

Inactivation of Cdk1/Cyclin B in metaphase-arrested mouse FT210 cells induces exit from mitosis without chromosome segregation or cytokinesis and allows passage through another cell cycle

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Abstract It is well known that inactivation of Cdk1/Cyclin B is required for cells to exit mitosis. The work reported here tests the hypothesis that Cdk1/Cyclin B inactivation is not only *necessary* but also *sufficient* to induce mitotic exit and reestablishment of the interphase state. This hypothesis predicts that inactivation of Cdk1 in metaphase-arrested cells will induce the M to G1-phase transition. It is shown that when mouse FT210 cells (in which Cdk1 is temperature-sensitive) are arrested in metaphase and then shifted to their non-permissive temperature, they rapidly exit mitosis as evidenced by reassembly of interphase nuclei, decondensation of chromosomes, and dephosphorylation of histones H1 and H3. The resulting interphase cells are functionally normal as judged by their ability to progress through another cell cycle. However, they have double the normal number of chromosomes because they previously bypassed anaphase, chromosome segregation, and cytokinesis. These results, taken together with other observations in the literature, strongly suggest that in mammalian cells, inactivation of Cdk1/cyclin B is the trigger for mitotic exit and reestablishment of the interphase state.

Introduction

M-phase Promoting Factor (MPF) is a complex of cyclin-dependent kinase 1 (Cdk1; also known as Cdc2 kinase) and cyclin B whose activation in late G2-phase of the cell cycle

triggers the onset of mitosis (Norbury and Nurse 1992). Cdk1/cyclin B activation leads to the phosphorylation of many proteins (e.g., Gurley et al. 1978; Stukenberg et al. 1997), which in turn leads to the characteristic events of mitosis such as reorganization of the cytoskeleton, nuclear envelope breakdown, chromosome condensation, etc. Nuclear envelope disassembly, for instance, is accompanied by phosphorylation of nuclear lamins by Cdk1 (Ward and Kirschner 1990; Heald and McKeon 1990; Peter et al. 1990).

In this paper, “exit from mitosis” will refer to the actual process of leaving mitosis and moving to G1-phase, not necessarily accompanied by anaphase, chromosome segregation, or cytokinesis. During mitotic exit, the events that occurred during the onset of mitosis are reversed, presumably as a result of dephosphorylation of the same proteins. For example, reassembly of the nuclear envelope involves dephosphorylation of the same lamin proteins that were phosphorylated at the start of mitosis (Glass and Gerace 1990; Peter et al. 1991; Marshall and Wilson 1997).

Exit from mitosis requires inactivation of Cdk1/cyclin B (Murray et al. 1989; Ghiara et al. 1991; Luca et al. 1991; Gallant and Nigg 1992; Luo et al. 1994; Rimmington et al. 1994). In normal mitosis, this comes about when the anaphase promoting complex (APC), a specific ubiquitin ligase, marks cyclin B and other proteins for destruction by the proteasome (King et al. 1995; Hixon and Gualberto 2000). Once cyclin B is destroyed, Cdk1 becomes inactive.

The purpose of this work was to test the hypothesis (Paulson et al. 1996, 1997) that inactivation of Cdk1/cyclin B is not only *necessary* but also *sufficient* to trigger exit from mitosis. One could imagine that other mitotic events, independent of Cdk1/cyclin B inactivation, might also be required for a cell to return to interphase, but this hypothesis states that exit from mitosis requires only the

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“upstream” events that lead to Cdk1/cyclin B inactivation (such as APC activation and cyclin B degradation) and “downstream” events that follow from Cdk1/cyclin B inactivation (such as protein dephosphorylation).

The hypothesis predicts that inhibition or inactivation of Cdk1/cyclin B in metaphase-arrested cells, by any available means, should induce exit from mitosis. This prediction has been tested and confirmed using temperature-sensitive mutants in yeasts (Ghiara et al. 1991; He et al. 1997) and in *Drosophila* (Sigrist et al. 1995; Onischenko et al. 2005), but such specific tests have not been done with mammalian cells. We previously tested the prediction by treating metaphase-arrested HeLa cells with inhibitors of Cdk1, and we observed rapid reassembly of interphase nuclei, decondensation of chromosomes, and dephosphorylation of histone H1 (Paulson et al. 1996). Similar effects of Cdk1 inhibitors on mammalian cells have been reported by Nakamura and Antoku (1993), Th'ng et al. (1994), Hall et al. (1996), and Potapova et al. (2006). However, these protein kinase inhibitors are not completely specific for Cdk1 (Gadbois et al. 1992; Vesely et al. 1994; Meijer 1996; Kitagawa et al. 1993), so the results leave open the possibility that inactivation of other protein kinases, such as Cdk2, might also be required for mitotic exit. Furthermore, in none of these cases was it demonstrated that the resulting interphase cells are normal in the sense of being capable of continued progression through the cell cycle. Noton and Diffley (2000) showed that inactivation of Cdk1 in metaphase-arrested *Saccharomyces cerevisiae* is sufficient for origin resetting, re-replication of DNA, and passage through another cell cycle. However, the situation could be more complex in mammalian cells where re-replication of DNA also requires (apparently independently of Cdk1 inactivation) the APC- and proteasome-dependent destruction of geminin (McGarry and Kirschner 1998).

In the work reported here, the above hypothesis was tested using mouse FT210 cells (Mineo et al. 1986) in which Cdk1 is temperature-sensitive (Th'ng et al. 1990). The hypothesis predicts that heat treatment of metaphase-arrested FT210 cells should lead to exit from mitosis, and this prediction is confirmed. Moreover, the interphase cells produced in this way are able to complete another cell cycle. These results support the notion that Cdk1/cyclin B inactivation in metaphase-arrested mammalian cells is by itself sufficient to trigger reestablishment of the interphase state.

