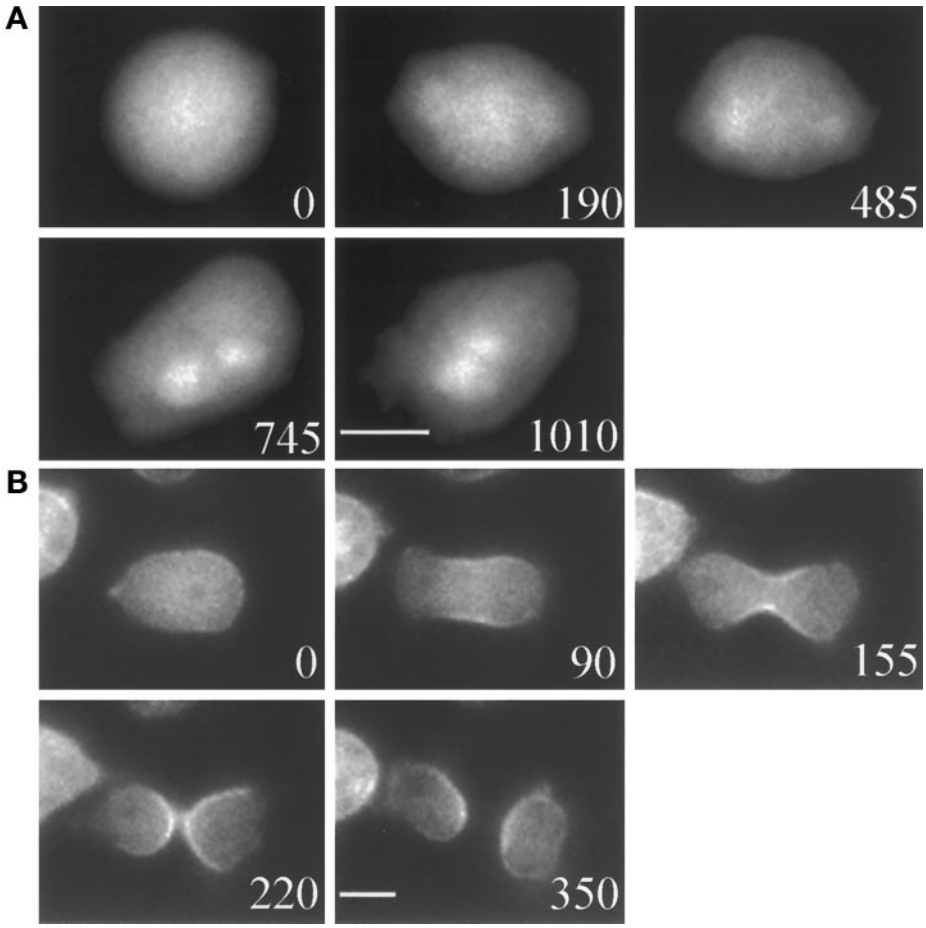

Figure 3. Myosin-null cells cannot divide on a hydrophobic surface. (A) A myosin-null cell expressing NLS-GFP fails to form a furrow on such a surface. Times of the panels are 0, 190, 485, 745, and 1010 s. (B) A cell expressing GFP-myosin divides successfully on a hydrophobic surface. Times of the panels are 0, 90, 155, 220, and 350 s. Bar, 5 μm.

