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Myosin II-independent processes in mitotic cells of *Dictyostelium discoideum*: redistribution of the nuclei, re-arrangement of the actin system and formation of the cleavage furrow

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SUMMARY

Mitosis was studied in multinucleated and mononucleated mutant cells of *Dictyostelium discoideum* that lack myosin II (Manstein et al. (1989) *EMBO J.* 8, 923-932). Multinucleated cells were produced by culture in suspension, mononucleated cells were enriched by growth on a solid surface (DeLozanne and Spudich (1987) *Science* 236, 1086-1091). The multinucleated cells disclosed interactions of mitotic complexes with the cell cortex that were not apparent in normal, mononucleated cells. During the anaphase stage, entire mitotic complexes consisting of spindle, microtubule asters, and separated sets of chromosomes were translocated to the periphery of the cells. These complexes were appended at a distance of about 3 μm from the cell surface, in a way that the spindle became orientated in parallel to the surface. Subsequently, lobes of the cell surface were formed around the asters of microtubules. These lobes were covered with tapered protrusions rich in coronin, an actin associated protein that typically accumulates in dynamic cell-surface projections (DeHostos et al. (1991) *EMBO J.* 10, 4097-4104). During their growth on a solid surface, mononucleated myosin II-null cells passed through the mitotic cleavage stages with a speed comparable to wild-type cells. Cytokinesis as linked to mitosis is distinguishable by several parameters from traction mediated cytofission, which results in the pinching off of pieces of a multinucleated cell in the interphase. The possibility is discussed that cells can cleave during mitosis without forming a contractile ring at the site of the cleavage furrow.

Key words: *Dictyostelium*, Mitosis, Myosin, Microtubule function

INTRODUCTION

During mitotic division, various activities of a eukaryotic cell are coordinated to guarantee equal distribution of chromosomes to the daughter cells, and to separate these cells from each other. Coordination of chromosome condensation and replication, organisation of the spindle and of the asters of microtubuli at the spindle pole bodies, segregation of the chromosomes by tubulin-based molecular motors, and formation of a cleavage furrow in the cell cortex, need to take place in the right temporal and spatial order. Finally, constriction of the cleavage furrow leads to separation of the daughter cells.

The question of how a cell cleaves during mitosis and how this cleavage is linked to separation of the chromosomes is being addressed in cells of *Dictyostelium discoideum* by the search for proteins that are localised to specific sites in dividing cells, and by the analysis of mutants that are impaired in cytokinesis. Three proteins of the actin system have been reported in *D. discoideum* to accumulate at specific loci in the cortical region of mitotic cells. Myosin II, the conventional double-headed myosin, becomes enriched, under certain conditions, in the form of a ring in the cleavage furrow (Fukui et al., 1989, 1990; Yumura et al., 1984; Yumura and Fukui, 1985). Actin is also accumulated in that ring and produces filaments that extend from the furrow towards the bodies of the incipient daughter cells (Fukui and Inoué, 1991). In addition, actin is enriched in protrusions that are formed at the distal regions of these cells. The third protein, coronin, becomes specifically concentrated at these distal regions (Gerisch et al., 1995). Coronin, a member of the WD40-repeat family of proteins, has previously been shown to play a role in the dynamic changes of the actin system that are crucial for the rapid motion of *Dictyostelium* cells (Gerisch et al., 1995; DeHostos et al., 1993).

The role of myosin II and coronin in cytokinesis has been analysed by the use of mutants that lack either one of these proteins. The most characteristic feature of myosin II-null cells is their inability to divide in suspension (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). Without a supporting surface, these mutant cells grow up into large masses of cytoplasm that are not subdivided by membranes and may contain 50 or more nuclei before they eventually die. When attached to a solid surface, these giant cells are capable of dividing by ‘traction-mediated cytofission’ (Spudich, 1989). This type of pseudocleavage is not coupled to mitosis (Fukui et al., 1990). It is based on the forces exerted by multiple leading edges in a multinucleated cell; since each edge continues to move on the surface to which the cell is attached, portions of the cell are pulled into different directions, hence

causing these portions to be drawn out into thin cytoplasmic bridges. Sometimes these bridges become disrupted, giving rise to smaller cells with an irregular number of nuclei (DeLozanne and Spudich, 1987; Manstein et al., 1989) or even without a nucleus (Warrick and Spudich, 1987).

Cells lacking coronin tend to become multinucleated, similar to myosin II-null cells, but the increase in cell size and number of nuclei is less dramatic in coronin-null cells when these cells grow in suspension rather than attached to a surface (DeHostos et al., 1993). The difference in anchorage dependence between myosin II-null and coronin-null cells has been interpreted to signify that myosin II plays a role in contraction of the cell at the cleavage furrow, an activity that is essential in suspension, and that coronin plays a role primarily in enhancing cell motility, which assists in separating the daughter cells on a substrate surface (DeHostos et al., 1993).

By analysing the F-actin and coronin distribution in mononucleated myosin II-null cells, we recognised shape changes in these cells that are linked to mitosis. The mutant cells turned out to be capable of cleaving by furrow formation when they are anchored on a substratum. This process will be designated as 'attachment assisted mitotic cleavage' in order to distinguish it from the traction-mediated cytofission, that is not coupled to mitosis and in interphase cells results in irregular fragmentation of the cell body.

In the giant, multinucleated cells produced in suspension cultures of myosin II-null mutants, nuclei divide synchronously (Fukui et al., 1990). In a search for periodic changes in the actin system that concur with the cell cycle, we realised that the giant cells are valuable tools to study interactions of the mitotic apparatus with the cell cortex. Nuclei were found to be translocated during mitosis from the center to the periphery of these cells, together with the spindle pole bodies and the system of microtubules linked to them. This translocation of entire mitotic complexes in the course of anaphase opened the possibility of studying cell-surface changes that are elicited at sites where these complexes become appended.

MATERIALS AND METHODS

Cell culture

Dictyostelium discoideum strains AX2-214 and AX4 were cultivated at 21-23°C axenically in nutrient medium to a density of not more than 5×10^6 cells per ml. The myosin II gene replacement mutant HS2205, a derivative of the AX2-214 strain (Manstein et al., 1989), was obtained from Dr Dietmar Manstein (MPI für Medizinische Forschung, Heidelberg), the myosin II gene disruption mutant producing heavy meromyosin (HMM) (DeLozanne and Spudich, 1987) was a gift from Dr James A. Spudich, Stanford University. The absence of revertants in the gene disruption mutant was confirmed by labelling aliquots of cells used for the experiments with myosin II-specific mAb 56-396-5. To obtain a high yield of mononucleated cells, the mutants were cultivated in plastic Petri dishes containing nutrient medium. To prepare large multinucleated cells, HS2205 was cultivated for 36 hours in shaken suspension.

The cells were synchronised by harvesting them from nutrient medium by gentle centrifugation (4 minutes at 130 ***g***) and resuspending them in 17 mM K/Na-phosphate buffer, pH 6.0. After shaking at 150 rpm at 21-23°C for 3 hours, the cells were allowed to settle on an HCl-cleaned coverslip for 30 minutes. Subsequently the phosphate buffer was replaced by nutrient medium. After 3 to 4 hours the synchronised population showed a high percentage of mitotic cells. Care was taken to avoid washing of the buffer flasks in a dishwasher, to avoid any interference of polyphosphates with cell attachment to the glass surface.

Wild-type NC4 was cultivated on SM agar plates on lawns of *Klebsiella aerogenes*. For synchronisation, NC4 cells collected from the borders of the colonies were suspended in the K/Na-phosphate buffer, washed in the buffer and shaken for 3 hours. To record mitosis under similar conditions as for axenically cultivated cells, cells were allowed to settle on a glass surface, and the buffer was replaced by nutrient medium supplemented with *K. aerogenes* bacteria and penicillin-streptomycin to limit their growth.

Labelling with antibodies, phalloidin or DAPI

For indirect immunofluorescence labelling the synchronised cells were fixed with picric acid/formaldehyde for 20 minutes at room temperature and post-fixed with 70% ethanol as described by Jungbluth et al. (1994), and subsequently processed for immunofluorescence labelling according to the method of Humbel and Biegelmann (1992). In the control experiment shown in Fig. 7, cells were fixed in methanol at –20°C for 10 minutes.

The following monoclonal antibodies were used to visualise protein distribution: mouse anti-coronin mAb 176-3-6 (DeHostos et al., 1993), mouse anti-myosin II mAb 56-396-5 (Claviez et al., 1982; Pagh and Gerisch, 1986), and rat anti-α-tubulin mAb YL-1/2 (Kilmartin et al., 1982), purchased from Dunn Labortechnik (53567 Asbach, Germany). As second antibodies, FITC- or CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, Pennsylvania, USA) or, in the case of α-tubulin, FITC- or CY3-conjugated goat anti-rat IgG antibodies (Sigma, St Louis, MO, USA) were used. F-Actin was labelled with TRITC-conjugated phalloidin (Sigma). Nuclei were stained for DNA with 4,6-diamidino-2-phenylindole (DAPI) purchased from Sigma.

For conventional fluorescence microscopy, an Axiophot microscope (Zeiss) equipped with a ×100/1.3 Plan-NEOFLUAR objective was used and the labelled cells were photographed on Fuji Neopan 400 ASA film. Confocal microscopy was performed on a Zeiss LSM 410. Digitalised data from the confocal sections were analysed and prepared for printing with the graphic software system AVS (Advanced Visual Systems Inc., Waltham, MA, USA). For colour printing a Tektronix Phaser IISD printer was used.

In vivo microscopy

For the recording of mitosis, cells were placed in 5 ml nutrient medium into an open chamber, which consisted of a plastic ring with an inner diameter of 40 mm, mounted with paraffin on a glass coverslip of 50 mm × 50 mm. The chamber was fixed on the stage of an inverted microscope.

