Induction of partial mitosis in BHK cells by 2-aminopurine

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Summary
The protein kinase inhibitor 2-aminopurine (2-AP) inhibits a subset of mitotic events in BHK cells. In the presence of the drug, these cells form a bipolar spindle in mitosis, but chromatin fails to generate functioning chromosomes. Cells in 2-AP progress through a partial mitosis, in which there is no observable metaphase, anaphase or telophase events. After 12 h of exposure to 2-AP the chromatin in mitotic cells fails to condense into discrete chromosomes, and is displaced by the spindle to form 'binucleate' cells and cells containing abnormally shaped nuclei in the subsequent interphase. Other mitotic modifications of nuclei, such as nucleolar and nuclear lamina disassembly, occur normally. Centromeres in these nuclei do not become engaged in the spindle, but instead show either no association or a lateral arrangement around the spindle. Cells treated with 2-AP are not arrested in mitosis. Therefore, mitotic exit is not inhibited by the failure of these cells to progress through the latter stages of mitosis. Further, nocodazole-arrested cells quickly exit mitotic arrest when 2-AP is added. We conclude that 2-AP interferes with a specific subset of mitotic events, and that it allows cells to overcome checkpoints that require spindle function for mitotic progression.

Key words: cell cycle, chromosomes, mitotic spindle, p34\textsuperscript{cdc2}, MPM-2.

Introduction
Entry of cells into mitosis characteristically involves coordinated and simultaneous events, which include cytoskeletal rearrangements, disassembly of the nuclear envelope and of nucleoli, and condensation of chromatin into chromosomes. Genetic, biochemical and morphological evidence implicate p34\textsuperscript{cdc2} kinase as the master switch phosphorylated by p34\textsuperscript{cdc2} kinase. These substrates of p34\textsuperscript{cdc2} include histone H1 (Langan et al. 1989), nucleolin and NO38 (Peter et al. 1990a; Belenguer et al. 1990), lamin B (Peter et al. 1990b) and caldesmon (Yamashiro et al. 1991).

The cytoskeleton of cultured cells entering mitosis is rearranged dramatically. Caldesmon, an actin-associated protein, has been shown to be a p34\textsuperscript{cdc2} kinase substrate (Yamashiro et al. 1991), and its phosphorylation may be involved in induction of M-phase-specific dissolution of actin cables. The interphase microtubule network disassembles, and is replaced by a mitosis-specific astral array emanating from centrosomes. This rearrangement has been correlated with the presence of mitosis-specific p34\textsuperscript{cdc2} kinase in cell extracts (Verde et al. 1990), and with increased MAP2 kinase activity (Gotoh et al. 1991).

Changes in nuclear structure during mitotic entry are also correlated with p34\textsuperscript{cdc2} activity. Chromatin condensation into chromosomes is accompanied by p34\textsuperscript{cdc2} kinase-induced phosphorylation of histone H1 (Langan et al. 1989), nuclear envelope dissolution is accompanied by p34\textsuperscript{cdc2}-specific phosphorylation of lamin B (Peter et al. 1990b), and nucleolar disappearance is coordinated with the p34\textsuperscript{cdc2}-dependent phosphorylation of nucleolin and NO38 (Peter et al. 1990a; Belenguer et al. 1990).

As mitosis progresses, the p34\textsuperscript{cdc2} kinase appears to trigger a cascade of downstream mitotic phenomena such as metaphase alignment of chromosomes, segregation of sister chromatids in anaphase and cleavage furrow formation. Little is known of the proximal triggers of these downstream events, either with respect to their identity, or with respect to the spectrum of their individual functions.

We here present evidence for the specific inhibition of a subset of mitotic events by 2-aminopurine (2-AP), which has been identified as a specific protein kinase inhibitor (Farrell et al. 1977; Mahadevan et al. 1990). After 12 h of exposure to 2-AP, BHK cells form a bipolar mitotic spindle, but chromatin does not condense into chromosomes nor interact with the spindle. As a result, the mitotic spindle deforms an apparently intact nucleus before the cell reverts to interphase. No 2-AP-treated mitotic cell has shown evidence of a metaphase, anaphase or telophase configuration. The deformed nuclei that result persist into the next interphase, and for the most part give cells the appearance of being 'binucleate'. The induction of apparent binucleate status in mammalian cells by exposure to 2-AP has been reported previously (Schlegel et al. 1990), and attributed to a failure of cytokinesis. However, we find that such binucleate cells appear to arise by lateral displacement and deformation of nuclei on either side of a bipolar mitotic spindle during mitosis. We characterize the failure of chromatin to transform into functioning chromosomes in otherwise normal mitotic cells as 'partial mitosis'.