undergo cytokinesis under these conditions. On an adhesive surface, large multinucleated myosin-null cells in interphase undergo a process termed "traction-mediated cytofission" (Spudich, 1989). Different parts of such a cell migrate in different directions, resulting in fragmentation of the cell into smaller cells with fewer nuclei. Cells that are kept on an adhesive surface for some time become largely mononucleated by this process. We also observed traction-mediated cytofission events that were coupled to mitosis, and those cell divisions appeared morphologically very similar to those that occurred in GFP-myosin cells (Figure 4). On an adhesive surface, the myosin-null cells rounded up, elongated, polar ruffled, and formed a cleavage furrow. The width of the furrow decreased and, ultimately, two daughter cells emerged. However, we did observe differences in behavior between myosin null cells and cells expressing functional myosin. The rate of cleavage furrow constriction in myosin-null cells was 26 $\pm$ 12 nm/s (mean $\pm$ SD), n = 27, about one-half the speed of GFP-myosin cells, which is 43 $\pm$ 11 nm/s (Sabry *et al.*, 1997). Occasionally, the furrows in myosin-null cells were not centered, resulting in daughter cells of unequal sizes (Figure 4C). Furthermore, unlike GFP-myosin cells (Sabry *et al.*, 1997), the myosin-null cells often did not show a linear decrease in the furrow width over time (Figure 5). Sometimes the furrowing process paused; other times, the furrow width constricted at an uneven rate. The mean r^2 value, which indicates how well the data fit to a straight line, was 0.98 for GFP-myosin cells (Sabry *et al.*, 1997), but only 0.94 for the myosin-null cells. Student's *t* test performed on these two groups of cells showed that the differences in r^2 values were statistically significant.

Cells in Which Wild-Type Myosin Was Replaced with a Mutant Myosin with Its Light Chain-Binding Domains Missing Are Capable of Dividing in Suspension and on a Nonadhesive Surface

In earlier work, Smith *et al.* (1996) disrupted what was thought to be the only myosin light chain kinase gene (*MLCK-A*) in *Dictyostelium* to examine the effect on cytokinesis. Those experiments, however, revealed that there is at least one other myosin light chain

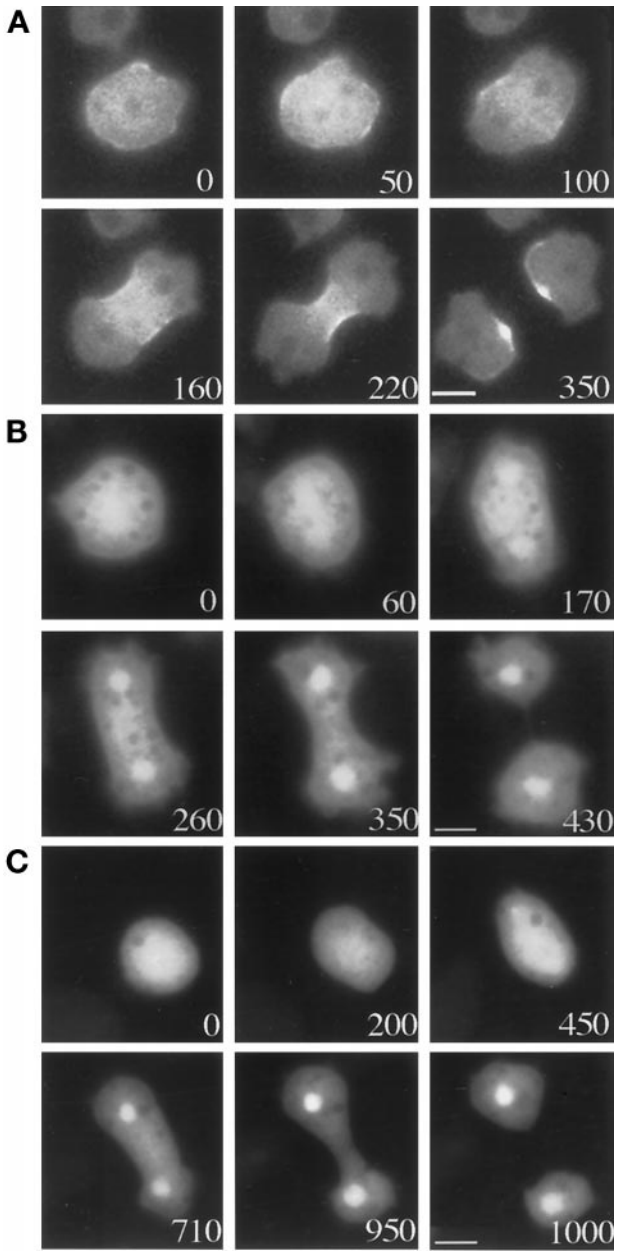

Figure 4. Myosin-null cells are able to divide on an adhesive surface, such as glass. (A) Cells expressing GFP-myosin are able to divide on a glass surface successfully. Times of the panels are 0, 50, 100, 160, 220, and 350 s. (B) Myosin-null cells can undergo mitosis-coupled division with morphologies similar to cells expressing functional myosin. Times of the panels are 0, 60, 170, 260, 350, and 430 s. (C) However, sometimes the cleavage furrow is not centered, resulting in unequal daughter cells. Times of the panels are 0, 200, 450, 710, 950, and 1000 s. Bar, 5 μm.

kinase in this organism. This finding complicated the issue of the role of light chain phosphorylation in cytokinesis, and so we decided to take another approach.