Video recording was performed at 23°C using a Zeiss Axiovert 135 TV inverted microscope with a ×100/1.3 Plan-NEOFLUAR objective, in combination with a CCD video camera and a Panasonic AG-6720A time-lapse video recorder. Micrographs were taken either on this microscope or on a Zeiss IM 35 inverted microscope with a ×100/1.3 Plan-Apochromat objective, using a Contax 167 MT camera and Fuji Neopan 400 ASA films for black and white prints.

RESULTS

Mitosis and traction-mediated cytofission in multinucleated myosin II-null cells

Myosin II-null cells were cultivated in suspension in order to produce multinucleated cells. For the synchronisation of mitosis, these cells were starved for 3 hours in a phosphate buffer and then re-transferred to nutrient medium, which was

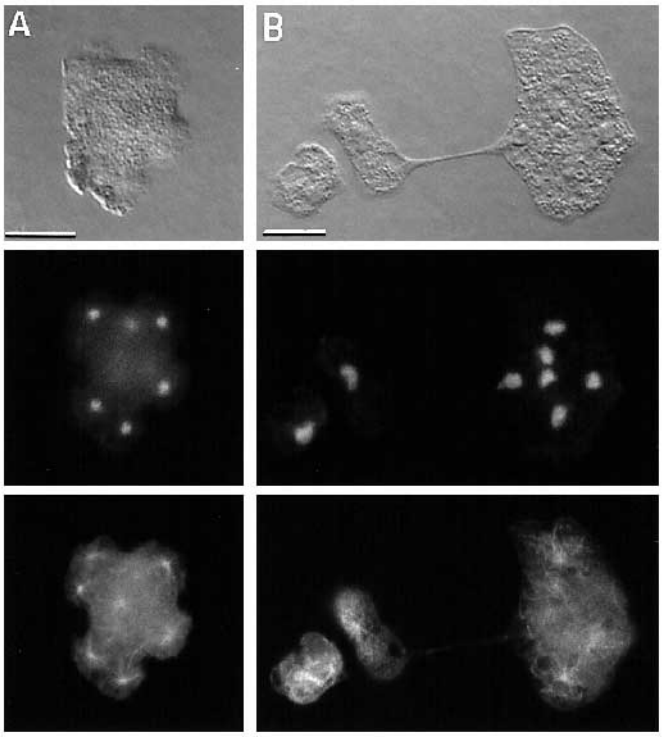

Fig. 1. Comparison of mitosis (A) and cytofission (B) in multinucleated myosin II-null cells. Fixed cells were stained with DAPI and labelled with antibody for α-tubulin. The panels show DIC images to visualise cell shape (top), DAPI staining of DNA (middle), and arrangement of microtubules (bottom). The cell in A displays typical features of a multinucleated cell in late anaphase. Its surface is subdivided into lobes. The separated sets of chromosomes form compact, DAPI-stained clusters. The microtubule system consists of asters arising from spindle pole bodies that are located in the center of the lobes, and of rod-like spindles. The cell in B contains seven nuclei, one of them residing in a fragment that is connected with the major portion of the cell through a dilated thread of cytoplasm. All nuclei are in the interphase state, as is the single nucleus in the small cell on the left. The nuclei are large and appear irregular in shape. Several microtubule systems centered to MTOCs in the vicinity of nuclei are distinguishable, although the microtubules of these systems are often interpersed. Bars, 10 μm.

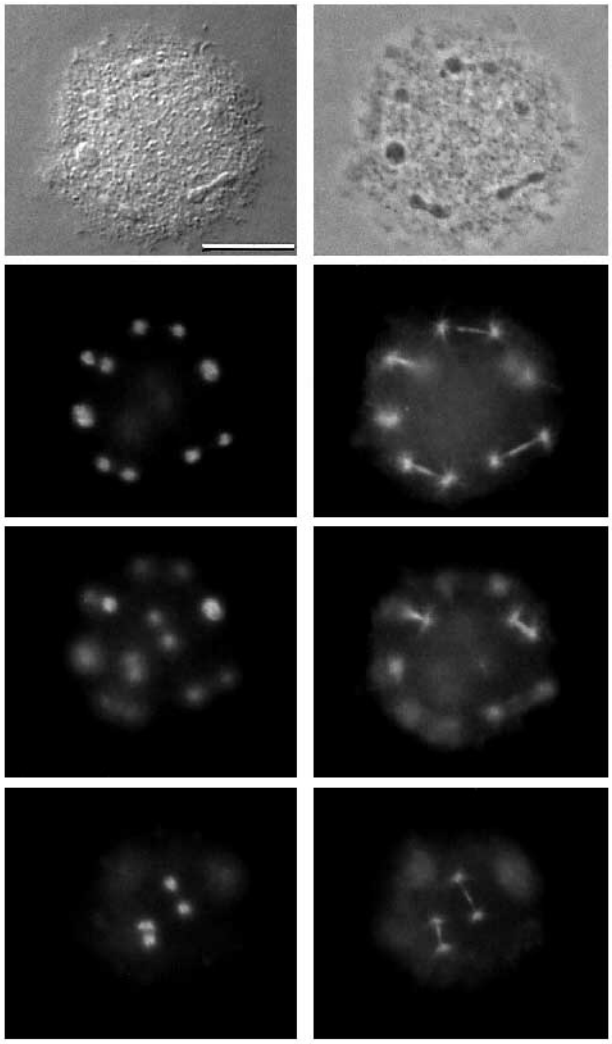

Fig. 2. Array of spindles beneath the cell surface of a mitotic multinucleated cell. A myosin II-null cell fixed in anaphase was stained with DAPI and labelled with α-tubulin antibody. This cell contained eight mitotic complexes, each consisting of two sets of chromosomes, two asters, and one spindle. Positions of some of these complexes relative to the border of the cell is recognisable with DIC or phase contrast optics (left and right panels on top). Fluorescence images were taken at three different planes of focus from the lower to the upper portion of the cell (panels from top to bottom). In the left row, DAPI stained clusters of chromosomes are distinguishable. In the right row, orientation of the spindles can be identified. All the spindles are located in the peripheral region of the cell. Two mitotic complexes, which in vertical projection seem to be located in the central region, are in fact near to the upper cell surface. In the middle of each spindle, a thickened zone is visible, which corresponds to the region of overlap between microtubules of opposite polarity (Moens, 1976; McIntosh et al., 1985). Bar, 10 μm.

poured as a fluid layer on a glass coverslip. After 3 to 4 hours, a maximal number of cells in various mitotic stages was obtained. At this time the cells were fixed, stained with DAPI, and labelled for α-tubulin, F-actin, or coronin.

Since stages of mitosis and traction-mediated cytofission were present in the same preparations, they could be immediately compared. Fig. 1A illustrates two characteristics of a multinucleated myosin II-null cell near to the end of mitosis. First, the center of the cell is depleted of nuclei, which are concentrated in a layer about 3 μm beneath the cell surface. Second, the surface is folded around the asters of microtubules in a way that the spindle pole bodies become located in the middle between the folds. Fragmentation into portions that are connected by extended cytoplasmic bridges, as shown in Fig. 1B, is characteristic of cells undergoing cytofission. Staining with DAPI revealed multiple nuclei in the interphase state,which were often concentrated in the central region of the cells. In accord with the presence of several nuclei, separate systems of microtubules, each arising from one MTOC, were distinguishable. Video recording confirmed that cells of a shape as shown in Fig. 1B can give rise to completely separated fragments. More often, however, the threads of cytoplasm connecting the portions of such a cell became shortened after a while, and the portions fused together into one compact body.

