Transitions Regulating the Timing of Cytokinesis in Embryonic Cells

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Summary

Anaphase, mitotic exit, and cytokinesis proceed in rapid succession, and while mitotic exit is a requirement for cytokinesis in yeast [1, 2], it may not be a direct requirement for furrow initiation in animal cells [3, 4]. In this report, we physically manipulated the proximity of the mitotic apparatus (MA) to the cell cortex in combination with microinjection of effectors of the spindle checkpoint and CDK1 activity to determine how the initiation of cytokinesis is coupled to the onset of anaphase and mitotic exit. Whereas precocious contact between the MA and the cell surface advanced the onset of cytokinesis into early anaphase A, furrowing could not be advanced prior to the metaphase-anaphase transition. Additionally, while cells arrested in anaphase could be induced to initiate cleavage furrows, cells arrested in metaphase could not. Finally, activation of the mitotic checkpoint in one spindle of a binucleate cell failed to arrest cytokinesis induced by the control spindle but did inhibit the formation of furrows between the arrested MA and the control, nonarrested MA. Our experiments suggest that the competence of the mitotic apparatus to initiate cytokinesis is not dependent on cyclin degradation but does require anaphase-promoting complex (APC) activity and, thus, inactivation of the mitotic checkpoint.

Results and Discussion

Precocious Induction of Cytokinesis

Previous studies have demonstrated that altering the geometric relationship between the mitotic apparatus and the cell cortex advanced the timing of cytokinesis relative to unmanipulated controls [3, 5]. Presumably, this reduction in the distance between the spindle poles and cell cortex decreases the distance required for the asters to grow and contact the cortex. However, neither study accurately measured these changes in the timing of cytokinesis relative to the onset of sister chromatid separation. In an effort to define precisely the earliest window during mitosis in which cytokinesis may be initiated, blastomeres of the sand dollar *Echinarachnius parma* and the sea urchin *Lytechinus pictus* were drawn into incrementally smaller capillary pipettes prior to nuclear envelope breakdown (NEB), such that the spherical cells were reshaped into a cylinder with the mitotic appa-

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ratus aligned along the long axis of the cell. Cells manipulated in this fashion allowed for accurate measurement of the time interval between the initiation of chromosome separation and cytokinesis onset. Whereas normal spherical controls began dividing 7–7.5 min following anaphase onset (Figure 1A), a 2-fold reduction in the distance between the spindle poles and the cell cortex (from ∼55 μm to 25 μm) resulted in precocious furrows forming no less than 3.5 min after the initiation of chromosome separation (n = 6), with identical results obtained for *E. parma* and *L. pictus*. In the cell shown in Figure 1B, the spindle poles were between 10 and 15 μm from the cell surface, with the asters kept in constant contact with the cell surface. The earliest contractile behavior indicating cytokinesis onset was detected 3.5 min following the initiation of chromosome segregation (Figure 1Bf; also see Movie 1 in the Supplementary Material available with this article online), but well before the normal manifestations of mitotic exit (expansion of the spindle poles, astral microtubule elongation, karyomere formation). Sister chromatids had separated less than 8 μm from each other at the time of furrow initiation, whereas chromatid separation in controls typically occurred 20 μm before furrowing activity could be detected (data not shown). Measurements of poleward chromosome movement reveal that chromosomes migrate toward the poles at approximately 1.2 μm/min in both cylindrical and spherical cells (data not shown), suggesting that anaphase chromosome movements and mitotic exit were unaffected by the extreme physical constraints placed upon these cells. However, despite the 4-fold reduction in the distance between the spindle poles and the surface, no furrowing could be detected in these or any other geometrically altered cell prior to 3.5 min past the anaphase onset. This lag phase between anaphase onset and cytokinesis in manipulated cells closely matched earlier measurements for the minimal period of time between astral fiber contact with the cortex and furrow initiation [6]. Thus, even under conditions that favor precocious furrow initiation, furrowing cannot be initiated until an inhibitor of cytokinesis is inactivated, either concomitantly or immediately preceding the metaphase-anaphase transition.