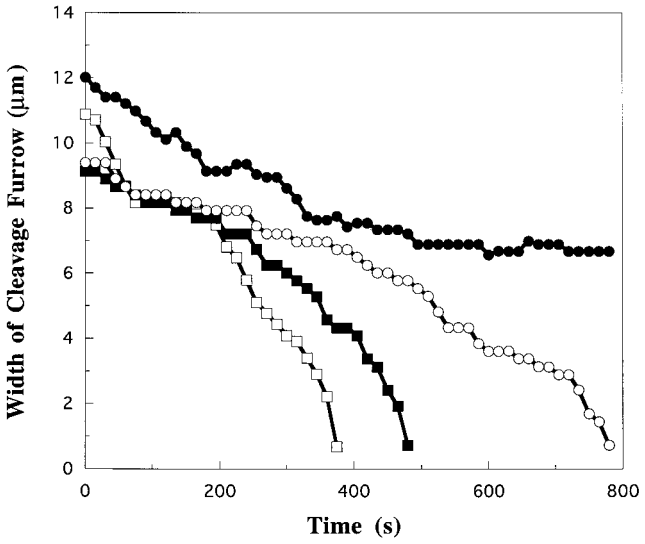

Figure 5. The rate of furrowing for myosin-null cells dividing on a glass surface is not constant. The width of the cleavage furrow vs. time is presented for four representative cells. One cell (closed circles) failed to divide into two.

To determine unambiguously whether the light chain-binding region of the myosin is essential for normal spatial and temporal control of assembly of the contractile ring and for normal constriction of the cell, a plasmid was created to encode a gene for a fusion protein, consisting of GFP attached to the N terminus of *Dictyostelium* myosin heavy chain with an internal deletion of both light chain-binding sites (ΔBLCBS-myosin). This plasmid was transformed into a *Dictyostelium* cell line that lacks its sole endogenous copy of the myosin heavy chain gene, and independent clones were isolated and assayed for GFP-ΔBLCBS-myosin expression. In these cells, GFP-ΔBLCBS-myosin was expressed at about 3 times the wild-type myosin level (Figure 6, A and B). It migrated slower than wild-type myosin, yet slightly faster than GFP-myosin, consistent with the deletion of 57 amino acids in the light chain-binding region. Indeed, when purified GFP-ΔBLCBS-myosin was analyzed by SDS/PAGE, only the myosin heavy chain was present, whereas both the light chains and the heavy chain were present in the case of wild-type myosin (Figure 6D). Furthermore, immunoblots of *Dictyostelium* whole cell lysate probed with anti-GFP antibody revealed only one band in cells expressing GFP-ΔBLCBS-myosin, demonstrating that the GFP fluorescence we detect is due to the fusion protein (Figure 6C).

Cells expressing GFP-ΔBLCBS-myosin, like those expressing GFP-myosin, can also divide on an adhesive glass surface (Figure 7A). Since GFP-ΔBLCBS-myosin, like GFP-myosin, is excluded from the nuclei, the nuclei appeared as areas of reduced fluorescence in the images, and we observed nuclear divisions. On

