2-Aminopurine overrides multiple cell cycle checkpoints in BHK cells

(Purine analogues/mitosis/S phase)

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ABSTRACT BHK cells blocked at any of several points in the cell cycle override their drug-induced arrest and proceed in the cycle when exposed concurrently to the protein kinase inhibitor 2-aminopurine (2-AP). For cells arrested at various points in interphase, 2-AP-induced cell cycle progression is made evident by arrival of the drug-treated cell population in mitosis. Cells that have escaped from mitomycin G2 arrest, from hydroxyurea or aphidicolin S-phase arrest, or from VM-26-induced G2 arrest subsequently have all the hallmarks of mitosis—such as a mitotic microtubule array, nuclear envelope breakdown, and chromatin condensation. In a synchronous population, the time course of arrival in mitosis and its duration in 2-AP-treated cells that have escaped drug-induced cell cycle blocks is indistinguishable from control cells. Cells arrested in mitosis by nocodazole or taxol quickly escape mitotic arrest and enter interphase when exposed to 2-AP. 2-AP by itself does not influence the timing of cell cycle progression. We conclude that 2-AP acts to override checkpoints in every phase of the cell cycle, perhaps by inhibiting a protein kinase responsible for control of multiple cell cycle checkpoints.

The progression of a cell through the mitotic cycle is controlled by an array of proteins. Central among them is p34cdc2, a serine/threonine protein kinase that determines induction of a variety of specific mitotic events (for reviews, see refs. 1 and 2). The p34cdc2 kinase, or a closely related variant, is also involved in induction of DNA replication in S phase (3–5). The activity of p34cdc2 is, in turn, controlled by a series of specific phosphorylation and dephosphorylation events on p34cdc2 (2) and by association of p34cdc2 with various cyclins (for reviews, see refs. 1, 2, and 6), proteins that oscillate in amount as the cell cycle advances.

The progression of the cell to the next stage of its cycle is under the control of factors that act as checkpoints to assure that the previous stage has been completed before the subsequent stage ensues (7). The cell contains exquisitely sensitive feedback-control circuits that can, for example, prevent exit from S phase when a fraction of percent of DNA remains unreplicated (8) and can block advance into anaphase in mitosis until all chromosomes are aligned on the metaphase plate (9). The nature of these checkpoints and how they act to block cell cycle progression is unknown.

Various mutants have been isolated that escape specific cell cycle-control circuits and progress inappropriately to the next cell cycle stage. They include wee1 mkl1 double mutants (10), pim1 (11), and rad9 (12) in yeast; bimE7 in Aspergillus (13); and the RCC1 mutant tsBN2 in mammalian BHK cells (14). All of these mutants exhibit an uncoupling of entry into mitosis from the completion of DNA replication or DNA repair. In addition, drug treatments, such as combining exposure to the DNA replication inhibitor hydroxyurea with exposure to caffeine, a purine analogue, can cause normal mammalian cells to enter mitosis without completing S phase (15). Recently, mutations in Saccharomyces cerevisiae, BUB (16), and MAD (17) have been isolated that fail to arrest in mitosis with microtubule-destabilizing drugs.

The purine analogue 2-aminopurine (2-AP), a specific protein kinase inhibitor (18, 19), can cause S-phase arrested cells to inappropriately enter mitosis (20). Recently, we found that this analogue also causes BHK cells in mitotic arrest to rapidly exit mitosis (21). Also, recently, 2-AP has been reported (22) to permit cells to overcome a G2 block induced by γ irradiation. As this drug has the capacity to advance cells inappropriately past checkpoints at distinct parts of the cell cycle, this result suggested that an underlying common factor might be responsible for the various inhibitory controls of the cell cycle and that the capacity of 2-AP to override cell cycle blocks is universal. We have therefore tested the ability of 2-AP to inappropriately advance the cell cycle after cell blockage by a variety of stage-specific inhibitors. We report here the striking result that 2-AP causes cells to override every cell cycle-block point examined, regardless of whether the arrest point is in G1, S phase, G2, or mitosis. Further, cells exposed continuously to 2-AP alone apparently exit S phase without completion of replication and can exit mitosis without metaphase, anaphase, or telophase events. Therefore, we now believe an underlying commonality does exist, perhaps at the level of a specific 2-AP-sensitive protein kinase. The present work thus extends previous findings (20–22) and now unequivocably shows that 2-AP can universally override cell cycle blocks.