Materials and methods

Media and chemicals

Tissue culture media and components were obtained from Gibco BRL (Rockville, MD) or Atlanta Biologicals

(Atlanta, GA). The caspase inhibitor zVAD-fmk (benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone, or caspase inhibitor 1) was obtained from Calbiochem, dissolved at 50 mM in dimethyl sulfoxide, and stored at -20°C . Other reagents were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture and metaphase arrest

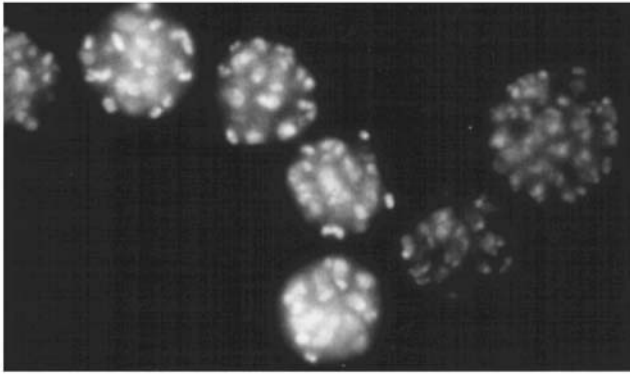
FT210 and FM3A cell lines (Mineo et al. 1986) were kindly provided by Dr. P. M. Yau and Dr. E. M. Bradbury of the University of California-Davis. Cells were grown in suspension at 32°C in 75-cm² polystyrene tissue culture flasks in RPMI-1640 medium supplemented with penicillin, streptomycin, 10% heat-inactivated newborn calf serum, and 20 mM Na⁺-Hepes pH 7.4. Cultures were maintained at between 15 and 60 ml per flask and were diluted daily to $2\text{--}3 \times 10^5$ cells/mL. For metaphase arrest, cultures were treated with 0.25 $\mu\text{g}/\text{mL}$ nocodazole (from a 5 mg/mL stock solution in dimethyl sulfoxide) at 32°C for 24–26 h. This method typically yields cultures with mitotic indices of 70–90% for FT210 and 60–80% for FM3A, with little or no loss of viability. HeLa cells were grown in suspension at 37°C , synchronized by treatment with thymidine, and arrested in metaphase with nocodazole as previously described (Paulson 1982), except that the cultures were shifted to 32°C at the time of addition of nocodazole.

Cell viability was determined using Trypan blue (Patterson 1979). To determine mitotic index, a 200- μL sample of cell culture was mixed with 200 μL of water containing 20 $\mu\text{g}/\text{mL}$ Hoechst 33342. After 5 min at room temperature, 40 μL of freshly prepared fixative (three volumes methanol, one volume acetic acid) was added. To concentrate the sample, fixed cells were pelleted at $735 \times g$ for 2 min in an Eppendorf 5415 C microcentrifuge and gently resuspended in a small portion (about 30 μL) of the supernatant. Finally, the cells were viewed by epifluorescence in a Nikon Labophot microscope. Cells containing individual condensed chromosomes and no nuclear envelope were scored as mitotic (cf. Fig. 1a); cells in which the chromatin exhibited a smooth border indicative of a nuclear envelope were scored as interphase (cf. Fig. 1b). At least 200 cells were counted for each mitotic index determination.

Heat treatments of metaphase-arrested cells

For experiments involving determination of mitotic indices only, culture aliquots were treated in a circulating water bath at the prescribed temperature ($\pm 0.2^{\circ}\text{C}$) in stoppered tubes or small Erlenmeyer flasks. However, for experiments involving histone extraction or determination of the percentage of cells in S-phase, larger quantities of cells were needed. In these cases, culture aliquots were incubated

a M-arrested, before treatment



b After heat treatment

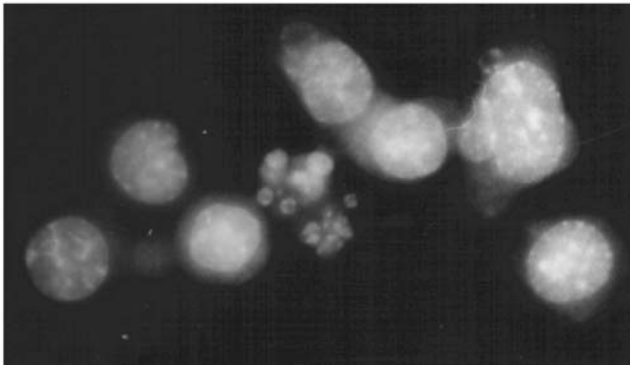


Fig. 1 Heat treatment of metaphase-arrested FT210 cells induces reassembly of interphase nuclei and decondensation of chromosomes. A culture of FT210 growing at 32.0°C was arrested in metaphase by treatment with nocodazole and then shifted to 40.6°C for 90 min. Chromosomes and nuclei were visualized by staining with Hoechst 33342 (see “Materials and methods”). **a** Before heat treatment, most of the cells are in metaphase arrest (mitotic index=80%). **b** After heat treatment, nuclei have reassembled and chromosomes have decondensed in virtually all cells. Cell viability, determined by Trypan blue exclusion, was little affected by the heat treatment

in 250- or 500-mL media bottles containing 1½ or 2-in. Teflon-coated magnetic stirring bars and stirred at 60–80 rpm. For the experiment shown in Fig. 6, cells were heat-treated in 500-mL bottles, but were then placed in 75-cm² flasks at 32°C for the remainder of the experiment.

Determination of S-phase cells

For the determination of the percentage of cells in S-phase (Fig. 6), 5-bromo-2'-deoxyuridine (BrdU) was prepared as a 50-mM stock solution in water and stored in the dark at 2–4°C. Anti-BrdU-FLUOS (Boehringer-Mannheim), a fluorescein-labeled antibody specific for BrdU-containing DNA, was dissolved in water and diluted with incubation buffer (Boehringer-Mannheim). Hanks balanced salt solution (HBSS) consisted of 8.0 g NaCl, 0.4 g KCl, 0.06 g KH₂PO₄, and 0.0621 g Na₂HPO₄ per liter of water. Microscope slides were cleaned with 70% ethanol, air

dried, coated by covering for 5 min with 50 µL/cm² of 0.1 mg/mL poly-L-lysine (Sigma, P-7890) in water, washed several times with water, and thoroughly air-dried.

To label S-phase cells, 25 mL culture aliquots were incubated in the dark at 32°C with 10 µM BrdU in 75-cm² flasks. After 30 min, the cells were pelleted by centrifugation for 5 min at 500×g in a bench top swinging bucket centrifuge, washed twice with fresh culture medium, and resuspended to approximately 5×10⁷ cells/mL. Of this cell suspension, 50 µL was smeared over a clean, poly-L-lysine-coated slide using a second clean slide. The slide was blotted at the edges and air-dried.

