Staurosporine Overrides Checkpoints for Mitotic Onset in BHK Cells

Sun W. Tam and Robert Schlegel

Department of Molecular and Cellular Toxicology, Division of Biological Sciences, Harvard School of Public Health, Boston, Massachusetts 02115

Abstract

Under normal conditions, mammalian cells will not initiate mitosis in the presence of either unreplicated or damaged DNA. We report here that staurosporine, a tumor promoter and potent protein kinase inhibitor, can uncouple mitosis from the completion of DNA replication and override DNA damage-induced G2 delay. Syrian hamster (BHK) fibroblasts that were arrested in S phase underwent premature mitosis at concentrations as low as 1 ng/ml, with maximum activity seen at 50 ng/ml. Histone H1 kinase activity was increased to approximately one-half the level found in normal mitotic cells. Inhibition of protein synthesis during staurosporine treatment blocked premature mitosis and suppressed the increase in histone H1 kinase activity. In asynchronously growing cells, staurosporine transiently increased the mitotic index and histone H1 kinase activity but did not induce S phase cells to undergo premature mitosis, indicating a requirement for S phase arrest. Staurosporine also bypassed the cell cycle checkpoint that prevents the onset of mitosis in the presence of damaged DNA. The delay in mitotic onset resulting from gamma radiation was reduced when irradiation was followed immediately by exposure to 50 ng/ml of staurosporine. These findings indicate that inhibition of protein phosphorylation by staurosporine can override two important checkpoints for the initiation of mitosis in BHK cells.

Introduction

Staurosporine is a potent general protein kinase inhibitor that can suppress in vitro the activity of phospholipid-Ca2+-dependent and cyclic nucleotide-dependent serine/threonine protein kinases as well as the tyrosine kinases p60c-src and epidermal growth factor receptor (see Ref. 1 for review). This microbial alkaloid has been used to investigate the role of protein phosphorylation in numerous cell processes, including, but not limited to, differentiation and development (2–5), carcinogenesis (6–8), mitogenic signaling pathways (9–12), and cell cycle control (13–15). Staurosporine has recently been found to reversibly arrest mammalian cells in both the G1 and G2 phases of the cell cycle (13–15). The phase of the cell cycle affected is dependent on the concentration of staurosporine used, with low concentrations (1–10 ng/ml) leading to G1 arrest and higher concentrations (50–100 ng/ml) superimposing a G2 arrest. Transformed cells appear to differ markedly from nontransformed cells in their response to staurosporine. Of five different transformed cell lines examined, all were resistant to staurosporine-induced G1 arrest but retained sensitivity in the G2 phase of the cell cycle (14, 15).

Considering the known effects of staurosporine on cell growth and cell cycle control, it was of interest to determine whether this chemical could bypass normal checkpoints for mitotic onset that ensure the proper timing of mitosis. The eukaryotic cell cycle contains mitotic checkpoints which prevent cells from initiating mitosis in the presence of either unreplicated or damaged DNA (see Refs. 16 and 17 for reviews). Mitotic checkpoints are important for maintaining DNA integrity, and loss of these controls dramatically reduces cell survival. The requirement for the completion of DNA replication before the onset of mitosis can be bypassed in mammalian cells by several different chemicals. Caffeine (18), the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine (19), and the protein phosphatase inhibitor okadaic acid (20, 21) have been shown to induce mitosis in hamster cells that are arrested in S phase. Caffeine (21) and okadaic acid (20) indirectly dephosphorylate p34cdc2, the catalytic subunit of the p34cdc2/cyclin B histone H1 kinase complex, leading to the active mitotic form of the kinase.

In this report, it is shown that staurosporine bypasses normal checkpoints for mitotic onset and leads to the activation of cyclin-dependent histone H1 kinase. This potent protein kinase inhibitor induces premature mitosis in cells that contain incompletely replicated genomes and suppresses G2 delay in cells that have suffered DNA damage.

