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Cytokinesis Failure in Clathrin-Minus Cells Is Caused by Cleavage Furrow Instability

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The role of membrane traffic during cell division has only recently begun to be investigated. A growing number of trafficking proteins seem to be involved in the successful completion of cytokinesis. Clathrin was the first trafficking protein to be shown to be essential for cytokinesis in *Dictyostelium*. Here we investigate the nature of the cytokinesis defect of *Dictyostelium* clathrin null cells. We found that adherent clathrin null cells do form cleavage furrows but cannot maintain a consistent rate of furrow ingression. Clathrin null cells are completely defective in cytokinesis when placed in suspension. In these conditions, the cells develop an abnormal division morphology that consists of two lateral "furrows" on either side of a bulging equatorial region. Cells expressing GFP-myosin II were examined at various stages of cytokinesis. Clathrin null cells show multiple defects in myosin organization and localization that parallel the striking failure in furrow morphology. We postulate that this morphology is the result of contraction at the rear of the presumptive daughter cells in concert with incomplete furrow ingression. Cell Motil. Cytoskeleton 48:213–223, 2001.

Key words: membrane traffic; cell division; contractile ring; *Dictyostelium*

INTRODUCTION

Clathrin is a component of the protein coat system involved in vesicle budding from the plasma membrane and the trans-Golgi [Brodsky, 1997]. At the plasma membrane, clathrin coats are involved in the endocytosis of surface receptors. At the trans-Golgi, clathrin coats are involved in the selective sequestration and budding of proteins targeted to the lysosome. The clathrin heavy chain and associated light chain form a hexameric triskelion that assembles into polyhedral lattices on membranes The lattice structures form coated pits that invaginate and scission from the membrane as clathrin-coated vesicles.

Mutants in the *Dictyostelium* clathrin heavy chain have been generated by antisense RNA inhibition and homologous recombination [O'Halloran and Anderson, 1992; Ruscetti et al., 1994]. These clathrin null mutants have defects in processing and sorting of lysosomal enzymes, and have markedly reduced rates of fluid-phase uptake. In addition to lysosomal and endocytic defects, the formation of the large compartments of the contractile vacuole is affected in clathrin mutants [O'Halloran

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QuickTime movies of the images in this manuscript can be accessed at http://www.zo.utexas.edu/faculty/delozanne/gerald2001.html

Abbreviations used: DAPI = 4,6-diamidino-2-phenylindole; GFP = green fluorescent protein.

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and Anderson, 1992]. As a result, cells deficient in clathrin function have defects in osmoregulation.

Clathrin null cells also have defects in the *Dictyostelium* developmental cycle. Part of the defect is attributed to improperly differentiated stalk and spore cells in the fruiting body [Niswonger and O'Halloran, 1997a]. In addition, clathrin null cells stream into mounds more slowly than wild-type cells. Three-dimensional analysis of streaming clathrin null cells indicates that motility is severely impaired [Wessels et al., 2000]. Clathrin null cells fail to maintain cellular polarity during streaming, and produce multiple pseudopods in the Z-axis.

Interestingly, clathrin was also found to be required for cytokinesis [Niswonger and O'Halloran, 1997b]. Clathrin null cells become large and multinucleate in suspension culture possibly due to a lack of myosin II localization in a clearly defined contractile ring [Niswonger and O'Halloran, 1997b]. This defect is not due to a general inability to localize myosin II since clathrin null cells can localize myosin II to the rear of a moving cell, and can also execute other myosin-dependent activities. Since clathrin does not localize to the region of the cleavage furrow [Damer and O'Halloran, 2000], it must exert its function elsewhere during cytokinesis. This function remains unknown.

In this study, we closely examined clathrin null cells to determine how they fail to divide during cytokinesis. We found that dividing clathrin null cells develop an abnormal cleavage morphology that consists of two lateral "furrows" and a bulging equatorial region. In addition, adherent clathrin null cells cannot maintain a constant rate of cleavage furrow ingression. Clathrin null cells display abnormal myosin localization, but these cells do localize myosin to regions of plasma membrane constriction. We postulate that this morphology results from incomplete furrow ingression accompanied by contraction at the rear of the daughter cell.

MATERIALS AND METHODS

Cell Culture

Cells were cultured on 100-mm Petri dishes in HL5 media. For clathrin null cells, we used the 5E2 cell line in which the clathrin heavy chain gene was deleted by homologous recombination [Ruscetti et al. 1994]. As control cells, we used 4F9 cells, which contain a random insertion of the knockout plasmid. In addition, DH1 and AX3 were used as control cells in some experiments. Cells expressing a GFP-myosin II construct were maintained under selection with 10–20 μg/ml G418 [Moores et al., 1996].

In preparation for microscopy, cells in log phase growth were removed from Petri dishes by gentle pipetting, and washed twice in fresh HL5 as published previously [Gerald et al., 1998]. For observation of cells in adherent conditions, cells were allowed to attach to coverslips in HL5 for 4–5 h. For observation of cells in suspension conditions, cells were cultured in HL5 in Erlenmeyer flasks shaking at 220 rpm. Cells were allowed to acclimate to these conditions for 4–5 h, and then samples were taken at various time points for microscopy.

Video Microscopy

All video observations were performed with a slide chamber consisting of a glass coverslip and a gas permeable Teflon membrane to prevent anoxia [Kiehart et al., 1994]. For viewing attached cells, the chamber was loaded with about 0.1 ml HL5 media and a coverslip with adherent cells was gently placed on top. Cells were viewed on a Zeiss Axiophot (Zeiss, Oberkochen, Germany) upright microscope with a 100× 1.3 NA Plan NeoFluor phase contrast objective. A 600-nm red filter was used to reduce light toxicity. For viewing nonattached cells, aliquots from shaking culture were mixed 2:3 with a solution of 0.1% low-melt agarose in HL5 media. Approximately 0.1 ml of this mixture was immediately loaded into the slide chamber, and a clean coverslip was placed on top. The cells in suspension were observed with a 20× 0.7 NA phase contrast objective.