Several of the inhibitors used here to induce cell cycle arrest (hydroxyurea, VM-26, and taxol) are used experimentally or therapeutically for cancer treatment (for reviews, see refs. 23–25). None of these drugs are of themselves lethal to culture cells during short exposure. However, inappropriate exit from an arrested state, induced by 2-AP, is ultimately lethal to the cell. Therefore, our results suggest that binary therapy, combining a drug such as VM-26 or taxol with 2-AP or another purine analogue, will cause inappropriate escape from cell cycle blockage and may have a synergistic destructive effect on tumors.

MATERIALS AND METHODS

Cell Culture and Synchronization. Baby hamster kidney (BHK) cells were grown as monolayers in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% defined fetal bovine serum (HyClone), and were maintained in a humid incubator at 37°C in a 5% CO2 environment.

Abbreviations: 2N, diploid (number of chromosomes); 4N, tetraploid (number of chromosomes); 2-AP, 2-aminopurine.

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Drug Treatment. 2-AP was obtained from Sigma as a nitrate salt. A stock of 100 mM 2-AP was kept frozen 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. Aphidicolin, hydroxyurea, and mimosine were obtained from Sigma and used at final concentrations of 5 μM 2-AP, 2 mM, and 200 μM, respectively. VM-26 (4′-demethyl-epipodophyllotoxin) (Bristol-Myers Squibb) was used at 0.45–0.50 μg/ml. For examination of the response of mitotic blocks to 2-AP treatment, nocodazole was used at 0.06 μg/ml, and taxol (from M. Suffness, National Institutes of Health) was used at 5 μM.

Determination of Mitotic Index. For testing mitotic blockage with nocodazole and taxol, cells were grown a minimum of 16 hr on polylysine-coated glass coverslips before drug treatment. Cells were fixed at intervals and stained with antibodies to detect lamin B and counterstained with propidium iodide to assay chromosome condensation. To test cell cycle blocks in interphase, cells were synchronized in mitosis by adding nocodazole (Sigma) to a final concentration of 0.05 μg/ml from a 1 mg/ml stock in dimethyl sulfoxide. After 12-hr arrest, the mitotic subpopulation was isolated by shake-off from the culture plate. After applying cell cycle-blocking drugs to or 2-AP, cells were fixed at intervals, prepared for indirect immunofluorescence with antitubulin antibodies, and counterstained with propidium iodide. All data time points represent averages of three counts of >150 cells each.

SE was never >1.5% on the ordinate scale.

Immunofluorescence Microscopy. Fixation, permeabilization, incubation with antibodies, and mounting of cells grown on coverslips were done as described (21). Primary antibodies included mouse anti-β-tubulin antibody (Eastacres Biologicals, Southbridge, MA), diluted 25-fold for use, and anti-lamin B human autoimmune serum (26), from J.-C. Courvalin, diluted 200-fold for use. Secondary antibodies included fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse and anti-human antibodies, applied at 8.5 μg/ml. Secondary antibodies were from Tago. Cells were counterstained with propidium iodide as described (21). Samples were recorded by using a MRC-500 laser scanning confocal apparatus (Bio-Rad Microscience) attached to a Nikon Optiphot microscope.