Results

Staurosporine Uncouples Mitosis from the Completion of DNA Replication. Fig. 1 illustrates the changes in cell morphology and chromatin structure of S phase-arrested BHK cells following exposure to staurosporine. Fig. 1a shows untreated BHK cells that were arrested in S phase for 8 h with hydroxyurea. When staurosporine (50 ng/ml) was added to the medium for the final 5 h of S phase arrest, numerous cells assumed the rounded morphology typical of mitotic cells and could be accumulated in the presence of the microtubule polymerization inhibitor nocodazole (Fig. 1b). Many of these cells also displayed extensive cytoplasmic blebbing (indicated by arrow). Concomitantly, with cytoplasmic morphology changes, the chromatin of these cells underwent premature conden-
sation. These rounded cells were selectively removed from the dishes by gentle physical detachment and prepared for chromosome analysis. The chromatin in these cells (Fig. 1c) had the condensed and "pulverized" appearance characteristic of S phase cells forced into mitosis prematurely by chemicals (18, 19) or by fusion with other mitotic cells (22). If cells undergoing staurosporine-induced premature mitosis were not collected in the presence of nocodazole, interphase morphology often returned, and the nuclear envelope reformed around decondensed and fragmented chromatin to produce numerous micronuclei (Fig. 1d).

**Dose Response for Staurosporine-induced Premature Mitosis.** Fig. 2 shows a dose response for staurosporine-induced premature mitosis. When exposed for 8 h, BHK cells showed a slight, but significant [P < 0.01 by the binomial comparison described by Kastenbaum and Bowman (23)], increase in premature mitosis at doses as low as 1 ng/ml, with maximum effectiveness at 50 ng/ml. Approximately one-third of the total cells responded at the optimal concentration. The effective concentrations in vivo are similar to those reported as inhibitory in in vitro protein kinase assays (1). Decreased activity was found above 50 ng/ml, with less than 10% of cells undergoing premature mitosis at 100 ng/ml (data not shown). This decreased response was likely due to inhibition of p34<sup>++</sup>, an essential kinase for initiation of mitosis, at high concentrations. To test this hypothesis, activated histone H1 kinase was precipitated from mitotic BHK cells on p13<sup>-</sup>-<sup>++</sup> beads and examined for in vitro H1 kinase activity in the absence or presence of various concentrations of staurosporine (Fig. 3, A and B). The p13<sup>-</sup>-<sup>++</sup> beads bind to the cyclin-dependent kinases, cdc2 and cdk2, with cdc2 accounting for the vast majority of precipitated kinase activity from mitotic cell extracts. Kinase activity was reduced to 8% of control in the presence of 100 ng/ml staurosporine, with an IC<sub>50</sub> of approximately 20 ng/ml (~40 nm). This finding is consistent with earlier reports showing that concentrations of staurosporine above 50 ng/ml arrested cells in G<sub>2</sub> (13–15). Activation of H1 kinase activity by staurosporine, therefore, requires a compromise between concentrations of staurosporine that lead to indirect activation (see below) and those that directly inhibit H1 kinase.

**Requirements for RNA and Protein Synthesis and for S Phase Arrest.** The kinetics of premature mitosis induced in BHK cells by 50 ng/ml of staurosporine are shown in the accompanying figure (Fig. 1). The effects are both rapid and transient. The onset of premature mitosis was near instantaneous; cells were arrested in mitosis within 1 h of exposure to staurosporine. The duration of the mitotic arrest also depended on the concentration of staurosporine used; the higher the concentration, the shorter the mitotic arrest. The majority of cells arrested in mitosis were viable, and the physical appearance of the cells was normal, with round nuclei and distinct nuclear envelopes. It is possible that staurosporine arrested cells in anaphase or mitotic prophase, so that cell separation was blocked, leading to cell rounding.

**Fig. 1. Changes in cell and chromatin morphology during staurosporine-induced premature mitosis.** BHK cells were arrested in S phase with hydroxyurea (2.5 mM) for 8 h, and premature mitotic cells were accumulated in the presence of nocodazole (0.25 μg/ml) during the final 5 h. A, phase contrast micrograph of cells arrested in S phase. ×200. B, phase contrast micrograph of "rounded up" premature mitotic cells. Conditions were identical to those in A except that cells were exposed to 50 ng/ml staurosporine during the last 5 h. ×200. C, chromosome preparation stained with Hoechst 33258 showing "pulverized" chromatin of S phase cells that underwent staurosporine-induced premature mitosis. Cells were exposed to staurosporine for 8 h. Otherwise, treatment was identical to that in B. ×1000. D, chromatin decondensation and micronucleation of cells treated as in B, except that cells were not exposed to nocodazole and were fixed in absolute methanol and stained with Hoechst 33258 10 h after addition of staurosporine. ×1000.