Differential Cytokinetic Capacity of Cell Cycle-Arrested Cells

To more precisely identify the cell cycle transition required for the induction of cytokinesis in these embryonic cells, we injected blastomeres with proteins that arrested the cell cycle in either metaphase or anaphase, and we assessed their ability to form cleavage furrows following micromanipulation. One cell of a two-cell embryo was injected either with injection buffer alone (Control), nondegradable cyclin B (Δ90 cyclin B), the N-terminal domain of cyclin B (1–102 cyclin B), or the spindle checkpoint protein, Mad2 (Mad2) (Figure 2A). Following cytokinesis of the uninjected blastomere (lower blastomere for each panel), injected blastomeres were scored
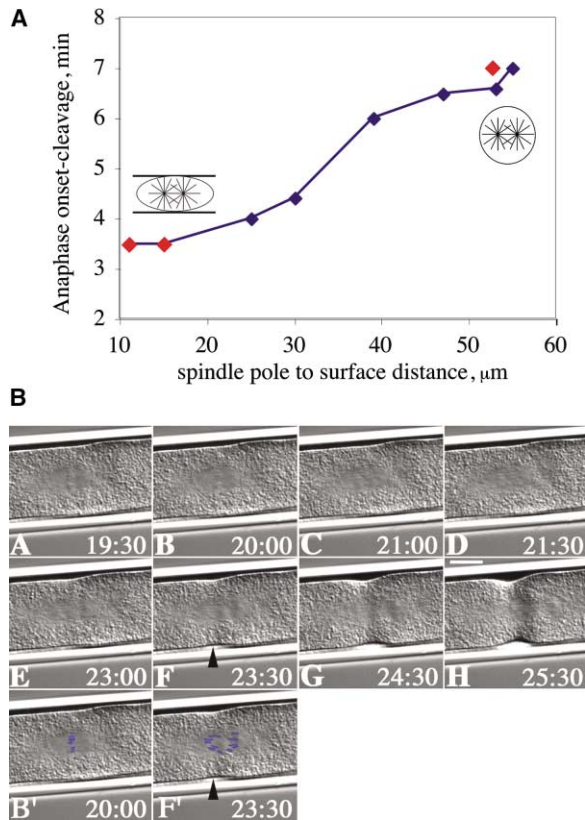
Figure 1. Reducing the Distance between the Spindle Poles and the Cell Cortex Alters the Timing of Cytokinesis in Cylindrical Blastomeres

(A) Fertilized *Echinarachnius parma* and *Lytechinus pictus* eggs were stripped of their fertilization membranes and hyaline layers, cultured through the first division, and one blastomere of a two-cell embryo was drawn into a capillary pipette just before nuclear envelope breakdown. The timing between anaphase onset (detected by Hoescht staining) and the furrow initiation was recorded and plotted against the distance between the spindle poles and the nearest cell surface. Data points for *E. parma* are shown in blue, and data points for *L. pictus* are shown in red.

(B) One blastomere of a two-cell *Lytechinus pictus* embryo was drawn into a narrow, thin-walled capillary (i.d. 22 μm) following the first cell division and was then followed by time-lapse microscopy. In these extremely thin cells, the chromosomes were easily discernable, and the lag period between anaphase onset (b) and the first evidence of furrow ingression (arrow in [F]) was 3.5 min. The average distance between the spindle poles and the surface was 11 μm. Times denote minutes past nuclear envelope breakdown. The chromosomes in (b′) and (f′) have been pseudocolored blue to highlight the positions of the sister chromatids. The scale bar represents 10 μm. For a QuickTime movie, see Movie 1 in the Supplementary Material.
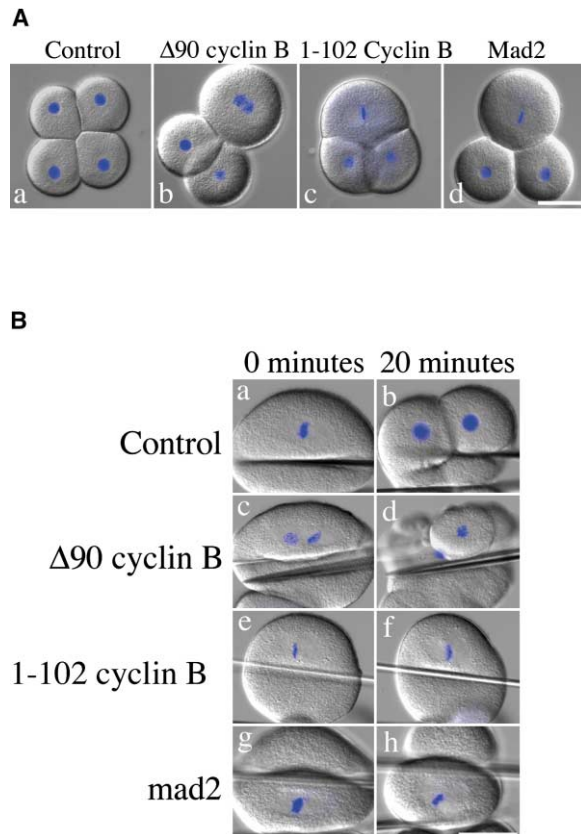