Flow Cytometric Analysis of DNA Content. Samples were fixed 30 min on ice with 70% ethanol/1× phosphate-buffered saline (136 mM NaCl/2 mM KCl/10.6 mM Na2HPO4/1.5 mM KH2PO4, pH 7.4) and then stored at 4°C until preparation for staining. Before analysis, cells were washed twice with phosphate-buffered saline and then stained by using the optimal procedure reported by Tate et al. (27). Measurements of propidium iodide fluorescence signal were made with an Epics 753 analyzer (Coulter) on 10⁴ cells.

RESULTS

Cells arrested in S phase by hydroxyurea have been shown to escape the S-phase block when exposed to 2-AP (20). Further, we have recently reported that BHK cells blocked in mitosis by nocodazole rapidly exit mitosis upon 2-AP addition (21). We have tested to determine whether these effects result directly from interference with the cell cycle by 2-AP alone. For this test, BHK cells were synchronized by shake-off of the mitotic subpopulation and assayed for timing of progression through the cell cycle by flow cytometric analysis of DNA content (Fig. 1A) and the time of arrival in the subsequent mitosis (Fig. 1B), while constantly exposed to 10 mM 2-AP.

Mitotic shake-off of nocodazole-arrested cells resulted in a starting population of mitotic cells with tetraploid (4N) DNA content (Fig. 1A). The drug 2-AP was applied 4 hr after shake-off and recovery from nocodazole arrest, when most cells had reentered G1 (Fig. 1A). S phase appears to initiate at approximately the same time in 2-AP-treated and control cells, ~6 hr after synchronization (data not shown). Results of analyzing DNA content are shown at 12 hr after synchronization, when control cells are approaching 4N DNA content and the first mitotic cells are visualized morphologically, and also at 18 hr, when mitosis is largely completed in control cells. Treatment with 2-AP markedly slows DNA replication: DNA synthesis lags behind control cells at 12 hr and remains incomplete for the population 18 hr after synchronization (Fig. 1A). Interestingly, many of the 2-AP-treated BHK cells apparently fail to complete S phase and yet arrive in mitosis at approximately the same time as control cells (Fig. 1B). For unknown reasons, the mitotic index is lower for 2-AP-treated cells than for control populations. These results show no acceleration of S phase or of mitosis with 2-AP but suggest that many cells in 2-AP do not respect the requirement for completion of S phase before proceeding to mitosis.

G1, S-Phase, G2, and M-Phase Cell Cycle Blocks Are Overridden by 2-AP. Mammalian culture cells may be blocked in various parts of the cell cycle by specific inhibitors. Mimosine, an amino acid analogue, has been reported to block cells at a point in G1 that precedes S phase by ~2 hr. Aphidicolin and hydroxyurea, inhibitors of DNA polymerase α (29) and ribonucleotide reductase (30), respectively, block cells in early S phase. VM-26, a topoisomerase II inhibitor (31), induces a cell cycle block in G2 (32). Nocodazole and taxol, drugs that interfere with normal microtubule-assembly behavior (33, 34), block cells in mitosis.
As 2-AP will overcome an S-phase block (20) and induces nocodazole-blocked cells to exit mitosis (21), we have tested the ability of 2-AP to override any of a variety of different cell cycle blockages specific to different stages. For each experimental test, except blockage at mitosis, cells were first synchronized by shake-off in a nocodazole-arrested mitotic state and then allowed to recover from nocodazole and exposed to the blocking agent, either in the presence or absence of 10 mM 2-AP. In each case, the cell cycle behavior of drug-treated cells was compared with the behavior of untreated control cells.

Cells were tested for their ability to overcome mimosine G1 arrest in 2-AP by assaying the mitotic index at time points after drug addition. BHK cells treated with both mimosine and 2-AP exhibited the same time course and amplitude of arrival in mitosis as untreated controls (Fig. 2 A); approximately 80% of both cell populations proceeded through mitosis. In contrast, cells treated with mimosine alone did not pass through mitosis (Fig. 2 A). Mimosine plus 2-AP-blocked cells did not engage in DNA replication but remained diploid (2N) until mitosis, as assayed by flow cytometry (Fig. 2 B). Mimosine-treated cells, either with or without 2-AP, also showed no intranuclear accumulation of proliferating cell nuclear antigen (35), an S-phase marker (data not shown). Thus, entry into mitosis did not result from simple reversal of the mimosine block. Mimosine plus 2-AP-treated cells were determined to be in mitosis by the criteria of rounding, condensation of chromatin, loss of nucleolar structures, and loss of the interphase microtubule array. Instead of a fully formed mitotic spindle, these cells exhibited a single small aster adjacent to the chromatin (Fig. 2 C).

