ABSTRACT

At any point during the progression of many tumor types, cells can develop a hyperploid DNA content. Hyperploid tumors are significantly more aggressive, with a higher growth rate and a poor patient prognosis. Yeast genetics have implicated three important genes involved in DNA ploidy changes: cdc2, cyclin b, and a specific inhibitor of the p34^{cd}c2/cyclin B kinase, run1. Mutations in these genes uncoupled the dependence of mitosis on DNA replication in the fission yeast, *Saccharomyces pombe*. It was proposed that the inactivation of the mitotic kinase complex, p34^{cd}c2/cyclin B, induces a G1 state wherein the cells re-replicate their DNA without an intervening mitosis. We show in this report that treatment of only M phase-arrested mouse cells, with the protein kinase inhibitor staurosponine, induced polyploidy. Nocodazole-arrested metaphase F210 cells were pulsed with 100 ng/ml of staurosopine for 1 h. This 1-h treatment results in the inhibition of the mitotic p34^{cd}c2 kinase. The inhibition of the mitotic kinases leads to a reduction in the histone H1 and H3 mitotic-associated phosphorylations, chromosome decondensation, and nuclear membrane reformation. When released into normal growth medium, these cells are reset to a G1 state, re-replicate their DNA without completing mitosis, and become octoploid.

INTRODUCTION

Two important events in the eukaryotic cell cycle are responsible for the genetic fidelity of the daughter cells. The first, DNA replication, correctly duplicates the genetic material and the second, mitosis, ensures that each daughter cell receives a complete set of chromosomes. These two phases are tightly coordinated such that the initiation of one is dependent on the completion of the other (1). In the event that the checkpoint controls for these phases are compromised, an abnormal or skipped mitosis could occur, resulting in polyploid cell formation (2).

It has been suggested that mutations that increase the frequency of whole chromosome abnormalities (genetic instability) may be particularly important to cancer progression (1, 3). The induction of polyploidy in continually cycling cultured cells may induce genetic instability in the daughter cells by facilitating the formation of viable aneuploid cells (2) and may play the same role during tumor formation. Over one-half of all solid tumors in humans demonstrate a hyperploid DNA content (4, 5), which results in an increased aggressiveness and poor patient prognosis (6–9). Studies of proneoplasias have demonstrated that hyperploidy can even occur prior to cellular transformation, in essentially “normal” cells (10–12). Because polyploidy can change so early in oncogenesis, it suggests the involvement of very few or possibly even one gene directly involved in ploidy control. The loss of cell cycle checkpoint controls does not directly produce polyploidy but instead increases the susceptibility (predisposition) to ploidy changes following additional genetic or externally induced changes in the cell cycle (13). Because polyploidy/hyperploidy can occur in the very early stages of cancer, the study of the molecular control of the cellular mechanism(s) of polyploidy may be pivotal to our understanding of the progression of cancer.

There are situations, however, during normal cellular growth and differentiation when M phase is uncoupled from S phase. Successive S phases must take place without an intervening or completed mitoses in some polyploid mammalian tissue cell types (14–16). Conversely, meiosis requires that two rounds of segregation take place without intervening S phases to produce haploid gametes. These examples imply that cells are naturally capable of uncoupling M and S phases and contain appropriate control pathways for this process, possibly through the controlled inactivation of the p34^{cd}c2 kinase (17–22).

In *Saccharomyces pombe*, the cdc2, cyclin b, and run1 genes have been shown to play a direct role in DNA ploidy change (17–20). The cdc2 and cyclin b gene products constitute the eukaryotic growth-associated H1 or M phase kinase complex p34^{cd}c2/cyclin B. The loss of p34^{cd}c2 kinase activity, either through complete degradation of the p34^{cd}c2 protein (18), through insufficient expression of the cyclin B subunit (19), or through the overexpression of the inhibitor p2^run1 (17, 20) in yeast mutants, leads to polyploidy. These studies clearly link the loss of p34^{cd}c2/cyclin B kinase activity with the generation of polyploidy in yeast.

The p34^{cd}c2 kinase is highly conserved between yeast and higher eukaryotes (23) and in mammalian cells may be required for a number of mitotic functions additional to those in yeast. These include chromosome condensation (24–27), nuclear envelope disassembly (28–31), sister chromatid separation (32–34), and cytokinesis (35–38). Alterations in any or all of these events can lead to polyploidy, further suggesting that changes in cellular control of the p34^{cd}c2 kinase activity, during or prior to mitosis, may control cellular mechanisms involved in mammalian cell ploidy change (2). Recently, there have been indications that the loss of the p34^{cd}c2 kinase may be responsible for polyploidy in some higher eukaryotes, including mammals (21, 22).

Polyploidy can be induced in mammalian cells by the use of many drugs, including inhibitors of the spindle apparatus (39), deactylases (40–42), topoisomerases (43), and kinases (44, 45). These drugs induce a cell cycle arrest during G2 or M phase. After an extended period of time (~24–30 h), the cells begin to enter G1 without undergoing a complete mitosis. The periodicity of cell cycle protein expression is generally maintained, indicating that the loss of checkpoint controls on some unknown “clock mechanism” allows normal cyclic gene expression throughout the drug arrest (44). These drug studies may examine loss of checkpoint controls (predisposition) and not the gene(s) directly controlling mammalian cell ploidy change.