To visualize BrdU-labeled cells, slides were rehydrated in HBSS for 15 s, placed in fixative (70% ethanol and 50 mM glycine, pH 2) for 45 min, washed twice for 2 min in HBSS, treated 10 min with 4 M HCl to denature DNA, incubated 5 min in each of four changes of HBSS, and incubated 10 min in incubation buffer (Boehringer-Mannheim). After removal of incubation buffer, Anti-BrdU-FLUOS working solution was added along with a coverslip, and the slides were placed in a humidified incubator at 37°C for 45 min. Finally, labeled cells in randomly selected fields were counted by epifluorescence (515 nm long pass filter) in a Nikon Labophot microscope. Total cells were counted in the same fields under phase contrast.

Determining the number of metaphase chromosomes per cell

Chromosome spreads from metaphase-arrested cultures were prepared as described by Musio et al. (1997). Cells were pelleted, incubated in 75 mM KCl for 20 min at 37°C, fixed with a freshly prepared mixture of methanol and acetic acid (3:1 by volume), splashed dropwise onto cold wet slides from a height of about 20 cm, and finally air-dried. Unstained chromosome spreads were photographed under phase contrast using a 20× objective. Micrographs were printed at a final magnification of about 1,000×, and the chromosomes in each cell were counted on the prints.

To minimize errors due to clumped chromosomes, overlapping chromosome spreads from different cells, loss of chromosomes due to cell breakage, or addition of stray chromosomes from other broken cells, the chromosomes of a given cell were only counted if the following criteria were met: (1) The cell is not badly distorted but is basically round; (2) The chromosomes are confined to the visible limits of the cell; (3) The cell is well separated (at least two cell diameters) from other nearby metaphase spreads; (4) Individual chromosomes are visible and well separated from one another; and (5) There are no chromosomes in the background between cells. Slides were scanned on a raster under phase contrast, and every metaphase spread which came into view was examined. Cells that clearly failed to meet the criteria

were not photographed. After printing, when each cell could be examined more closely, the criteria were applied again. Only if a cell met all five criteria were its chromosomes counted and the number included in the data set.

Extraction and gel electrophoresis of histones

For analysis of histone phosphorylation, cell culture aliquots containing $1.0\text{--}1.5 \times 10^7$ cells were quickly chilled by mixing with three volumes of cold 0.9% NaCl and then incubated on ice for 30 min. The cells were pelleted, washed twice with cold 0.9% NaCl solution, and lysed in 3 mL of 10 mM Na⁺-Hepes pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.5 M sucrose, and 0.1% Non-idet P40. The lysis solution also contained 2 mM *p*-chloromercuriphenyl sulfonate or *p*-hydroxymercuribenzoate to prevent histone dephosphorylation (Paulson 1980). After pelleting chromosomes and nuclei, histones were extracted with 1.0 mL 0.2 M H₂SO₄, precipitated with four volumes of ethanol overnight at -20°C , washed with acetone, dried in a desiccator and redissolved in 1 mM HCl.

For acid-urea polyacrylamide gel electrophoresis, aliquots of extracted histones were freeze-dried in 1.5 mL microcentrifuge tubes in a Centrivap (Labconco), redissolved in sample buffer, and run on polyacrylamide gels containing 2.5 M urea, 5.4% acetic acid, 15% acrylamide, and 0.1% bisacrylamide as described by Panyim and Chalkley (1969), except that the gel wells were filled during pre-electrophoresis with a solution containing 2.5 M urea, 5.4% acetic acid, and 10% (*w/v*) polyethylene glycol 8,000 (Sigma) to prevent distortion of the wells (Paulson and Higley 1999). Acid urea gels were stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol, 10% acetic acid and destained in 5% methanol, 10% acetic acid.

SDS polyacrylamide gels were run as described by Laemmli and Favre (1973), except that the gels contained 12% acrylamide and 0.321% piperazine diacrylamide (Bio-Rad). Gels were first stained with ProQ Diamond fluorescent phosphoprotein-specific stain (Molecular Probes), imaged using a BioRad FX fluorescence imager, and subsequently stained with Coomassie Brilliant Blue R250.

Results

Heat treatment of metaphase-arrested FT210 cells induces reassembly of interphase nuclei, decondensation of chromosomes, and dephosphorylation of histones H1 and H3

It has been shown that the Cdk1 component of MPF is temperature-sensitive in the mouse mammary carcinoma

cell line FT210 (Th'ng et al. 1990). To test whether inactivation of Cdk1/cyclin B in mitotic cells is sufficient to trigger exit from mitosis and reassembly of interphase nuclei, FT210 cells were arrested in metaphase with nocodazole at their permissive temperature (32.0°C) and then shifted to non-permissive temperatures (39.0°C or higher). At various times, samples were removed, treated hypotonically, stained with Hoechst 33342, and fixed for examination in the fluorescence microscope.

Figure 1 shows clearly that heat treatment of metaphase-arrested FT210 cells induces exit from mitosis. Before heat treatment, the majority of cells contain condensed metaphase chromosomes (Fig. 1a). However, after treatment for 90 min at 40.6°C , more than 90% of the cells contain interphase nuclei with decondensed chromosomes (Fig. 1b). This result cannot be explained by preferential death of metaphase-arrested cells, as the cell viability and cell concentration dropped by less than 10% during the heat treatment.

Note that the reassembly of nuclei observed in Fig. 1 occurs in the continued presence of nocodazole and, thus, in the absence of a mitotic spindle. Cells therefore exit mitosis without chromosome segregation or cytokinesis. They clearly do not divide, as the cell population does not increase and metaphase plates, anaphase or telophase cells, and G1-pairs are never seen. Presumably, nuclear envelopes simply reassemble around the clustered chromosomes. Interestingly, about 20% of the cells contain multiple micronuclei; an example is seen near the center of Fig. 1b. This is most likely due to reassembly of nuclear envelopes around individual chromosomes or small groups of chromosomes.