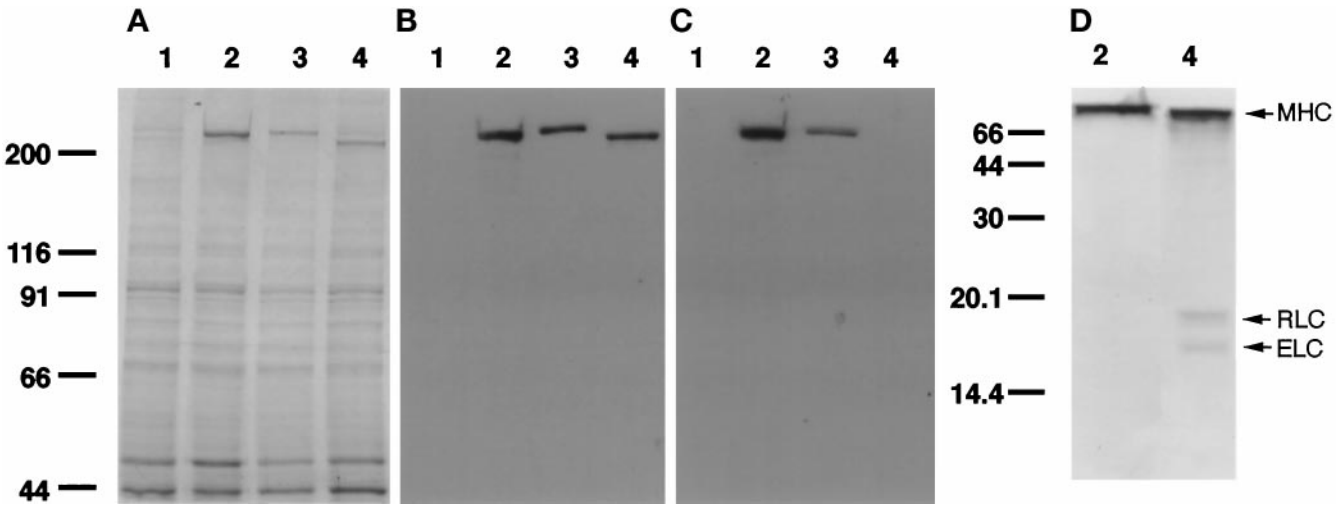

Figure 6. Expression of GFP-ΔBLCBS-myosin in comparison to that of myosin and GFP-myosin. (A–C) Whole cell lysates were run on 7.5% SDS-PAGE gels. Lane 1, myosin null cells; lane 2, cells expressing GFP-ΔBLCBS-myosin; lane 3, cells expressing GFP-myosin; lane 4, cells expressing wild-type myosin. (A) Coomassie-stained gel. (B) Immunoblot probed with antimyosin antibody. (C) Immunoblot probed with anti-GFP antibody. (D) Neither light chain binds to GFP-ΔBLCBS-myosin. Purified GFP-ΔBLCBS-myosin protein and wild-type myosin were run on a 15% SDS-PAGE gel and subsequently Coomassie stained to visualize the light chains. MHC, myosin heavy chain; ELC, essential light chain; RLC, regulatory light chain.

a nonadhesive surface (Figure 7B) and in suspension (Zang and Spudich, unpublished observations), cells expressing GFP-ΔBLCBS-myosin underwent quiescence, elongation, furrowing, polar ruffling, and daughter cell separation after mitosis. All 21 cells we observed on the hydrophobic surface divided successfully.

Wild-type *Dictyostelium* myosin moves at 3 μm/s in vitro, ~fivefold faster than ΔBLCBS-myosin, presumably due to its longer lever arm (Uyeda *et al.*, 1996). We compared the rate of furrow constriction for cells expressing wild-type myosin, GFP-myosin, and the slower motor, GFP-ΔBLCBS-myosin. The rate of change of cleavage furrow width over time for GFP-ΔBLCBS-myosin cells on an adhesive surface showed a good fit to a straight line (Figure 8), as was observed previously for GFP-myosin cells on a glass surface (Sabry *et al.*, 1997). The average r^2 value of this regression analysis is 0.97. In suspension, the mean rate of furrow constriction was 57 ± 12 nm/s (n = 27) for wild-type cells and 36 ± 10 nm/s (n = 26) for GFP-ΔBLCBS-myosin cells. On an adhesive surface, the mean rate of furrow constriction was 43 ± 11 nm/s for GFP-myosin cells (Sabry *et al.*, 1997) and 32 ± 7 nm/s (n = 43) for the GFP-ΔBLCBS-myosin cells. Thus, both on an adhesive surface and in suspension, the rate of constriction of cleavage furrows of GFP-ΔBLCBS-myosin cells was similar to that of wild-type or GFP-myosin cells.

