The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle

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The cytokinesis phase, or C phase, of the cell cycle results in the separation of one cell into two daughter cells after the completion of mitosis. Although it is known that microtubules are required for proper positioning of the cytokinetic furrow [1,2], the role of pre-anaphase microtubules in cytokinesis has not been clearly defined for three key reasons. First, inducing microtubule depolymerization or stabilization before the onset of anaphase blocks entry into anaphase and cytokinesis via the spindle checkpoint [3]. Second, microtubule organization changes rapidly at anaphase onset as the mitotic kinase, Cdc2–cyclin B, is inactivated [4]. Third, the time between the onset of anaphase and the initiation of cytokinesis is very short, making it difficult to unambiguously alter microtubule polymer levels before cytokinesis, but after inactivation of the spindle checkpoint. Here, we have taken advantage of the discovery that microinjection of antibodies to the spindle checkpoint protein Mad2 (mitotic arrest deficient) in prometaphase abrogates the spindle checkpoint, producing premature chromosome separation, segregation, and normal cytokinesis [5,6]. To test the role of pre-anaphase microtubules in cytokinesis, microtubules were disassembled in prophase and prometaphase cells, the cells were then injected with anti-Mad2 antibodies and recorded through C phase. The results show that exit from mitosis in the absence of microtubules triggered a 50 minute period of cortical contractility that was independent of microtubules. Furthermore, upon microtubule reassembly during this contractile C-phase period, ~30% of the cells underwent chromosome poleward movement, formed a midzone microtubule complex, and completed cytokinesis.

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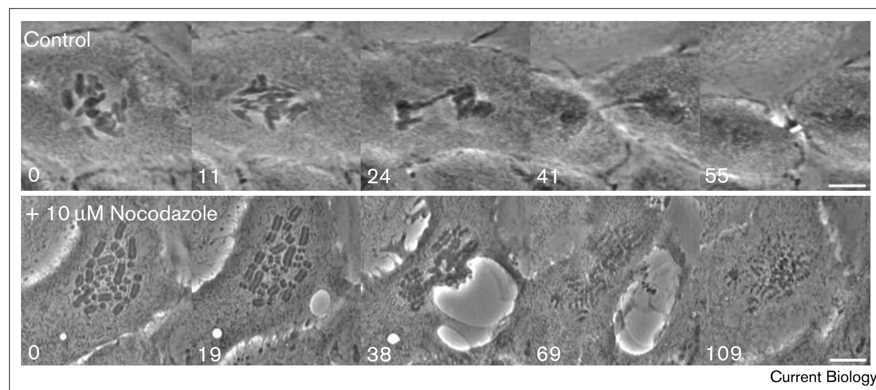
Results and discussion

Cells injected with anti-Mad2 antibodies while in nocodazole enter and exit C phase

C phase and M phase have traditionally been grouped together, with cytokinesis as the final step of mitosis. However, in some organisms, such as *Schizosaccharomyces pombe* [7] and *Saccharomyces cerevisiae* [8], cytokinesis can be uncoupled from M phase. Furthermore, M phase is not always followed by C phase. Early *Drosophila* embryos, for example, undergo multiple rounds of mitosis without cytokinesis, resulting in the formation of a syncitium. Presumably, one of the essential steps in C phase is to prepare the cell cortex for the state of contractility associated with cytokinesis. We found that the timing and progression of microtubule and actin cytoskeletal changes are normal in PtK_1 cells injected with anti-Mad2 antibodies (J.C.C. and E.D.S., unpublished observations; see also [5,6]). Therefore, we sought to define the contractile behavior of the cortex after anaphase onset in the absence of microtubules.

Prophase and prometaphase PtK_1 cells were pretreated with 10 μM nocodazole to depolymerize all microtubules (data not shown), microinjected with anti-Mad2 antibodies, and then recorded by time-lapse phase-contrast microscopy. Control cells were not pretreated with nocodazole before antibody injection and they underwent normal cytokinesis, finishing about 30 minutes after anaphase onset (Figure 1, upper row). However, the cortex of nocodazole-pretreated cells became contractile soon after anaphase onset (Figure 1, bottom row). Although a few cells formed transient circumferential furrows (data not shown), the majority of the cells contracted without obvious direction. About 50 minutes after the onset of contractility (52 ± 9 minutes, $n = 6$; see Figure 1), the cells respread without dividing, indicating exit from C phase. This was not merely an artifact of extended recording, as uninjected cells treated with nocodazole did not respread (data not shown). Interestingly, by preventing cytokinesis with cytochalasin treatment, which inhibits actin polymerization, others have found a similar time-frame for the cytokinetic ability of mammalian cells [9]. These cells were able to undergo cytokinesis if cytochalasin was washed out within one hour after anaphase onset, but not if drug washout occurred after an hour had elapsed. The fact that the anti-Mad2 antibodies induced cortical contractility for a finite period in mammalian cells treated with nocodazole is of particular interest for defining C phase as a discrete phase of the cell cycle. The timing of C phase exit in nocodazole-treated cells was longer than the timing in cells induced to enter C phase without nocodazole pretreatment