The rate of conversion of metaphase-arrested FT210 cells to interphase cells depends upon the temperature at which they are treated. For example, the process is half complete in about 20 min at 42.0°C , 30 min at 41.2°C , and 50 min at 40.4°C (Fig. 2). This temperature dependence is not surprising, as one would expect the heat-labile Cdk1 of FT210 (Th'ng et al. 1990) to be inactivated more rapidly or more completely at higher temperatures. Reassembly of nuclei is not observed in cells left at 32.0°C (Fig. 2).

Histone H1 becomes highly phosphorylated at the onset of mitosis, but is dephosphorylated during exit from mitosis (Gurley et al. 1978). The mitosis-specific phosphorylation of H1 significantly lowers its mobility in acid-urea polyacrylamide gel electrophoresis (Panyim and Chalkley 1969; Gurley et al. 1978) and, thus, the mitotic and interphase forms of H1 can be easily distinguished by this technique. Analysis of FT210 histones on acid-urea gels shows that histone H1 is dephosphorylated during the course of heat treatment, roughly in parallel with reassembly of interphase nuclei. For example, in metaphase-arrested cells treated at 42.0°C , dephosphorylation of H1

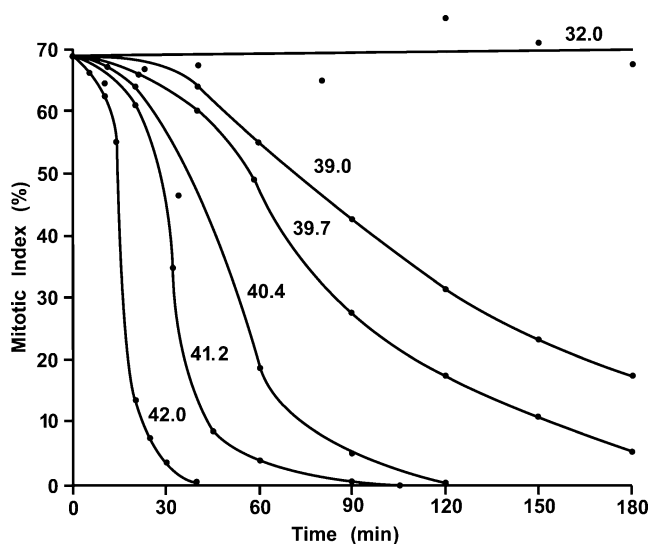


Fig. 2 Rate of reassembly of interphase nuclei during heat treatment of metaphase-arrested FT210 cells at various temperatures. A culture of FT210 growing at 32.0°C was arrested in metaphase with nocodazole (mitotic index=69%), and aliquots were then incubated at 32.0 (control), 39.0, 39.7, 40.4, 41.2, and 42.0°C. Mitotic indices are plotted as a function of the time of treatment at the indicated temperatures

is nearly complete after 25 min (Fig. 3a), at which point the mitotic index has dropped from its initial value of 75% to less than 10% (cf. Fig. 2).

Like histone H1, histone H3 also becomes phosphorylated at the onset of mitosis and is dephosphorylated during exit from mitosis (Gurley et al. 1978; Paulson and Taylor 1982). The acid-urea gel in Fig. 3a shows that H3 also becomes dephosphorylated during heat treatment of metaphase-arrested FT210 cells. Dephosphorylation of both histones H1 and H3 during the heat-treatment has been confirmed by SDS polyacrylamide gel electrophoresis followed by staining with a fluorescent phosphoprotein-specific stain (Fig. 3b).

Effects of heat treatment on metaphase-arrested mouse FM3A cells and HeLa cells

One could argue that perhaps heat treatment of metaphase-arrested cells would induce exit from mitosis with any mammalian cell line and that the results described above are unrelated to the temperature-sensitivity of Cdk1 in FT210. To test this possibility, FM3A cells and HeLa cells were also examined (Figs. 4 and 5). As before, cultures were arrested in metaphase at 32.0°C and then shifted to higher temperature. Samples were taken at various times for determination of mitotic index and for histone extraction.

FM3A is the parent cell line from which FT210 was produced by mutagenesis (Mineo et al. 1986). When metaphase-arrested FM3A cells are treated at higher temperature, reassembly of interphase nuclei and histone