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Negative regulators of downstream mitotic events, called checkpoints, have been identified by genetic criteria (Hartwell and Weinert, 1989). These regulators require the completion of prerequisite events before advancement in the cell cycle. Such checkpoints occur to ensure the fidelity of chromosomal segregation to daughter cells (Hartwell and Weinert, 1989). Cells treated with 2-AP apparently can overcome mitotic checkpoints, as they are not arrested in partial mitosis. Mitotic exit signals are still capable of functioning despite incomplete mitosis. Many, and perhaps all, cell types are arrested in mitosis by drugs that depolymerize microtubules, such as nocodazole (Zieve et al. 1980). We find that in cells arrested in mitosis by nocodazole the addition of 2-AP is able to overcome mitotic arrest, and cause a reversion of cells to interphase. When nocodazole-blocked cells are released into 2-AP, such cells retain chromosomes while a bipolar spindle forms, but their chromosomes exhibit no capacity to engage in the mitotic spindle. Thus, even when chromosomes can be made to coexist with the mitotic spindle in 2-AP-treated cells, they fail to function.

The observation that selective mitotic events can be specifically abolished by 2-AP indicates that 2-AP-treated cells will serve as a useful model system for the biochemical analysis of the control of chromosome behavior in mitosis. As 2-AP permits cells to exit from mitosis without a metaphase or anaphase, and to escape nocodazole-dependent mitotic arrest, the drug will also prove useful for biochemical analysis of the regulators of downstream checkpoints in mitosis.

Materials and methods

Cell culture and synchronization

Baby Hamster Kidney (BHK) cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% defined fetal bovine serum (Hyclone Laboratories, Logan, UT), and were maintained in a humid incubator at 37°C in a 5% CO₂ environment. Cells were synchronized in mitosis by addition of nocodazole (Sigma Chemical Co., St. Louis, MO) to a final concentration of 0.06 μg ml⁻¹, from a 20 μg ml⁻¹ stock in dimethylsulfoxide.

Antibodies

Anti-lamin B human autoimmune serum (Guilly et al. 1987a), a gift from Dr J.C. Courvalin, was used at a 200-fold dilution. Anti-centromere serum from a human patient (J.D.) with a CREST scleroderma autoimmune disorder was supplied by Dr Barbara Hamakalo and used at a 500-fold dilution. MPM-2 mouse monoclonal antibody, reactive with mitosis-specific phosphoproteins (Davies et al. 1983), was used at a 500-fold dilution of the ascites fluid. MPM-2 antibody was supplied by Dr P.N. Rao. Mouse anti-β-tubulin antibody (Eastacres Biologicals, Southbridge, MA) was diluted 25-fold for use.

Immunofluorescence microscopy

In preparation for microscopy, cells were grown for a minimum of 12 h on polylsine-coated glass coverslips. Unless otherwise noted, cells were fixed for 20 min with 2% paraformaldehyde in PBS (136 mM NaCl, 2 mM KCl, 10.6 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.4) at 37°C. The coverslips were then washed 3 times, 5 min each, with PBS. Primary and secondary antibodies were applied in PBS, also containing 3% bovine serum albumin, 0.05% Tween-20 and 0.1% sodium azide. Incubation with primary antibodies (60 min in a humid chamber at 37°C) was followed by three washes with PBS, as above. Secondary antibodies were then applied for 30 min at 37°C in a humid chamber. They included FITC-conjugated affinity-purified goat anti-human and anti-mouse antibodies, applied at 8.5 μg ml⁻¹, and Texas Red-conjugated goat anti-mouse antibodies, applied at 14 μg ml⁻¹. All secondary antibodies were from Tago, Inc. (Burlingame, CA). Coverslips were then washed twice with PBS, both before and after a 5 min incubation with propidium iodide (1 μg ml⁻¹ in PBS), or three times in PBS if propidium iodide staining was omitted.

MPM-2 antibody was applied to cells as described above, except that cells were preclarified and fixed according to the technique of Vandre and Borisy (1989). Cells were permeabilized for 90 s with 0.5% Triton X-100 in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.9) at 37°C and then fixed 15 min with 0.7% glutaraldehyde in PHEM at 37°C. Following a PBS wash, autofluorescence was quenched by two 15-min reductions with 1 mM NaBH₄ in 10 mM Tris-HCl, 125 mM NaCl, pH 7.2. Coverslips were then washed 3 times with PBS, and the antibody incubations and washes were thereafter done as described above.

For microscopy, coverslips were mounted with 25 mg ml⁻¹ 1,4-diazabicyclo[2.2.2]octane (Kodak, Rochester, NY) in 90% glycerol/PBS, pH 8.6 (Johnson et al. 1982), and samples were observed using a MRC-500 Laser Scanning Confocal apparatus (Bio-Rad Microscience, Cambridge, MA) attached to a Nikon Optiphot microscope (Nikon, Inc., Torrance, CA). Photographs were taken on Ilford XP1 400 film (Ilford Ltd., Cheshire, England) at an exposure of f/5.6, for one second. Prints were made on Ilford MC Rapid paper (Ilford).

2-Aminopurine treatment

2-Aminopurine was obtained from Sigma Chemical Company (St. Louis, MO), as either a free base or a nitrate salt. A stock of 100 mM 2-AP was kept in 100 mM Hepes buffer at pH 7.2. In experiments where 2-AP was applied, control cells received identical final concentrations (10 mM) of Hepes buffer.