Video sequences were recorded in real time to videotape with a CCD camera (DAGE MTI, Inc., Michigan City, IN) and a Sony S-VHS VCR model SVO-9500MD (Sony, Park Ridge, NJ). Taped sequences were digitized and processed for time-lapse sequence using a Scion (Scion Corp., Frederick, MD) frame grabber card driven by NIH Image 1.61 software (available at http://rsb.info.nih.gov). In addition, digital still images were acquired directly on a Photometrics cooled CCD camera (Photometrics Ltd., Tucson, AZ) at 5-, 6-, or 10-sec intervals using MetaMorph software (Universal Imaging Co., West Chester, PA). Digital sequences were cropped and assembled into image stacks or QuickTime movies using NIH Image 1.61 software. QuickTime movies were compressed and flattened by Movie Cleaner Lite 1.2 (Terran Interactive, Inc., San Jose, CA). These movies are archived at http://www.zo.utexas.edu/faculty/delozanne/gerald2001.html. Select digital images were cropped, aligned, and contrast adjusted in Adobe PhotoShop 5.0 (Adobe, San Jose, CA). A high pass filter was applied to images from suspension culture to suppress image background.

Measurements

Measurements of cell equatorial width were obtained as pixel values from lines drawn directly on digital frames in NIH Image 1.61. The initial equatorial width

was determined when a cell was fully elongated, but just before equatorial constriction began. All subsequent measurements were normalized by the initial equatorial width.

Fixation

Cells expressing GFP-myosin II were fixed with picric acid as described [Gerald et al., 1998; Humbel and Biegelmann, 1992; Neujahr et al., 1997a]. Briefly, adherent cells were fixed with 2% paraformaldehyde, 15% concentrated picric acid, 10 mM piperazine-N,N′-bis-2-ethanesulfonic acid (PIPES), pH 6.5 for 30 min, followed by 10 min post-fixation with 70% ethanol. For cells in suspension, an equal volume of cells were added to 2× fix solution (4% paraformaldehyde, 30% picric acid, 10 mM PIPES, pH 6.5) and inverted gently for 15 min. Cells were spun down and allowed to settle onto poly-L-lysine coverslips in the presence of fix for 10–15 min, and these coverslips were then treated as adherent samples.

After fixation, cell nuclei were stained with 10 μM 4,6-diamidino-2-phenylindole (DAPI), 50 mM ammonium chloride in phosphate buffered saline (PBS) for 10 min, and the coverslips were mounted in 50% glycerol in PBS with 100 mg/ml 1,4-diazabicylclo-[2.2.2]octane (DABCO; Sigma Chemical Co., St. Louis, MO).

Images of fixed samples were acquired on a Zeiss Axiophot microscope using a Star I cooled CCD camera (Photometrics Ltd., Tucson, AZ) and a 1.3 NA 100× Pan Neofluor oil objective. Images were taken and converted to PICT format with IP Lab software (Signal Analytics Corp., Vienna, VA) and transferred to Adobe PhotoShop 5.0 for contrast adjustment.

Confocal Microscopy

For live cell imaging, cells were grown in HL-5 in a 60-mm Petri dish with a coverslip glued to a hole cut in the bottom of the dish. Before imaging, the HL-5 was replace with PDF buffer (11 mM K_2HPO_4, 13.2 mM KH_2PO_4, 20 mM KCl, 1 mM $CaCl_2$, and 2.5 mM $MgSO_4$, pH 6.4). Cells were imaged using a laser scanning confocal microscope (Zeiss Microsystem LSM, Thornwood, NY) with an excitation wavelength of 488 nm and emission filter of 488/568 nm and attenuation set at 10%. Cells were scanned every 15 sec with a scan length of 4 sec. Images were captured by Carl Zeiss LSM-PC version 3.5. Selected imaged were cropped, aligned, and adjusted for contrast in Adobe Photoshop 5.0.

RESULTS

Clathrin Null Cells Form Abnormal Cleavage Furrows But Complete Cytokinesis on a Substrate

To understand the role of clathrin in cytokinesis, we characterized in detail the ability of clathrin null cells to achieve cell division. We first used time-lapse video-light microscopy to observe cells attempting cell division on a substrate. All wild-type cells (2 strains, n = 20) displayed a sequence of events well characterized for cytokinesis [Fukui and Inoue, 1991; Neujahr et al. 1997a]. Specifically, in the early stages of cytokinesis, the cells elongated and formed membrane ruffles at the poles. The cells formed a single equatorial furrow (Fig. 1A, 108 sec) that quickly and evenly constricted into a thin cytoplasmic bridge (Fig. 1A, 174 sec). The cells then severed these bridges, and separated into daughter cells. As described previously [Sabry et al., 1997], we found that wild-type cells constricted their cleavage furrows at a nearly linear rate (Fig. 2A).

Analysis of dividing clathrin null cells attached to a substrate revealed striking defects in cytokinesis (Fig. 1B–D. Like wild-type cells, clathrin null cells elongated and formed membrane ruffles at their poles. However, most clathrin null cells (14/24) failed to construct a single furrow in the midline of the cell. Instead, these cells developed two abnormal constrictions that appeared on either side of a pronounced swelling, or bulge. Some of these attached clathrin null cells (9/24) began division with a single constriction similar to wild-type cells, but subsequently developed double constrictions. Other clathrin null cells (5/24) displayed two furrowing constrictions from what appeared to be the earliest stages of furrowing.

For example, the cell shown in Figure 1C formed a single equatorial furrow that began to constrict at a wild-type rate (Fig. 1C, 120 sec, arrowhead; Fig. 2C). However, this furrow did not constrict to completion, and the cell had difficulty remaining attached to the substrate (see QuickTime movie at http://www.zo.utexas.edu/faculty/delozanne/gerald2001.html). Subsequently, the initial equatorial constriction receded, and two lateral constrictions abutting a bulging equator formed (Fig. 1C, 414 sec, arrowheads; Fig. 2C). Despite this abnormal shape, the cell ultimately completed cytokinesis. One of the constrictions (Fig. 1C, 432 sec, arrowhead) contracted further and separated the daughter cells (Fig. 1C, 444 sec).

In the cell shown in Figure 1D, two membrane constrictions flanking the equator were apparent as the cell began to elongate (Fig. 1D, 132sec, arrowheads). Both constrictions remained visible as the entire equatorial region ingressed (Fig. 1D, 192 sec, arrowheads). As in the cell shown in Figure 1C, one constriction contracted dramatically to complete separation of the cells (Fig. 1D, 228sec, arrowhead; 252 sec).