1 Supported by Grant GM45890 from the NIH USPHS, Grants FS10 and ERWF510 (to E. M. B.) from the Department of Energy Program, and a grant from Lady Davis Foundation, Jewish General Hospital (to J. P. H. T.).

2 To whom requests for reprints should be addressed, at Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616. Fax: (916) 752-3516; E-mail: lhall@ucdavis.edu.

3 The abbreviations used are: stsp, staurosporine; MI, mitotic index; RLF, replication licensing factor.

Received 11/13/95; accepted 5/28/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by Grant GM45890 from the NIH USPHS, Grants FS10 and ERWF510 (to E. M. B.) from the Department of Energy Program, and a grant from Lady Davis Foundation, Jewish General Hospital (to J. P. H. T.).

2 To whom requests for reprints should be addressed, at Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616. Fax: (916) 752-3516; E-mail: lhall@ucdavis.edu.
inhibitor in vitro, normal cells have only two stsp arrest points during the cell cycle, in G1 and G2 (51–53), and most transformed cells have only the G2 arrest point (45, 52). This suggests that there may be fewer cell cycle kinases sensitive to stsp in vivo than was previously thought, and that only the mitotic kinases, responsible for the G2-M transition, remain sensitive to stsp in transformed cells.

Here we report that a 1-h pulse of stsp administered to mouse cells arrested at metaphase leads to polyploidy when released from the drug. This premature exit from mitosis into G1 may result from the inhibition of the p34^cd2 kinase during mitosis. Our results suggest that in mammalian cells, the reset of mitotic cells to G1 phase may be controlled by the relative activity of the p34^cd2 kinase and the resulting changes in substrate phosphorylations.

MATERIALS AND METHODS

Cell Culture Conditions. The mouse mammary tumor cell line, FT210, was maintained at a density of about 5 x 10^6 cells/ml in RPMI 1640 supplemented with 10% calf serum. The cell density was increased to 1 x 10^6 cells/ml for all experiments.

Cell Synchronization. The synchronization protocol was based on that described in Th'ng et al. (54). The asynchronously growing cells were arrested in G1, with isoleucine-deficient RPMI 1640 supplemented with 10% heat inactivated and dialyzed calf serum for 16 h (55). The cells were released into normal growth medium containing aphidicolin (2.5 μg/ml) for 9 h to arrest the cells at the G1-S boundary. Then the cells were washed once with PBS and transferred to normal growth medium for 18 h with nocodazole (50 ng/ml) added for the last 12 h, where cells with a mitotic index of 60–70% were obtained. Since the cells take ~12.9 h to traverse the cell cycle from the G1-S transition to metaphase, the majority of the cells are arrested at metaphase for only about 5–6 h before treatment.

stsp Treatment. Synchronized cells arrested in M phase with 100 ng/ml nocodazole were treated with 100 ng/ml (0.2 μM) of stsp (Sigma Chemical Co.) for 1 h while still in nocodazole. Subsequently, they were washed twice with PBS and then transferred to drug-free medium. At specific time intervals, as indicated in the figures, samples were taken for analysis. Control cells that were similarly arrested in M phase with nocodazole were mock treated and released into drug-free media. Samples were then taken in parallel with the stsp-treated cells.

Mitotic Index. Cells were fixed either with 3.7% formaldehyde in PBS then stained with 0.1% Hoechst 33342 as described in Th’ng et al. (54) or stained with 0.1% Hoechst 33342 without fixation while cells were in normal growth medium for 1 h. Mitotic indices were determined under a Leica fluorescence microscope. A minimum of 200 cells were scored for each sample. Only cells showing clearly defined chromosomes and absent nuclear membranes were scored as mitotic cells (prometaphase or metaphase).

Histone Phosphorylation. Histones were extracted as described by Th’ng et al. (54). Cells were incubated in [32P]orthophosphoric acid 12 h prior to harvesting. The cells were then collected by centrifugation, washed once with ice-cold PBS, and lysed in nuclei buffer [0.25 M sucrose, 0.2 mM NaCl, 10 mM Tris (pH 8.0), 2 mM MgCl2, 1 mM CaCl2, and 1% Triton X-100]. Histones were then extracted with 0.4 N H2SO4 and precipitated in 20% trichloroacetic acid. The precipitate was washed with acetone, resolubilized in acid-urea gel sample buffer (methylene/green; 8 M urea, 10% glycerol, and 5% acetic acid) and then loaded onto a 12% acrylamide gel containing 8 M urea. Histone phosphorylation was determined by autoradiography after separation of a 17.5% SDS-PAGE gel.

Flow Cytometric Analysis. Cells treated as described above were harvested by centrifugation and washed once with PBS before fixation in 70% ETOH. Cells were stained with mithramycin A (Pfizer, Inc.) and analyzed on a FMF II single argon laser system. All flow analysis was carried out at the National Flow Cytometry and Sorting Resources at the Los Alamos National Laboratory Life Sciences Division.

RESULTS

The Induction of Polyploidy by stsp Pulse Treatment. stsp treatment has been shown to inhibit the p34^cd2 kinase in vivo.5 The effect of stsp inhibition on the p34^cd2 kinase in metaphase-arrested mouse cells and the resulting cell cycle progression was studied. The mouse mammary tumor cell line FT210 was synchronized through sequential cell cycle arrests, with a final arrest in M phase with nocodazole (typically, a MI of between 65 and 75% was obtained for FT210 cells). Half of the nocodazole-arrested cells were treated with a 1-h pulse of 100 ng/ml stsp, while the other half remained in nocodazole only. Both cell populations were then washed twice in PBS and released into drug-free medium. Samples were taken every 2 h following release and were analyzed by flow cytometry (Fig. 1).