Figure 2. Cells Arrested in M Phase Differ in Their Ability to Induce Cleavage Furrows

(A) One blastomere of a two-cell *Lytechinus pictus* embryo was injected with (a) injection buffer alone (containing fluorescein-dextran as an injection marker) or with buffer containing (b) purified recombinant Δ90 cyclin B, the (c) N-terminal 102 amino acids of cyclin B, or (d) human Mad2 protein. (b) Note that while injection of Δ90 cyclin B arrested the injected blastomere in anaphase, (c and d) injection of 1–102 cyclin B or Mad2 resulted in metaphase arrest. The scale bar represents 50 μm.

(B) Blastomeres of two-cell *Lytechinus pictus* embryos were injected either with (a and b) control buffer, (c and d) Δ90 cyclin B, (e and f) 1–102 cyclin B, or (g and h) Mad2. Needles were applied to the injected blastomere after the control blastomere had begun or completed cleavage, such that the spindle poles were placed in close proximity to the cell surface. Cells were followed by time-lapse video microscopy, and 20 min after the application of the needles, the cells were scored for furrow formation and chromatin condensation (shown in blue). An oblique furrow formed in the cell arrested with Δ90 cyclin B (d), whereas no furrowing was detected in cells arrested in metaphase with 1–102 cyclin or Mad2. The scale bar represents 50 μm.

for sister chromatid segregation and cleavage. Control injections resulted in normal cytokinesis (59/60 cells) that was synchronous with the uninjected blastomere (Figure 2Aa). Δ90 cyclin B-injected cells underwent normal anaphase A chromosome segregation (22/24 cells) but arrested with condensed chromatin and metaphase-like asters (Figure 2Ab). Injection of either recombinant protein or in vitro-transcribed mRNA resulted in anaphase arrest, with spindle pole splitting and cell death observed with cells injected with higher concentrations

of mRNA (~1mg/ml), as reported previously [3, 7]. In contrast, cells injected with either the 1–102 cyclin B (37/44 cells) or Mad2 protein (21/25 cells) failed to progress into anaphase or mitotic exit and arrested with small metaphase-like asters and chromosomes aligned at the spindle midzone (Figures 2Ac and 2Ad). The N terminus of cyclin B inhibits mitotic progression by acting as a competitive inhibitor of cyclin and securin destruction [8], whereas Mad2 directly inhibits the targeting of substrates to the anaphase-promoting complex [9]. Both 1–102 cyclin B- and Mad2-injected cells were sta-

ble for up to 4 hr, beyond which cells injected with the 1–102 cyclin B underwent anaphase and mitotic exit and divided normally (data not shown). While spindle pole splitting was observed in both 1–102 cyclin B- and Mad2-injected cells, neither exhibited the aberrant morphology and cell death observed in Δ90 cyclin B-arrested cells.

To assess whether cells injected with Δ90 cyclin B, 1–102 cyclin B, or Mad2 were capable of initiating furrows, arrested blastomeres were manipulated with needles to place the mitotic apparatus directly adjacent to the cell surface to compensate for the lack of astral microtubule elongation that normally occurs when cells exit mitosis to initiate cytokinesis [3]. Cells were then scored for cleavage 20 min after the application of needles (Figure 2Bb). Control-injected embryos manipulated with needles underwent normal anaphase, mitotic exit (as evidenced by chromatin decondensation, Figures 2Ba and 2Bb), and cytokinesis (5/5 cells). Manipulation of cells arrested in anaphase with Δ90 cyclin B mRNA or recombinant protein also initiated cytokinetic furrows, but without normal chromatin decondensation and nuclear envelope reformation (7/13 cells), with higher concentrations of Δ90 cyclin B resulting in furrow regression. In the cell shown in Figures 2Bc and 2Bd, the furrow formed obliquely to the plane of the needle and coverslip, leaving one daughter cell out of the plane of focus. In contrast, no furrowing activity could be detected in manipulated cells arrested in metaphase with either 1–102 cyclin B (n = 8) or Mad2 (n = 9) (Figures 2Be–h). Four cells arrested with 1–102 cyclin B and subsequently manipulated with needles formed cleavage furrows, and in each case, the cells had exited M phase, as evidenced by chromatin decondensation and nuclear envelope reformation. These results indicated that the initiation of cytokinesis was dependent upon proteolytic events associated with anaphase onset, but not MPF inactivation.