Some attached clathrin null cells (10/24) completed cytokinesis with a single furrow (Fig. 1B). Regardless of whether they formed one constriction or two, clathrin null cells failed to constrict their furrows at a consistent

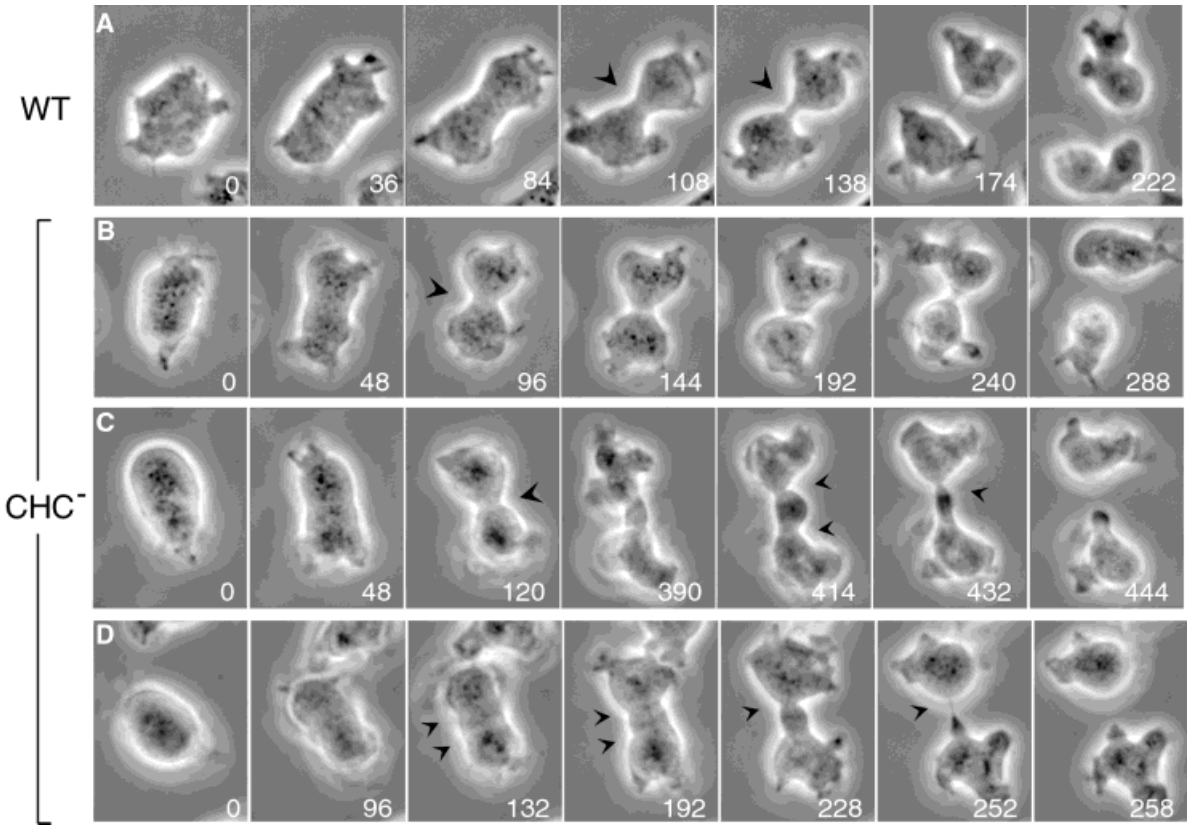

Fig. 1. Adherent clathrin null cells can initiate cleavage furrow formation but with frequent aberrant morphologies. **A:** A wild-type cell completes cytokinesis on a substrate (t = sec). Mitotic wild-type cells elongate, then rapidly divide by means of an equatorial cleavage furrow (*arrowhead*). **B:** Some adherent clathrin null cells can divide with wild-type attributes. About 42% (10/24) of mitotic attached clathrin null cells divided successfully with equatorial furrows (*arrowhead*, 96 sec). **C:** 37% (9/24) of attached clathrin null cells began division with wild-type furrow morphology (120 sec, *arrowhead*), but developed an equatorial bulge during later stages (414 sec). The equatorial swelling was bounded by two constrictions in the plasma membrane (414 sec, *arrowheads*). **D:** About 21% (5/24) of the attached clathrin null cells initiated cell division with the formation of two constrictions (132 sec, *arrowheads*). In these cases, both constrictions remained visible throughout division, although one constriction progressed significantly to separate the cells (228 sec, *arrowhead*). In total, 14 out of 24 adherent cells formed an equatorial bulge. Three of these cells failed to divide.

rate. These cells often began constriction at a rate similar to wild-type cells, but failed to maintain this rate throughout division. For example, the cell from Figure 1B began furrowing at a wild-type rate, but then slowed dramatically (Fig. 2B, 100–150s). After this delay, furrowing resumed to separate the daughter cells (Fig. 2B, 150–250s).

Clathrin Null Cells Display Two Lateral Furrows Around a Bulging Equator as They Fail to Divide in Suspension

In the absence of cell adhesion, cytokinesis becomes dependent on the activity of myosin II and other molecules for furrow formation. To analyze cytokinesis in this stringent condition, we examined cells floating in a suspension of low melting-temperature agarose [Gerald et al., 1998]. While in suspension, wild-type cells (n = 10) behaved similarly to cells adhered to a substrate, beginning cytokinesis with an elongated shape and polar membrane ruffling (Fig. 3A). Subsequently, these cells established equatorial furrows (Fig. 3A, 35 sec, arrowhead), which rapidly constricted at a linear rate (Fig. 4A).