We also measured the speed of nuclear migration and of leading edge advancement for GFP-ΔBLCBS-myosin cells on a surface (Figure 8). The rate of nuclear migration was 38 ± 8 nm/s (n = 42), and the rate of leading edge advancement was 36 ± 7 nm/s (n = 40). These values are quite similar to those of the GFP-myosin cells, whose rate of nuclear migration was 40 ±

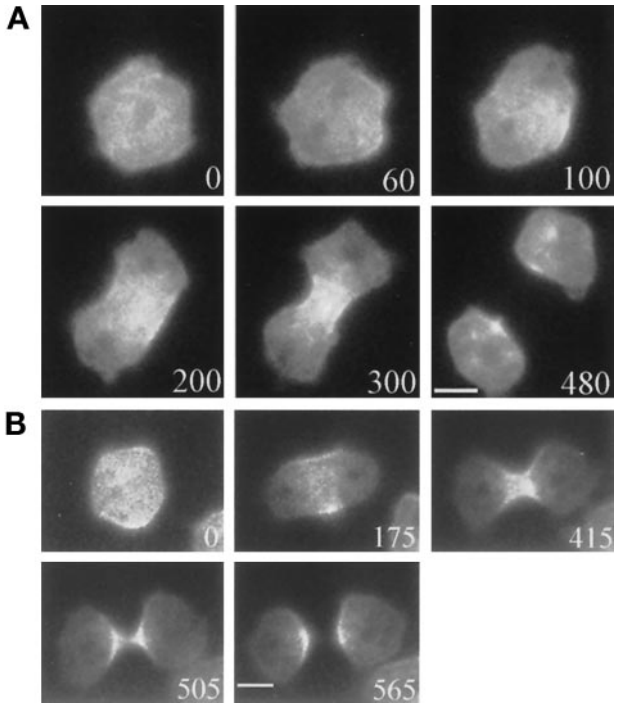

Figure 7. Cells expressing GFP-ΔBLCBS-myosin are able to divide successfully on an adhesive surface (A) or on the hydrophobic surface (B). The number in the lower right corner indicates the progression of division in seconds. Bar, 5 μm.

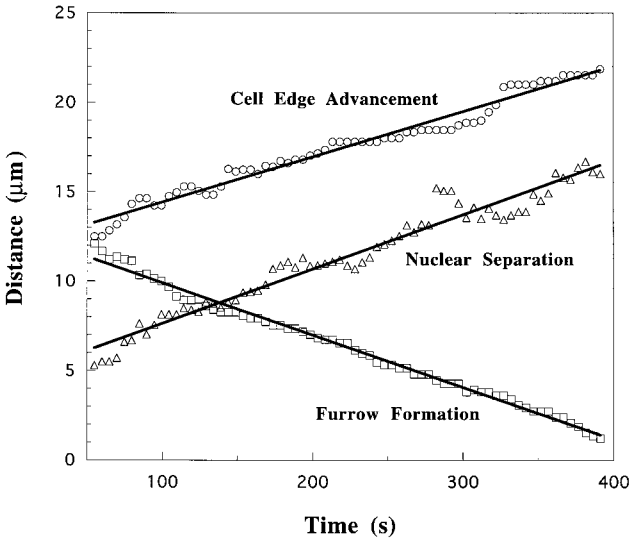

Figure 8. Analysis of rate of cleavage furrow constriction, nuclear separation, and cell edge advancement for GFP-ΔBLCBS-myosin cells. Cleavage furrow constriction in cells expressing GFP-ΔBLCBS-myosin is linear.

17 nm/s and whose rate of leading edge advancement was 43 ± 21 nm/s (Sabry *et al.*, 1997).

While the localization of myosin seemed to be relatively normal in cells expressing GFP-ΔBLCBS-myosin, abnormal cell shapes were seen both in suspension and on a surface. Sometimes cells expressing GFP-ΔBLCBS-myosin stretched out into an asymmetric or bent cylindrical shape, rather than the more uniform, cylindrical shape that we generally saw with cells expressing wild-type myosin or GFP-myosin. Furthermore, the general appearance of the myosin was more filamentous, possibly due to its overexpression.