Figure 1



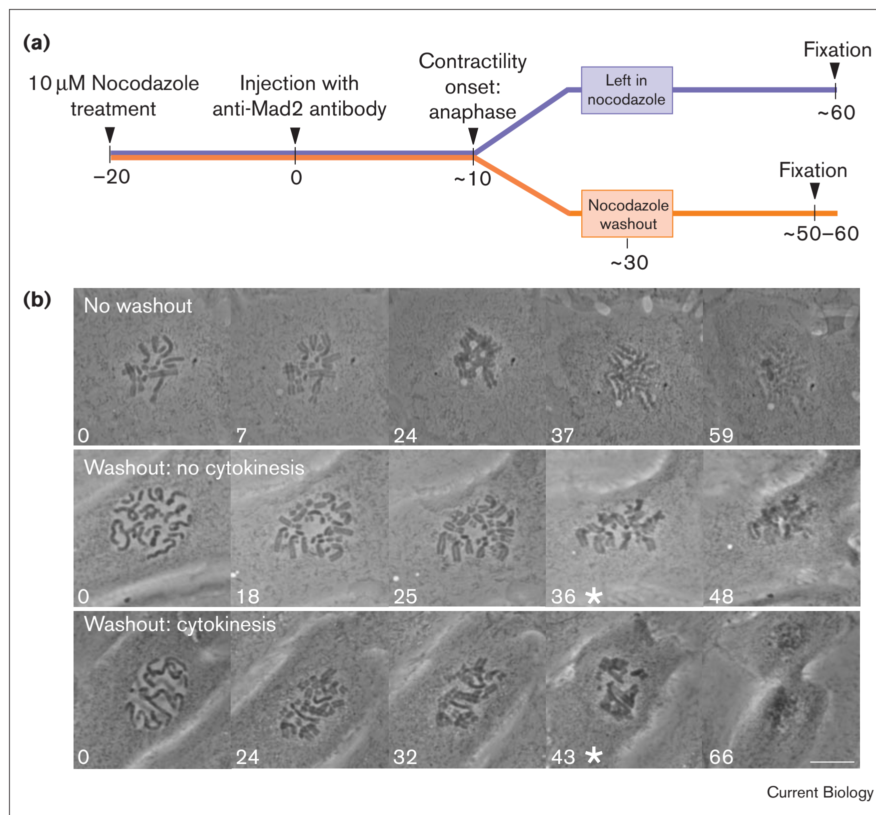
Microtubules are not required for C phase. Time-lapse images of PtK_1 cells injected with an anti-Mad2p antibody entering C phase under control conditions (top row) and in nocodazole (bottom row). Time is indicated in min after microinjection. The scale bar represents 7 µm.

(Figure 1). Perhaps this difference permits the correction of mistakes in cytokinesis or furrow positioning, allowing cells to form a cytokinetic furrow in the correct plane (as was reported for echinoderm embryos [10,11]).

Nocodazole washout of anti-Mad2 antibody-injected cells after anaphase onset but before the exit from C phase

