The Kinase Inhibitor Staurosporine Induces G₁ Arrest at Two Points: Effect on Retinoblastoma Protein Phosphorylation and Cyclin-dependent Kinase 2 in Normal and Transformed Cells

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ABSTRACT

Staurosporine (ST), a protein kinase inhibitor, at a concentration of 20 nm arrests normal diploid fibroblasts 3 h into G₁ (H. A. Crissman et al., Proc. Natl. Acad. Sci. USA, 88: 7580–7584, 1991; K. Abe et al., Exp. Cell Res., 192: 122–127, 1991). ST (2 nm) induces a new G₁ arrest point at 6 h into G₁. Partial phosphorylation of the retinoblastoma protein was observed at the 2 nm ST arrest point, whereas the retinoblastoma protein was unphosphorylated or underphosphorylated at the 20 nm arrest point. This correlated with the activity of the cyclin-dependent kinase 2 (CDK2) and the phosphorylation of the Thr₁60 residue of p32CDK2. The cyclin E and cyclin D1/2 levels were reduced at the 20 nm ST arrest point. In HeLa cells that do not arrest in G₁ in response to 2 or 20 nm ST, the retinoblastoma protein and CDK2 phosphorylations and CDK2 activity were not affected by ST. These results suggest that ST inhibits one or more G₁-regulating protein kinases, which lie upstream of CDK2.

INTRODUCTION

In normal cells, growth factors bind to cell surface receptors and initiate signals that induce cell proliferation. Omitting particular growth factors leads to growth arrest at defined points in G₁ of the cell cycle which are called restriction points (1). Intracellular signal transduction and cell cycle progression involve protein phosphorylation cascades. In tumor cells, protein kinase genes are frequently mutated and have been identified as oncogenes (2). The importance of protein kinases in cell cycle regulation has also been shown by the use of protein kinase inhibitors. For example, low concentrations (2–20 nm) of the protein kinase inhibitor ST cause cell cycle arrest in G₁ of normal diploid fibroblasts (3, 4). These cells arrest in both G₁ and G₂ at the higher concentration of 100–150 nm (3–5). Consequently, cell cycle arrest points in G₁ were mapped for more specific kinase inhibitors that are structurally related to ST (6, 7). These arrest points were found at different times in G₁, suggesting that several protein kinases regulate the progression of normal cells through G₁. Interestingly, in a number of transformed cell lines the G₁ arrest was not induced by low concentrations of ST, although the G₂ arrest by the higher concentration of ST was always observed in both normal and transformed cells (3, 6). The lack of response of many transformed cells to the protein kinase inhibitors is similar to the lack of response to growth factors and indicates that ST and the other related inhibitors may affect similar regulatory pathways.

The activities of several proteins that are involved in checkpoint controls of the cell cycle are regulated by phosphorylation and dephosphorylation. For example, active Rb protein is unphosphorylated and in this form causes the arrest of cells in G₁ (8). Rb becomes inactivated by phosphorylation in mid- to late G₁ to allow progression through G₂ and remains hyperphosphorylated during S and G₂–M (9–13). Rb is dephosphorylated when cells enter G₀–G₁ (9–11). One of the enzymes, which phosphorylates the Rb protein, is CDK2 (14). The catalytic subunit p33CDK2 is phosphorylated at least at two residues, Tyr₁5 and Thr₁60, by different enzymes (15). These phosphorylations regulate the activity of CDK2.

To determine further the action of ST in cell cycle regulation, we have mapped a new ST arrest point in normal diploid fibroblasts in G₁ and analyzed the effect of ST on the Rb phosphorylation and CDK2 activity at the ST arrest points in both normal and transformed cells. We find differences in the Rb protein phosphorylation that correlate with the CDK2 activity. ST does not arrest HeLa cells in G₁ and does not affect Rb protein phosphorylation or CDK2 activity.

MATERIALS AND METHODS

Cell Culture. Human diploid fibroblasts strain 55 (HSF55; kindly provided by Dr. D. Chen, Los Alamos National Laboratory) was derived from neonatal foreskin samples. Cells from passage 6 to 10 were used in these studies. For the analysis of the ST arrest points, HSF55 cells were cultured at 37°C and 5% CO₂ in a minimum essential medium (Gibco BRL) containing 10% bovine calf serum (Hyclone). For all other experiments, HSF55 and HeLa cells were cultured in the same conditions but in H-(2-hydroxyethyl)-1-piperazinepropane sulfonic acid-buffered RPMI 1640 supplemented with 10% fetal calf serum (JRH Biosciences) or 10% fetal calf serum (Sigma). All cells were determined to be free of Mycoplasma contamination.