*The abbreviations used are: IC<sub>50</sub>, 50% inhibitory concentration; HEPES, 4-[2-hydroxyethyl]-1-piperazinethanesulfonic acid; EGTA, ethyleneglycol bis(oxyethyl) ether-2-aminoethylester-N,N',N''-tetraacetic acid.*
Actinomycin D is not shown). Fig. 6. Approximate 2 h of exposure were required before premature mitosis was apparent, and the process was gradual, with the percentage of responding cells increasing over at least 6 h. New protein and RNA synthesis were required for staurosporine-induced premature mitosis. Premature mitosis was strongly inhibited when cells were exposed to either cycloheximide (10 μg/ml) or actinomycin D (2 μg/ml) beginning 30 min before the addition of staurosporine. The above concentrations of cycloheximide and actinomycin D inhibited protein and RNA synthesis, respectively, by 90–95%, as measured by [%1S]methionine and [%H]uridine incorporation (data not shown).

Okadaic acid can induce premature mitosis in nonarrested cells (20), but caffeine, 2-aminopurine, and 6-dimethylaminopurine elicit this response only when cells are arrested in the S or G2 phases of the cell cycle (18, 19, 21). To assess any requirement for cell cycle arrest, exponentially growing BHK cells were exposed to 50 ng/ml of staurosporine. Chromosome preparations that were made after 2, 4, and 8 h of treatment showed no evidence of premature mitosis among the 500 mitotic cells examined at each time point, as indicated by the lack of S phase "pulverization" or G2 chromosome morphology. Continuous arrest in S phase is therefore a requirement for uncoupling mitosis from the completion of DNA replication. These results are consistent with previous work, in which the mRNA needed for caffeine-induced premature mitosis was shown to become unstable when cells were actively synthesizing DNA (24).

Staurosporine Activates Cyclin-dependent Histone H1 Kinase in Vivo. The onset of mitosis in eukaryotic cells is controlled by a serine/threonine protein kinase that is composed of a catalytic subunit, p34cdc2, and a regulatory subunit, cyclin B. The activity of this kinase for the substrate histone H1 is increased dramatically as cells progress from interphase to mitosis (25). To determine whether staurosporine induces premature mitosis by activating this regulatory kinase in vivo, p34cdc2/cyclin B was affinity precipitated on p13met1 beads from whole cell extracts of control and staurosporine-treated cells and assayed for histone H1 kinase activity. Although p13met1 beads are known to bind another cyclin-dependent kinase, cdk2, the kinase activity precipitated is due predominantly to cdc2.

Staurosporine (50 ng/ml) caused a rapid increase in cyclin-dependent histone H1 kinase activity in S phase-arrested BHK cells (Fig. 5, A and B). This increase was readily detectable within 1 h, reached a maximum 6 h after treatment, and began to decline thereafter. Peak activity was approximately 55% of that seen in normal mitotic cells. Control cells showed no appreciable change in activity during this period, remaining at approximately 10% of the activity of mitotic cells. At 8 h after drug treatment, staurosporine and caffeine, both of which induce premature mitosis, displayed equivalent kinase activity. New protein synthesis is required for staurosporine- and caffeine-induced premature mitosis, and exposure of cells to either of these drugs in combination
with cycloheximide suppressed the activation of histone H1 kinase (Fig. 5, A and B). Although cycloheximide did not completely eliminate drug-induced kinase activation, levels rose to only 16% of that seen in normal mitotic cells.

Staurosporine did not uncouple mitosis from the completion of DNA replication in asynchronously growing cells. It was therefore surprising to find that staurosporine rapidly activated histone H1 kinase activity in asynchronous cultures (Fig. 5, A and B). This activation was of lower magnitude and of shorter duration than that in S phase-arrested cells, however. Maximum H1 kinase activity did not exceed 40% of that in normal mitotic cells, and activity declined after 2 h of exposure. A transient increase in the mitotic index of asynchronous cells did not accompany this moderate increase in H1 kinase activity, however (Fig. 6). After a slight delay, the mitotic index paralleled kinase activity, reaching a maximum of 3-fold higher than control after 4 h of exposure. Induction of kinase activity was seen 1 h prior to an increase in mitotic cells, supporting a causative role for staurosporine-induced H1 kinase activity in the increased mitotic index and indicating that higher kinase activity was not just an indirect consequence of a larger population of mitotic cells.