Without cell adhesion, the defects in cytokinesis exhibited by clathrin null cells were exacerbated. All mitotic clathrin null cells observed in suspension formed two lateral constrictions, an equatorial bulge, and subsequently failed to divide (n = 16). Most of these clathrin null cells (11/16) began cell division with an elongate shape and a single initial furrow, but subsequently formed a second furrow in later stages of cytokinesis. An example is shown in Figure 3B. This clathrin null cell began division with a clearly defined furrow on the cell equator roughly symmetrical to the cell poles (Fig. 3B, 0 sec, arrowhead). In this cell, the furrow appeared to lose

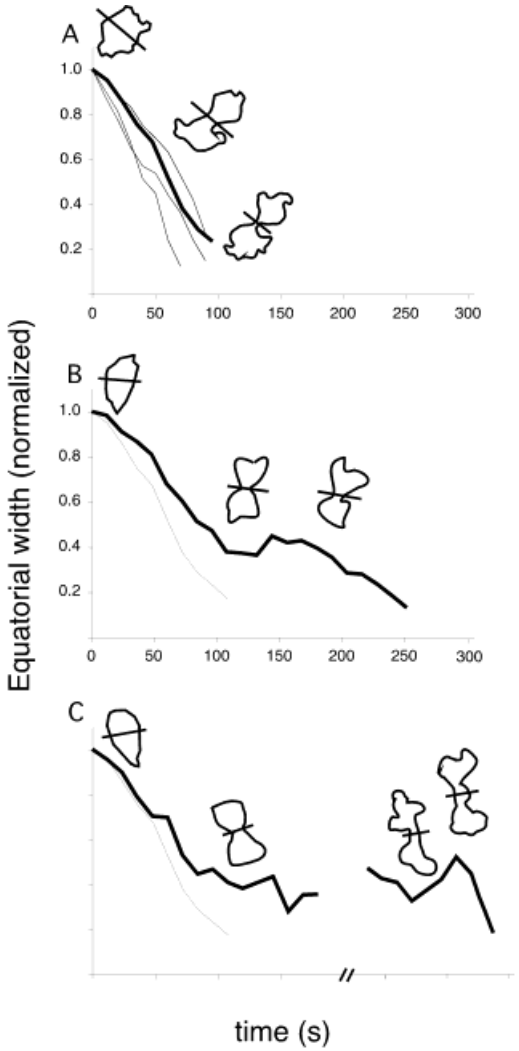

Fig. 2. Adherent clathrin null cells do not maintain consistent rates of furrow ingression. Equatorial width was measured over time on elongated mitotic cells. **A:** Wild-type cells constrict their cleavage furrows at a similar linear rate. (Four examples out of 10 cells are shown, bold line indicates measurement for cell depicted in Figure 1A). **B:** Clathrin null cell from Figure 1B (bold line) begins furrowing at a wild-type rate, but slows between 100 and 150 seconds. A wild-type cell is co-plotted (fine line) for comparison. **C:** Clathrin null cell from Figure 1C (bold line) begins furrowing at wild-type rate, but slows between 90 and 200 sec. No measurements were possible between 200 and 325 sec, as the cell became partially detached and the furrow remained out of focus (see QuickTime movies at http://www.zo.utexas.edu/faculty/delozanne/gerald2001.html). The peak that appears at 415 sec on the graph (dotted lines) represents the growth and constriction of the equatorial bulge described in Figure 1C.

its symmetrical position at the cell equator, and the constriction began to "migrate" towards one of the poles (Fig. 3B, 60 sec, arrowhead). Subsequently, a second constriction formed near the opposite pole of the cell (Fig. 3B, 90 sec, arrowhead). These two "furrows" constricted to varying degrees in a non-coordinated fashion, and the equatorial region swelled between them into a bulge. The bulge remained visible throughout an extended period of dramatic cell shape changes. Ultimately, cytokinesis failed, the cell rounded up and became binucleated (Fig. 3B, 590 sec).

Without adhesion, some clathrin null cells (5/16) displayed two sets of membrane constrictions even at early stages of cytokinesis. These cells failed to establish a single centrally placed furrow, but initiated two membrane constrictions, one on each side of the equator (Fig. 3C, 55 sec, arrowheads). Again, an equatorial bulge emerged between the alternating contractions of the two constrictions (Fig. 3C, 175–235 sec), and the cells rounded-up after cytokinesis failure. Regardless of their initial stages of aberrant furrow formation, all cells failed to divide.

When the equatorial width of cells in suspension was plotted, we found that clathrin null cells failed to maintain any significant rate of equatorial ingression when compared to wild-type cells (Fig. 4B). In fact, clathrin null cells displayed a net increase in equatorial width throughout the events leading to cytokinesis failure.

Localization of Myosin II in Dividing Clathrin-Null Cells

Previous studies had suggested that the cytokinesis defect of clathrin null cells was due to the inability of these cells to assemble myosin II into a functional contractile ring in the cleavage furrow [Niswonger and O'Halloran, 1997b]. However, the initiation of constriction in the absence of cell adhesion suggested that clathrin null cells possessed a contractile activity associated with these aberrant furrows. Thus, we decided to examine the distribution of myosin II in clathrin null cells under different conditions.

We first fixed adherent cells expressing GFP-myosin II and analyzed them by epifluorescence. As previously reported [Fukui and Inoue, 1991; Gerald et al., 1998; Yumura and Uyeda, 1997; Zang and Spudich, 1998], wild-type cells ($n = 27$) concentrated myosin II in an equatorial cleavage furrow (Fig. 5A–C). Most of the clathrin null cells (22/28) localized GFP-myosin II to a plasma membrane constriction (Fig. 5D–F, G–I). However, instead of the strong and exclusive concentration of myosin II into the furrowing region seen in wild-type cells, myosin II in clathrin null cells was diffusely distributed along the cortex and not restricted to the constriction. The GFP-myosin II was "patchy" and diffuse in clathrin null cells with one constriction or with two. Myosin II often extended along the cortex of these cells to regions of the plasma membrane around the poles (Fig. 5F).