Results

After 12 h of exposure to 2-aminopurine (2-AP), a substantial proportion of baby hamster kidney (BHK) cells appear binucleate (Fig. 1A), and one's first impression on observing these cells is that the presence of 2-AP has caused the cells to abort in cytokinesis. Many 2-AP-treated cells appear truly binucleate on close examination (Fig. 1A,B). However, many of the cells in a population treated with 2-AP are not binucleate, but contain a single nucleus with various characteristic distortions in shape. Of these, a substantial number of cells have a lobed nucleus, with the two lobes connected by a narrow isthmus of nuclear material (Fig. 1B,C). Another rarer distortion is a toroid, or doughnut-shaped, nucleus (Fig. 1D). Among other morphologies observed are interphase nuclei with multiple lobes, or asymmetric lobes (Fig. 1A, and data not shown). We will show that the origin of these distorted nuclei is due to a 'partial mitosis' rather than a failure of cytokinesis. Partial mitosis is defined as the failure of cells containing a mitotic spindle to form functioning chromosomes, and by the exit of these cells from mitosis without a metaphase, anaphase or telophase.

Partial mitosis in 2-AP-treated cells

After 12 h of exposure to 2-AP, one can readily identify a subpopulation of treated cells in mitosis, as defined by anti-tubulin staining. These cells have lost their interphase tubulin array and instead display the tight astral foci of microtubules and the bipolar spindle arrays characteristic of mitosis (Fig. 2). The proportion of the cell population with mitotic microtubule arrays remains at a constant 4–5% of the total through 12 h, which is not appreciably different from the mitotic index of untreated cells (see Fig. 10).

When counterstained with propidium iodide, 2-AP-
treated mitotic cells exhibit a remarkable chromatin morphology. Although mitotic spindles are present, chromatin is not condensed into discernible chromosomes, but remains a contiguous mass (Fig. 2). We term such chromatin in otherwise mitotic cells 'nuclear bodies'. Some mitotic character is present in these mitotic nuclear bodies, in that they contain no apparent nucleoli, as determined by the lack of distinctive nucleolar staining with propidium iodide (Fig. 2), and by the failure of nuclei to stain with anti-nucleolar antibodies (data not shown). The chromatin appears somewhat condensed relative to interphase nuclei, but never displays discrete chromosomal structures.

Optical sectioning with a confocal microscope reveals that, in 2-AP-treated cells in mitosis, nuclear bodies are displaced laterally or are pierced by spindle microtubules. The displacement of the nuclear bodies in mitotic cells yields distinctive polymorphic nuclei. In one class of mitotic cell, one finds a bipolar spindle lying between two spherical nuclear bodies laterally arranged on either side of the spindle (Fig. 2A). Other commonly observed classes include mitotic nuclear bodies pierced by narrow channels that contain spindle microtubules, and have a sharp boundary of chromatin bordering the channel (Fig. 2B). Also, there are kidney-shaped nuclear bodies (Fig. 2C), or nuclear bodies with two or more lobes connected by a narrow isthmus of chromatin crossing the spindle equator (see Fig. 1B for an interphase cell with this nuclear configuration). One occasionally finds toroid nuclear bodies. In these cells, the central overlap zone of the bipolar spindle appears to puncture through to the center of the mitotic nuclear body to create a doughnut or torus shape. Serial optical sections of one such mitotic cell are shown in Fig. 3.

Images of 2-AP-treated mitotic cells suggest that the mitotic spindle actively deforms the nuclear body. In support of this conclusion, a quantitative analysis of the proportion of the 2-AP-treated cells with distorted nuclei shows that the percentage of the population with apparent binuclear appearance (including lobed nuclei) rises linearly to approximately 50% of the population over 12 h of exposure to the drug (Fig. 4A). The rate of accumulation of 'binucleate' cells is similar to the rate of accumulation of cells in mitotic arrest following exposure to nocodazole (see Fig. 10), consistent with a requirement for passage through mitosis in order to form distorted nuclei. The requirement for a mitotic spindle to create distorted nuclei is also supported by our observation that simultaneous exposure to nocodazole prevents formation of binucleate cells by 2-AP, causing cells to remain mononucleate over 12 h of drug exposure (Fig. 4A, B).

It is worth noting that binucleate cells begin accumulating immediately upon exposure to 2-AP (Fig. 4). This observation suggests that the partial mitotic effect of 2-AP results from the drug acting directly on mitosis and does not result from mutagenic effects of 2-AP (Caras et al. 1982; Schaff et al. 1990; Speit et al. 1990) during S-phase. However, some effect of S-phase on chromatin behavior is suggested by observations (data not shown) that some M-phase cells have distinct but nonfunctioning chromosomes after short times of exposure (3 h) to 2-AP.