**Staurosporine Suppresses Radiation-induced Mitotic Delay.** Damage to cellular DNA typically prolongs the G2 phase of the cell cycle, producing a delay in the onset of mitosis and a rapid decrease in the mitotic index of the cell population (26). Chemicals such as caffeine, 2-amino purine, and 6-dimethylaminopurine, which are able to uncouple mitosis from the completion of DNA replication (18, 19), can also suppress mitotic delay following DNA damage (21, 27–29). We therefore wanted to determine whether staurosporine displayed both of these activities (Fig. 7). Asynchronously growing BHK cells that received 250 rads of gamma radiation rapidly underwent G2 arrest, which was reversed without further treatment approximately 3 h after irradiation. This delay was greatly attenuated when 50 ng/ml of staurosporine were added to the culture medium immediately after irradiation. A partial G2 arrest was evident 1 h after combined treatment with radiation and staurosporine, but this arrest was completely reversed within 2 h. As described earlier, treatment with staurosporine alone increased the mitotic

---

**Fig. 4.** Protein and RNA synthesis requirements for staurosporine-induced premature mitosis. BHK cells were arrested in S phase and at the indicated times were quantitated for premature mitosis as in Fig. 2. Cells either were treated with hydroxyurea alone (Δ) or were exposed to hydroxyurea and 50 ng/ml staurosporine (○) at T = 0, with or without simultaneous addition of 2 μg/ml actinomycin D (●) or 10 μg/ml cycloheximide (■).

**Fig. 5.** A, in vivo activation of histone H1 kinase by staurosporine. BHK cells were exposed to 50 ng/ml staurosporine during S-arrest as in Fig. 2, during S-arrest with simultaneous treatment with cycloheximide (CHM) (10 μg/ml), and during log phase growth. At the indicated times after initiation of staurosporine treatment, whole cell extracts were prepared, and histone H1 kinase activity was visualized by autoradiography of the phosphorylated histone H1 substrate after separation on polyacrylamide gels, as described under "Materials and Methods." Extracts from mitotic cells accumulated in nocodazole for 8 h (M), from S-arrested cells treated for 8 h with 5 mM caffeine (CF), and from untreated controls taken 8 h after the T = 0 control (C) were also included. B, same procedures were used as in A, except that kinase activity was quantitated by excising histone H1 protein from the gel and measuring radioactivity by Cerenkov counting. Cells were exposed to 50 ng/ml staurosporine during S-arrest (C), during S-arrest with simultaneous treatment with 10 μg/ml cycloheximide (○), and during log phase growth (Δ). Kinase activity of staurosporine-treated cells was compared to that seen in mitotic cell extracts. The specific activity of the M phase H1 kinase was ~30 pmol phosphate/mg protein/min in whole cell extracts.
index when compared with untreated cells. This increase was evident after 2 h of exposure and was unaffected by irradiation.

Discussion

The onset of mitosis in all eukaryotic cells studied is controlled by the serine/threonine protein kinase p34^cdk2. The activity of this kinase is regulated by the formation of complexes with other cellular proteins as well as by the phosphorylation status of the kinase (25). For full kinase activity at mitosis, mammalian p34^cdk2 must be complexed with cyclin B, dephosphorylated at threonine 14 and tyrosine 15, and phosphorylated at threonine 161. The protein kinase Wee1 maintains the inhibitory phosphorylation on p34^cdk2 at tyrosine 15, whereas the protein phosphatases cdc25 and p65 dephosphorylate this site (see Refs. 17 and 30 for reviews). Modulation of cyclic AMP-dependent protein kinase activity in mammalian somatic cells and *Xenopus* oocytes (31, 32) and deletion of a yeast protein kinase C homologue (33) have also been shown to affect the onset of meiosis and mitosis. Protein phosphorylation, therefore, plays a major role in the regulation of mitosis.