Microscopic examination indicated that the MI for the control, nocodazole-arrested cells in Fig. 1 was ~70% prior to release. Flow cytometric analysis showed that over 70% of the control cells had entered G1 by 2 h after release (Fig. 1, row A), and by 6 h, over 95% of the cells had passed through mitosis and entered G1, as shown by the appearance of cells with a 2C DNA content. Microscopic examination of the cells shortly after the release from nocodazole arrest revealed a reduction in the percentage of cells with chromosomes aligned at the metaphase plate (MI) and the appearance of cells in anaphase and telophase. Flow analysis revealed that by 6 h after release, the control cells had initiated DNA replication, as indicated by a broadening of the G1 peak (Fig. 1, row A, filled arrow), and the majority were in mid-S phase by 12 h, with no production of polyploid cells.

When 100 ng/ml of stsp were added to the nocodazole-arrested FT210 cells, microscopic examination showed that the MI decreased rapidly from 70% to less than 5% after a 1-h incubation. Microscopic examination of the cells shortly after stsp treatment revealed a reduction in the percentage of cells with defined chromosomes, an increase in cells showing partially decondensed chromosomes, reforming nuclei, and no cells in anaphase or telophase. These changes indicate that stsp treatment causes a direct transition from metaphase morphology to interphase morphology, similar to that described by Th’ng et al. (54). Flow cytometric analysis of the cells released into a drug-free medium (Fig. 1, row B) shows that 70% of the stsp-treated cells remained tetraploid, as shown by a 4C DNA peak, even after 4 h incubation in drug-free medium. The remaining cells (30%) had completed mitosis and entered G1 phase (2C) 6 h after release, initiated DNA replication by 8 h, and were in mid S phase by 12 h. In contrast, over 50% of the cells in the tetraploid population started to re-replicate between 4 and 6 h following release (Fig. 1, row B, open arrow), almost 3 h ahead of the 30% cells entering G1 phase, and these cells became fully octaploid (8C) by 12 h. In summary, these flow cytometric analyses demonstrate that mitotic cells pulsed for 1 h with 100 ng/ml stsp prematurely exit mitosis, and when released from all drugs, re-replicate their DNA as polyploids. This experiment


4 R. A. Tobey, unpublished data.
Fig. 1. Row A, flow cytometric analysis of nocodazole-arrested FT210 control cells that were released into normal growth medium for 0–12 h and produced no polyploid cells. arrow, initiation of DNA replications. The first histogram (column A, row A) shows asynchronous FT210 cells. Row B, flow analysis of FT210 cells that were synchronized at mitosis with nocodazole, treated with 100 ng/ml stsp for 1 h, and then released into drug-free medium for 0–12 h showed generation of polyploid cells. arrowhead, replicating cells. Row C, flow analysis of FT210 cells synchronized at mitosis by an overnight treatment with nocodazole (18 h) and held for an additional 12 h in nocodazole failed to produce polyploid cells. arrow, no replication by 12 h. Row D, flow analysis of nocodazole-arrested FT210 cells treated with 100 ng/ml stsp for 1 h before release back into nocodazole-containing medium still showed polyploidy by 12 h. arrow, replicated cell population after step-staged cells were released back to nocodazole-containing medium. The bars under the histograms indicate either 2C, 4C, or 8C DNA content. The first bar corresponds to the G1 DNA content of 2C, the second bar corresponds to the G2 DNA content of 4C, and the last bar corresponds to the second G2 (+) DNA content of 8C.

Microscopic Analysis of Polyploid Cells Induced by stsp Treatment. The chromosomes of the resulting octaploid cells were examined for any obvious replication abnormalities (partially replicated chromosomes or fragments). Nocodazole-arrested M-phase cells were treated with stsp for 1 h and then released into nocodazole-containing medium for 24 h. This captures the octaploid cells in the following M phase. Samples were taken during the initial metaphase arrest, after the 1-h stsp treatment, and during the following metaphase arrest for microscopic examination. The unfixed cells were stained with 0.1 mg/ml Hoechst 33342 and examined under a fluorescence micro-

Fig. 2. Mitotic cells were treated with stsp to induce re-replication as described in Fig. 1A. Nocodazole was added to arrest the cells at metaphase, and the chromosomes were visualized under a fluorescent microscope after staining with Hoechst 33342. A and B show nocodazole-arrested mitotic untreated tetraploid FT210 cells. A, cells stained with Hoechst; B, the same cells under phase contrast. C and D, nocodazole-arrested FT210 cells during a 1-h stsp treatment. C, DNA-stained cells; D, the phase contrast of these cells. E, the resulting octaploid cells arrested in metaphase 24 h after release from stsp. F, some octaploid FT210 cells showing classic endo-reduplicated chromosomes. arrowheads, two paired re-replicated sister chromosomes (end-reduplication).
scope. Fig. 2, A and B, shows nocodazole-arrested mitotic (4C) cells prior to stsp treatment, displaying condensed chromosomes and no nuclear membranes. This field shows an interphase nuclei as well (30% of total population). A 1-h treatment with 100 ng/ml stsp results in the decondensation of the metaphase chromosomes and the reformation of defined nuclear membranes, as shown in Fig. 2, C and D. The reformation of interphase nuclei occurs during nocodazole arrest; therefore, no separation of chromosomes (anaphase or telophase) was observed under fluorescence microscope. However, we do observe that some cells become multinucleated, which is most likely due to the disorder of the chromosomes upon decondensation. This is consistent with previous published data by Th'ng et al. (54). The stsp-induced chromosome decondensation also occurs in other cell lines tested, including other mouse cells (FM3A and L-cells), hamster cells (BHK), and human cells (HSF and HeLa). Octaploid (8C) FT210 cells that were arrested in the following metaphase with nocodazole 24 h after stsp treatment are shown in Fig. 2E. Although these octaploid cells were generally bigger than tetraploid metaphase cells, they showed normal looking metaphase chromosomes (no fragmentation) and no nuclear membranes. Further examination of the octaploid-arrested cells showed normal looking metaphase chromosomes and production of interphase nuclei, which then re-replicate their DNA-producing octaploid cells. These octaploid cells showed typical metaphase chromosomes when arrested in the following M phase and some endo-reduplication.