DISCUSSION

It is intriguing that while *Dictyostelium* cells lacking functional myosin II cannot divide in suspension, they can propagate if given an adhesive surface, such as a plastic Petri dish or a glass coverslip. To resolve this paradox, we have devised two new approaches to examine cells undergoing cytokinesis to avoid complications from adhesive surfaces: filming cells in a hanging drop and on a hydrophobic surface. To our knowledge, division of cells under nonadhesive conditions has generally not been explored. The hanging drop method provides one way to do so. The second method, using a hydrophobic surface, offers several advantages. It is less labor intensive, and it results in images of better quality.

In studies using fluorescence microscopy to follow myosin dynamics in living cells, we could detect the separation of nuclei, since GFP-myosin or GFP-ΔBLCBS-myosin molecules are large enough to be excluded from nuclei. Thus, we are looking at mitosis-coupled division. To identify mitosis in myosin-null cells, we devised NLS-GFP. This NLS-GFP does not localize into the nucleus during early stages of mitosis, even though the nuclear envelopes remain intact (Moens, 1976). This may indicate that import of protein is suspended during early mitosis; alternatively, protein may be able to diffuse out of the fenestrations in the nuclear envelope through which the spindle passes.

Myosin II Is Not Required for Cell Rounding during Mitosis or Polar Ruffling during Cytokinesis

Dictyostelium cells, like most mammalian cells, round up during mitosis (Fukui, 1990; Mitchison, 1992; Cramer and Mitchison, 1997). Furthermore, like mammalian cells, their contractile vacuoles and their Golgi apparatus disperse at prophase/metaphase with the breakdown of microtubules (Fukui, 1990; Zhu *et al.*, 1993). This is reflected morphologically since the cell surfaces become very smooth and quiescent.

Myosin-null cells also undergo quiescence during mitosis. In addition, they round up during mitosis both on a surface and in suspension (Figures 1, 3, and 4). This result is consistent with the finding in mammalian tissue culture cells that mitotic rounding is independent of myosin II (Cramer and Mitchison, 1997). However, interphase myosin-null cells are unable to generate the cortical tension necessary to round up in response to cAMP or to the application of azide (Pasternak *et al.*, 1989; Fukui *et al.*, 1990). This observation suggests that the organization and functioning of the actin cytoskeleton of mitotic cells are different from those of interphase cells. It is known that in mammalian cells, caldesmon, an actin-modulating protein, is phosphorylated during mitosis, causing it to disassociate from the actin cytoskeleton (Yamashiro *et al.*, 1990, 1991). Other cytoskeletal proteins could be in different activation states during mitosis, thereby causing cell-cycle–dependent changes in the properties of the actin cytoskeleton.

Myosin-null cells are capable of polar ruffling in suspension, even though cleavage furrows are not formed. Similarly, on a hydrophobic surface with reduced adhesion, they polar ruffle while remaining relatively round. Polar ruffling, which manifests in the extension of pseudopods in the polar regions, coincides with the proximal presence of the nucleus as if the nucleus somehow induces this action. On a glass surface, the ruffling or the pseudopod extensions probably serve to aid in the formation of the daughter cells since, after division, these polar pseudopods become the leading edge of the daughter cells as they migrate in opposite directions. This behavior is observed in both the null cells and in wild-type cells. In cells that express myosin, myosin is found in the pos-

terior of the migrating daughter cells (Figures 4A and 7A) (Sabry *et al.*, 1997), just as it is found in the back of a migrating cell during interphase (Moores *et al.*, 1996). Thus, myosin may aid the establishment of cell polarity for migration but is clearly not required for it.

Myosin II Is Required for Stretching Out and Forming a Cleavage Furrow for Cell Division in the Absence of Cell Adhesion, but Not for Cell Division on an Adhesive Surface

Unlike GFP-myosin cells and GFP-ΔBLCBS-myosin cells, myosin-null cells failed to stretch out to a cylindrical shape in suspension or on a hydrophobic surface, demonstrating that this elongation process is myosin dependent. This result is not surprising because, during anaphase, myosin starts to concentrate along the cortex of the impending cleavage furrow (Figure 4A) (Fukui, 1990; Sabry *et al.*, 1997). Therefore, myosin is probably acting to change the cell's shape before furrow constriction.