The mitotic spindle that forms in 2-AP-treated cells (Fig. 2A, B, C) is characteristically narrower than the typical spindle found in untreated cells (Fig. 2D) and usually has a more prominent array of astral microtubules. This morphology possibly arises due to lack of stabilization of kinetochore microtubules by chromosome capture (Mitchison and Kirschner, 1985; Hayden et al. 1990). Nonetheless, it appears that discrete chromosomes may not be required in order to generate a bipolar spindle morphology in these cells.

Mitotic progression in 2-AP

Upon entry into mitosis, the lamin proteins that demarcate the nuclear periphery disassemble. Lamin B undergoes a p54<sup>crf</sup> kinase-specific phosphorylation that has been functionally linked to nuclear lamina breakdown (Peter et al. 1990h). From <i>in vitro</i> model systems with <i>Xenopus</i> oocytes, it is evident that lamin disassembly can be temporally distinct from both chromosome condensation and nuclear envelope dissolution (Newport and Spann, 1987). In 2-AP-treated cells identified as mitotic by the presence of a mitotic spindle, lamin B has generally disappeared from the periphery of the nuclear bodies (Fig. 5), although in some rare cases a remnant of the lamin border can be detected on nuclear bodies in mitotic cells (see Fig. 5A, arrow). Interphase cells in 2-AP, in contrast, contain intact lamin B borders around their nuclear peripheries.
Fig. 2. Condensed mitotic chromosomes fail to form during mitosis in 2-AP, and uncondensed chromatin is deformed by the mitotic spindle. (A–C) Mitotic BHK cells from a cycling population, treated with 2-AP for 12 h, and (D) a similarly processed mitotic control, are shown stained with anti-tubulin (left column), counter-stained with propidium iodide (middle column) and as merged images (right column). A,B,D are horizontal optical sections, while C is a projection generated by summation of serial horizontal sections. Propidium iodide staining shows a lack of discrete chromosomes in 2-AP-treated cells (A2, B2, C2). (A) Chromatin is displaced laterally by the spindle into two discrete nuclear bodies. (B) Microtubules pierce the chromatin and form narrow channels through it (arrow). (C) A kidney-shaped nuclear body surrounds the spindle. (D) In a control cell, metaphase chromosomes are aligned at the center of the spindle.

nuclei (see Fig. 1A). It is not known whether mitotic nuclear bodies retain a distinct semi-permeable border in the absence of lamin B. However, where microtubules of the spindle appear to pierce through the interior of the nuclei, one always finds a distinct boundary separating the polymer from the nuclear interior (see Fig. 2B).

We have also assayed for the distribution of the mitosis-specific antigen MPM-2, which has been shown to represent mitosis-specific phosphorylation of a subset of mitotic proteins (Davis et al. 1983). MPM-2 antigens are normally present in the cytoplasm of mitotic cells, and are detected localized to centrosomes and chromosomes follow-
Fig. 3. Serial sections of a toroid nuclear body. Selected horizontal 0.2 μm sections through a mitotic cell are shown, proceeding from top to bottom at intervals of 0.6–0.8 μm. Anti-tubulin indirect immunofluorescence staining is shown in the left-hand column, and propidium iodide staining of DNA is shown at the right. The chromatin is discontinuous only in the central horizontal sections of the cell, where microtubules penetrate the nuclear body.

We have presented images of mitotic cells in 2-AP showing the absence of condensed chromosomes, and the lack of distinct mitotic stages. A quantitation of the mitotic stages observed in 2-AP cells in mitosis versus the distribution of mitotic stages in control cells (Table 1) demonstrates unequivocally the complete failure of 2-AP-treated cells to progress through the normal stages of mitosis. No cell observed to be in mitosis, as defined by the presence of a mitotic spindle, gives evidence of normal mitotic chromosomes, or of chromatin aligned as for metaphase or later stages of mitosis. All the surveyed cells contain a spindle and a discrete nuclear body with chromatin that has failed to condense into individual chromosomes.

Using anti-centromere antibodies one finds that the centromeres of mitotic 2-AP-treated cells are often dispersed at random in the mitotic nuclear bodies and do...
Fig. 4. Accumulation of multinucleate and lobed nuclear cells is linear with time during 2-AP treatment, but is prevented by the presence of nocodazole. (A) Multinucleate/lobed nuclear cells accumulate during treatment with 10 mM 2-AP (filled squares), but not when treated with either nocodazole (filled circles), or nocodazole plus 2-AP (open squares). Untreated controls (open circles) are also shown. Nuclear morphology was assayed both by epifluorescence with anti-lamin B antibodies, and by propidium iodide staining. All points represent the average of three independent counts of greater than 100 cells each. Standard deviation was less than 2.5% of the ordinate for all data points. (B) Cells treated for 12 h with nocodazole and 2-AP remain mononucleate, as assayed with anti-lamin B (upper frame), and with propidium iodide (lower frame).