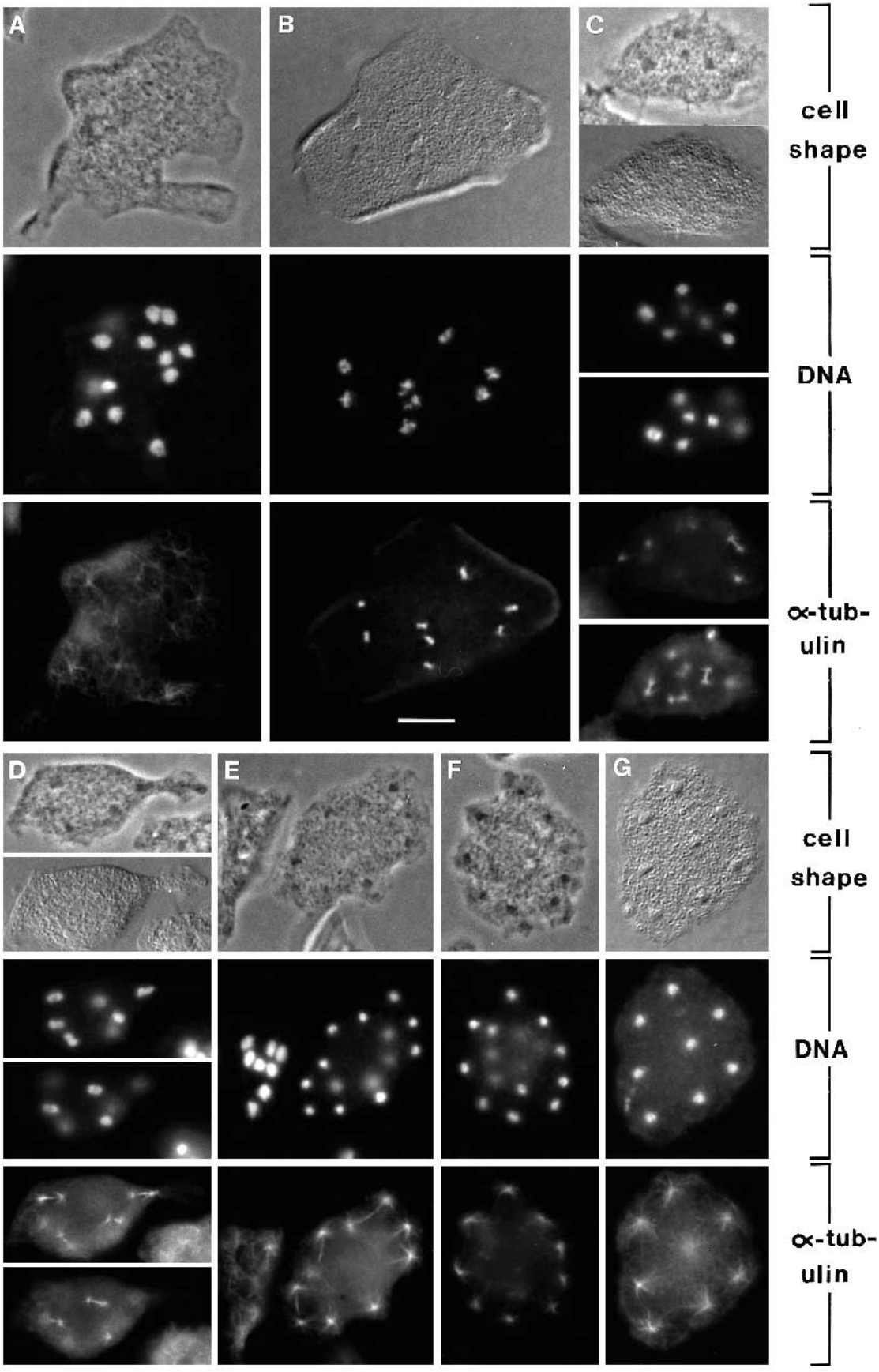

Fig. 3. Series of mitotic stages revealing translocation of mitotic complexes to the periphery of multinucleated myosin II-null cells. (A) A reference cell in interphase, (B) a cell in metaphase, (C,D) cells in early anaphase, (E) a cell in late anaphase, and (F,G) cells in telophase. The top panel of each triple set shows a cell in phase contrast (A,E,F) or DIC optics (B,G), or in both (C,D). The middle panel of each set displays nuclei or chromosomes stained with DAPI, and the bottom panel organisation of the microtubule system, as revealed by labelling with α-tubulin specific antibody. In C and D, pairs of fluorescence images focussed to lower and upper portions of the cells are shown (upper and lower micrographs). The cell near to the left border of E allows immediate comparison of the positions and sizes of nuclei in an interphase cell with the mitotic cell on the right. Bar, 10 μm.

Re-localisation of nuclei and orientation of spindles in multinucleated myosin II-null cells

Analysis of the cell shown in Fig. 1A suggests that in the course of mitosis, nuclei are being localised to the periphery of a multinucleate cell, and spindles become orientated relative to the cell surface. Micrographs taken at different planes of focus through a cell in late anaphase established this localisation and orientation of mitotic complexes. The central region of the cell shown in Fig. 2 is depleted of nuclei, and the long axes of the spindles are in a position parallel to the cell surface.

Stages of the re-localisation of nuclei are compiled in Fig. 3. Mitotic stages were put in an order of succession according to the length of the spindles and the distances between the two clusters of segregated chromosomes (McIntosh et al., 1985; Fukui and Inoué, 1991). As a reference, a multinucleated myosin II-null cell in the interphase state is shown (Fig. 3A). This cell displays the typical location of nuclei in its central region and contains multiple systems of microtubules. During metaphase and early anaphase, translocation of nuclei was not yet obvious (Fig. 3B-D); the translocation became clearly recognisable during the late anaphase stage (Fig. 3E). The entire complexes of spindle, immature asters, and chromosomes became translocated to the periphery of the cells. In parallel to the translocation of these complexes, the microtubules in the asters increased in length. After arrival of the mitotic complexes at the periphery, the cell surface became folded into lobes around the asters of microtubules (Fig. 3F,G). These lobes were separated by folds, that indented the cell surface either on top of the spindles or at areas between the individual mitotic complexes (Fig. 3E).

Localisation of coronin relative to microtubule asters in the multinucleated cells

The spatial relationship between asters and sites of coronin accumulation in multinucleated myosin II-null cells was analysed by double labelling with anti-coronin and anti-α-tubulin antibodies. Each aster of microtubules arising from a spindle pole body tended to reach a symmetrical arrangement relative to the cell surface, with the spindle forced to point towards the center of the cell. The fact that a spindle connects two asters prevents it, however, from assuming this orientation. As a consequence, spindles were bent as seen in Fig. 4A.

Confocal sections revealed that the positions of asters at telophase coincided with areas of coronin accumulation in the cell cortex. The coronin-rich areas were spread over the ends of the microtubules that formed the asters (Fig. 4). No accumulation of coronin was apparent in the folds between the asters. The translocation of coronin from the cytoplasm to specific sites beneath the cell surface indicates that the cell cortex is organised during mitosis in conjunction with the microtubule system.

Characteristics of cytokinesis in mononucleated myosin II-null cells

Mitotic stages were recognisable in mononucleated myosin II-null mutant cells by two criteria. (1) Since *D. discoideum* lacks a G_1 phase (Weeks and Weijer, 1994), the segregated sets of chromosomes in the anaphase and telophase can be distinguished in DAPI stained preparations by their smaller size and half the DNA content of interphase nuclei. (Chromosomes are hard to distinguish during anaphase under the conditions used.) (2) In meta- and anaphase, cytoplasmic microtubules are replaced by the spindle, and by asters of microtubules arising

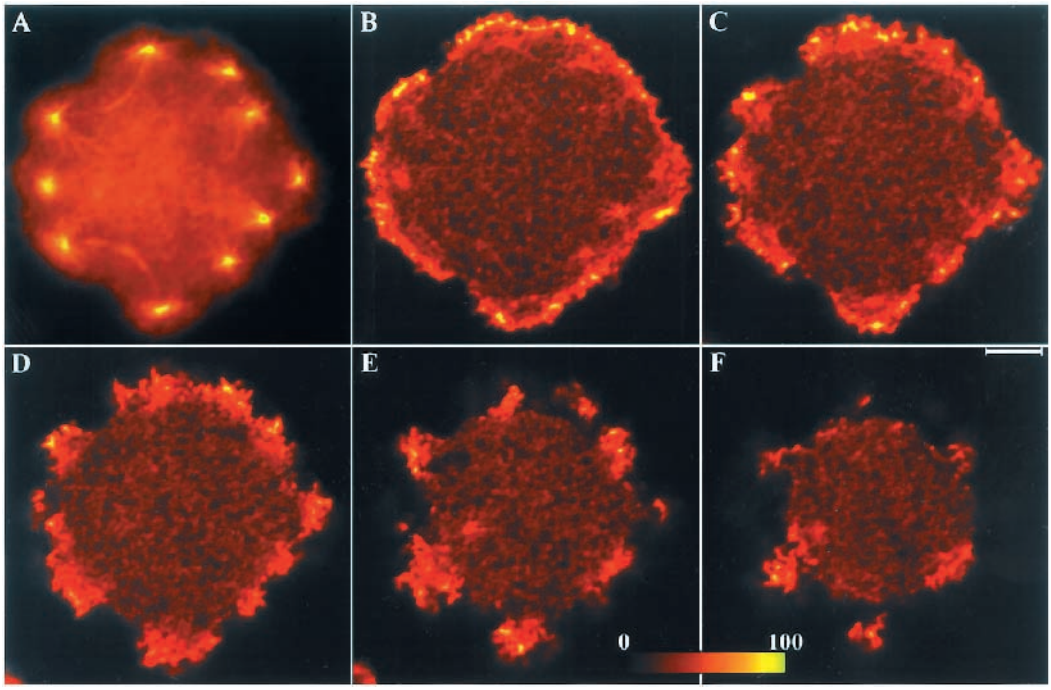

Fig. 4. Positions of microtubule asters and spindles relative to surface protrusions rich in coronin. A multinucleated myosin II-null cell in telophase, double-labelled for α-tubulin and coronin, was analysed by confocal microscopy. The image in A shows the α-tubulin label photographed in the non-confocal mode. Positions of the centers of microtubule asters and two full-length spindles are discernible. (B-F) The distribution of coronin in confocal sections from the bottom (B) to the top (F) of the same cell. Near to the supporting glass surface, the cell periphery is almost continuously decorated with coronin (B). The lobes are formed at the lateral and top areas of the cell surface. Tipped protrusions of these lobes are most intensely labelled (C to F). These lobes envelop the microtubule asters, as revealed by comparing A with C-F. The indentations of the cell surface on top of the middle region of the spindles are depleted of coronin. Distances in the *z*-axis between the confocal sections are 1 μm. The colour code at the bottom indicates fluorescence intensities on a linear scale. Bar, 5 μm.