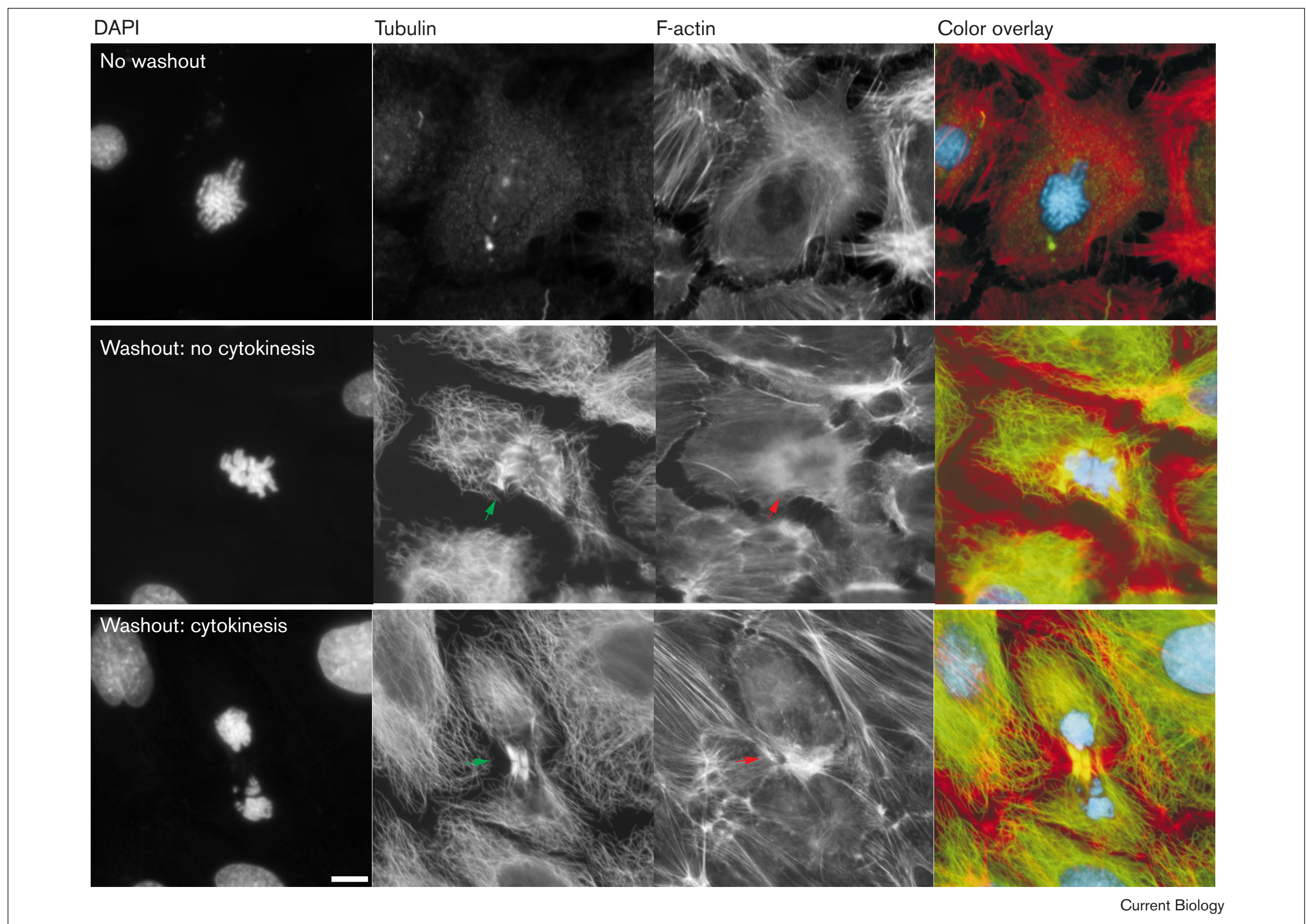
To test whether cells can accomplish cytokinesis without a pre-anaphase spindle and/or astral microtubules, prophase and early prometaphase PtK_1 cells were treated with 10 µM nocodazole for 10–20 minutes to depolymerize all of the microtubules (data not shown), microinjected with anti-Mad2 antibodies to induce anaphase onset, and recorded by time-lapse phase-contrast microscopy. Cells were then maintained in nocodazole for ~20–30 minutes to allow for progression into C phase, and then released from nocodazole treatment during the middle of C phase (Figure 2a). Cytokinesis was observed

Figure 2



Cytokinesis does not require the pre-anaphase spindle. **(a)** Schematic diagram showing the experimental design. Cells were treated with nocodazole, injected with anti-Mad2 antibodies after 20 min, allowed to enter anaphase, and then either left in nocodazole or rinsed with culture medium to remove nocodazole. **(b)** Time-lapse images of anti-Mad2p antibody-injected cells treated according to the scheme outlined in (a). Cells entering C phase in 10 µM nocodazole were either allowed to progress through C phase in nocodazole (top row), or released from nocodazole by drug washout after anaphase onset (center and bottom rows). Cells left in nocodazole (top row) exhibited cortical contractions but not cytokinesis. Some cells did not form a cytokinetic furrow following washout of nocodazole (center row), but about 30% of cells underwent chromosome separation, midzone complex formation, and cytokinesis following release from nocodazole in anaphase (bottom row). Time is indicated in min after microinjection. Washout required ~6 min off the microscope. The asterisks indicate the first images taken after nocodazole washout. The scale bar represents 10 µm.