Spindle Checkpoint Activation in Cells Containing Two Spindles

Direct manipulation of the cell cycle established that, while cells arrested in anaphase were capable of initiating cleavage furrows, cells arrested in metaphase could not (Figure 2). To confirm and extend these results, we explored the relationship between the mitotic checkpoint and cytokinesis by examining furrow formation in binucleate cells with asynchronous spindles. Rappaport demonstrated that, in cells containing two spindles, cleavage furrows may be induced between two asters regardless of whether there is an intervening central spindle or chromosomes [10, 11]. We reasoned that, if the spindle checkpoint acted locally as previously reported [12], then checkpoint activation in one spindle should not inhibit cytokinesis induced by the other spindle. To ask if furrows could form between control and checkpoint-activated spindles, we generated binucleate cells by suppressing the first cleavage with 50 mM urethane, which inhibits astral microtubule growth and thus cytokinesis [13]. After the removal of urethane, the daughter nuclei were then separated by gently pushing a glass ball through the center of the cell. In cells manipulated in this fashion (termed "donut" cells), both nuclei undergo NEB and mitosis synchronously. And, following anaphase onset, furrows form not only over the spindle midzone at the normal plane of the metaphase plate, but also at the midpoint between unrelated asters (see Movie 2 in the Supplementary Material). These "secondary" furrows normally initiated later than the primary furrows and failed to initiate or retracted if the distance between the aster centers was greater than 110 μm (data not shown).

To create donut cells with asynchronous spindles, one spindle pole was gently pulled off the nucleus with a microneedle prior to NEB (Figure 3B), and the needle was kept in the cell to block the aster from returning to the nucleus. Spindles manipulated in this manner resulted in one monopolar spindle (denoted by an asterisk), a free aster, and a control, bipolar mitotic apparatus (Figures 3E–3L). The control, bipolar spindle underwent normal anaphase and mitotic exit (as evidenced by elongated astral microtubules and decondensed chromatin; Figures 3F–3H and 3J–3L) and induced cytokinesis. The monopolar spindle remained arrested in mitosis with condensed chromatin and metaphase-like asters (Figures 3F–3H and 3J–3L). In six cells manipulated in this manner, monopolar spindles remained arrested for up to 45 min past NEB before finally exiting mitosis. In cases in which the cut aster migrated back to the monopolar spindle to reform a bipolar spindle (n = 6), the injured spindle was able to proceed through anaphase and induce cytokinesis with a 5- to 20-min delay (data not shown).

The spindle checkpoint activated in these monopolar spindles did not appear to be freely diffusible and thus was unable to arrest the control bipolar spindles contained within a common cytoplasm (Figure 3; [12]). However, in all cells examined (n = 12), no secondary furrows formed between normal telophase asters and checkpoint-arrested monasters, regardless of the pole-to-pole distance. In the cell illustrated in Figure 3, the monopolar spindle was within 75 μm of the nearest telophase aster, but no furrowing could be detected between these spindle poles. The free aster originally cut from the nucleus was greater than 100 μm from the nearest telophase aster, and while some cortical contractility was detected near the free aster, no sustained furrowing activity was detected in this region of the cell.

In contrast to yeast, it is unlikely that mitotic exit is the direct timer for cytokinesis in animal cells. This is based on our own results ([3]; Figure 2) demonstrating that manipulated sea urchin blastomeres arrested with nondegradable cyclin B are capable of initiating cleavage furrows as well as recent findings in *Drosophila*, where expression of nondegradable cyclin B3 results in cytokinesis in the absence of mitotic exit [4]. And while the metaphase furrows of the syncytial *Drosophila* embryo do not ingress to completion before the 14th division [14], these furrows contain all the normal components of cytokinesis and assemble in the presence of elevated MPF activity [15, 16]. It should be noted, however, that late events in cytokinesis, such as new membrane addition, do require CDK1 inactivation [10]. In yeast, there is a well-established requirement for mitotic exit before the contractile ring may function. In both fission and
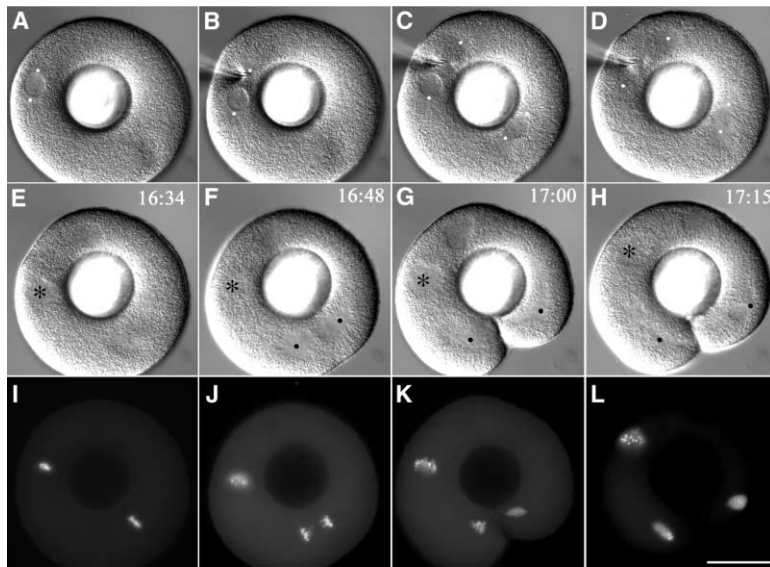
Figure 3. Cleavage Furrow Formation in Donut-Shaped Cells with Asynchronous Spindles