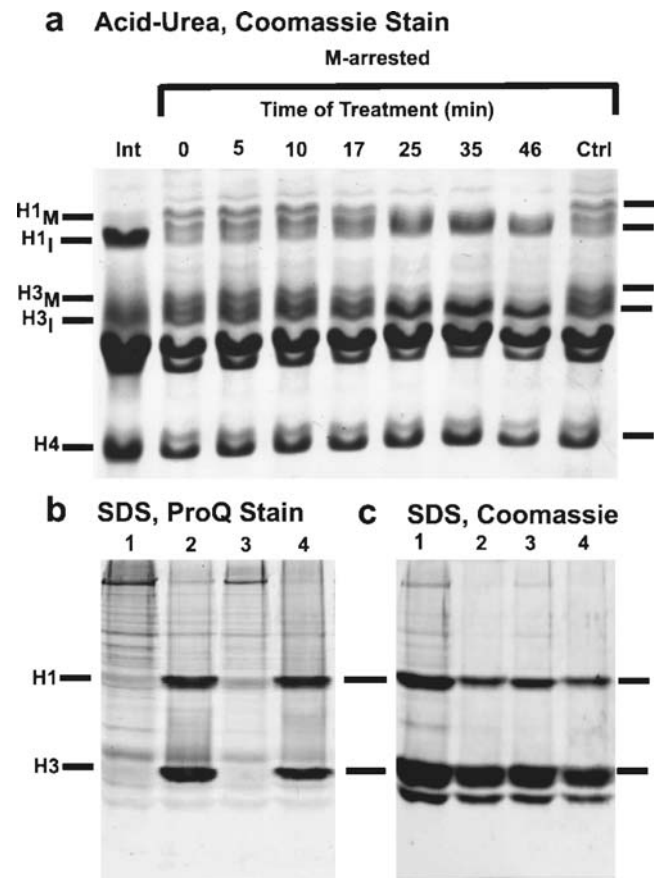


Fig. 3 Heat treatment of metaphase-arrested FT210 cells induces dephosphorylation of histones H1 and H3. **a** Dephosphorylation observed by acid-urea polyacrylamide gel electrophoresis (Panyim and Chalkley 1969): Cells were arrested in metaphase with nocodazole (mitotic index, 75%), and histones were acid-extracted after various times of treatment at 42.0°C. The control (*Ctrl*, lane 9) remained at 32.0°C, and histones from unsynchronized (predominantly interphase) cells are shown for comparison in lane 1 (*Int*). The positions of some major histone species are shown at the left. *H1_M* and *H3_M* indicate the positions of mitotic (phosphorylated) histones, and *H1_I* and *H3_I* indicate the positions of the interphase forms. The gel was stained with Coomassie blue. **b** Dephosphorylation observed by SDS polyacrylamide gel electrophoresis followed by staining with the phosphospecific stain ProQ Diamond (Molecular Probes): Histones were acid-extracted from unsynchronized (predominantly interphase) cells (*lane 1*); from cells arrested in metaphase at 32°C (mitotic index, 80%; *lane 2*); from cells arrested as for lane 2 and then treated 80 min at 42.0°C (*lane 3*); and from cells arrested as for lane 2 and then incubated an additional 80 min at 32°C (*lane 4*). **c** Same gel as in (b), but stained with Coomassie blue

H1 dephosphorylation are observed. However, in nine repetitions of this experiment, these changes were always significantly delayed in comparison with FT210 cells treated in parallel (e.g., Fig. 4). Heat treatment of FM3A has been shown to lead to loss of Cdk1 kinase activity, although less rapidly than in FT210, and this is thought to be due to a heat-shock stress effect rather than a result of the temperature-sensitivity of Cdk1 itself (Th'ng et al. 1990; Hall, Guo, Bradbury, personal communication).

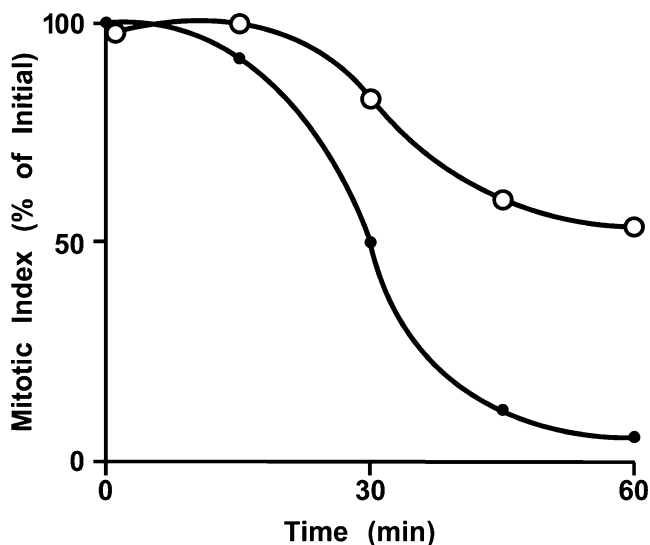


Fig. 4 Comparison of the effects of heat treatment on metaphase-arrested FT210 and FM3A cells. Cultures of FT210 (●—●) and FM3A (○—○) were arrested in metaphase with nocodazole at 32°C and then treated in parallel at 41.2°C. For ease of comparison, the *ordinate* shows the mitotic index as a percentage of the initial mitotic index, which was 90% for FT210 and 79% for FM3A

With metaphase-arrested HeLa cells, the mitotic index also falls during heat treatment, although much more slowly than with FT210 cells treated in parallel (Fig. 5a). However, in this case, one does not observe reassembly of interphase nuclei. Instead, many cells are seen with highly blebbed plasma membranes and with highly condensed and fragmented nuclear material, suggesting apoptosis. Histone H1 dephosphorylation is observed (data not shown), but this is also characteristic of apoptosis (Kratzmeier et al. 2000). The occurrence of apoptosis rather than nuclear reassembly was confirmed by carrying out the heat treatment in the presence of 200 μ M zVAD-fmk (caspase inhibitor 1), which blocks apoptosis (Fearhead et al. 1995; Pronk et al. 1996; Thornberry and Lazebnik 1998). In the presence of zVAD-fmk, the morphological changes characteristic of apoptosis do not occur, and the HeLa cells remain in metaphase arrest after the heat treatment (Fig. 5b). By contrast, zVAD-fmk has no effect on reassembly of nuclei during heat treatment of metaphase-arrested FT210 cells (compare Fig. 5a and b). The occurrence of apoptosis as a result of heat treatment of metaphase-arrested HeLa cells has been further confirmed by detection of active Caspase 3 and other experiments (Kecskeméti et al. 2002).

Reentry of the cell cycle following heat treatment of metaphase-arrested FT210 cells

The results described above show that heat treatment of metaphase-arrested FT210 cells leads to dephosphorylation of histones, reassembly of nuclei, and decondensation of