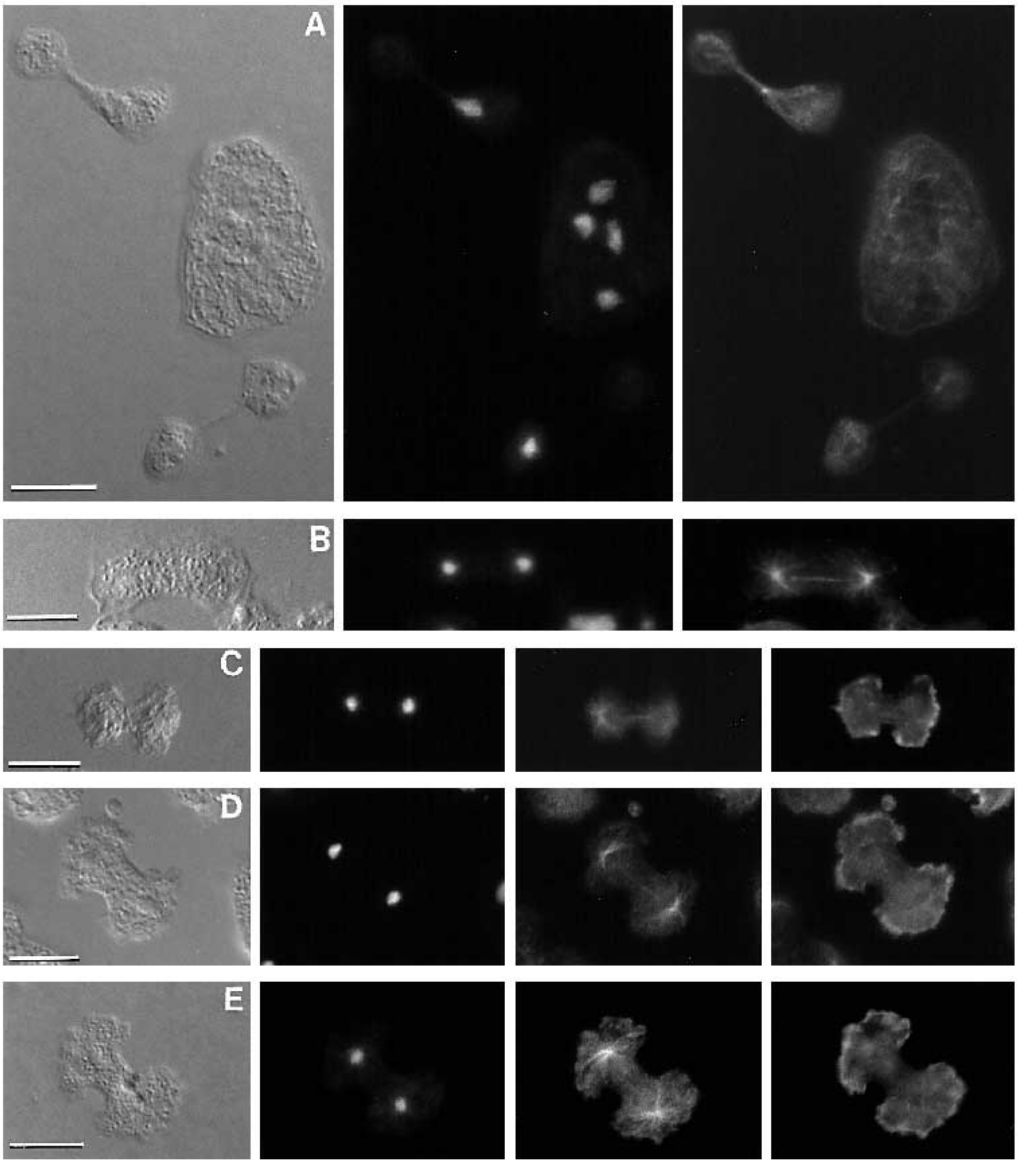

Fig. 5. Two stages of traction mediated cytofission (A) compared with four stages of mitotic cleavage (B-E) in mononucleated myosin II-null cells. (A,B) Shape of the cells as revealed by DIC optics, nuclei stained with DAPI, and microtubules labelled with antibody are shown from left to right. The two mononucleated cells in A are divided into two portions that are connected by a stretch of cytoplasm. The nuclei of these cells are large and of irregular shape, as are the four nuclei of the big interphase cell in the same field. Microtubules are present in both the nucleated and non-nucleated portions of the small cells. The cell on top reveals microtubules extending from an MTOC near to the tapered end of the nucleus, into the other portion of the cell, thereby passing the cytoplasmic connection. The cell in B represents an early mitotic cleavage stage with a clearly discernible spindle and two well developed asters. (C-E) For the more advanced mitotic stages, cell shape as revealed by DIC optics, staining of nuclei with DAPI, labelling of microtubules, and either F-actin with phalloidin (C) or coronin with antibody (D,E), is shown from left to right. The labelling of microtubules in C revealed the presence of an intact spindle in a cleavage stage more advanced than that in B. In D, remnants of the spindle are still seen. In the most advanced cleavage stage of E, the spindle is no longer detectable and the microtubule organisation is being turned into the interphase state. Both the F-actin and coronin labels sharply discriminate between the distal portions and the central shaft of the dividing cells. Bars, 10 μm.

from the two spindle pole bodies, one at each end of the spindle (Moens, 1976). The spindle is mostly intranuclear in *D. discoideum* (Moens, 1976; Roos and Camenzind, 1981; McIntosh et al., 1985), and appears in fluorescence images as a compact stick of microtubules. In interphase cells, the microtubules are spread throughout the cytoplasm, many of them ending close to the plasma membrane and some being bent back, giving the system of microtubules a rosette-like appearance.

During traction mediated cytofission of mononucleated myosin II-null cells, the nucleus remained undivided in one portion of the bipartite cell (Fig. 5A), and the microtubules displayed typical features of the interphase state. The microtubules extended into the long stretches of cytoplasm connecting the fragments of the cell body. Typically, these stretches smoothly broadened into the portions of the cell body that they connected.

Mitosis in mononucleated myosin II-null cells was distinguished by a sequence of characteristic changes in cell shape from the fission of interphase cells. In the course of anaphase, symptoms of cleavage became recognisable. A constricted region grew into a central shaft connecting the distal portions of a dividing cell (Fig. 5B-E). At both ends of this shaft, the cells broadened abruptly into their distal portions, which were covered at their surface by rounded or tipped protrusions. In their interior, these distal portions of the cells contained the asters of microtubules.

Localisation of F-actin and coronin in mitotic stages of mononucleated myosin II-null cells

Labelling of the cells in late anaphase and telophase with phalloidin or anti-coronin antibody gave characteristic patterns that accentuated the distinction between the central shaft and distal portions of a cell (Fig. 5C-E). Both F-actin and coronin were strongly localised to protrusions of the

distal portions, with a sharp decline in abundance at the borders to the central shaft. Since these labelling patterns correlated with the presence of two small clusters of chromosomes in a cell, they are considered to be diagnostic of cytokinesis as linked to mitosis.

The F-actin and coronin distribution was semi-quantitatively analysed in anaphase cells by confocal microscopy (Fig. 6). F-Actin proved to form a shallow cortical layer along the central shaft. The accumulation of F-actin in the distal portions of a dividing cell, as seen in the whole-mount preparation of Fig. 5C, was established by confocal sectioning (Fig. 6A,B). Labelling with anti-coronin antibody revealed a pattern similar to that of F-actin. The difference between the central shaft and the distal portions of the cell body was even more extreme: no accumulation of coronin was detectable by confocal microscopy in the cortical region of the shaft. The surface extensions at the distal portions of the cells were distinctly marked, including crown-shaped protrusions that pointed from the upper cell surface into the free fluid space (Fig. 6C).

Sequence of shape changes during mitosis of mononucleated myosin II-null and wild-type cells

To unequivocally identify mitotic cleavage stages in myosin II-null mutants, synchronised cells were stained with DAPI and labelled with a myosin II-specific antibody. This antibody clearly distinguished wild-type from myosin II-null cells in immunofluorescence images and immunoblots. As a positive control, wild-type cells were added to the myosin II mutant cells. Cells in cleavage stages corresponding to those of Fig. 5B-E were recognized by the antibody as being definitely myosin II-null. The series of stages shown in Fig. 7 indicates that cleavage proceeded in myosin II-null cells to an almost terminal step.

To see whether cells attached to a substrate can fully divide in the absence of myosin II, individual live cells were followed by video recording or serial micrographs (Fig. 8). The vast majority of cells passed through shape changes that are typical of mitosis, as ascertained in fixed preparations (Figs 5, 7).

A characteristic feature of mitotic cleavage was the absence of an extensive elongation during cleavage furrow formation (Fig. 8). This behaviour is in variance to that of interphase cells undergoing cytofission, for which continuous stretching of cytoplasmic bridges up to their break is essential. During mitosis, the diameter of myosin II-null cells increased in their long axis on an average by only 9 per cent between an early stage with a broad shaft of cylindrical shape (150 seconds panel in Fig. 8A), and a late stage with almost completely separated daughter cells (300 seconds panel of Fig. 8A). In 12 out of 35 cells measured, no significant increase in length was found during formation and progression of the cleavage furrow.

Among a total of 76 mitotic myosin II-null cells we have recorded under our standard conditions on a glass surface, 66 cells completed cytokinesis by constriction of the cleavage furrow, 7 cells showed abortive cytokinesis, and 3 cases were questionable for various technical reasons. In order to assess that cytokinesis is not a special feature of HS2205, the particular myosin II-null strain used in these experiments, we have also studied a mutant producing heavy meromyosin (DeLozanne and Spudich, 1987), whose cells behave like myosin II-null cells (Wessels et al., 1988). Cytokinesis in the *hmm* cells was similar as in HS2205, with 80 to 90 per cent of mononucleated cells proceeding through all stages of cytokinesis in a regular fashion.

Abortive cytokinesis in the absence of myosin II was mostly due to an imbalance in the motile activities of the two halves of a dividing cell, which resulted in resorption of one of the incipient daughter cells by the other, thus giving rise to a binucleated product. Sometimes cytokinesis was interrupted by another cell, which interfered with cleavage in a way of contact inhibition of the motile activity on one end of the dividing cell. These observations indicate that tension produced by the motile activity at the distal portions of a dividing cell is crucial for mitotic myosin II-null cells to divide. Although this pull did not result in a considerable elongation of the cells during their cleavage, it prevented the collapse of the cleavage furrow.