(A–H) Binucleate *L. pictus* eggs were manipulated with a glass ball to separate the nuclei ([A], the lower nucleus is out of the plane of focus). (D) Just prior to NEB, one centrosome was cut from the upper nucleus (centrosomes marked with dots), leaving a single centrosome associated with the nucleus during nuclear envelope breakdown. Whereas the control spindle underwent normal anaphase, cytokinesis, and mitotic exit, the monopolar spindle (marked with an asterisk) remained arrested in metaphase, with condensed chromatin and short metaphase-like asters. No secondary furrow formed between the telophase control aster and the monopolar metaphase aster. (G) A broad, shallow furrow was observed between the free aster and the other, telophase aster from the control spindle, which retracted.

(I–J) Chromatin staining of (E)–(H).

The scale bar represents 50 μm. See Movie 2 in the Supplementary Material for an example of secondary furrow formation in a control, manipulated sea urchin egg.

budding yeast, the cleavage plane is determined prior to mitotic exit, and proper timing of cytokinesis is likely achieved either by regulating constriction [17] or the recruitment of ring components to the bud neck [18, 19]. In animal cells, the cleavage site is not predetermined, nor is the contractile apparatus preassembled prior to anaphase onset. And while there appears to be flexibility in regards to how long furrow-inducing activity may extend following mitotic exit [20, 21], we find that furrowing cannot be induced prior to the metaphase-anaphase transition (Figure 1). Thus, the apparent block to cytokinesis in animal cells appears to be more closely linked to events associated with anaphase onset than to mitotic exit.

By the time the cell reaches the metaphase-anaphase transition, several critical cell cycle transitions have already occurred. Principle among these is the inactivation of the mitotic spindle checkpoint [22]. Upon attachment of the last unbound kinetochore to the mitotic spindle, the spindle checkpoint is inactivated, initiating the degradation of securins, cyclins, and kinesin-like proteins [23, 24]. Using two different experimental approaches, we find that the mitotic checkpoint regulates the timing of cytokinesis independently of mitotic exit. First, we used a defined effector of the mitotic checkpoint, Mad2, to generate blastomeres arrested in metaphase. And while cells arrested with stabilized cyclin B were able to initiate cytokinesis when the asters were placed in close proximity with the cell cortex, similarly manipulated Mad2-arrested cells failed to stimulate furrow formation (Figure 2). Using a different approach in tissue culture cells, Canman et al. [25] demonstrated that inactivation of Mad2 in nocodazole-treated cells induced cortical contractions, suggesting that Mad2 controlled the initiation of cortical contractility. As a second approach, we generated a binucleate cell in which one checkpoint-activated monopolar spindle and a normal, bipolar MA share a common cytoplasm. And while the bipolar control spindle progressed through mitosis

and induced cytokinesis normally, furrows that normally form between asters from unrelated spindles [10, 11] failed to initiate between normal and checkpoint-arrested spindles (Figure 3). Interestingly, inhibition of metaphase and anaphase by preventing the degradation of endogenous substrates of the anaphase-promoting complex (APC) with the D box-containing, N-terminal domain of cyclin B also inhibited furrow formation [8]. Together, these results indicate that, while the proximal signals regulating cytokinesis may not require mitotic exit, they are dependent upon spindle checkpoint inactivation and may also be dependent on the proteolysis of some other regulatory factor.

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