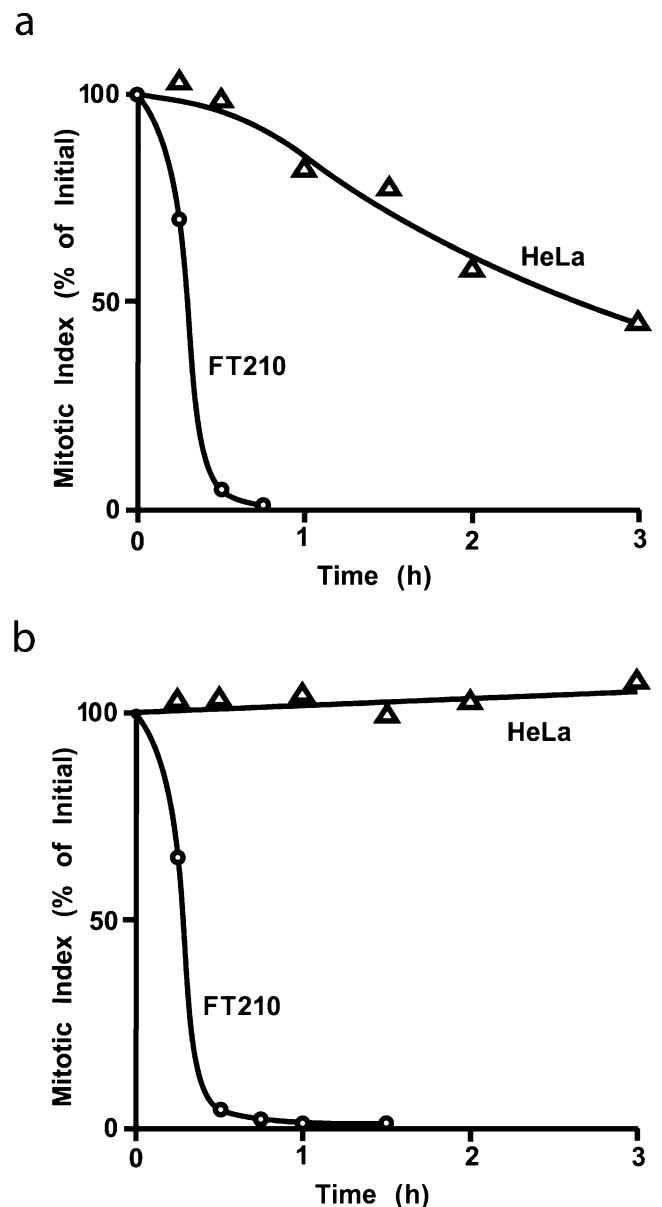


Fig. 5 Comparison of the effects of heat treatment on metaphase-arrested FT210 and HeLa cells. Cultures of FT210 (○—○) and HeLa (△—△) were arrested in metaphase with nocodazole at 32°C and then treated in parallel at 41.4°C either in the absence (a) or in the presence (b) of 200 μ M zVAD-fmk, a specific caspase inhibitor. For ease of comparison, the *ordinate* shows the mitotic index as a percentage of the initial mitotic index, which was 90% for FT210 and 70% for HeLa. In (a), the decrease in the percentage of metaphase-arrested FT210 cells is accompanied by an increase in the percentage of interphase cells, reflecting exit from mitosis. However, the decrease in the percentage of metaphase-arrested HeLa cells is due to induction of apoptosis, not reassembly of interphase nuclei. When apoptosis is blocked with zVAD-fmk (b), the percentage of metaphase-arrested HeLa cells does not decrease during the heat treatment

chromosomes, three clear indications of exit from mitosis. But are the nuclei which result from this treatment really functional? Or do they represent an abnormal state which, despite its appearance in the light microscope, is unable to carry out normal interphase functions?

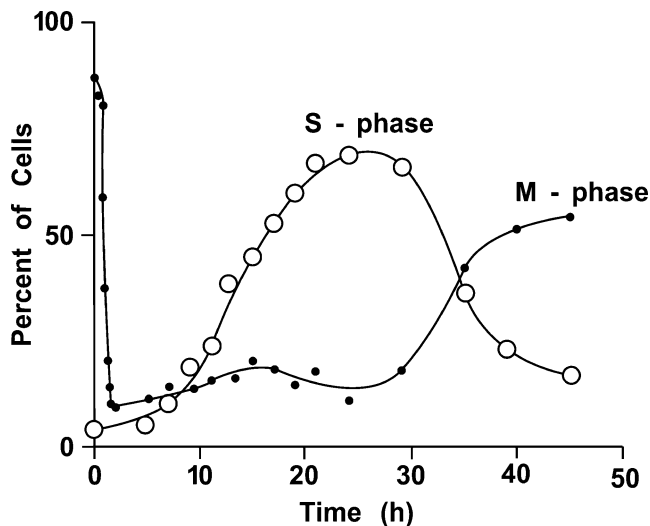


Fig. 6 Heat treatment of metaphase-arrested FT210 cells leads to reassembly of functional nuclei as demonstrated by the cells' ability to progress through another cell cycle. Nocodazole-arrested FT210 cells (mitotic index, 87%) were treated for 90 min at 40.6°C, causing the mitotic index to drop to less than 10%. The cells were then shifted back to 32.0°C and further incubated. At various times, the mitotic index (●—●) and the percentage of cells in S-phase (○—○) were determined

To answer these questions, the cells were tested for their ability to progress through another cell cycle after heat treatment. A nocodazole-arrested FT210 culture (mitotic index, 87%) was treated at 40.6°C for 90 min to induce reassembly of nuclei, and then shifted back to the permissive temperature of 32.0°C. At various times, mitotic index, cell concentration, cell viability, and percentage of cells in S-phase were determined.

Figure 6 shows that most cells entered S-phase between 8 and 20 h after the heat treatment. The percentage of cells in S-phase reached a maximum of nearly 70% after 24 h and then began to fall as cells completed DNA synthesis. Moreover, many cells which exited metaphase arrest during the heat treatment were able to progress to the next mitosis. As nocodazole was still present in the culture medium, they again arrested in metaphase, eventually reaching a mitotic index of more than 50% in this second metaphase arrest (Fig. 6).

As heat treatment of metaphase-arrested FT210 cells appears to cause exit from mitosis without chromosome segregation or cytokinesis, cells which reach the next metaphase would be expected to have double the normal number of chromosomes. This is indeed the case. Before heat treatment ($T=0$ in Fig. 6), metaphase-arrested FT210 cells typically have about 40 chromosomes (e.g., Fig. 7a), but after heat treatment and further incubation of the cells for 45 h at 32.0°C ($T=45$ in Fig. 6), a typical metaphase-arrested cell has about 80 chromosomes (e.g., Fig. 7b). The mean number of chromosomes was found to be 41.0 ± 1.4 ($n=68$) before heat treatment (Fig. 7c) and 81.1 ± 2.6 ($n=59$) at the next metaphase after heat treatment (Fig. 7d).

These results indicate that a majority of the metaphase-arrested FT210 cells which exit mitosis as a result of heat inactivation of Cdk1 are able to progress through another cell cycle. When arrested at the next mitosis, they have twice the usual number of chromosomes, confirming that the heat treatment caused them to return to interphase without dividing.