Myosin is essential for cytokinesis in suspension or on a nonadhesive surface. On an adhesive surface, however, traction forces, presumably produced by polar pseudopods exerting force on the adhesive surface, are clearly involved in causing cell shape changes and the constriction of the cleavage furrow. Such traction forces were measured in mammalian cells (Burton and Taylor, 1997). Actin, coronin, and myosin I are found in the polar ruffles in dividing cells (Fukui *et al.*, 1989; de Hostos *et al.*, 1993); they may play a role in generating these traction forces. Indeed, the deletion of the coronin gene, which codes for an actin-modulating protein, decreases the efficiency of division on a surface for *Dictyostelium* (de Hostos *et al.*, 1993). The ability of myosin-null cells to undergo successful division on an adhesive surface (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Fukui *et al.*, 1990; Neujahr *et al.*, 1997; this report) shows that cytokinesis is not powered by force generated by myosin alone. Rather, it is achieved by the cooperation of other cytoskeletal proteins.

Effects of Deletion of the Light Chain-Binding Sites on Cytokinesis

We examined the localization of GFP-ΔBLCBS-myosin and the speed of cleavage furrow constriction in cells expressing this mutant myosin. These cells undergo relatively normal cytokinesis, which is somewhat surprising given the severity of the mutation in the myosin. GFP-ΔBLCBS-myosin is not regulated by light chain phosphorylation, and the maxium speed at which it moves actin filaments is at one-fifth the maximum wild-type myosin speed (Uyeda *et al.*, 1996).

Our experiments support the idea that light chain phosphorylation regulation is not essential for cytokinesis in *Dicytostelium*. Cells expressing internally truncated myosin heavy chain without the RLC-binding site (ΔRLCBS-myosin) (Uyeda and Spudich, 1993) or both the RLC and the essential light chain-binding sites (ΔBLCBS-myosin) (Uyeda *et al.*, 1996) are able to grow in suspension. In a related study, Ostrow *et al.* (1994) found that cells expressing a mutant RLC whose activating phosphorylation site has been changed to an alanine are also able to divide in suspension. These findings seem to contradict the proposed function of myosin light chain phosphorylation in cytokinesis in mammalian cells (Satterwhite *et al.*, 1992; Yamakita *et al.*, 1994; Debiasio *et al.*, 1996). It should be noted, however, that *Dictyostelium* myosin may be regulated differently from mammalian myosins. For example, although the phosphorylation of *Dictyostelium* myosin at serine-13 (Ostrow *et al.*, 1994), which increases myosin ATPase and in vitro motility (Griffith *et al.*, 1987), is comparable to the phosphorylation of serine-19 in smooth muscle myosin, inhibitory sites comparable to the smooth muscle myosin serine-1 or serine-2 are not known. Although the signal for the timing of cytokinesis is not determined by the phosphorylation state of the regulatory light chain in *Dictyostelium*, light chain phosphorylation may play a role in cytokinesis. *Dictyostelium* cells in which the gene for myosin light chain kinase A (MLCK-A) is disrupted show an increased number of multinucleate cells, suggesting that MLCK-A contributes to, but is not essential for, cytokinesis (Smith *et al.*, 1996).

We also measured the speed of cleavage furrow constriction in cells expressing GFP-ΔBLCBS-myosin. These cells constricted their furrows nearly as fast as wild-type myosin (43 nm/s vs. 32 nm/s), both on a surface and in suspension. However, by in vitro motility assays, ΔBLCBS-myosin moves actin filaments only 20% as fast as wild-type myosin (3 μm/s vs. 0.6 μm/s). The reduction in in vitro motility rate is thus much greater than the observed reduction in rate of cleavage in vivo. This lack of strict correlation between the in vitro motility rate and the rate of furrow constriction could be accounted for in two ways. Both of these possibilities could also explain the 100-fold difference in the absolute rates of in vitro motility and furrowing.