Fig. 5. Distribution of lamin B in 2-AP-treated mitotic cells. The continuous rim of lamin B that forms the boundary on interphase nuclei (see Fig. 1A) is largely dispersed throughout the cytoplasm in 2-AP-treated mitotic cells (A). Small amounts of residual lamin B (arrow) sometimes remain associated with the chromatin border (chromatin is the clear zone in A). The cell is identified as mitotic by the presence of a mitotic spindle (A2), detected with anti-tubulin antibodies.

Mitotic behavior of nocodazole-treated cells in 2-AP
As randomly cycling cells exposed for 12 h to 2-AP contain only relatively decondensed chromatin during mitosis, it cannot be ascertained by observing these cells whether the bipolar spindle could engage chromosomes if they were present, and permit normal mitotic function. To create this experimental condition, cells were blocked in mitosis with nocodazole and released from nocodazole block into 2-AP.

not appear to interact with the spindle (Fig. 7A) in contrast to the characteristic integration of centromeres into the spindle in control cells (Fig. 7C). However, a number of mitotic nuclear bodies exhibit centromeres clustered in a 'shell' at the periphery of the spindle (Fig. 7B), giving evidence of an apparent limited capacity of centromeres in some cells to associate with microtubules.
Table 1. Progression of 2-AP-treated cells through mitosis

<table>
<thead>
<tr>
<th>Stage</th>
<th>2-AP (%)</th>
<th>Control (%)</th>
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<tbody>
<tr>
<td>Prophase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear bodies</td>
<td></td>
<td></td>
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<tr>
<td>(no chromosomes):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifurcated</td>
<td>27.7±2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lobed</td>
<td>62.4±2.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Undeformed</td>
<td>9.6±0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Chromosomes</td>
<td>0.3±0.5</td>
<td>37.3±2.0</td>
</tr>
<tr>
<td>Metaphase</td>
<td>0.0</td>
<td>40.8±0.3</td>
</tr>
<tr>
<td>Anaphase</td>
<td>0.0</td>
<td>9.2±1.3</td>
</tr>
<tr>
<td>Telophase</td>
<td>0.0</td>
<td>12.5±0.9</td>
</tr>
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</table>

All values represent percentage of mitotic cells, as scored by the presence of a mitotic spindle detected by immunofluorescence with anti-tubulin antibodies after exposure to 2-AP for 12h. Stages of mitosis were classified by the distribution of DNA stained by propidium iodide, relative to the spindle. Metaphase was defined as the alignment of chromosomes/chromatin in a plate at the center of the spindle, and anaphase as chromosomes/chromatin separated in two distinct sets along the axis between the poles. Telophase was defined by the presence of a cleavage furrow, detected by propidium iodide staining of cytoplasmic RNA. All other distributions of DNA are designated as prophase. Values are averages of 3 counts of 100 or more cells each.

In these cells, chromosomes persist for at least 20 min but appear to have no capacity to become engaged in the bipolar spindle that forms (Fig. 8). In a subset of cells, the chromosomes remain as a spherical shell around an aster before they decondense. In the remainder, chromosomes either border each side of the spindle, as shown here (Fig. 8), or surround the spindle in a 'figure of 8' pattern. Centromeres appear to lie proximal to the spindle on its periphery (data not shown), reminiscent of the frequently observed proximal orientation of centromeres on spindles in cells treated with 2-AP alone (Fig. 7B). In no case have we observed chromosome or centromere orientations that suggest metaphase or anaphase in these cells. Finally, when these cells re-enter interphase, they often have H-shaped or figure of 8 nuclei. In contrast, control cells released from nocodazole block proceed through a normal mitosis (data not shown).

If nocodazole-blocked cells are exposed to 2-AP without first releasing them from nocodazole, it is evident that 2-AP induces mitotic exit signals that overcome the mitotic arrest (Figs 9 and 10). Prior to addition of 2-AP, nocodazole-treated cells, blocked in mitosis, display a typical array of condensed chromosomes when stained with propidium iodide (Fig. 9B1). When counterstained with anti-lamin B antibody, there is only a dispersed cytoplasmic stain in these cells (Fig. 9B1). Within 30 min of addition of 2-AP to these cells, still in nocodazole, their chromatin has decondensed (data not shown). After 60 min of 2-AP treatment, nucleoli are evident by propidium iodide staining (Fig. 9A1), and lamin B has re-formed around the nucleus (Fig. 9A2), despite the continuous presence of nocodazole. As shown here (Fig. 9A2), lamin B often occurs transiently as disconnected cytoplasmic 'sheets' during re-entry into interphase.

A quantitative analysis of a cell population blocked with nocodazole, then additionally exposed to 2-AP shows that 2-AP rapidly causes a reversion of the cells in mitotic arrest to an interphase state (Fig. 10). We have made this analysis, scoring for either decondensed chromatin or for deposition of a lamin B border around nuclei, with comparable results. A careful examination of these nocodazole-treated cells with reconstituted nuclei reveals that they are truly in interphase, and can initiate another round of DNA replication without having segregated their chromatin (Andreassen and Margolis, unpublished data). We conclude that 2-AP not only prevents chromosome condensation and function in mitosis, but also actively reverses mitotic arrest.