The octaploid peak is lost 27 h after release from stsp treatment in the FT210 cells. Microscopic examination of the octaploid cells traversing mitosis in the following M phase show multipolar mitoses, bipolar mitoses, and some cell death. This suggests that the loss of the G₂ polyploid peak may be through either multipolar mitoses or cell death or both.

Nocodazole Has No Effect on Polyploidy Production. Nocodazole is known to induce polyploidy in some cell lines (39), either by continuous treatment (>20 h) or upon release from the drug due to loss of checkpoint controls like p53 (13). Therefore, the potential effects that nocodazole may have on checkpoint controls and the induction of polyploidy in the FT210 cell line was assessed. As shown in Fig. 1, row A, synchronized FT210 cells, released from overnight nocodazole arrest into normal growth medium, do not form polyploid cells. Continuous incubation of nocodazole-arrested M phase cells in nocodazole for an additional 12 h also failed to produce polyploid cells (Fig. 1, row C, double arrow). In contrast, when nocodazole-arrested M phase cells were treated with stsp for 1 h and then released back into nocodazole-containing media, over 50% of the cells remained tetraploid and became octaploid by 12 h (Fig. 1, row D, line arrow). These data suggest that nocodazole alone has no effect on the polyploidy of these cell lines during this time period, and that these cells maintain sufficient spindle checkpoints (13).

Continuous stsp Treatment Has Little Effect on Polyploidy Production. Continuous incubation in 5–10 ng/ml stsp has been shown to induce polyploidy in a significant (40–50%) population of MOLT-4 cells by 24 h (45). To determine the effect of long-term treatment with stsp, FT210 cells were incubated in the presence of 10, 30, 50, 70, and 100 ng/ml stsp for up to 30 h. Cells incubated in less than 50 ng/ml stsp for 17 h do not seem to show a significant arrest in G₂-M, by flow cytometric analysis (Fig. 3). In MOLT-4 cells, only these lower concentrations produced polyploid cells. However, the FT210 cells treated with less than 50 ng/ml stsp may be slowly traversing M phase without producing polyploid cells, even after 30 h of continuous incubation. Only cells incubated in stsp concentrations higher than 50 ng/ml display significant G₂-M phase arrest by 17 h. When stsp concentrations reached 100 ng/ml, over 80% of the cells became arrest in G₂. Less than 5% of the total cell population become polyploid by 30 h at these higher concentrations. In addition, FT210 cells incubated in the presence of stsp for 30 h showed extensive cell death, as evidenced by a gradual decrease in DNA content (to the left of the 2C or 4C peaks) and by microscopic examination. Even at stsp concentrations as low as 10 ng/ml, some cell death is seen. The absence of significant polyploidy suggests that continuous stsp treatment in this cell line does not cause the majority of the cells to skip M phase and re-replicate their DNA. Instead, it may cause a complete cell cycle arrest in the majority of the cell population, resulting in eventual cell death.

Mitotic Requirement for Cells to Undergo Polyploidization. An interesting observation from the data in Fig. 1, row B, is that the number of cells arrested in metaphase (~70% MI) prior to the stsp treatment corresponds to the number of cells that remain tetraploid upon release. It is from these tetraploid cells that the octaploid cells arise. The remaining 30% of the cells that traverse a regular mitosis to produce diploid cells corresponds to the percentage of cells that were still in G₂ at the time of stsp treatment. This suggests that the only cells capable of becoming polyploid with stsp treatment are those in M phase.

To verify whether only the mitotic cells are able to become polyploid, FT210 cells were arrested in nocodazole for 9, 10, and 12 h to produce cell populations with 30, 50, or 70% of the cells arrested in M phase. These cells were then treated with 100 ng/ml stsp for 1 h before release into drug-free medium for up to 10 h. Flow cytometric analysis of treated cells with either 30, 50, or 70% MI show that by 10 h after release, the proportion of polyploid cells reflect the MI (Fig. 4, rows A, B, and C, respectively). The number of cells entering S phase by 10 h with larger than 2C DNA content were roughly 20, 30, and 45%, respectively. This experiment suggests there is a require-
STAUROSPORINE INDUCES PREMATURE METAPHASE EXIT

0 h 4 h 6 h 8 h 10 h

A
B
C

Fig. 4. FT210 cells were arrested in M phase with nocodazole for 15, 17, and 19 h before a 1-h treatment with 100 ng/ml stsp (rows A, B, and C, respectively). The MIs for each sample set before treatment were 52, 54, and 70%, respectively. Cells were released into drug-free medium for up to 10 h and analyzed by flow cytometry. The G1 DNA content of 2C, the G2 DNA content of 4C, and the second G2 (+) DNA content of 8C are indicated under the X axis of the last row of histograms.