Similarly, BHK cells overcame S-phase arrest upon 2-AP addition (Fig. 3). This ability to override S-phase arrest, as assayed by ability to undergo mitosis, was independent of whether the arrest was induced by hydroxyurea (Fig. 3 A) or by aphidicolin (Fig. 3 B), which are unrelated inhibitors of DNA synthesis. Again, both timing and amplitude of mitosis were comparable to controls (Fig. 3 A and B). Cells treated with hydroxyurea plus 2-AP differed from mimosine plus 2-AP-treated cells in that they typically exhibited a small bipolar spindle (Fig. 3 C). This bipolar spindle was arranged either parallel (as shown) or perpendicular to the chromatin mass. Neither of these spindle orientations occurs in cells treated only with 2-AP (21), and the spindle is smaller than in 2-AP-treated cells. Flow cytometric analysis showed the cells that overrode S-phase blockage did not proceed through S-phase replication but remained 2N at mitosis (data not shown).

BHK cells are blocked in G2 by VM-26, as determined by failure to enter mitosis (Fig. 4 A) and by flow cytometry (Fig. 4 B). Simultaneous treatment with VM-26 and 2-AP causes these cells to override the G2 block and enter mitosis (Fig. 4 A and C). The time of arrival in mitosis is the same as for untreated control cells. A full mitotic spindle forms, but chromosomes do not integrate into the spindle. Cells treated in this manner exhibit a phenomenon we have termed partial mitosis (21) in that they exit mitosis without proceeding through metaphase, anaphase, or telophase and, thus, fail to divide into daughter cells. Given that VM-26 acts on topoisomerase II, a major component of the chromosome scaffold (36), and is believed to prevent mitotic entry by inhibition of chromatin condensation (37), it is surprising that chromatin is clearly condensed into chromosomal elements during partial mitosis in VM-26 plus 2-AP (Fig. 4 C).

2-AP also overrides mitotic arrest, independent of the drug used to block cells in mitosis. With either taxol or nocodazole, BHK cells arrest in mitosis (Fig. 5). Upon 2-AP...
We have that as nocodazole does it cycle these typical mitotic of independent conduction, determined was in VM-26 plus 2-AP treated cells were synchronized were 2-AP plus 2-AP; the rate of DNA replication appears somewhat retarded relative to that in control cells, but 2-AP-treated cells exit S phase and arrive at mitosis at the appropriate time regardless of incomplete DNA replication in the population. Further, as we have reported (21), BHK cells exit mitosis in 2-AP without respecting the checkpoint (9) that requires alignment of their chromosomes in a metaphase plate.

It is of obvious interest now to determine whether, as the data suggest, 2-AP acts always on the same effector at different points in the cell cycle. The same or similar enzymes may be imagined to act as negative regulators at each of several points in the cell cycle, blocking progress until certain conditions are met. 2-AP could inhibit the activity of this enzyme, therefore causing the cell to bypass checkpoint controls. This purine analogue is a highly selective inhibitor of protein kinase activity in vivo (19), as 2-AP down-regulates phosphorylation of a very limited subset of the phosphoproteins in the cell. The identities of the phosphorylated substrates and of the protein kinases inhibited by 2-AP in vivo are presently unknown.