Comparison of cytokinesis in myosin II-null and wild-type cells

In order to relate cytokinesis in the myosin II-null cells to the division of wild-type cells, we have employed NC4, the prototype strain of *D. discoideum* (Raper, 1935), as well as its axenically growing derivative AX2, which is the parent of the myosin II-null mutant HS2205 (Manstein et al., 1989). Since cells of these strains overlap in their behaviour, we show only NC4 as a wild-type reference (Fig. 9). Some of the bacteria-grown NC4 cells were more strongly contracted than was regularly observed in myosin II-null cells, and their distal portions appeared more rounded, with a sharply separated shaft between them (Fig. 9B). However, often the sequence of shape changes during mitosis was indistinguishable in these wild-type cells from those in myosin II-null or *hmm* cells (Fig. 9A and C). In particular, the formation of the cleavage furrow was accompanied by the active extension of protrusions at the distal portions of the dividing cells.

The two halves of a dividing cell were often observed to freely exchange cytoplasm through the cleavage furrow, which acted as a tube with fluid cytoplasm. Depending on the actual differences in tension produced by the two distal portions of a cell, cytoplasm did alternately stream in one or the other direction through the tube. Continuous streaming into one direction was occasionally observed in myosin II-null cells during retraction of one of their leading edges. When, as a consequence, one of the two daughter nuclei happened to pass through the furrow, cytokinesis was going to fail in that cell.

In most of the wild-type or myosin II-null cells, a filamentous connection persisted at the end of cytokinesis, which was finally disrupted by independent movement of the daughter cells (Figs 8A and C, 9A to C). This is similar to what has previously been seen at the end of mitotic cleavage in *Dictyostelium* wild-type cells (Gerisch, 1964; Fukui and Inoué, 1991), and occurred regularly in any myosin II minus and plus cell type used in this study.

The time myosin II-null cells needed to pass through cleavage proved to be more variable than for wild-type cells (Fig. 10). The time required by AX2 and NC4 cells sharply peaked at 2 to 3 minutes under our conditions. More than half of the myosin II-null cells finished cleavage within the same span of time as NC4 cells, but a portion of the population needed 2 to 3 times longer.

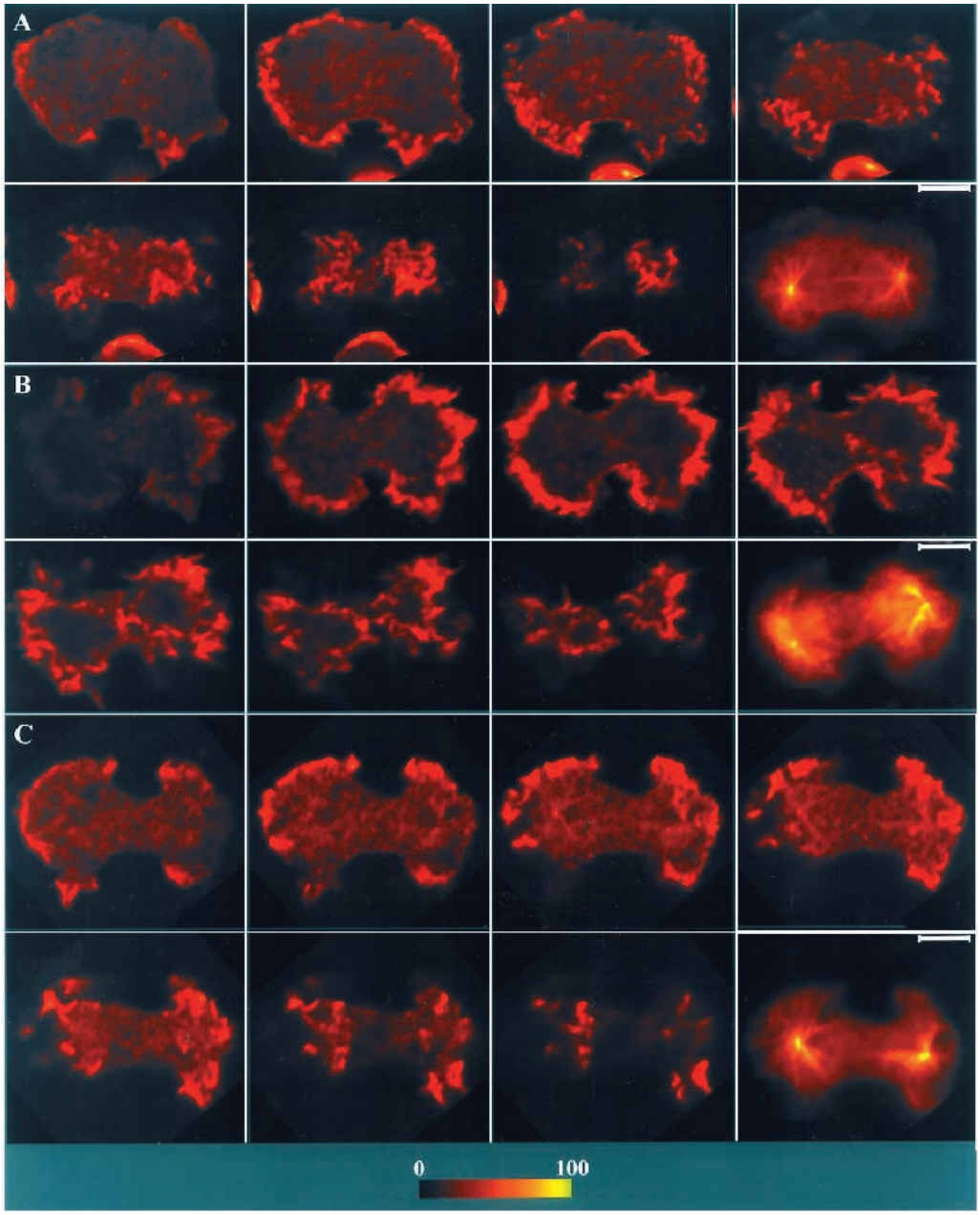

Fig. 6. Series of confocal sections through mononucleated myosin II-null cells in mitosis. Cells in A and B were labelled with TRITC-conjugated phalloidin, the cell in C was labelled with antibody against coronin. In addition, the cells were labelled for α-tubulin in order to depict, at the end of each series, the organisation of the microtubule system in a non-confocal mode. The cell in A has an elongated spindle and well developed asters at its ends, as it is typical of telophase. In post-telophase (B), the microtubule system is in an advanced stage of re-organisation to the interphase state. (C) A state between A and B. In A and B, F-actin is shown to be most strongly enriched in cell-surface protrusions formed at the distal portions of the dividing cells. Along the central shaft, F-actin decorates the flanks of the cells in a shallow layer. (B) Well developed crown-shaped extensions on the upper surface of each of the incipient daughter cells. The label in C indicates that coronin is distributed within the cytoplasm and is enriched only at distal portions of a cell. Confocal sections were taken from the lower surface, where the cells are attached to glass, to the upper surface at a section-to-section distance of 0.5 μm. Linear colour scale is shown at the bottom. Bars, 5 μm.

DISCUSSION

Mitotic cleavage in myosin II-null cells is distinct from traction-mediated cytofission

The present study focuses on the functional interphase between the microtubular system and the actin-rich cell cortex in mitotic cells of *Dictyostelium discoideum*. The microtubular system, as organised by the spindle pole bodies, consists in mitotic cells of the spindle and two asters of microtubules. Since the forces responsible for separation of the daughter cells are generated in the cell cortex, any lack of coupling between the microtubule-based movement of the chromosomes and the formation of a cleavage furrow in the cortex will give rise to multinucleated cells.

We have applied two criteria to unequivocally distinguish mitotic cleavage from cytofission of interphase cells. First, the small size and low DNA content of nuclei, as revealed by DAPI staining, proved to be the most reliable criterion to identify mitotic cells. Since *D. discoideum* has no conspicuous G_1 phase (Weeks and Weijer, 1994), a low DNA content of nuclei defines cells in ana- and telophase. The shape changes observed in living cells could be correlated with stages of mitosis in fixed and DAPI stained preparations. Mitosis was thus followed in wild-type and myosin II-null cells up to complete separation of the daughter cells by video recording and serial micrographs.