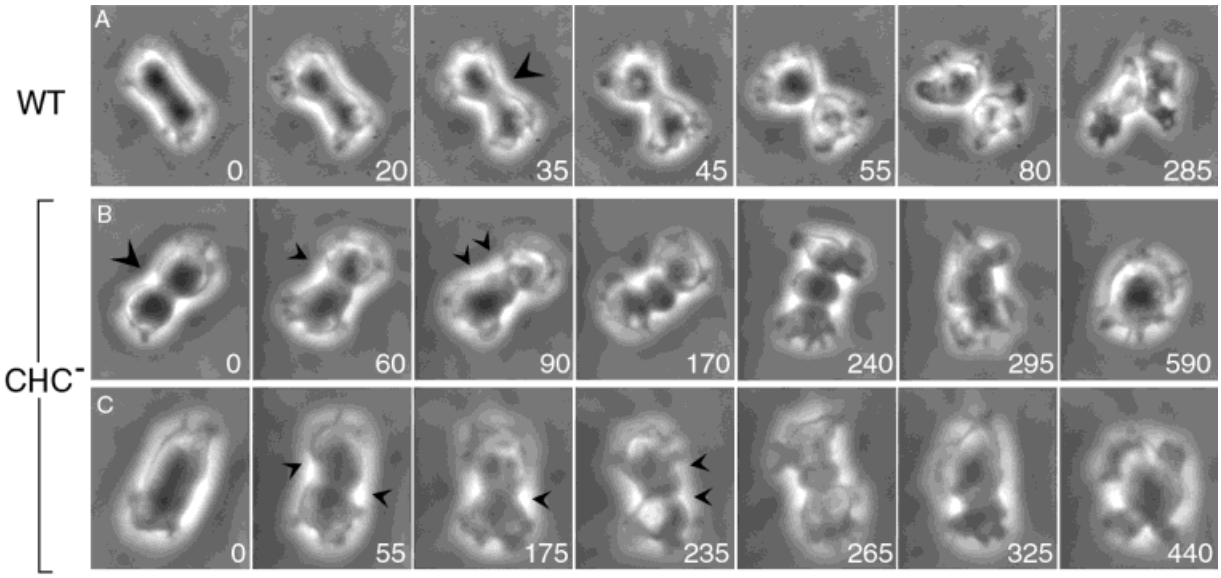

Fig. 3. Clathrin null cells form equatorial bulges and fail to divide in suspension. **A:** Wild-type cells (n = 10) in suspension conditions form an equatorial cleavage furrow that quickly constricts (*large arrowhead*, 35 sec). These cells cannot achieve the traction necessary to crawl away from one another and tumble around each other. **B:** Some clathrin null cells in suspension (11/16) initiate a single equatorial furrow (*large arrowhead*, 0 sec). However, the cells do not constrict this furrow, or maintain its equatorial position (*arrowhead*, 60 sec). A second site of constriction causes the equatorial region to bulge (90–170 sec). The bulge remains visible throughout minutes of violent cell shape changes and extensions, but in the end cytokinesis fails (590 sec). **C:** Some clathrin null cells (5/16) initiate two regions of constriction from the earliest stages of cytokinesis (*arrowheads*, 55 sec). The equatorial bulge grows as both regions alternately constrict and relax (*arrowheads*, 235 sec). Despite their initial morphology, all mitotic clathrin null cells in suspension bulge and fail to divide.

Interestingly, in a subset of cells (6/28), GFP-myosin II was absent from the constricting furrow at the equator of the cell (Fig. 5J–L). Instead, GFP-myosin II localized to regions of the plasma membrane at either side of the equatorial midline (Fig. 5L). All of these cells were at late stages of cytokinesis.

Since we found that the cytokinesis defect of clathrin null cells was much more penetrant in suspension, we decided to investigate the distribution of myosin II in these conditions. We fixed cells expressing GFP-myosin II in suspension and observed them by epifluorescence microscopy. In contrast to other reports in the literature [Neujahr et al. 1997a], non-attached wild-type cells (n = 25) localized GFP-myosin II to the plasma membrane at the center of an equatorial furrow (Fig. 6A–C). Wild-type cells fixed at the early stages of cytokinesis localized GFP-myosin II to the equator. Cells fixed at mid stage or late-stage furrow progression localized GFP-myosin II to the most constricted region of the cell.

Consistent with our observations on adherent cells, clathrin null cells fixed in suspension displayed sparse and poorly organized GFP-myosin II on the cell cortex (n = 27) (Fig. 6F,I,L). Similar to adherent cells, the clathrin null cells localized myosin II to regions of plasma membrane constriction, but myosin II was neither strongly concentrated nor restricted to the area of constriction.

Some mitotic clathrin null cells (5/24) without cell adhesion displayed early-stage or mid-stage, single furrows at their equators. These cells localized GFP-myosin II to their furrows, although the GFP-myosin II was less concentrated than in wild-type cells (Fig. 6D–F). The majority of the mitotic clathrin null cells fixed in suspension displayed evidence of two furrows. In these cells, myosin II was somewhat concentrated to the two constrictions, but its localization was patchy and also found in the polar regions (Fig. 6I,L,N). The association of myosin II with the bulging midpoint was variable: in some cases myosin II was excluded from the bulge (Fig. 6I,N) and in other cases not (Fig. 6L). The abnormal myosin II localization in clathrin null cells was not an artifact caused by the GFP fusion protein because identical results were obtained by immunofluorescence. Cells lacking GFP-myosin II were fixed in suspension and stained for myosin II. Wild-type cells localized myosin II to an equatorial cleavage furrow (Fig. 6M). Clathrin null cells displayed myosin II localization in an asymmetrical strip near the equator accompanied by abnormal localization at the poles (Fig. 6N).

Indirect immunofluorescence using anti-myosin II antibodies showed that the assembly of myosin II into a functional contractile ring was abnormal in clathrin-null cells. To examine the dynamic distribution of GFP-myosin II, we also observed live cells using confocal mi-

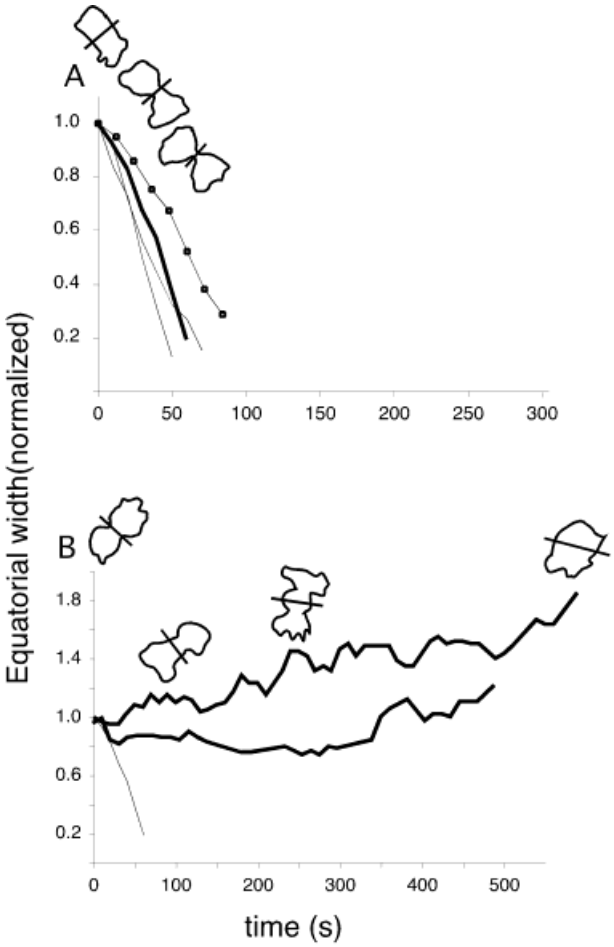

Fig. 4. Clathrin null cells in suspension do not significantly constrict the equatorial region. Equatorial width was measured over time in cells under suspension conditions. **A:** Wild-type cells in suspension furrow at a rate similar to adherent wild-type cells. Measurements for an attached cell (*circles*) were co-plotted with three cells in suspension (*no circles*). (The *bold line* represents the cell depicted in Figure 3A). **B:** The equatorial width of clathrin null cells (*bold lines*) depicted in Figure 3B and C show a net increase over time. A wild-type cell in suspension is co-plotted (*fine line*) for comparison.