Fig. 5. FT210 cells were synchronized in G2 by incubation at the nonpermissive temperature of 39°C for 18 h. The MI for these cells was 0%. These G2-arrested cells were treated with 100 ng/ml stsp for 1 h before release into normal growth medium at 32°C for 0, 4, 8, and 12 h. A, flow cytometric analysis of an asynchronous cell population. Samples were taken every 4 h for flow cytometric analysis. The same results were obtained for cells arrested in G2 with Hoechst 33342 followed by step treatment. The G1 DNA content of 2C and the G2 DNA content of 4C are indicated under the X axis of all histograms. The arrowhead indicates that there are no octoploid cells by 12 h after release.

ment for the cells to be in M phase for stsp to induce polyploidy, and that G2 phase cells may not become polyploid with stsp treatment.

To test this possibility, FT210 cells were synchronized in G2 with either 175 ng/ml of Hoechst 33342 (data not shown) or by incubation at 39°C before treatment with 100 ng/ml stsp (Fig. 5). The MI for both types of G2 arrests was 0%, confirming the G2 arrest. Following the 1 h incubation in stsp, these G2-arrested cells were released into normal growth medium for up to 12 h, and samples were taken at regular intervals. Flow cytometric analysis (Fig. 5) shows that when the cells were released from the 39°C block after stsp treatment, over 50% of the cells had divided and entered G1 phase by 8 h, with no polyploidy production. Arrest in G2 by either 39°C or 4°C substantially inhibited the p34cdc2 kinase activity after stsp treatment. No other G2-arrested control cells arrested in G2 with Hoechst 33342 produced identical results. The data demonstrate that G2-phase FT210 cells cannot bypass M phase and become polyploid following stsp treatment.

Tetraploid Cells Traverse an Almost Complete G1 Phase before Commencing Re-Replication. Normal G1 in the FT210 cell cycle takes approximately 6.8 h to complete, whereas M phase takes close to 30 min. The initiation of DNA re-replication in the tetraploid (4C) cells (Fig. 5, row B) occurs approximately 1 h in advance of the nocodazole-treated control cells (Fig. 5, row A) as shown in Fig. 1. This indicates that these cells may initiate G1 directly upon release, whereas the control cells must complete mitosis first. This is further substantiated by using isoamylase-deficient (Ile-) RPMI 1640, which arrests FT210 cells in very early G1, possibly at the M-G1 transition (55). When the stsp-treated, FT210 cells were released into Ile- RPMI 1640, the tetraploid cells did not arrest, and re-replication continued to take place, as shown in Fig. 1. This suggests that upon release from stsp treatment, these cells re-enter the cell cycle after the M-G1 transition, skip the Ile- arrest point, and traverse a G1 phase truncated by less than 1 h.

p34cdc2 Activity Is Lost with stsp Treatment. The presence of stsp is known to dramatically inhibit the activity of p34cdc2 and minimally inhibit the p33′cdc2 kinase in vivo. To follow the changes in the p34cdc2 kinase activity after stsp treatment, the kinase complexes were immunoprecipitated from cell lysates and assayed for activity using histone H1 as substrate. Fig. 6 shows a graph of the p34cdc2 kinase activity compiled from densitometer readings of four different autoradiograms. These experiments show that the presence of stsp results in a significant reduction in activity in the p34cdc2 kinase to approximately 10% of the original (100%) M-phase level. Interestingly, this kinase activity remains below 20% activity after release from both drugs (Fig. 6, O) for the duration of the experiment. The activity of the p34cdc2 kinase in the nocodazole-arrested control cells drops to ~5% of the original 100% level after release and remains below 5% for the duration of the experiment (Fig. 6, []). These data confirm that the ability of the immunoprecipitated p34cdc2 kinase complex to 32P-label histone H1 decreases dramatically with stsp treatment and remains reduced after release from the drug for up to 12 h. Metaphase cells treated for 1 h with 100 ng/ml of stsp results in the reduction of p34cdc2 kinase activity to the approximate levels found in control cells following nocodazole release.

Histone H1 Hyperphosphorylation Is Reduced following stsp Treatment. Hyperphosphorylation of histone H1 and phosphorylation of histone H3 have been correlated with the final stages of

Fig. 6. Nocodazole-arrested FT210 cells were treated with 100 ng/ml stsp for 1 h before release into normal growth medium for 12 h. Nocodazole-arrested FT210 cells, not treated with stsp, were used as controls. The p34cdc2 kinase complex was immunoprecipitated from the cell lysates using antibodies against a COOH-terminal peptide of the human p34cdc2 protein. The activity of the kinase was assayed using 32P and histone H1 as the substrate. The histone H1 was then run on SDS-PAGE before autoradiography. Densitometer readings on the autoradiograms of four repeated experiments were obtained. The average and SDs for each time point are plotted for the nocodazole-released control cells ([]) and for the stsp-treated/released cells (O). The first time point shows the initial M phase percentage of kinase activity (— 1 h time point). stsp was added for 1 h, or the control cells were not treated for 1 h before a sample was taken (0 h time point). [], the percentage of p34cdc2 kinase activity during a normal nocodazole release from 0—12 h after release. O, the percentage of p34cdc2 kinase activity in the cells released after a 1-h stsp treatment (0—12 h).