As a second criterion of mitosis, the organisation of the microtubule system has been employed. Early anaphase stages are clearly distinguishable from the interphase stage by the presence of a spindle and by straight radial microtubules forming asters around the spindle pole bodies. The problem in using microtubule organisation to identify mitotic stages at the

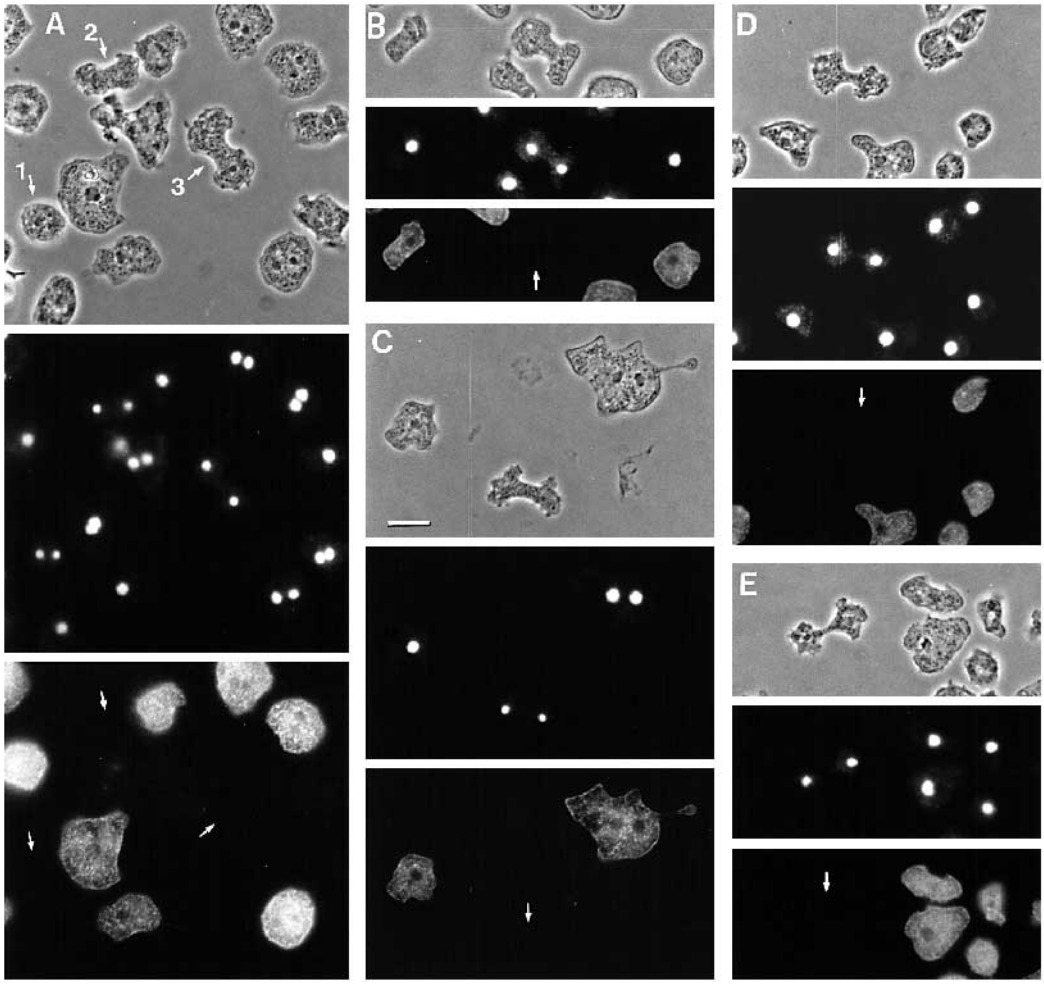

Fig. 7. Stages of mitotic cleavage in the absence of myosin II. Cells of mutant HS2205 were mixed with AX2 wild-type cells as an internal control for the absence of myosin II in the mutant cells. Each of the five sets of three micrographs (A-E) shows cells in phase contrast (top panels), DAPI-stained nuclei (middle), and myosin II labelled with mAb 56-396-5 (bottom). This antibody recognizes myosin II in both its monomeric and filamentous states (Claviez et al., 1982; Pagh and Gerisch, 1986). Arrows point to myosin II-null cells in mitotic or post-mitotic stages. In A, three mitotic stages, labelled (1 to 3), from early anaphase to telophase are present. Bar, 10 μm.

time the cleavage furrow is formed, resides in the late appearance of the furrow (Fukui and Inoué, 1991). The furrow is formed at a stage in which the microtubular system is already turning into the interphase state. Only rarely is an advanced cleavage furrow seen in a cell together with typical asters and remnants of the spindle (Fig. 5C,D). These results indicate that the time window is small at which spindle and cleavage furrow co-exist.

The sequence of shape changes observed in mononucleated myosin II-null cells undergoing mitosis proved to be similar to the typical changes observed in *Dictyostelium* wild-type cells (Gerisch, 1964; Fukui and Inoué, 1991; and Fig. 9 of the present paper). At an early anaphase, the central region of the cells becomes elongated and assumes a cylindrical shape. Subsequently it becomes concave, turning into a furrow, as cleavage proceeds to complete the separation of the daughter cells (Fig. 8A,C and 9). At the final post-mitotic stage, pulling by migration of the daughter cells is instrumental in disrupting a thin thread of cytoplasm that is sometimes kept between the daughter cells, both in the wild-type and the myosin II-null mutants. This final disruption by cell migration probably replaces in *Dictyostelium* cells the terminal closure of a 0.5 μm channel between bud and mother cell, which in the immobile yeast cells may be brought about by septins (Sanders and Field, 1994).

Traction-mediated cytofission is observed when multinucleated myosin II-null cells are allowed to attach and to move on a solid surface (Warrick and Spudich, 1987). In contrast to cytokinesis, this fission is not a programmed process. The shape of interphase cells undergoing cytofission is variable, and often the long threads of cytoplasm connecting portions of these cells will contract, thus giving rise to a re-united giant cell. It may be of relevance in this context that cytofission of interphase cells is not unique to myosin II-null cells. It occurs also in large, multinucleated wild-type cells of *D. discoideum* that are generated by electrofusion and subsequently allowed to spread on a surface (Neumann et al., 1980; G. Gerisch and R. Neujahr, unpublished results).

Cytokinesis is distinguished from cytofission by its coupling to mitosis and by a regular succession of cleavage stages. As shown in this paper, mitotic cleavage co-exists with traction-mediated cytofission in myosin II-null cells that are attached to a surface. This result indicates that cytofission, resulting in the pinching off of portions of an interphase cell, does not replace but supplements mitotic cleavage in mutants lacking myosin II. Because traction-mediated cytofission and mitotic cleavage are both anchorage dependent in these mutants, we have suggested the term ‘attachment assisted mitotic cleavage’ to characterise the latter.

Myosin II function in cytokinesis

Mitotic cleavage has been suggested to be due to active contraction, resulting in the development of an ingrowing furrow,

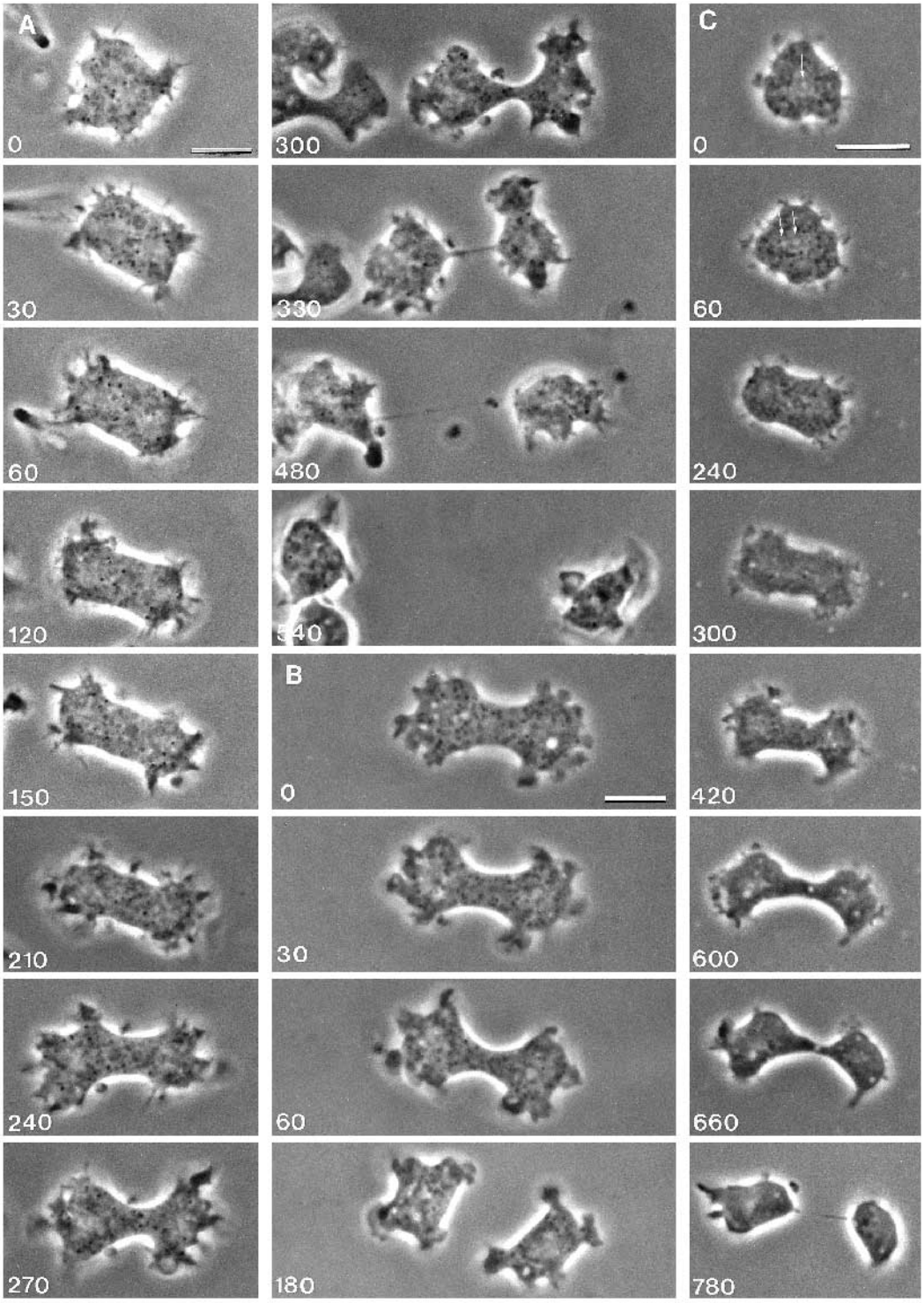

Fig. 8. Sequence of mitotic cleavage stages in three myosin II-null cells (A-C). Serial micrographs of living cells were taken with phase contrast optics. Numbers denote time in seconds starting with the first photograph of each series. Examples show two rapidly cleaving cells (A and B) and a slowly dividing cell (C). Nuclei of the mitotic cells are recognisable in some of the stages as indistinct, greyish areas. In the 0 and 60 second frames of C, nuclei are marked by arrows to indicate the segregation of two clusters of chromosomes. Bars, 10 μm.