The first possibility is that the actin–myosin interaction is not rate limiting in cytokinesis. The furrow is a dynamic structure. There are undoubtedly many rearrangements of the actin cytoskeleton in the furrow region as the size of the furrow decreases. For example, actin filaments are presumably being disassembled or relocalized as the furrow volume reduces (Schroeder, 1972). Phalloidin, a drug that stabilizes actin filaments, inhibits furrow progression (Hamaguchi and Mabuchi, 1982). This kind of rearrangement of the cytoskeleton, rather than the actin–myosin interaction per se, could be rate limiting for the rate of cleavage.

The second possibility is that myosin is under a relatively heavy load during cytokinesis. It is known

that in muscle, myosin's velocity is greatly reduced under a load compared with its velocity in the unloaded situation (Huxley, 1974). Furthermore, GFP-ΔBLCBS-myosin is overexpressed in cells. More myosin molecules in the furrow region may produce more force. Because the in vitro motility assay measures the velocity at which myosin molecules move actin filaments in the unloaded situation, the exact correspondence between in vivo and in vitro velocity would depend on the force–velocity curves of the motors and on the force being applied in the cleavage furrow.

Cytokinesis A and Cytokinesis B

The fact that *Dictyostelium* myosin-null cells cannot form a furrow in suspension or on a nonadhesive hydrophobic surface supports the idea that an actin-myosin–based contractile ring is responsible for cleaving the mother cell into two daughter cells (Schroeder, 1973). However, in the absence of myosin, cytokinesis can occur if the cells are attached to an adhesive surface, such as glass. This was apparent in early studies with myosin-depleted cells (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Spudich, 1989; Fukui *et al.*, 1990) and has been recently emphasized by Neujahr *et al.* (1997) and also shown here. Therefore, cytokinesis is driven by two mechanisms, which we call Cytokinesis A and B.

Cytokinesis A is a myosin-dependent active furrowing and is the sole means of division under circumstances where cells cannot adhere to a surface, such as in suspension or on a hydrophobic surface. Thus, myosin-null cells fail to divide because they cannot form a furrow, whereas cells expressing functional myosin can form an active contractile ring to constrict the furrow and cleave the cell. Cytokinesis A would be the only mechanism of division in cells that do not adhere to the surface, such as eggs. Therefore, the injection of an antimyosin antibody into starfish blastomeres inhibits myosin function and thus furrow formation (Mabuchi and Okuno, 1977).

Cytokinesis B is myosin independent and possibly results from the traction forces generated by polar pseudopods exerting force on an adhesive surface. Such mechanical forces have been demonstrated and measured in adhesive mammalian cells (Burton and Taylor, 1997). *Dictyostelium* myosin-null cells dividing on an adhesive surface are morphologically very similar to cells expressing functional myosin (Figure 4) (Neujahr *et al.*, 1997). They appear to have a furrow, and actin has been found in this region by immunofluorescence (Fukui *et al.*, 1990). However, unlike cells expressing functional myosin, the reduction in furrow width seen in myosin-null cells dividing on an adhesive surface does not occur at a constant rate (Figure 5). Furrowing in myosin-null cells is presumably a result of the pulling of the polar pseudopods in opposite directions, although some as yet unknown adhesion-dependent mechanism of cleavage may be operating, as suggested by Neujahr *et al.* (1997). In early works, we coined the term traction-mediated cytofission (Spudich, 1989; Fukui *et al.*, 1990) to refer to this adhesion-dependent cell division event. In our view, this process can occur in both mitotically dividing cells, where it is specifically coupled to mitosis and daughter cell separation, and in interphase cells, where more random traction-mediated cytofission events result in cell fragmentation. In the case of mitotically dividing cells, we called this traction-mediated cytofission Cytokinesis B.

For cells expressing myosin and dividing on an adhesive surface, Cytokinesis A and B presumably act cooperatively to cleave the mother cell in two. Thus, just as there are two different mechanisms, anaphase A and anaphase B, for separating chromosomes during mitosis, eukaryotic cells may generally use two different mechanisms for cytokinesis as well.

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