6 R. A. Tobey and E. M. Bradbury, unpublished data.
These cells showed diffuse chromatin and defined nuclear membranes. Cells continue to maintain interphase chromatin structure, by microsopic examination. The flow cytometric data in Fig. 1. A 1-h treatment with 100 ng/ml stsp is clearly absent, histone H1 is hyperphosphorylated during and after release from stsp treatment. Chromosome condensation seen at mitosis (57–59). Since histone H1 is the putative substrate of p34cdc2, we studied the changes in histone phosphorylations during and after release from stsp treatment.

During metaphase, the chromosomes are maximally condensed, the nuclear membrane is clearly absent, histone H1 is hyperphosphorylated (Fig. 7B, arrow), and histone H3 is phosphorylated. Previous studies from this laboratory have shown that stsp (50–100 ng/ml) added to nocodazole-arrested metaphase cells leads to decondensation of chromosomes and dephosphorylation of histone H1 and H3 (54). Fig. 7 shows a similar effect following a 1-h treatment of metaphase-arrested cells with 100 ng/ml stsp. The phosphorylation levels on histones H1 and H3 were reduced to interphase levels. These decreases in histone H1 and H3 phosphorylations correlate directly with the decondensation of the metaphase chromosomes, as illustrated by a drop in MI from 70% to less than 5% by microscopic examination. These cells showed diffuse chromatin and defined nuclear membranes (Fig. 2, C and D). When the cells were transferred to regular growth medium, there was a partial recovery of the hyperphosphorylated chromosome and dephosphorylation of histone Hi and H3 (54).

A 1-h treatment with 100 ng/ml stsp induces dephosphorylation of the histones H1 and H3 to the levels seen during interphase concurrent with the decondensation of the metaphase chromosomes to interphase chromatin. This dephosphorylation of histones H1 and H3 persists after release from the drugs as the cells continue to maintain interphase chromatin structure, by microscopic examination.

DISCUSSION

There have been numerous reports linking polyploidy with carcinogenesis (4, 5, 60–63). A key initiating event in polyploid cell production must be an absent or abnormal mitosis, which allows the cells to enter G1 with an increased DNA content (2). To date, the gene(s) directly responsible for producing polyploidy in mammalian cells remain to be identified. In yeast, the loss of the p34cdc2 kinase, either through mutation or through the activity of its inhibitor p25crim1, has been shown to be involved in polyploid cell formation (18–20), mainly through the dual role the kinase plays in controlling the onset of S and M phases (18, 19). Although p34cd2 is not required for G1 or S phase progression in higher eukaryotes, a correlation was found between the loss of p34cdc2 kinase activity, in some cycling plant and mammalian cells, and the initiation of polyploid cell formation (21, 22). In this report, we show that a brief exposure of metaphase-arrested mouse cells to stsp results in: (a) a complete and irreversible loss of the p34cdc2 kinase activity (Fig. 6); (b) loss of the mitotic phosphorylations of histones H1 and H3 (Fig. 7); (c) decondensation of the metaphase chromosomes to interphase chromatin (Fig. 2); and (d) reformation of nuclear membranes and exit from metaphase without completion of anaphase, telophase, or cytokinesis (Fig. 2). Upon removal of nocodazole and stsp from the cells, they traverse an almost complete G1 phase as polyploids (4C) before re-replicating their DNA to become octaploid in the following G1 (Fig. 1B).

Stsp has been shown to reversibly inhibit a wide range of kinases in vitro (46–50), as well as a few kinases during interphase in vivo, including p34cdc2 (52–54). However, in this report, we show that an irreversible inhibition of p34cdc2 is produced in mitotic FT210 cells (Fig. 6), which may involve cyclin B degradation. Commercially available cyclin B antibodies (from Santa Cruz Biotechnology, Upstate Biotechnology, Inc., and PharMingen) failed to identify cyclin B protein in FT210 cells. However, the same experiments performed in mitotic HeLa cells resulted in a rapid degradation of cyclin B upon stsp treatment. Since both cell lines produce irreversible p34cdc2 kinase inhibition upon stsp treatment, which result in polyploid cell formation, we assume that stsp also induces cyclin B degradation in the FT210 cell line. Additionally, in the FT210 cell line, there is no change in the migration of the p34cdc2 protein on an SDS-PAGE gel, indicating that the irreversible loss of the p34cdc2 kinase activity is not due to inhibitory phosphorylations on the protein. The continued loss of the kinase after release from the drug suggests that further downstream events may have been activated that triggered the "end" of M phase and entry to G1, as found for a normal completed mitosis. It is not clear whether the degradation of cyclin B after stsp treatment is through the ubiquitin degradation pathway (64–67). This ubiquitin-mediated inactivation of p34cdc2/cyclin B usually triggers the exit of M phase and the entry into G1 in normal cell cycle. While the kinase remains active, there is no entry into G1, as found for a normal completed mitosis. If the loss of the kinase activity upon completion of mitosis is the final downstream checkpoint that triggers the entry into G1 phase during a normal cell cycle, then the premature loss of the kinase activity during metaphase by stsp treatment may be sufficient to trigger exit into G1, as shown in this study.