which finally causes the cell body to divide into two symmetrical portions. One postulate in this view is that myosin II forms a ring in the cell cortex, which contracts by interaction with actin filaments that are linked to the plasma membrane (reviewed by Fishkind and Wang, 1995). This hypothesis is supported by the fact that, under certain experimental conditions, myosin II strongly accumulates in cells of *D. discoideum* at the cleavage furrow, and F-actin accumulates in close association with the myosin (Kitanishi-Yumura and Fukui, 1989; Fukui and Inoué, 1991). Similar results on the accumulation of myosin II at the cleavage furrow have been obtained in other cells, including sea urchin blastomeres (Mabuchi and Okuno, 1977; Kiehart et al., 1982) and mammalian HeLa or 3T3 cells (Fujiwara and Pollard, 1976; DeBiasio et al., 1996). Earlier work showing inhibition of cleavage furrow formation by the microinjection of antibodies against cytoplasmic myosin into starfish blastomeres provided support for a role of the myosin in cytokinesis (Mabuchi and

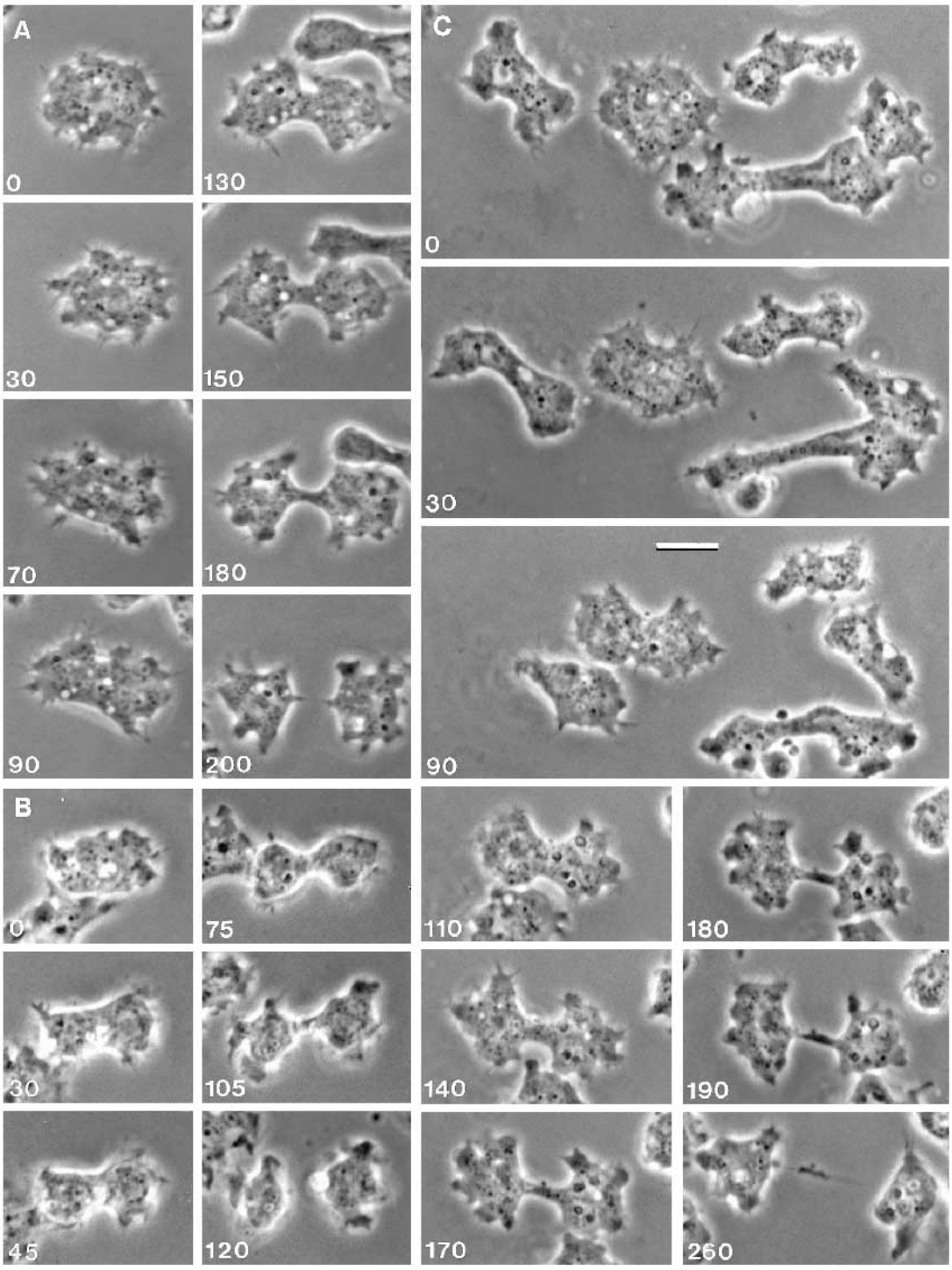

Fig. 9. Sequence of mitotic cleavage stages in three NC4 wild-type cells (A-C). Micrographs are numbered as in Fig. 8. Shape changes in two of the cells (A and C) closely resemble cytokinesis in myosin II-null cells. One cell is more strongly rounded up (B). In the first two frames of A nuclear division is seen. The mitotic cell in C is flanked by two interphase cells whose shape resembles in some way mitotic stages. As the sequence of the first three frames of C shows, these similarities are transient, in contrast to the regular sequence of shape changes during and after mitosis. Bar, 10 μm.

Okuno, 1977; Kiehart et al., 1982). Injection of anti-myosin antibodies into epitheloid kidney cells did delay but not prevent formation or constriction of the cleavage furrow (Zurek et al., 1990).

Direct proof for a role of myosin II in mitotic cleavage has been provided by the finding that mutants of *D. discoideum* that lack this motor protein are unable to divide in a shaken suspension culture (Knecht and Loomis, 1987; DeLozanne and Spudich, 1987; Manstein et al., 1989). These results are in accord with deficiencies seen in yeast cells of *Saccharomyces cerevisiae* that produce truncated MYO1, a myosin homologue (Watts et al., 1987; Rodriguez and Paterson, 1990). The mutant yeast forms chains of cells as a result of imperfect cell division due to abnormal cell wall organization at the mother-bud neck. Irregular numbers of nuclei in these cells indicate that nuclear migration is also impaired. The impairment of cleavage furrow formation in a mutant of the *spaghetti-squash* gene of *Drosophila*, which encodes a myosin light chain kinase, points in the same direction (Karess et al., 1991).

Our results showing cytokinesis in myosin II-null cells of *D.*

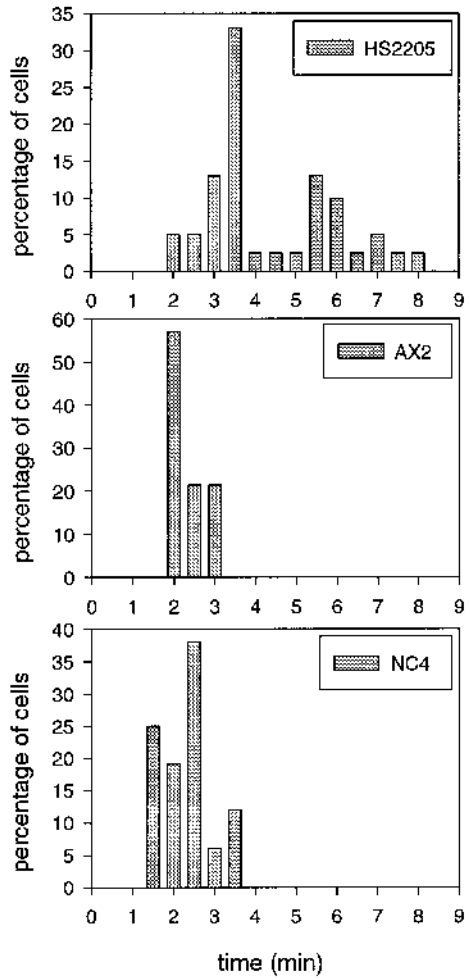

Fig. 10. Times required for mitotic cleavage in myosin II-null and wild-type cells. Time intervals were determined from the stage of an elongated cell just before the beginning of constriction, which corresponds to early telophase, up to the end of the cleavage process, when only a thin thread of cytoplasm remained between the independently moving daughter cells. (See Fig. 8A, 60 second frame for example of the starting point and Fig. 9A, 200 seconds frame for the end of the interval.) The histograms summarise measurements on synchronized cells that divided within a period of 8 hours after transfer from phosphate buffer into nutrient medium. Measurements were made using video-recordings of mononucleated cells that were identified as undergoing mitosis by observing the typical changes in nuclear structure. Number of cells analysed per strain: 39 for HS2205; 14 for AX2; and 16 for NC4.

discoideum that are anchored to a surface indicate that myosin II is not essential for constriction of the cleavage furrow. What then is the function of this motor protein in attachment assisted mitotic cleavage? Several pieces of evidence indicate that myosin II plays a supporting role in increasing the reliability and probably also the speed of mitotic cleavage under these conditions.

The presence of a mixture of mononucleated and multinucleated cells of various size during growth on a solid surface indicates that cells, even when they are attached to a surface, more often fail to complete mitotic cleavage in the absence of myosin II than in its presence. Under the conditions used in this study, only a minor fraction of myosin II-null cells, about 10 per cent of the mononucleated cells entering mitosis, failed to complete cytokinesis. In these cases of abortive cytokinesis, a cleavage furrow was formed, but finally one incipient daughter cell was resorbed by the other, yielding a binucleated product.