croscopy. In wild type cells, at the start of cytokinesis, GFP-myosin II localized to the equatorial region of the elongated cell (Fig. 7A, 0 sec). As cytokinesis progressed, myosin II remained tightly associated with the constricting furrow (Fig. 7A, 75 sec). Once division was completed, myosin was visible at the rear of the daughter cells as they crawled away from one another (Fig.7A, 270 sec).

Similar to the fixed cells, live clathrin null cells (n = 11) displayed an abnormal and patchy GFP-myosin II localization compared to wild-type cells. Five cells completed cytokinesis successfully. In one of these cells, myosin II was visible but not heavily concentrated in the equatorial region of the early furrow (Fig. 7B, 15–75 sec). As the furrow progressed, myosin II appeared to be more localized to two regions of the plasma membrane that flanked the equatorial mid-line (Fig. 7B, 105–120 sec). These regions of myosin II localization constricted, while the less brightly stained equatorial region bulged out slightly (Fig. 7B, 105 sec, arrow). As cytokinesis progressed, the GFP-myosin II remained most visible at the two constrictions on both sides of the equatorial midline. These regions of myosin localization coincided with the retracting backs of the daughter cells after the separation of the cytoplasmic bridge.

Figure 7C shows a clathrin null cell that became partially detached from the substrate and did not complete cytokinesis but tried several times to establish a cleavage furrow over 15 min. Initially, myosin II was localized to the cortex of the cell and was visible in the incipient cleavage furrow (Fig. 7C, 0 sec). However, as cytokinesis proceeded, a bulge emerged from the middle of the cell. This bulge lacked perceptible myosin II (Fig. 7C, 90 sec, arrow). Subsequently, myosin II was associated with the cortical regions adjacent to the equator of the cell (Fig. 7C, 210 sec). Ultimately, cytokinesis failed and myosin II resumed an uneven and diffuse distribution on the cortex of the cell (Fig. 7C, 285–420 sec, arrows).

DISCUSSION

Previous work established an essential role for clathrin in cytokinesis [Niswonger and O'Halloran, 1997b]. To better understand this novel role, we observed cells undergoing cell division in a variety of conditions. This analysis revealed that myosin II can drive constrictions during cytokinesis in clathrin null cells. However, the loss of clathrin function profoundly affects both the dynamic placement and assembly of a persistent and functional contractile apparatus.

Abnormal Cleavage Furrows in Clathrin Mutant Cells

While clathrin null cells have a complete cytokinesis defect when grown in suspension, cells can be propagated when attached to a substrate [Niswonger and O'Halloran, 1997b]. We first assumed that these cells divided on a substrate by a myosin II-independent process similar to that found in myosin-null mutants [Neujahr et al., 1997b]. However, upon close inspection we found that the clathrin null cells could begin to form a cleavage furrow even in suspension, where myosin II is indispensable for furrowing. Most frequently, the clathrin null cells established an equatorial furrow initially, but the maintenance and successful constriction of this furrow were impaired. Often, the furrow "drifted" away from the equatorial mid-line towards one of the poles and

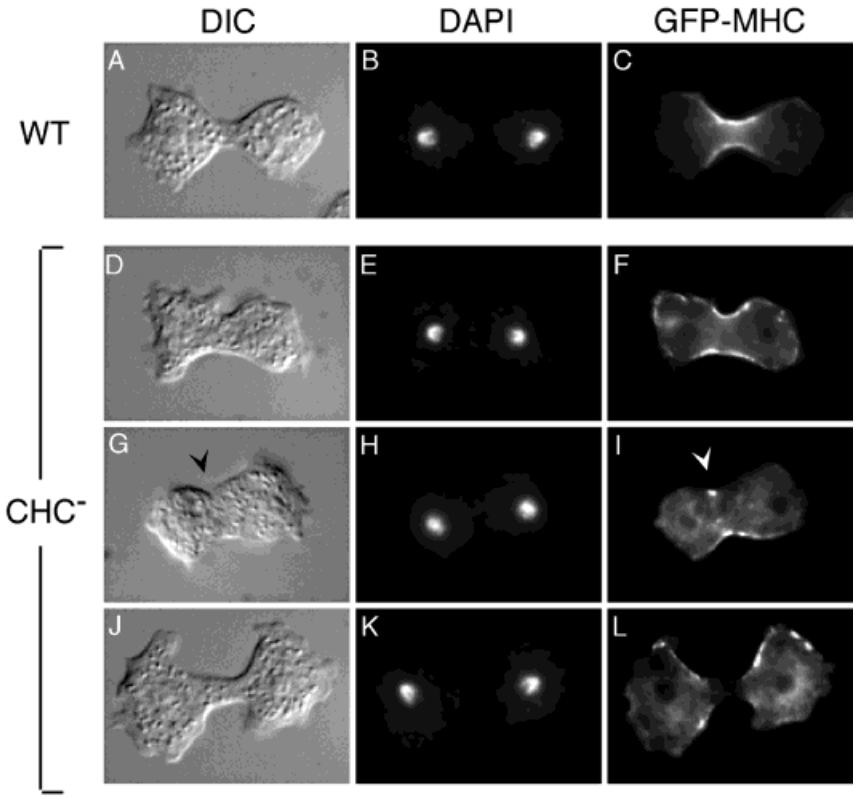

Fig. 5. Clathrin null cells fixed on a substrate display a range of myosin II mislocalization during cytokinesis. **A–C:** Fixed wild-type cells strongly concentrate GFP-myosin II to the cleavage furrow (n = 27). **D–F, G–I:** 79% (22/28) of clathrin null cells generally localized GFP-myosin to a furrow or region of constriction. However, GFP-myosin II was not tightly restricted to these regions, and the overall GFP-myosin signal was punctate compared to wild-type cells. **G–I:** Clathrin null cell with slightly asymmetrically placed constriction. Punctate GFP-myosin signal is most localized to regions of constriction (G, I, *arrowheads*). **J–L:** 21% (6/28) of adherent clathrin null cells did not have a clear localization of myosin to the middle of the furrow. All 6 of these cells were fixed at a very late stage of furrow constriction.

a second furrow formed near the opposite pole of the cell. In other instances, two furrows were formed from the beginning of cytokinesis. In either case, the region between the furrows formed a large "bulge" that changed in volume depending on the degree of constriction of the surrounding furrows. While attached cells often managed to divide despite their bulging equators, cells without adhesion rounded up and failed in cytokinesis.