Discussion

The control of the onset of mitosis involves activation of p34(cdc2) kinase (Murray and Kirschner, 1989; Nurse, 1990; Draetta, 1990), which in turn appears to act as a master switch inducing downstream mechanisms that complete the mitotic process (Dunphy and Newport, 1988). In prophase, a number of events occur coordinately, including dissolution of nuclear structures such as lamins and nucleoli, condensation of chromatin antigens, separation of...
Fig. 7. Centromeres interact abnormally with the spindles of 2-AP-treated cells. During mitosis in the presence of 2-AP, centromeres do not align as for metaphase or anaphase, but instead either show no association with the spindle as in (A), or form a shell around the outside of the spindle, as in (B). For contrast, centromere alignment in a typical metaphase array is shown in an untreated mitotic cell (C). For each cell, anti-centromere indirect immunofluorescence staining is shown in the left column, anti-tubulin staining is shown in the center column, and a merge of the anti-centromere and anti-tubulin images is shown in the right column.

Fig. 8. Chromosomes present in cells released from a nocodazole block into 2-AP do not engage in the mitotic spindle. Cells were blocked with nocodazole for 12 h, recovered from blockage by washing into fresh medium, and 10 mM 2-AP was added. After 40 min in 2-AP, the cells were fixed and processed for indirect immunofluorescence. (A1) Chromosomes stained with propidium iodide. (A2) Anti-tubulin counterstain of same cell. (A3) Merged image of propidium iodide and anti-tubulin stain, demonstrating that the chromosomes lie adjacent to the spindle.

centrosomes, induction of kinetochore elements on chromosomes, disassembly of the cytoskeletal systems and reassembly of microtubules into a bipolar spindle.

We have demonstrated here that the presence of 2-aminopurine (2-AP) causes cells to proceed through an aberrant partial mitosis. Partial mitosis is characterized by the transient expression of a bipolar mitotic spindle, but a failure of chromatin to form functioning chromosomes and of the cells to proceed through the recognizable mitotic phases of chromatin separation. All other morpho-
Fig. 9. Nuclear re-formation following the addition of 2-AP to nocodazole-blocked cells. Propidium iodide staining of DNA is shown in the left column, and indirect immunofluorescence staining of lamin B is shown in the right column. The addition of 2-AP to mitotic cells in the continued presence of nocodazole induces the re-formation of nuclei that are bordered by lamin B ($A_1$, $A_2$). In nocodazole-blocked cells treated with 2-AP lamin B reassembles not only at the periphery of the nucleus, but also frequently in the cytoplasm independently of chromatin, as seen here. Otherwise untreated cells blocked in mitosis by nocodazole typically have clustered chromosomes and a dispersed lamin B stain ($B_1$, $B_2$).

Fig. 10. 2-AP induced reversion of nocodazole-blocked mitotic cells to interphase. The mitotic index of a population of cells increases in the continuous presence of nocodazole (filled squares). When 10 mM 2-AP is added to cells in the continued presence of nocodazole (arrow), the mitotic index of this population drops precipitously (filled circles). Cells were judged to be in mitosis by the absence of nuclear lamina (determined with anti-lamin B antibodies); and by the presence of discrete chromosomes, or the absence of nucleoli, both as assayed by propidium iodide staining. Control cell populations were treated continuously with 10 mM 2-AP (open squares), or were untreated except for exposure to DMSO and the Hepes used for drug treatment (open circles).

It has been shown that 2-AP is a protein kinase inhibitor (Farrell et al. 1977; Mahadevan et al. 1990), and that the inhibitory effect of 2-AP on kinase activity in vivo is quite selective. When interphase mammalian cells are exposed to 2-AP, phosphorylation is altered in a very restricted subset of proteins (Mahadevan et al. 1990). In this context, it does not appear that partial mitosis results from inhibition of p34$^{cdk2}$ kinase itself, since mitotic entry appears to occur normally, with the exception of chromosome condensation. The selectivity of 2-AP inhibition is also indicated by the failure of 2-AP to visibly alter mitotic phosphorylation detected by MPM-2 antibodies. We therefore propose that 2-AP selectively inhibits a protein kinase activated by p34$^{cdk2}$ during mitosis.

While we have not yet identified a particular protein kinase inhibited by 2-AP in mitosis, it is reasonable to postulate that there are protein kinases with activity downstream from p34$^{cdk2}$. Other protein kinases with increased activity during mitosis have been identified in echinoderm oocytes (Pelech et al. 1988), and in mammalian cells (Chackalaparampil and Shalloway, 1988; Liu et al. 1990). Microinjection of antibodies to cyclic AMP-dependent protein kinase type II and a specific inhibitor of this kinase (Browne et al. 1987) has yielded evidence that a kinase other than p34$^{cdk2}$ is involved in progression through mitosis. There is a possibility that p34$^{cdk2}$ activates a cascade of downstream protein kinases during mitosis, as suggested by Dunphy and Newport (1988). This possibility is supported by the p34$^{cdk2}$-dependent phosphorylation of the c-src and c-abl kinases in vitro at the same sites as modified in vivo (Shenoy et al. 1989; Morgan et al. 1989; Kipreos and Wang, 1990), as well as phosphorylation and activation of casein kinase II by p34$^{cdk2}$ in vitro (Mulner-Lorillon et al. 1990). As further support, Gotoh et al. (1991) have recently demonstrated that MAP2 kinase activity is directly linked to the mitosis-specific display of microtubules.