According to previous data, wild-type cells need between 6 and 20 minutes from the beginning of elongation to the final stage of cleavage (Fukui and Inoué, 1991; Kitanishi-Yumura and Fukui, 1989). The myosin II-null cells needed 2 to 8 minutes under our conditions, which is somewhat less. However, under the same conditions wild-type cells seemed to be even faster, with a quite precisely fixed cleavage time of 2 to 3 minutes (Fig. 10).

One possibility how myosin II acts is suggested by the observation of cytoplasmic streaming through the central shaft of a dividing cell, which indicates that a balance of tension between both sides of a dividing cell is necessary to hinder one half of the cell to vanish by streaming of its cytoplasm, together with the nucleus, into the other half. Myosin II may stabilize the shape of the cells at both flanks of the furrow. This stabilization is obviously crucial for a suspended cell, whereas an attached cell can produce tension by the motile activity of its distal portions to stabilize the bipartite configuration.

What is the mechanism of cytokinesis in the absence of myosin II?

One question raised by the reported results concerns force generation at the cleavage furrow. For cells attached to a surface, a requirement for actin and actin-based motor proteins in the cleavage furrow is neither established nor excluded. *D. discoideum* contains, in addition to a single myosin II gene, at least 12 different genes encoding other myosins (Titus et al., 1994). One or several of these myosins may take over the role of myosin II as long as actin filaments are present. In mammalian fibroblasts, myosin I is not only localised to the distal edges of the cells at late cytokinesis, but also enriched in the cleavage furrow (Breckler and Burnside, 1994).

F-actin forms only a shallow cortical layer at the cleavage region, but this may be sufficient for a role of actin in constriction. According to the solation-contraction hypothesis of Hellewell and Taylor (1979), contraction is prevented when long filaments of actin are connected by crosslinking proteins into a rigid fabric. With this hypothesis, contraction is said to be favoured at sites of a cell where actin filaments of moderate length are loosely connected by few crossbridges. The cleavage furrow with its thin actin layer may be such a region predisposed to undergo contraction.

To adopt an alternative view, one might assume that cleavage in cells attached to a surface is primarily due to destabilisation of the actin cortex along the shaft that connects the incipient daughter cells. In this view, the area of the cell cortex that is not stabilised by the asters of microtubules would be subject to resorption rather than to active contraction in the absence of myosin II. The sequence of shape changes in a cell dividing on a substrate surface is, to say the least, not inconsistent with this possibility.

The basic message our results transmit is that one has to search for mechanisms that bring about, in the absence of myosin II, constriction of the cleavage furrow and separation of the daughter cells. One factor involved in the mitotic cleavage of myosin II-null cells is anchorage of these cells to

a surface of optimal adhesiveness. A clean glass surface coated with serum albumin has been shown to allow *D. discoideum* cells to firmly adhere without preventing their movement (Schindl et al., 1995; Weber et al., 1995). The conditions used in the present study were similar, with albumin being replaced by the peptone and yeast extract of the nutrient medium in which the cells are cultivated.

Another factor that contributes to cytokinesis of attached cells is the motile activity that tends to draw the distal portions of a dividing cell in opposite directions. This activity does not lead to a substantial elongation of a dividing cell during progression of its cleavage furrow (Fig. 8). However, the tension produced at the distal portions of the dividing cell will prevent the daughter cells still connected by the furrow region from sliding towards each other, which would cause the furrow region to collapse.

It follows from the results presented in this paper that at least five activities are involved in mitotic shape changes of myosin II-null cells: (1) rounding up of a cell during the pro- and metaphase, (2) stretching of the cell in parallel to spindle elongation during ana- and telophase, (3) appropriate adhesion to a substratum in combination with (4) tension produced by the two motile regions at the distal portions of a late mitotic cell, and (5) the unknown mechanism responsible for constriction of the cleavage furrow in the absence of myosin II. These results may provide a basis for future research on proteins that are involved in the formation of a cleavage furrow. Cells appropriately attached to a surface, as used in the present study, can be employed to screen, in a myosin II-null background, for mutations that affect cytokinesis. If this approach is combined with the REMI technique of mutagenesis (Kuspa and Loomis, 1992), sequence information can be obtained of the genes inactivated in the mutants of interest.

F-actin and coronin accumulate in myosin II-null cells outside of the cleavage furrow

The distribution of F-actin observed during the mitosis of myosin II-null cells is not fully consistent with the F-actin distribution previously found in wild-type cells (Fukui and Inoué, 1991). Both in wild-type and mutant, the F-actin is highly enriched in extensions of the distal regions of the cells, but only in wild-type has it been seen to form a ring of longitudinally orientated actin filaments that extend from the cleavage furrow outwards (Fukui and Inoué, 1991). From these results it may be inferred that myosin II is required for the parallel array of filamentous actin in the region of the cleavage furrow, probably by forming a scaffold at the cytoplasmic phase of the plasma membrane at which actin filaments can assemble.

A sharp boundary is seen between the distal areas of the cell cortex, where the actin-rich protrusions are formed, and the central area where the furrow is made, giving the cells a ‘double-cauliflower’ appearance. The area of the furrow, distinguished in myosin II-null cells by its thin cortical layer of F-actin (Fig. 6A,B) has a mostly smooth surface (Fig. 8). The double-cauliflower appearance of anaphase cells is especially clear in cells labelled for coronin (Fig. 5D,E). Coronin-rich areas are restricted to the distal portions of a dividing cell: no accumulation of coronin has been detected in the cleavage furrow (Fig. 6C). This distribution of coronin in mitotic myosin II-null cells coincides with its observed distribution in the anaphase of wild-type cells (DeHostos et al., 1993; Gerisch et al., 1993), indicating that coronin localisation in the course of cytokinesis does not require myosin II.

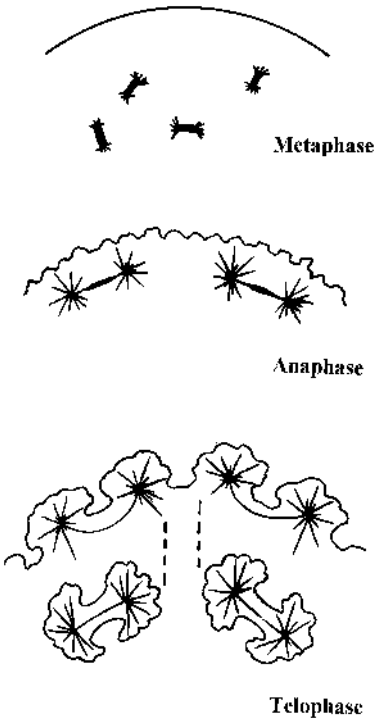

Fig. 11. Diagram of spatial relationships between the microtubule system and the cell cortex in mitotic stages of multinucleated myosin II-null cells. The diagram is based on data shown in Figs 1, 3, and 4. At the bottom, two mononucleated cells are depicted for comparison of their bilateral cleavage with the unilateral furrowing of multinucleated cells.

Results previously obtained with coronin-null mutants underline the importance of motile activity in the distal portions of a dividing cell where coronin is accumulated. Elimination of the coronin by gene replacement is known to slow down cell motility, and an impairment of cytokinesis found in the coronin-null mutants is most likely due to this motility defect (DeHostos et al., 1993).

The location of cleavage furrows in a multinucleated cell is linked to the final positions of microtubule asters

Multinucleated myosin II-null cells are convenient tools to study the dependence of cleavage furrow formation on microtubule organisation. Mitotic complexes, each one consisting of a spindle, the chromosomes, and two microtubule asters extending from the spindle pole bodies, are translocated during mitosis to the periphery of the large myosin II-null cells.

Superficially, this process resembles the translocation of nuclei during blastoderm formation of the early insect embryo. After 14 rounds of cleavage, nuclei dispersed within the cytoplasm are translocated to the periphery of the early *Drosophila* embryo, in order to form there a layer before the compartmentation into separate cells commences. In the first phase of their translocation, the transport of nuclei is mediated by the actin system, thereafter it becomes microtubule dependent (Sullivan and Theurkauf, 1995; Baker et al., 1993).

Mitosis in normal *Dictyostelium* cells does not appear to require nuclear migration. Nevertheless, the multinucleated myosin II-null cells unveil a process that most likely also takes place in mononucleated cells. The normal role of this process is probably to adjust the nucleus in the cell's center at the onset of mitosis, bringing it into an optimal position for proper coupling of chromosome segregation with symmetrical cleavage of the cell. Indications for such a positioning of the nucleus can be seen in video recordings of dividing wild-type cells of *Dictyostelium* (Gerisch, 1964).

During anaphase of the multinucleated myosin II-null cells, nuclei are arrested at a distance of about 3 μm from the plasma membrane, with the spindle assuming a tangential position relative to the cell surface. In that way nuclei are necessarily brought into an asymmetrical relationship to the surface of these large cells. This means, the mitotic machinery associates with the cortex of the cell by a mechanism that acts in an unilateral fashion. Since in the multinucleated cells a cleavage furrow cannot surround the spindle in a circular manner, the cell surface folds only at one side of the spindle. Later in anaphase, asters tend to assume a symmetrical position relative to the cell surface, even at the expense of bending the spindle (Figs 1A and 4A). 'Assuming a symmetrical position' implies that a fold is not only made at the proximal side of an aster, this means on top of the spindle, but also at the distal side of each aster (Fig. 11). The observed bending of the spindle suggests that force is generated by the interaction of the microtubule asters with the cell cortex. In this view the asters, rather than the spindle, play a dominating role in cytokinesis, and the region where the cell eventually cleaves is that part of a dividing cell which is not seized by the asters.

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