The uncoupling of mitosis from the completion of DNA replication by staurosporine is in many ways similar to that seen with caffeine and the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine (18, 19). In all cases, the onset of premature mitosis was a relatively slow and gradual process, suppression of new protein synthesis blocked the response, and little or no activity was apparent in exponentially growing cells. It appears that all of these chemicals induce premature mitosis by an indirect mechanism and that arrest in S phase makes cells more susceptible to chemically induced aberrations in mitotic control, most likely due to the accumulation of the cyclin B/p34^cdk2 complex (21). Staurosporine, like these other chemicals, was unable to induce premature mitosis in human HeLa cells (data not shown), consistent with earlier work showing that HeLa cells do not form the required cyclin B/p34^cdk2 complex during arrest in S phase (21).

The extent to which staurosporine raised the mitotic index of asynchronously growing cells distinguishes this chemical from caffeine and the kinase inhibitors 2-aminopurine and 6-dimethylaminopurine. These latter compounds have little or no effect on the mitotic index (21). Since chromosome preparations of asynchronous staurosporine-treated cells showed no evidence of G1 or S phase premature mitosis, these mitotic cells either were recruited from G2 or were delayed in their return to interphase. Time-lapse videomicroscopy and perhaps FACS analysis could be used to distinguish between these possibilities.

DNA damage prolongs the G1 phase of the cell cycle (26). This delay allows additional time for repair of DNA before mitotic onset and increases cell survival (see Ref. 16 for review). Staurosporine was also able to override this important cell cycle checkpoint. Indirect evidence suggests that both S phase arrest and DNA damage prevent the onset of mitosis by similar regulatory pathways. To date, all chemicals that uncouple mitosis from the completion of DNA replication also suppress DNA damage-induced mitotic delay. It has also been shown in mammalian cells that arrest in either the S or G2 phase leads to the accumulation of the inactive phosphorylated form of p34^cdk2, which is subsequently dephosphorylated and activated when these arrest points are bypassed by chemical treatment or by cell cycle mutants (20, 21, 34).

Staurosporine inhibits a wide spectrum of protein kinases. This nonselective inhibitory activity, coupled with the numerous kinases regulating mitotic onset, makes it difficult to predict the staurosporine substrates that normally act to couple mitosis to the completion of DNA.
replication and to the repair of damaged DNA. Recently, however, experiments using Xenopus oocyte extracts showed that caffeine inhibited the tyrosine phosphorylation of p34^{cdk2}, thereby leading to the accumulation of the unphosphorylated and activated form of the kinase (35). It is certainly possible that staurosporine acts through a similar mechanism. Since low doses of the protein kinase inhibitor staurosporine activate, rather than inhibit, the p34^{cdk2} kinase in p phase-arrested cells, the kinases maintaining p34^{cdk2} in an inactive form may be more sensitive to staurosporine than is p34^{cdk2}. Results presented in this report are consistent with this hypothesis. Whereas reported in vitro IC_{50} levels for staurosporine are in the range of 3–8 nM for cyclic nucleotide- and Ca^{2+}-dependent serine/threonine kinases and for certain tyrosine kinases (1), the in vitro IC_{50} for histone H1 kinase from BHK cells was found to be approximately 40 nM. It is interesting to note that while screening for antimitotic compounds, Rialet and Meijer (36) found staurosporine to inhibit H1 kinase from starfish oocytes with an in vitro IC_{50} of 3–4 nM. It is possible, therefore, that susceptibility of H1 kinase to chemical inhibition and activation may differ significantly among species.

The current findings, together with the work of other investigators, indicate that staurosporine has numerous effects on cell growth and is an important tool for investigating the regulation of cell cycle events. Depending upon the experimental model used, staurosporine has been shown to have both inhibitory (13–15) and stimulatory (present study) effects on cell cycle progression. This potent kinase inhibitor can block cells at the G_{1}-S and G_{2}-M transitions and can promote premature mitosis in cells arrested in S or delayed in G_{1}.