Figure 3



Fixed images of cells in C phase after washout of nocodazole. Cells were either fixed in nocodazole (top row), or fixed after post-anaphase washout of nocodazole (center and bottom rows). Cells were stained with anti-tubulin antibodies, Alexa 488–phalloidin, and DAPI to visualize microtubules, f-actin and DNA, respectively. The resulting images were color-encoded to determine the position of each polymer relative to the others: DNA, blue; tubulin, green; f-actin, red. The cells that were not released from nocodazole did not have a microtubule array at the time of fixation (top row). Cells that were released from nocodazole treatment were able to reform microtubules (center and bottom rows). Some cells did not complete cytokinesis following drug release (center row), yet there appeared to be bundles of microtubules associated with the chromosomes (see center far right color image). However, ~30% of cells that were released from nocodazole after anaphase onset were capable of forming cytokinetic furrows (bottom row). DNA was often separated into two masses in cells that underwent cytokinesis (bottom row). Green and red arrows indicate stem body microtubule bundles and actin respectively. The scale bar represents 10 μm.

in about 30% of these cells (Figure 2b, bottom row), but 70% of cells did not complete cytokinesis after nocodazole washout (Figure 2b, center row). No cytokinesis was seen in cells not released from nocodazole (Figure 2b, upper row).

Without nocodazole washout during C phase, cells did not grow microtubules or form stem bodies (Figure 3, upper row). To test whether cells could form a midzone microtubule complex after release from nocodazole in C phase, cells were fixed ~25 minutes after nocodazole washout (Figure 2a). In cells that underwent cytokinesis, a normal midzone microtubule complex was observed, as determined by anti-tubulin staining (Figure 3, bottom row). Interestingly, in cells that did not complete cytokinesis, stem body microtubule bundles were observed in the area around the chromosomes (Figure 3, center row). In addition, we found colocalization of filamentous (f)-actin in the general area of these bundles (Figure 3, center row). This indicates a potential link between the position of the stem bodies and f-actin localization, but further experiments must be done to test this hypothesis. Together, the above results demonstrate that cells are capable of forming a cytokinetic furrow and a midzone complex or stem bodies without a pre-anaphase spindle and/or astral microtubule array (Figures 2b and 3).

It is not clear why some cells undergo cytokinesis after nocodazole washout, but others do not. In PtK_1 cells treated with nocodazole, the centrosomes are not always physically separated (data not shown). As monopolar cells do not undergo cytokinesis [5], it is possible that the centrosomes must be a certain distance apart from one another in order to form a bipolar microtubule structure. This could explain why some cells underwent cytokinesis upon nocodazole washout, while others did not. To support this hypothesis, cells that underwent cytokinesis also separated their chromosomes into two masses, indicating the formation of a bipolar structure.

The current model for the formation of the midzone microtubule complex in anaphase involves the sliding of pre-existing microtubules along spindle fibers toward the equator [12]. It is also possible that the midzone microtubule complex is formed *de novo*, as suggested by the immunolocalization of γ-tubulin at the midzone microtubule complex [13]. Our results indicate that a normal midzone microtubule complex can form in cells that never had a pre-anaphase spindle and/or astral array. However, these cells did form a bipolar microtubule structure after drug washout (Figure 3, bottom row). Therefore, we cannot rule out the possibility that the midzone microtubule complex forms via sliding of polymerized microtubules after anaphase onset.

Materials and methods

PtK_1 cells were grown as described previously [6]. Antibodies were affinity purified against XMad2 [14] as described in [6]. To be effective at inducing anaphase, some fractions of antibody required the addition of 1–5 mg/ml bovine serum albumin to decrease antibody absorption by the injection needle. Cells were filmed by time-lapse phase-contrast microscopy, as described previously [6]. All nocodazole experiments were done by pre-treating coverslips for 10–20 min with 10 μM nocodazole before injection. These conditions are sufficient to depolymerize all pre-anaphase microtubules, as determined by microtubule immunofluorescence assays (data not shown). For cell fixation, all rinses were done by three or more changes of buffer within 15 min. All permeabilizations, fixations, and rinses were done in coplin jars. Cells were permeabilized in PHEM (18.14 g PIPES pH 7.0, 6.5 g Hepes, 3.8 g EGTA, 0.99 g $MgSO_4$ in 1 l) containing 0.5% Triton X-100 (PHEM-T) for 5 min. The cells were then fixed for 20 min in fresh 4% formaldehyde and 0.5% EM grade glutaraldehyde made in PHEM. After rinsing, the glutaraldeyde fixation reaction was quenched with 1 mg/ml of $NaBH_4$ in PHEM. After further rinsing, the coverslips were blocked with 5% boiled donkey serum for 1 h at 37°C or overnight at 4°C. Primary anti-tubulin antibodies, Alexa 488–Phalloidin (Molecular Probes) and DAPI were used to visualize microtubules, f-actin and DNA, respectively. The coverslips were mounted in mounting media (20 mM Tris pH 8.0, 0.5% *N*-propyl gallate, 90% glycerol). Optical sections of fluorescently labeled cells were obtained using a multi-mode digital imaging system as described previously [15].

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