At the molecular level, control of progression in the cell cycle is best understood at the point of entry into mitosis. At this point, the phosphorylation state of p34cdc2 and its association with cyclin B are evidently critical to the induction of a variety of mitosis-specific events by p34cdc2 kinase activity (1, 2). If p34cdc2 is phosphorylated on Tyr-15, it must be dephosphorylated for entry into mitosis to ensue in a process dependent on cdc25 (40), a gene that encodes a protein tyrosine phosphatase (41, 42). Okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (43), can induce premature entry into mitosis by dephosphorylation of p34cdc2 and promotes exit from mitosis by degradation of cyclin B (44). Okadaic acid appears to act directly on p34cdc2 rather than on checkpoint controls because 0.5 μM okadaic acid induces a rapid premature entry into mitosis of unblocked mid-S-phase cells (44).

In yeast, wee1 and mkl1 are implicated in the inhibitory regulation of p34cdc2, probably by direct or indirect control of the phosphorylation state of Tyr-15 (10). Both wee1 and mkl1 encode protein kinases that appear to act on cdc2 (10), and wee1 has the reported unusual property of being a serine/tyrosine protein kinase (45). In mitosis, wee1 mkl1 double mutants behave similarly to mammalian cells exposed to 2-AP. As seen in BHK cells (21), the yeast double mutants undergo an aberrant mitosis at restrictive temperature, characterized by abnormal patterns of chromosome segregation and septum formation (10). Of greatest importance, the wee1 mkl1 double mutant, combined with various cdc cell cycle-
arrest mutants, does not respect checkpoints in G1, S-phase, or G2 at restrictive temperatures (10).

Because 2-AP overrides checkpoints throughout interphase, as well as at mitosis, it is possible that 2-AP may act to inhibit the mammalian homologues encoded by wee1 (46) or mik1. The two protein kinases share substantial sequence homology, and both kinases contain ATP-binding sequences unlike the common motif shared by other protein kinases (10) and may, therefore, be specifically inhibitable by particular purine analogues.

The pim1 gene encodes an RCCI homologue in yeast (11). Mutation of RCCI in mammalian cells or of pim1 in yeast leads to premature chromosome condensation in the absence of DNA replication. This result suggests that checkpoint mechanisms, like p34cdc2 regulation of DNA replication. This crotubule-organizing centers. phase or with the replication process, treatment is effective on G1, hinders G1, G2, and G2/M checkpoints. The dissociation of cell cycle progression from stage-specific events is revealed here by the combined treatment of BHK cells with stage-specific blocks and 2-AP. For instance, the mimosine G1 block interferes with the induction of S phase or with the replication process, but signals nonetheless induce the expression of the cell at the appropriate time. There also appears to be a stage-specific maturation of the mitotic microtubule-organizing centers. G1-blocked cells, treated with 2-AP, typically transit mitosis with a single small aster, S-phase-blocked cells exhibit a small “safety pin” spindle, whereas G2-blocked cells exhibit an apparently mature spindle.

Previous data from in vitro models and from VM-26 treatment of intact cells have strongly implicated topoisomerase II as requisite for chromosome condensation (37, 47). Surprisingly, we find discrete chromosome structures in mitotic cells treated with VM-26 and 2-AP. Our results, therefore, suggest that VM-26 does not directly inhibit topoisomerase II-dependent chromosome condensation. VM-26 thus appears to inhibit a checkpoint of topoisomerase II activity required for exit from G1 that is independent of any role of topoisomerase II in chromosome condensation.

Overriding checkpoints in yeast by wee1 mik1/ double mutants has been reported (10) to result in a lethal M phase. Similarly, it is evident from our recent observations that overriding any of several cell cycle blocks by 2-AP is ultimately lethal to the cell (unpublished observations). Several drugs that arrest cells at specific stages of their cycle, such as VM-26, taxol, and vinblastine, have been used with success in tumor therapy. Because overriding cell cycle arrest is lethal, our results suggest that a combination of VM-26 or taxol therapy with a purine analogue, such as 2-AP, might represent a highly effective binary treatment of sensitive tumors.

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