Kinase Inhibitor Studies. Stock solutions of ST (Kamiya Biomedical Company, Thousand Oaks, CA) were prepared in dimethyl sulfoxide and stored at −20°C. For the ST arrest point studies, cells were plated in 75-mm flasks (24 h before the addition of ST) at a density that allowed cells to grow logarithmically throughout the experiment. The cells were collected 18 h after ST addition for FC analysis. For release experiments, cells were first treated with ST for 18 h to allow cells to accumulate in G₁. ST-containing medium was removed and monolayers were washed twice with phosphate-buffered saline (130 mM NaCl-10 mM Na₂HPO₄/NaH₂PO₄, pH 7.2) before adding ST-free medium. Samples were collected at intervals after release by trypsinization and analyzed using FC. For all other experiments, exponentially growing cells were prepared in 100-mm Petri dishes and treated for 22 h with different concentrations of ST. Cells were collected by scraping, washed once with phosphate-buffered saline, and used for FC and/or immunoblot analysis.

Flow Cytometric Analysis. Cells were fixed with 70% ethanol, cellular DNA was stained with mithramycin, and fluorescence was measured using a flow cytometer at 457 nm (17). For arrest point determination in HSF55, the percentage of cells in each phase of the cell cycle was calculated by computer-fit analysis (18).

Immunoblot Analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 μmol okadaic acid, and 1 mM sodium o-vanadate. The lysate was cleared by centrifugation and the supernatant saved for gel analysis. Rb protein was separated by 7.5% and p33CDK2 by 10% acrylamide/ bisacrylamide sodium dodecyl sulfate-gel electrophoresis according to the method of Laemmli (19). Proteins were blotted on Immobilon membrane (Millipore) in Tris-glycine buffer. The membrane was incubated with blocking B (4% w/v) nonfat dry milk, 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, and 0.1% Tween 20. The anti-Rb antibody was purchased from Triton and diluted to 0.4 μg/ml.
polyclonal antibodies against p33Cdc2 and cyclin E were purchased from Santa Cruz Biotechnology and the cyclin D antibody was purchased from UBI. All antibodies were diluted to 1 µg/ml. Detection of the immunoreaction was done by the enhanced chemiluminescence system (Amersham) with horseradish peroxidase coupled to a secondary antibody.

**Immunoprecipitation and Kinase Assay.** For immunoprecipitation and determination of CDK2 activity, cells from two 100-mm Petri dishes for each ST concentration were lysed in 0.5 ml 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 µmol okadaic acid, and 1 mM sodium o-vanadate buffer. The lysates were centrifuged in a microfuge for 5 min and the protein content in the supernatant was determined. Forty µg protein A-Sepharose 4B (Pharmacia) were incubated with 2 µg anti-p33Cdc2 antibody. The supernatant which contained sodium azide from the antibody solution was removed before adding equal amounts of total cell extract for each cell line. The tubes were gently rocked for 1 h at 4°C. Then the cell extracts were removed and the Sepharose was washed twice with Buffer A containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.1 mM Tween 20 and twice with Buffer A lacking Tween 20. The kinase assay was performed in 50 µl containing H1 kinase buffer (50 mM Tris-HCl (pH 7.4)), 10 mM MgCl2, 10 mM β-glycerophosphate, 10 mM β-mercaptoethanol, 1 mM EDTA, 10 µCi [α-32P]ATP (3000 Ci/mmol), 100 µM ATP, and 1.3 µg calf thymus H1] at 30°C for 15 min. The reaction was stopped by placing the tubes on ice and adding 20 µl 4-fold sodium dodecyl sulfate-loading buffer (19). Ten µlwere applied on a 12% PAGE gel. The gel was stained with Coomassie blue and dried for autoradiography. The H1 bands were cut out and counted in a scintillation counter. The counts were normalized to the total amount of radioactivity.