Discussion

Heat treatment of metaphase-arrested mouse FT210 cells induces exit from mitosis without chromosome segregation or cytokinesis and allows passage through another cell cycle

The results presented above show that heat treatment of metaphase-arrested mouse FT210 cells leads to reassembly of interphase nuclei (Figs. 1 and 2), decondensation of chromosomes (Fig. 1), and dephosphorylation of histones H1 and H3 (Fig. 3), all of which are clear signs of exit from mitosis. On the other hand, segregation of sister chromatids and cytokinesis do not occur. In the presence of the spindle poison nocodazole, nuclear envelopes simply reassemble around the clustered, condensed chromosomes (Glass and Gerace 1990; Marshall and Wilson 1997), and the cells return to interphase without dividing. Apparently, nuclear envelopes sometimes reassemble around individual chromosomes or small groups, giving rise to cells with multiple micronuclei. This phenomenon has also been observed previously in other situations where mammalian cells escape from metaphase arrest (Nakamura and Antoku 1993; Paulson et al. 1996; Ajiro et al. 1996; Brito and Rieder 2006).

After heat-induced exit from mitosis, the cells are able to replicate their DNA and progress through another cell cycle (Fig. 6), showing that the treatment does not simply induce an abnormal, interphase-like state. However, when arrested at the next metaphase, they have twice the normal number of chromosomes, confirming that they exited mitosis without dividing (Fig. 7). Endoreduplicated chromosomes are not observed, suggesting that even with an active spindle checkpoint, Cdk1 inactivation somehow leads to loss of chromosome cohesion, presumably by destruction of cohesins (Nasmyth et al. 2000). Potapova et al. (2006) have also shown that Cdk1 inhibition in mitotic cells leads to chromatid separation, provided that the proteasome is active.

Cdk1/cyclin B inactivation as the trigger for exit from mitosis and reestablishment of the interphase state

The purpose of these experiments was to test the hypothesis that specific inactivation of Cdk1 in mammalian cells is sufficient to trigger exit from mitosis. This hypothesis

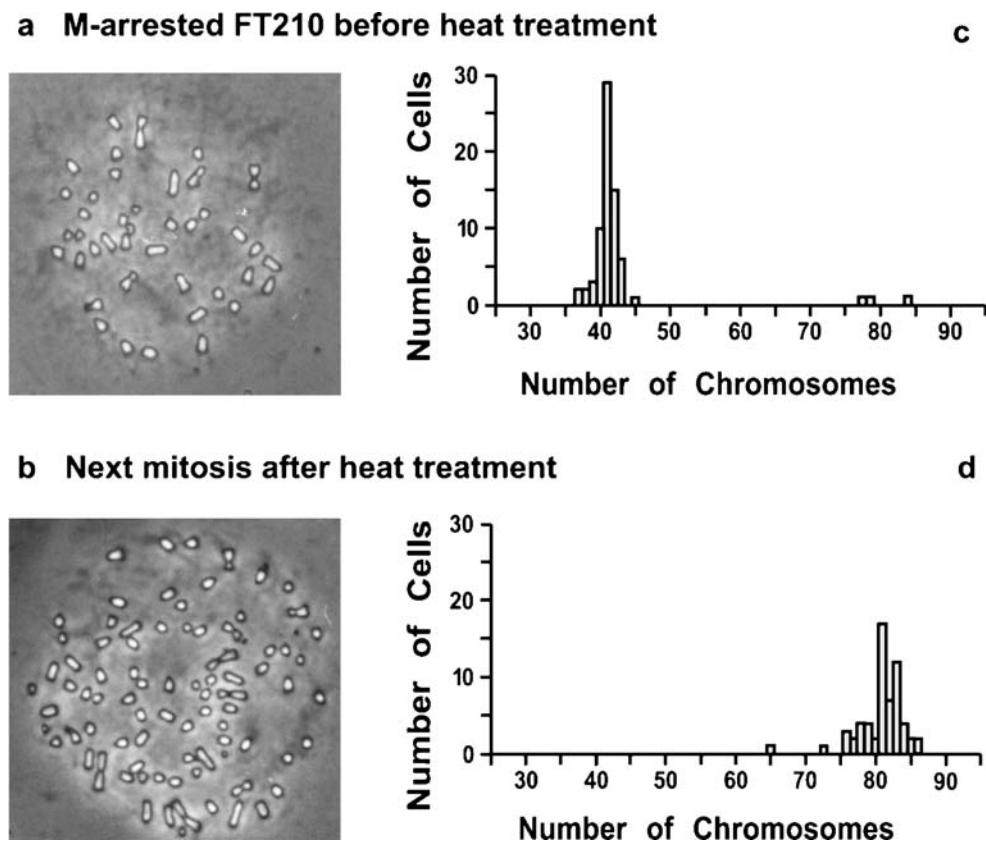


Fig. 7 Doubling of chromosome numbers following heat treatment of nocodazole-arrested FT210 cells. **a, b** Metaphase spreads viewed by phase contrast (*unstained*). **a** Spread chromosomes of a metaphase-arrested FT210 cell from $T=0$ in Fig. 6: This cell contains approximately 40 chromosomes. **b** Spread chromosomes of an FT210 cell which was arrested in metaphase, treated at 40.6°C for 90 min and then shifted to 32.0°C and allowed to progress to the next metaphase ($T=45$ h in Fig. 6): This cell contains approximately 80 chromosomes. **c, d** Distribution of

chromosome numbers in metaphase-arrested FT210 cells before heat treatment and at the next mitosis after heat treatment. **c** Before heat treatment ($T=0$ in Fig. 6): The majority of cells cluster in a peak with a mean chromosome number of 41.0 ± 1.4 ($n=68$), but a few cells with about 80 chromosomes are observed. **d** After heat treatment ($T=45$ h in Fig. 6): The majority of cells cluster in a peak with a mean chromosome number of 81.1 ± 2.6 ($n=59$), but a few cells with about 160 chromosomes are observed (not shown)

predicts that as Cdk1 is temperature-sensitive in FT210 (Th'ng et al. 1990), heat treatment of metaphase-arrested FT210 cells should lead to mitotic exit.

The results presented here tend to confirm the hypothesis for mammalian cells. In particular, they show that Cdk1 has a unique function in keeping cells in mitosis which cannot be substituted by Cdk2. Previous studies using protein kinase inhibitors to induce mitotic exit in mammalian cells could not draw this conclusion because those inhibitors would also have blocked Cdk2 (Gadbois et al. 1992; Vesely et al. 1994; Meijer 1996; Kitagawa et al. 1993). Studies in yeast cannot shed light on this problem, as they lack Cdk2 (Nasmyth 1993). Cdk1 may be an essential gene, whereas Cdk2 apparently is not, at least in part because only Cdk1 can keep cells in mitosis long enough for chromosome segregation to be executed successfully.