Once the M-phase kinase, p34cdc2/cyclin B, activity is lost and the cells are released from stsp, there are three possible outcomes for the cells: (a) the re-formation of the metaphase chromosomes and the completion of mitosis; (b) continuation of the cell cycle as G1 polyploid cells; or (c) irreversible cell cycle arrest and death. Since stsp-treated cells are now biochemically G1 cells, as regards kinase activity (low p34cdc2 and low p34cdk activity; Ref. 68) and have interphase chromatin structure, an organized re-condensation of the chromosomes may not be biochemically feasible. Furthermore, the

---

Fig. 7. FF210 cells were grown in the presence of 32Pi and then arrested in G0 with 10e RPMI 1640 (Lane G1), in G1-S with 2.5 mg/ml aphidicolin (Lane G1/S), and in M phase with 100 ng/ml nocodazole (Lane M). The metaphase-arrested cells were then treated for 1 h with 100 ng/ml stsp (Lane stsp) and released into normal growth media for 1, 2, 3, and 4 h (Lanes 1–4, respectively). Histones were extracted and run on acid urea gel electrophoresis before Coomassie staining and autoradiography. A, the Coomassie-stained gel; B, the resulting autoradiogram. Arrowhead, hyperphosphorylated histone H1 in mitotic cells.

3556
re-condensation of disorganized chromosomes may result in abnormal condensation and apoptosis. Mammalian cells are capable of undergoing multipolar mitoses during the following M phase and reconstituting the diploid DNA content in the resulting daughter cells (2). Thus, polyploidy may not even be permanent if all conditions remain normal for the next mitosis, which is the case for the stsp-induced polyploidy in the FT210 cell line. After the p34\(^{cdk2}\) kinase activity is lost in the metaphase-arrested cells and they exit to interphase, the most reasonable outcome for the cell is polyploid cell production, with the possibility of re-establishing a diploid DNA content in the next mitosis.

In this study, polyploidy could only be produced from metaphase-arrested FT210 cells but not from G\(_2\) phase-arrested cells treated with stsp. The reason for this observation is not known. However, mammalian cells have been shown to require the presence of a RLF in order to replicate (69–72). This requirement for the access of chromatin to RLF was met in the M phase-arrested FT210 cells before stsp treatment, enabling the cells to re-replicate after release. However, once G\(_2\) or M phase cells (4C) have skipped or prematurely exited mitosis, they are polyploid G\(_2\) phase cells (4C). Since these tetraploid cells have been reset to G\(_1\), they are already polyploid prior to DNA synthesis, which is where the RLF is required and where they re-replicate as polyploids. Therefore, RLF is not necessary for the G\(_1\) reset or required for the formation of polyploid cells. However, it is needed for those cells to re-replicate as polyploids.

G\(_2\) phase cells differ from mitotic cells in their relative p34\(^{cdc2}\) and p33\(^{cdk2}\) kinase activities. As shown by Gu et al. (68) in HeLa cells, the p34\(^{cdc2}\) kinase activity in G\(_1\), S, G\(_2\), and M phase cells are approximately 10, 5, 20, and 100\%, respectively. On the other hand, the p33\(^{cdk2}\) kinase activities in G\(_1\), S, G\(_2\), and M phase cells are 10, 40, 100, and 15\%, respectively. The stsp-induced loss of p34\(^{cdc2}\) kinase activity during mitosis was irreversible in the FT210 cell line. Previously, we have shown that the effect of stsp treatment on G\(_2\) phase cells to be completely reversible at the cellular level (54), and the inhibitory effect of stsp on the p33\(^{cdk2}\) kinase to be minimal in FT210 cells. Therefore, G\(_2\) phase-arrested FT210 cells treated with stsp are able to recover from the treatment. These G\(_2\) cells have high p33\(^{cdk2}\) activity (68), which is less sensitive to stsp treatment, and would either maintain or reconstitute the proper G\(_2\) phase p33\(^{cdk2}\) kinase activity after release from the drug, thereby maintaining the G\(_2\) kinase balance (high p33\(^{cdk2}\) and low p34\(^{cdc2}\); Ref. 68). These post-treated cells could then activate the M-phase kinase at the proper time and proceed normally through the G\(_2\)-M transition without a G\(_1\) reset and without polyploid cell production. Conversely, the dramatic and irreversible inhibition of the p34\(^{cdc2}\) kinase activity during mitosis, while the cells already contain low p33\(^{cdk2}\) kinase activity (68), would produce a G\(_1\)-phase kinase balance (low p34\(^{cdc2}\) and low p33\(^{cdk2}\); Ref. 68). These cells are then reset to G\(_1\), where they re-replicate their DNA as polyploids. Thus, only mitotic cells contain the appropriate kinase balance such that the inhibition of p34\(^{cdc2}\) kinase would produce G\(_1\) phase kinase activity, resulting in G\(_1\) phase-specific changes in the substrates and a G\(_1\) reset.

The changing balance of kinase activities during the cell cycle may link the phases of the cell cycle with chromosomes through the phosphorylation states of histone H1 and other substrates. Since there is a correlation between phosphorylation levels of some histones and the condensation state of chromatin during the cell cycle (57–59), the structural state of chromatin may also function as a cell cycle checkpoint to prevent the re-replication of the DNA or as a cell cycle “clock” controlling the timing of some phase-specific gene expression (73). In this study, when the M-phase cells were treated with stsp, the activity of the p34\(^{cdc2}\) kinase was reduced to approximately 10% of the original level. This is similar to the drop in activity seen in cells during the transition from M to G\(_1\) and may be reflected in the phosphorylation states of the phase-specific substrates. Thus, the changes in the phosphorylation states of the p34\(^{cdc2}\) substrates, such as histones and lamins, may trigger both the exit from mitaphase and the reset to G\(_1\).