**Materials and Methods**

**Cell Culture.** BHK (Syrian hamster fibroblasts) cells were grown in Dulbecco’s modified Eagle’s medium containing 10% iron-supplemented calf serum (HyClone) and glutamine (4 mM) in a water-saturated 10% CO_{2}-90% air atmosphere. New cultures were started from frozen stocks every 4–6 weeks. Cells were Mycoplasma-free based upon the routine staining with Hoechst 33258. Arrest in S phase was achieved by addition of 2.5 mM hydroxyurea to the medium. Staurosporine (Calbiochem) stock solution was prepared in dimethyl sulfoxide, diluted to the appropriate concentrations with phosphate-buffered saline, and added to the culture medium. The final concentration of dimethyl sulfoxide was kept below 0.1%. Cycloheximide (10 μg/ml), actinomycin D (2 μg/ml), and nocodazole (0.25 μg/ml) were purchased from Sigma and were added to the medium to inhibit protein synthesis, RNA synthesis, and microtubule polymerization, respectively.

**Quantification of Premature and Normal Mitosis.** The percentage of cells undergoing premature mitosis was determined as described previously (19). Briefly, nocodazole (0.25 μg/ml) was used to accumulate cells in mitosis, cells were fixed on the dishes in absolute methanol, and DNA was stained with Hoechst 33258 (1 μg/ml for 10 min). Premature mitosis was confirmed in chromosomes spreads by the breakdown of the nuclear envelope and by the condensed and pulverized chromatin morphology indicative of cells in S phase that are forced to undergo premature chromosome condensation. For each time point, at least 300 cells were examined at X400 magnification by fluorescence microscopy to determine the percentage of cells having undergone premature chromosome condensation.

Mitotic delay in G_{2} was induced by gamma irradiation from a ^{60}Co source. The percentage of mitotic cells was determined as above by fixing cells on the culture dishes and staining with Hoechst 33258. In this case, at least 1000 cells were examined for each time point.

**Preparation of Chromosome Spreads.** Premature mitotic cells were collected from flasks by shaking, swelled for 8 min in 0.075 M KCl, fixed for 10 min in methanol:acetic acid (3:1), and dropped on wet slides before staining with Hoechst 33258 as described above. Asynchronously growing cells were collected by scraping and prepared for chromosome analysis as before.

**Histone H1 Kinase Activity.** The histone H1 kinase assay was a modification of the procedures used by Yamashita et al. (20). Interphase cells collected by scraping and mitotic cells collected by physical detachment were washed twice with ice-cold phosphate-buffered saline, lysed in 20 mM HEPES-NaOH (pH 7.5), 12 mM 2-glycerophosphate, 5 mM NaF, 1 mM ATP, 1 mM Na_{2}VO_{4}, 2 mM EGTA, 250 mM NaCl, 15 mM MgCl_{2}, 1% Triton X-100, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, and 0.5 mM (p-aminophenol) methanesulfonfluoride, passed through a 25-gauge needle 10 times, kept on ice for 30 min, and clarified by centrifugation. Protein from the supernatant (10 μg as determined with the Pierce Micro BCA protein assay reagent kit) was mixed for 1 h at 4°C with 50 μl of p13^{51-52}-Sepharose beads, which were prepared as previously described using the p13^{51-52}-producing bacterial strain BL21(DE3)lySs, obtained from Dr. David Beach (37). The beads were then washed twice in 500 μl lysis buffer and three times in 500 μl of 20 mM HEPES-NaOH (pH 7.5), 15 mM EGTA, and 20 mM MgCl_{2}. Histone H1 kinase activity was determined by incubation of beads in 40 μl of 20 mM HEPES-NaOH (pH 7.5), 15 mM EGTA, 20 mM MgCl_{2}, 1 mM dithiothreitol, 0.01 μg/ml A kinase inhibitory peptide (Sigma), 0.5 μg/μl histone H1 (Boehringer Mannheim), and 50 μM ATP (5500 cpm/mm) for 20 min at 30°C. The reaction was terminated by addition of 40 μl of 2X Laemmli sample buffer (38) and boiled for 10 min. Proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide mini-gel, fixed in 25% trichloroacetic acid, and stained with Coomassie brilliant blue R. Following autoradiography, the histone H1 band was excised from the gel and quantitated for radioactivity by Cerenkov counting.

**Acknowledgments**

We thank David Beach for the p13^{51-52}-producing bacterial strain BL21(DE3)lySs and Bill Meikrantz for helpful comments.

**References**