Alternative but less likely explanations for the induction of partial mitosis include the possibility that 2-AP induces a partial inhibition of p34$^{cdk2}$ itself, and that events relating to chromosome behavior have a higher threshold of activation by the kinase. It is also possible that 2-AP, as an adenine analogue, may cause partial mitosis in 2-aminopurine.

Partial mitosis in 2-aminopurine
Mitotic spindle formation in 2-AP

Among cells treated with 2-AP, those in partial mitosis generate a bipolar mitotic spindle independent of chromosome condensation. The creation of a mitotic spindle requires that signals for mitotic initiation dissolve the interphase microtubule network, that centrosomes separate to the bipolar configuration and that microtubules emanating from each of the poles interdigitate and bundle in an antiparallel manner to form the bipolar spindle morphology.

The 2-AP-treated cells provide evidence that the spindle does not require discrete chromosomes in order to generate or maintain a bipolar morphology. This suggests that chromosomes are not required to engage the two half spindles or to keep them linked in their zone of overlap. The typical bipolar spindle in a 2-AP-treated cell is relatively thin compared to the more barrel-shaped spindles of control cells (see Fig. 2). This apparent paucity of microtubules between the spindle poles may arise from the fact that the entire class of kinetochore-to-pole microtubules is lacking, and only interpole microtubules survive. This observation suggests that there are two means of stabilizing spindle microtubules against inherent instability (Mitchison and Kirschner, 1984), thus ensuring their survival. Microtubules may be stabilized either by kinetochore capture (Mitchison and Kirschner, 1985; Hayden et al., 1990), or by antiparallel interactions with polymers arising from the other half spindle (Sawin and Mitchison, 1991).

Activation of mitosis-specific microtubule arrays assembling from centrosomes in vitro has been shown to be derived, directly or indirectly, from p34cdc2 kinase activity (Verde et al. 1990). It may be significant in this regard that p34cdc2 becomes associated with the centrosome in late G2 and remains concentrated at the centrosome through most of mitosis (Riabowol et al. 1989; Bailly et al. 1989). More recently, the mitosis-specific display of microtubules has been linked directly to MAP2 kinase activity (Gotoh et al. 1991). It appears, from our results, that these kinase activities are not appreciably affected by 2-AP treatment of cells.

Interaction of the spindle with the nucleus and with chromosomes

The bipolar spindle coexists with a structurally integral nuclear body in 2-AP-treated cells. The interaction of these two structures results in a curious phenomenon. The spindle microtubules appear to mold, deform or pierce the nuclear body. In order to generate a bipolar spindle with a spindle pole on either side of the nuclear body, the spindle poles must have separated. In doing so, their associated microtubules appear to have generated the requisite force to displace that part of the nuclear body that lay in the path of the growing spindle.

The fact that the nuclear body is deformable in 2-AP mitosis may relate to the observed loss of lamin B from the nuclear periphery in these cells. Lamin B is phosphorylated in mitotic cells by p34cdc2 and this phosphorylation seems intimately tied to lamin disassembly (Peter et al. 1990b). Lamin B is important to the maintenance of interphase nuclear morphology. It has recently been shown in some hematopoietic cells that lamin B alone, in the absence of lamins A and C, is sufficient to form a functional nuclear lamina (Guilly et al. 1987b; Rober et al. 1990). Having exited partial mitosis, the nuclei of 2-AP-treated cells retain their mitotic deformation. It is possible that the reassembled lamins will lock in whatever shape change is imposed on the mitotic nucleus when the cell reverts to interphase.

It is not uncommon that nuclei become deformed in a characteristic manner in various cells, and the deformation is sometimes related to association with microtubules at the nuclear surface. The nucleus of the spermatocyte serves as an example (McIntosh and Porter, 1967; Dooher and Bennett, 1974). The presence of a lobed nucleus, cupped around a centrosome, is characteristic of neutrophils and monocytes (Murphy, 1976). These may represent examples in nature of transient alterations in the state of phosphorylation of lamins, allowing for microtubule-dependent molding of the nuclear exterior.

Although there is no meaningful movement of chromatin in 2-AP-treated cells, centromeres are often seen to aggregate at the periphery of the spindle. Some interaction of centromeres with the spindle is therefore possible. This may perhaps be related to the recently observed prometaphase motility of chromosomes through lateral associations of kinetochores with spindle microtubules (Rieder and Alexander, 1990). It will be of interest to determine if chromatin in 2-AP-treated mitotic cells has generated a morphologically distinct kinetochore, and has incorporated the microtubule motor protein, cytoplasmic dynein (Paschal et al. 1987), which has been reported to be present on prometaphase centromeres (Pfarr et al. 1990; Steuer et al. 1990).