**RESULTS**

**G1 Arrest Times Induced by 2 and 20 nM ST.** It has been shown previously that 20 nM ST treatment of normal fibroblasts induces a G1 arrest from G0-G1, plus about 3 h through to the G1-S boundary (3, 4). However, concentrations of ST as low as 1 or 2 nM also induce cell cycle arrest in G1. Since ST is a potent inhibitor of several purified protein kinases, the question arises whether the arrest point induced by 20 nM ST is identical to the arrest point induced by 2 nM. The 2 nM ST arrest point was determined by releasing normal diploid fibroblast HSF55 cells from an 18-h treatment with 2 nM ST and measuring the time from release to the onset of DNA synthesis. Cells were harvested at different times and analyzed for DNA content using FCM. From the flow histograms (Fig. la), the percentages of cells in G0-G1 and S phases were determined. From these plots the time taken for the cells to progress from the 2 nM ST block point to the onset of DNA replication was determined to be between 6 and 9 h from the G0-G1 boundary. This is about 3 h later than induced by 20 nM ST (Fig. 1b; Refs. 3 and 6). Thus depending on its concentration, ST arrests normal cells at two points in G1.

**Rb Protein Phosphorylation at the ST Arrest Points.** The Rb protein is an important checkpoint protein in G1 of the cell cycle (8). This protein acts as a tumor suppressor through the response of cells to growth factors, a response that is lost in tumor cells lacking Rb (20). The function of the Rb protein is regulated by cell cycle-dependent phosphorylations (9-13, 21). Thus the phosphorylation of the Rb protein may serve as a marker for localizing the two ST arrest points in G1 phase.

Fig. 2a shows the cell cycle response of normal cells HSF55 and HeLa cells to 2 and 20 nM ST. Whereas HSF55 cells arrest in G1 at both concentrations of ST, the HeLa cell cycle progression is unaffected. HeLa cells, however, do show the typical G1 arrest at the 100 nM concentration of ST (3, 4). The states of phosphorylation of the Rb protein were analyzed by PAGE followed by immunoblotting. The multiple phosphorylated forms of Rb protein migrate more slowly than the underphosphorylated or unphosphorylated forms (9-11). In the control exponentially growing HSF55 cells, Rb protein is predominantly in the fully phosphorylated forms (Fig. 2b). In contrast, the Rb protein from the 20 nM ST G1-arrested HSF55 cells is predominantly in the unphosphorylated or underphosphorylated state. When HSF55 cells were treated with 2 nM ST to arrest cells about 6 h into G1, the Rb protein was distributed between unphosphorylated or underphosphorylated and phosphorylated states. No effect of either 2 or 20 nM ST was observed on the states of phosphorylation of Rb protein from HeLa cells. There is no problem with the ability of ST to enter HeLa cells, because 100 nM ST causes cell cycle arrest in G2 without affecting G1 progression. It would appear from these results that in HeLa cells the kinase acting directly on the Rb protein is not inhibited by ST; otherwise there would be an effect on its states of phosphorylations unless the HeLa Rb protein kinase is mutated and no longer sensitive to ST. Because the kinase activity of CDK2 is coordinated with Rb protein phosphorylation, CDK2 is a probable candidate for Rb protein kinase. The activity of CDK2 is determined by its states of phosphorylations and complex formation with cyclins D and E (15, 22, 23).

**Effects of ST on Cyclins D1/2, E, and CDK2.** One explanation for the inhibition of Rb protein phosphorylation in HSF55 cells by ST may be through reduced expressions of the cyclin D and E genes that limit active kinase complex formation with cyclins D and E. Levels of cyclins D1/2 and E in HSF55 cells at 0, 2, and 20 nM ST were analyzed by immunoblotting (Fig. 3). No effect was observed with 2 nM ST, arguing against limiting amounts of cyclin E as a factor in controlling CDK2 kinase activity. There appears also to be little effect of 2 nM ST on the levels of cyclins D1/2. However, the treatment of HSF55 cells with 20 nM ST causes a reduction of cyclin E, in particular of the more slowly migrating band and also of cyclins D1/2, that correlates with the timing of this arrest point, since the levels of cyclins D1/2 are lower earlier in G1 (24). However, the reduction or absence of Rb protein kinase activity cannot be explained by the cyclin levels alone.
cells were incubated in the absence (0) or presence of 2 and 20 nM ST for 20 h, harvested, and analyzed for their DNA content using FCM. b. Effect of ST on Rb phosphorylation.

We, therefore, investigated the effects of ST on the states of phosphorylation of p33CDK2. Thr160 phosphorylation is required for CDK2 activity (15). Human p33CDK2 migrates more slowly in PAGE than its phosphorylated form. Fig. 4a shows the effect of 0, 2, and 20 nM ST on the states of phosphorylation of p33CDK2 in HSF55 and HeLa cells. For the control HSF55 cells, p33CDK2 is predominantly in its active phosphorylated form. For HSF55 cells treated with 20 nM ST, p33CDK2 is predominantly in its inactive unphosphorylated state. For the 2 nM ST-treated HSF55 cells, most of the p33CDK2 is found in its unphosphorylated inactive state. The HeLa cell p33CDK2 protein is largely in its active phosphorylated form with a minor fraction in the inactive unphosphorylated form and this ratio is unaffected by exposure of cells to 2 or 20 nM ST.