Many cell cycle-inhibitory drugs have been shown to induce polyploidy in a number of mammalian cell lines after prolonged exposure to the drug (39–45). These long-term cell cycle arrest studies mainly follow the effects of cell cycle arrest on loss of checkpoint controls between species or during cellular transformation and not on gene(s) directly regulating ploidy change (74, 75). Cell cycle checkpoint loss does not usually produce polyploidy directly but instead predisposes the cell to ploidy changes following additional genetic or externally induced changes in the cell cycle (e.g., the p53 checkpoint and spindle inhibition by drugs; Ref. 13). The production of polyploidy through changes in the activity or abundance of gene product(s) that are directly involved in the cellular control of ploidy change should not be dependent upon additional loss of checkpoint controls. In the FT210 cell line, we tested for possible loss of common cell cycle “clock” checkpoints by prolonged incubation in M phase with nocodazole (Fig. 1C) and G\(_2\) phase with stsp (Fig. 3). No significant polyploidy was produced with these long-term treatments, suggesting that the majority of the cell population may still maintain sufficient cell cycle “clock” checkpoints during G\(_2\) and mitosis. Additionally, although the FT210 cells had been exposed to nocodazole for 12 h, they had been presynchronized, which means that the majority of the population was arrested at metaphase for only 5–6 h before the brief 1-h treatment with stsp and the resulting polyploidy production. Thus, there was no long-term (24–30 h) cell cycle arrest at metaphase, which makes it unlikely that the loss of a cell cycle “clock” checkpoint is involved. Instead, the induction of polyploidy may be due to the inactivation of an important protein product directly involved in the control of mammalian cell ploidy change, such as the p34\(^{cdk2}\) kinase.

In conclusion, although stsp may be acting on an unknown kinase that directly regulates mitotic and G\(_1\) phase control, our data suggest that the premature inhibition of the p34\(^{cdc2}\) kinase alone may be sufficient to trigger the G\(_1\) reset in mitotic cells. This trigger for the G\(_1\) reset may be due to the premature loss of the mitotic p34\(^{cdc2}\) kinase activity in a cell already containing G\(_1\) levels of the p33\(^{cdk2}\) kinase (68). Thus, the change from an M-phase to a G\(_1\)-phase balance of kinase activities is reflected in the phosphorylation levels of its phase-specific substrates, such as histone H1 and H3, nuclear membrane re-formation, and the state of chromatin condensation. This change in substrate phosphorylation may trigger entry into G\(_1\). No other known kinase, active during mitosis, controls as many M phase-specific activities known to be involved in ploidy change mechanisms as p34\(^{cdc2}\). Additionally, this kinase has already been shown to be involved in ploidy change in yeast and has been correlated with ploidy change in maize endosperm and rodent parotid glands. A recent publication has shown a direct correlation between loss of the p34\(^{cdc2}\)/cyclin B kinase activity and megakaryocyte (4C) cell formation (76).

ACKNOWLEDGMENTS

We thank Carolyn Bell-Prince and Harry Crissman of the Los Alamos National Flow Cytometry and Sorting Resources for the cell cycle analysis and advice.

REFERENCES

STAUROSPORINE INDUCES PREMATURE METAPHASE EXIT


63. Sandberg, A. A. A chromosomal hypothesis of oncogenesis. Cancer Genet. Cyto-
64. Gallant, P., and Nigg, E. A. Cyclin B2 undergoes cell cycle-dependent nuclear
translocation and, when expressed as a non-destructible mutant, causes mitotic arrest
vivo inhibition of cyclin B degradation and induction of cell-cycle arrest in mammal-
ian cells by the neutral cysteine protease inhibitor N-acetylleucylleucynorleucinal.
66. Sudakin, V., Ganoth, D., Dahn, A., Heller, H., Hershko, J., Luca, F. C., Ruderman,
J. V., and Hershko, A. The cyclosome, a large complex containing cyclin-selective
ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol.
69. Blow, J. J., and Laskey, R. A. A role for the nuclear envelope in controlling DNA
70. Leno, G. H., Downes, C. S., and Laskey, R. A. The nuclear membrane prevents
replication of human G2 nuclei but not G1 nuclei in Xenopus egg extract. Cell, 69:
required for cell cycle regulation of DNA replication in vertebrate cells. Nature
MCM-containing complex as a component of the DNA replication licensing system.
73. Lewin, B. Chromatin and gene expression: constant questions, but changing answers.
74. Hirschberg, J., and Marcus, M. Isolation by a replica-plating technique of Chinese
hamster temperature-sensitive cell cycle mutants. J. Cell. Physiol., 113: 159–166,
1982.
75. Handeli, S., and Weintraub, H. The ts41 mutation in Chinese hamster cells leads to
successive S phases in the absence of intervening G2, M, and G1. Cell, 71: 599–611,
76. Zhang, Y., Wang, Z., and Ravid, K. The cell cycle in polyploid megakaryocytes is
associated with reduced activity of cyclin B1-dependent cdc2 kinase. J. Biol. Chem.,
A Brief Staurosporine Treatment of Mitotic Cells Triggers Premature Exit from Mitosis and Polyploid Cell Formation

Lisa L. Hall, John P. H. Th'ng, Xiao Wen Guo, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/15/3551

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/56/15/3551. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.