A number of other studies in the literature can also be considered tests of this hypothesis (although few of them mention the idea of sufficiency explicitly and none of them

discusses it thoroughly). In all cases, inactivation or inhibition of Cdk1 leads to signs of exit from mitosis. First, inhibition of Cdk1 *in vivo* with various protein kinase inhibitors leads to exit from mitosis in metaphase-arrested mammalian cell cultures (Nakamura and Antoku 1993; Th'ng et al. 1994; Paulson et al. 1996; Hall et al. 1996; Potapova et al. 2006). Potapova et al. (2006) also showed that flavopiridol-induced exit from mitosis is reversible as long as cyclins are not degraded. Second, inhibitors of Cdk1 induce exit from meiotic metaphase II arrest in oocytes (Lee et al. 1999; Phillips et al. 2002). Third, treatment of metaphase-arrested cells with sodium vanadate, a Cdc25 inhibitor (Dunphy and Kumagai 1991), leads to inactivation of Cdk1/cyclin B via inhibitory phosphorylation of Cdk1 at Tyr-15, and this induces exit from mitosis in Chinese hamster tsTM13 cells (Ajiro et al. 1996) and in HeLa cells (J.R. Paulson, unpublished work). Vanadate also induces exit from meiotic metaphase-II arrest in pig oocytes (Lee et al. 1999). Fourth, inactivation of Cdk1/cyclin B by

heat-treatment of cells carrying appropriate temperature-sensitive mutations has been shown to induce mitotic exit in budding yeast (Ghiara et al. 1991), in fission yeast (He et al. 1997), and in *Drosophila* embryos (Sigrist et al. 1995; Onischenko et al. 2005). Finally, inactivation of Cdk1 (Cdc28) in metaphase-arrested *S. cerevisiae* carrying the *cdc28-as1* mutation, which makes Cdc28p uniquely sensitive to inhibition by an ATP analog (Bishop et al. 2000), leads to exit from mitosis in those cells (J.M. Keaton, B.G. Workman, L. Xie and J.R. Paulson, unpublished work).

None of these studies can by itself confirm the hypothesis with absolute certainty. With any single approach used to inactivate Cdk1/cyclin B, one cannot completely rule out the possibility that the treatment fortuitously triggers another event that is also required for mitotic exit. However, a wide variety of approaches, in several different systems, have given similar results. If there existed another event independent of Cdk1/cyclin B inactivation that was also required for mitotic exit, that event would have to be a chance by-product of all the treatments listed above. As this seems extraordinarily unlikely, we can confidently conclude that Cdk1/cyclin B inactivation alone is sufficient to trigger the mitosis to G1-phase transition.

What happens downstream from Cdk1 inactivation?

Evidence suggests that the dramatic cellular changes which occur at the onset of mitosis are most likely due to phosphorylation of proteins by Cdk1 (MPF) and secondary protein kinases, and that their reversal is due to dephosphorylation of the same proteins. How does Cdk1/cyclin B inactivation at the end of mitosis lead to protein dephosphorylation?

Evidence suggests that protein phosphatase 1 (PP1) is responsible for the dephosphorylation of proteins, and particularly nuclear proteins, at the end of mitosis. It localizes to mitotic chromosomes and is required for mitotic exit in mammalian cells (Fernandez et al. 1992) and in fission yeast (Kinoshita et al. 1991), and it is required downstream from Cdk1 inactivation during exit from mitosis in *S. cerevisiae* (J.M. Keaton, B.G. Workman, L. Xie and J.R. Paulson, unpublished work). During mitotic exit, PP1 is involved in nuclear reassembly and dephosphorylation of histone H1 (Paulson et al. 1996), dephosphorylation of nuclear lamin proteins (Peter et al. 1991; Thompson et al. 1997; Steen et al. 2000), dephosphorylation of histone H3 (Hsu et al. 2000), and dephosphorylation of the retinoblastoma protein (pRb) (Ludlow et al. 1993; Nelson and Ludlow 1997; Nelson et al. 1997; Puntoni and Villa-Moruzzi 1997a; Yan and Mumby 1999). Other evidence strongly suggests that PP1 is down-regulated during mitosis, possibly via phosphorylation of its catalytic

subunit by Cdk1/cyclin B (Yamano et al. 1994; Dohadwala et al. 1994; Ishii et al. 1996; Puntoni and Villa-Moruzzi 1997b,c; Kwon et al. 1997). Alternatively, PP1 may be controlled by phosphorylation of regulatory subunits (Ichikawa et al. 1996; Aggen et al. 2000) or by its localization in the cell (Andreassen et al. 1998; Haneji et al. 1998; Kotani et al. 1998).

These observations suggest a simple model in which PP1 is phosphorylated and down-regulated at the onset of mitosis by Cdk1/cyclin B or other protein kinases, and later dephosphorylated (by itself or other protein phosphatases) and reactivated after Cdk1 inactivation, thus leading to dephosphorylation of mitotic phosphoproteins and exit from mitosis. Indeed, inactivation of protein phosphatases may be just as important a feature of the onset of mitosis as activation of protein kinases. As initiation of mitosis involves protein kinases gaining the upper hand over protein phosphatases, this model would help explain the hysteresis observed in mitotic onset in which the level of Cdk1/cyclin B activity required to drive the cell into mitosis is greater than the level required to maintain the mitotic state (Sha et al. 2003; Pomerening et al. 2003).

Conclusions

Evidence has been presented that specific inactivation of Cdk1 in metaphase-arrested mammalian cells is sufficient to induce exit from mitosis and reestablishment of the interphase state. It is reasonable to suppose that Cdk1/cyclin B inactivation, via proteolytic destruction of cyclin B, is also the trigger for reestablishment of interphase at the end of normal mitosis, not only in mammalian cells but in all eukaryotes.

Heat treatment of metaphase-arrested FT210 cells could provide a tool for inducing mitotic exit quite synchronously in large populations of mammalian cells for biochemical and structural studies. Such a system would allow events downstream from Cdk1/cyclin B inactivation to be studied independently of the anaphase signal, chromosome segregation, and cytokinesis.

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