The activities of CDK2 from HSF55 and HeLa cells exposed to 0, 2, and 20 nM ST were determined following immunoprecipitation from cell lysates and using histone H1 as the substrate (Fig. 4b). The amount of total HeLa cell protein that was used for immunoprecipitation was about 1.5-fold more than the HSF55 total protein. For the control HSF55 cells the high activity of the phosphorylated form of p33CDK2 is demonstrated by the high level of H1 phosphorylation. The level of H1 phosphorylation is much reduced for the p33CDK2 from the 2 nM ST-treated cells and totally absent for the p33CDK2 from the 20 nM ST-treated cells. The levels of H1 phosphorylation parallel the states of phosphorylation of p33CDK2 shown in Fig. 4a. For HeLa cells there is little or no effect of 2 or 20 nM ST on the activity of p33CDK2 as shown by the hyperphosphorylated states of histone H1. These results accord well with the states of Rb protein phosphorylation in both HSF55 and HeLa cells (Fig. 2b).

DISCUSSION

We have found that 2 nM ST arrests normal cells about 6 h into G1, i.e., about 3 h later than the reported arrest point for 20 nM ST. We showed that there is no CDK2 activity and Rb protein is unphosphorylated or underphosphorylated at the 20 nM arrest point but there is residual CDK2 activity and Rb protein phosphorylation at the 2 nM ST arrest point. Cyclin E is present at the 2 nM arrest point but is greatly reduced at the 20 nM arrest point. Cyclins D1/2 and E. A similar ST-concentration-dependent study was done with mouse keratinocytes (25). While low (up to 2 or 4 nM ST) concentrations had little effect on the differentiation of normal keratinocytes, higher (10–20 nM ST) concentrations were effective and for tumor cell differentiation even higher ST amounts were required.

One explanation for concentration-dependent G1 arrest points is that 20 nM ST completely inhibits one protein kinase. The complete inhibition of this kinase by 20 nM ST causes cell cycle arrest about 3 h into G1, but the partial inhibition caused by 2 nM ST allows cells to progress to the ST concentration-dependent arrest point about 6 h into G1. A second more likely explanation is that two different kinases are inhibited by ST, of which one is much more sensitive to ST and is inhibited by 2 nM ST, whereas the other is inhibited by 20 nM ST. As a result of the inhibition of both kinases, the CDK2 activity would be impaired.

Recently G1 arrest points have been mapped using ST derivatives (6). Although it appears that the G1 arrest points are scattered, they are all located at two major time points within certain limits. One arrest point is located early at around 3 h in G1 for KT5926, K252b, and 20 nM ST, and the other arrest point is located at a point similar to 2 nM ST for KT5823 and KT5720. It seems that the ST derivatives are more specific with respect to one or the other arrest point than ST. For none of these inhibitors are the actual target proteins known in intact cells. At the transforming growth factor β arrest point it was shown that the cyclin E level was unchanged but CDK2 was inactive and p33CDK2 was unphosphorylated at Thr160 (26). This arrest point shows, therefore, similarities to the ST arrest points and it would be interesting to know whether overlapping pathways are affected by transforming growth factor β and ST.

We found that at the ST arrest points the CDK2 activity and Rb
phosphorylation was decreased or absent. This correlated with a change in the ratio of the Thr160 phosphorylated to unphosphorylated p33CDK2. At this point we are unable to directly link the effect of ST on CDK2 activity. It could well be that the effect of ST on the CDK2 phosphorylation is not a direct inhibition of the Thr160 phosphorylation kinase, CAK, but the inhibition of a protein kinase that lies upstream in the CDK2 activation pathway. Therefore, the arrests by ST offer a new model system to study the regulation of CDK2 activity and the role of oncogenes and tumor suppressor genes, in particular the Rb protein.

ST does not arrest HeLa cells in G₁ and does not reduce the amount of phosphorylated Rb nor the level of CDK2 activity. On the other hand as found for normal cells, HeLa cells are as tightly arrested in G₂ by 100 nM ST or higher. Therefore it appears that the protein kinase(s) that is inhibited in the G₁ phase of normal fibroblasts is not inhibited or is bypassed in HeLa cells by transformation.

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REFERENCES


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