These observations indicate that the mechanisms that establish a cleavage furrow are still operational in the absence of clathrin. However, it is clear that clathrin mutant cells cannot restrict the number and position of cleavage furrows.

Relationship of Myosin II to Abnormal Furrowing in Clathrin-Null Cells

Without myosin II, cells cannot form furrows or constrictions unless they are adhered to the substrate [Zang et al., 1997]. Thus, the presence of constricting equators in clathrin null cells dividing in suspension suggested that myosin II drove these abnormal furrows. Localization studies of myosin II during cytokinesis confirmed the presence of this protein at sites of constriction. In early stages of cytokinesis, myosin localized to the slightly furrowed equator, albeit less concentrated than wild-type cells. Myosin II was also found in the two constrictions that developed later in cytokinesis. These results provide strong evidence that myosin II is responsible for furrowing in clathrin null cells.

Despite their ability to form constrictions that contained myosin II, clathrin null cells displayed significant defects in furrowing. Whether attached or in suspension, wild-type cells ingress their furrows at a constant rate [Sabry et al., 1997; Zang and Spudich, 1998]. Almost all clathrin null cells attached to a substrate were able to achieve a rate of equatorial ingression similar to that of wild-type cells. However, these cells could not maintain a constant rate and their furrowing often slowed dramatically. Similarly, without attachment, clathrin null cells could initiate furrowing but then their furrows subsequently regressed and cytokinesis failed. This suggests that the defect in cytokinesis occurs at some point after the initiation of furrow ingression.

How can we explain the constrictions and bulges that are so striking in the later stages of the mutant phenotype? We propose that the dual furrows occur when the presumptive daughter cells localize myosin II to their posterior ends after failure of the contractile ring. This scenario predicts that the signals and cues used to localize myosin II to the backs of daughter cells are independent of those driving the contractile ring.

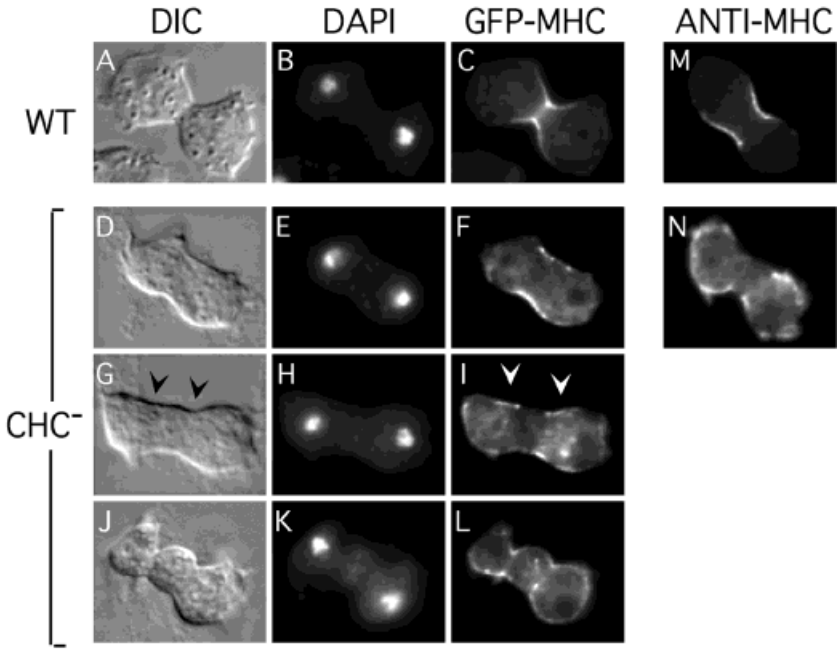

Fig. 6. Myosin II localizes to points of constriction during clathrin null cytokinesis failure in suspension. **A–C:** Wild-type cells fixed in suspension show strong GFP-myosin II concentration at the furrow (n = 25). **D–F:** 21% (5/24) of clathrin null cells were fixed with an early or mid-stage equatorial furrow. These cells localized GFP-myosin II to the equator in a punctate pattern. However, most mitotic clathrin null cells in suspension were fixed in various stages of bulge formation. **G–I:** 50% (12/24) of clathrin null cells displayed two sets of membrane constrictions on either side of the equator (G, *arrowheads*). In half these examples, GFP-myosin was dispersed throughout the equatorial region. In the remaining cells, GFP-myosin was visible in two strips on either side of the equator. In these cases, myosin II localization coincided with the membrane constrictions (I, *arrowheads*). **J–L:** 29% (7/24) of the clathrin null cells in suspension were fixed with extreme equatorial bulges. Although visible at the poles, GFP-myosin was localized to the points of constriction, and was not concentrated at the equatorial bulge. **M–N:** Cells lacking GFP fusion constructs were fixed in suspension, and stained for myosin II by immunofluorescence. Similar to GFP observations, wild-type cells localized myosin II to the center of the furrow (M). In clathrin null cells, myosin II localization was not restricted to the equatorial region, and often appeared in strips on either side of the equator (N).

Several observations lend support to this hypothesis. First, similar to wild-type cells, migrating clathrin null cells efficiently localize myosin to their posterior end [Niswonger and O'Halloran, 1997b]. Second, clathrin null cells with pronounced double constrictions often have decondensed nuclei typical of cells that have progressed beyond cytokinesis. Normally, decondensed nuclei are found in daughter cells that have already separated and display a strong localization of myosin II at their posterior end [Fukui and Inoue, 1991]. Third, we have never observed cells that have more than two clearly defined furrows. If the defect were simply the result of the inability to restrict the total number of cleavage furrows, then we might expect to see multiple furrows.