Recently, we have carefully examined mitosis in BHK cells treated for only 3 h in 2-AP (data not shown). After such relatively brief exposure to 2-AP a substantial fraction of mitotic cells contain condensed chromosomes. These chromosomes fail to engage in the spindle, and we observe no metaphase, anaphase or telophase mitotic figures. The condensation of chromosomes observed in some cells at early times suggests that the failure of chromatin condensation may result from incorporation of 2-AP into DNA during S-phase (Caras et al. 1982; Schaff et al. 1990; Speit et al. 1990). Partial mitosis as a result of 2-AP may therefore more properly be defined as the failure to form chromosomes that associate meaningfully with the spindle in mitosis, rather than as the failure of chromatin to condense into chromosomes.

We have also examined the fate of cells released from nocodazole block into 2-AP. In nocodazole-induced mitotic arrest the nucleus has dissolved and chromosomes are condensed. In this case, when the signal for chromosomal condensation has preceded 2-AP addition, the chromosomes remain condensed for at least 20 min. In some cells a bipolar spindle forms during nocodazole recovery and coexists with condensed chromosomes. In these cells the chromosomes exhibit little capacity to become engaged in the mitotic spindle other than by lateral association, and are never observed in typical metaphase or anaphase configurations.

Chromosomes are often arranged in a figure of 8, surrounding the bipolar spindle at its margin. As with nuclear bodies, one frequently finds centromeres proximal to the spindle margin. As these chromosomes have apparently lost their capacity to function in the mitotic spindle, they should prove to be an interesting model system for the in vitro re-establishment of kinetochore function by addition of mitotic factors.

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Mitosis exit signals and 2-AP

The mitotic index of a cell population is not altered measurably by 2-AP. There is thus no evidence that 2-AP-treated cells are hindered in transiting mitosis despite the lack of the distinct mitotic phases that may act as 'checkpoints' in mitotic progression (Hartwell and Weinert, 1989).

By criteria of chromatid and centromere distribution with respect to the spindle, 2-AP-treated cells never enter metaphase or anaphase. These cells also never exhibit furrowing indicative of telophase, and probably also exit mitosis without induction of cleavage furrows. Further evidence that 2-AP-treated cells do not undergo cleavage comes from the fact that these cells do not display the midbodies evident in control cells by tubulin antibody staining (data not shown).

Cells in 2-AP not only transit through mitosis without a requirement for completing downstream mitotic events, they can, in fact, overcome mitotic arrest. Cells blocked in nocodazole will remain in mitotic arrest for an indefinite period of time, but the addition of 2-AP to nocodazole-arrested cells induces a rapid reversal of the arrested state, as evidenced by re-formation of nuclei with lamin B borders in the continued presence of nocodazole.

The induction of cell cycle progression in M-phase-arrested cells is analogous to the effect of 2-AP and other purine analogues on S-phase-arrested cells. Cells can be arrested indefinitely in S-phase by drugs such as hydroxyurea (Tobey, 1975), but the addition of purine analogues to these cells induces escape from S-phase, leading to chromosome condensation (Schlegel and Pardee, 1986; Schlegel et al. 1990; Downes et al. 1990). Among the purine analogues with an effect on S-phase, we have found that 2-AP and 6-dimethylaminopurine, but not caffeine, are successful in overcoming nocodazole-induced mitotic arrest (P. Andreassen and R. Margolis, unpublished observations).

It has been shown that 6-dimethylaminopurine causes rapid decondensation of chromosomes at metaphase I of meiosis in oocytes of the mollusc Patella vulgata and in mouse oocytes (Neant and Guerrier, 1988; Rime et al. 1990). This decondensation is accompanied by substantial inhibition of protein phosphorylation specific to meiosis. We have found, in BHK cells, that 6-dimethylaminopurine causes partial mitosis events similar to those observed with 2-AP (unpublished observations). It is therefore probable, in view of our results, that 6-dimethylaminopurine induces a partial meiosis in the oocyte system.

Conclusions

The results reported here in BHK cells have been reproduced in HeLa cells (data not shown). We expect that the 2-AP induction of partial mitosis will prove to be widespread in occurrence. The ability to block certain mitotic events while other events proceed normally should prove valuable for biochemical dissection of the control of specific mitotic phenomena induced by p34^cdk2. Assuming that the mitotic effects of 2-AP are primarily due to its action as a specific kinase inhibitor (Farrell et al. 1977; Mahadevan et al. 1990), the drug may permit identification of specific down-stream mitotic kinases and their substrates. Further, our observations on 2-AP treatment of cells raise questions as to the interaction and interdependency of various events that occur in succession in a normal mitosis, and should permit the biochemical analysis of the molecular nature of mitotic checkpoints (Hartwell and Weinert, 1989) in mammalian cells.

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