What Is the Role of Clathrin in Cytokinesis?

Clathrin is a major participant in membrane traffic. Why does its loss have such significant impact on the action of the actomyosin cytoskeleton during cytokinesis? It is evident that membrane traffic plays a crucial role in cytokinesis since multiple trafficking proteins are required in cell division [O'Halloran, 2000]. For example, cytokinesis fails when the function of t-SNARE syntaxin is disturbed in *C. elegans* embryos [Jantsch-Plunger and Glotzer, 1999], *Drosophila* cellularization [Burgess et al., 1997], sea urchin embryos [Conner and Wessel, 1999], and plants [Lauber et al., 1997].

The simplest model postulates that clathrin is necessary for the remodeling of specific proteins or lipids at the membrane of the cleavage furrow. In this case, the loss of clathrin may alter the composition of membrane proteins at the furrow thereby affecting the contractile ring assembly and constriction. Moreover, the addition or removal of membrane surface could be necessary for furrow ingression. To directly address this question for clathrin function in *Dictyostelium*, a GFP-tagged clathrin that functionally rescues the null phenotype was observed in both live and fixed dividing cells. This tagged clathrin did not concentrate on the membrane of the cleavage furrow [Damer and O'Halloran, 2000]. Although a membrane interaction event might be rapid

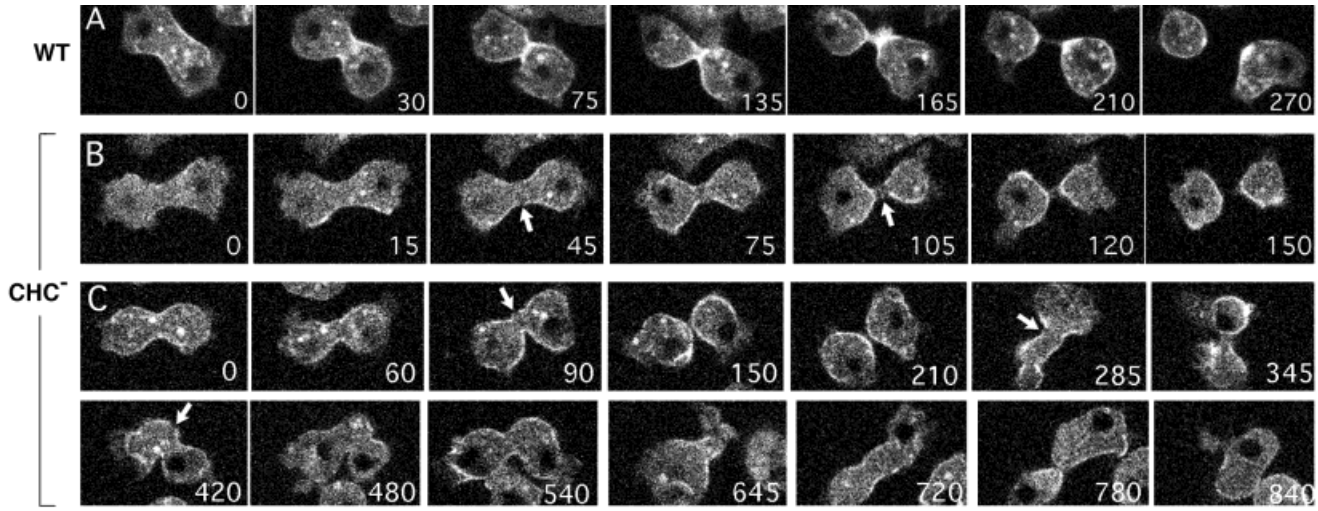

Fig. 7. Live dynamics of myosin II distribution in clathrin null cells. Adherent wild-type and clathrin null cells expressing GFP-myosin II were imaged every 15 sec using a confocal microscope. **A:** Wild-type cell exhibited tight GFP-myosin II localization to the cleavage furrow. **B:** Clathrin null cell completed cytokinesis with no perceptible increase of GFP-myosin II to the cleavage furrow (0–105 sec, *arrow*). However, GFP-myosin localized to the cortex on each side of the cleavage furrow and the backs of the resulting daughter cells (120–150 sec). **C:** Clathrin null cell became partially detached from the substrate and did not complete cytokinesis. GFP- myosin II was absent from small areas of the equatorial region (90, 285, 420 sec, *arrows*) and showed uneven cortical distribution (345–840 sec).

enough to be undetected by these techniques, this result strongly suggests that clathrin's function during cytokinesis happens at some place other than the membrane of the cleavage furrow.

Alternatively, the role of clathrin in cytokinesis could involve a more global function, which might not require an obvious clathrin localization at the cleavage furrow. Since there is evidence of cross-talk between membranous networks in *Dictyostelium* [Bush et al., 1996; O'Halloran and Anderson, 1992], loss of clathrin might affect the function of normally clathrin independent membrane processes. For example, newly synthesized phospholipids are transferred to the plasma membrane during cell streaming via a special population of uncoated vesicles [De Silva and Siu, 1981]. Mis-sorting of these vesicles, or any other perturbation in lipid traffic to the plasma membrane could have direct effects on cytokinesis.

Lipid composition alone can have effects on the curvature and internalization dynamics at the plasma membrane [Zha et al., 1998]. Furthermore, the correct distribution of phosphatidylethanolamine has been shown to be necessary both for furrow membrane ingression and the function of the contractile ring [Emoto et al., 1996; Emoto and Umeda, 2000]. This evidence highlights the importance of a particular composition of lipids for the membrane of the cleavage furrow. Depleting cells of clathrin could alter lipid traffic and thus affect the function of the cleavage furrow.

Recent studies suggest an interesting link to a novel membrane traffic pathway. The *Dictyostelium* LvsA protein is a novel protein required for cytokinesis and is related to the trafficking protein beige [Kwak et al. 1999]. Interestingly, lvsA mutants display the same double furrow defect as the clathrin null cells (Gerald and De Lozanne, personal observations). Furthermore, both mutations affect the structure and function of the contractile vacuole and as a result the mutant cells become osmosensitive. This suggests that LvsA and clathrin may participate in a common membrane traffic pathway that is required in cytokinesis. Investigation of the interplay between membrane traffic and cytoskeletal organization in *Dictyostelium* and other systems will provide a better understanding of the molecular basis of cytokinesis.

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