[CANCER RESEARCH 54, 5959–5963, November 15, 1994]

The Kinase Inhibitor Staurosporine Induces G_1 Arrest at Two Points: Effect on Retinoblastoma Protein Phosphorylation and Cyclin-dependent Kinase 2 in Normal and Transformed Cells[1]

Joachim B. Schnier,[2] Donna M. Gadbois, Kayoko Nishi, and E. Morton Bradbury

Department of Biological Chemistry, Medical School, University of California Davis, Davis, California 95616 [J. B. S., K. N., E. M. B.], and Life Science Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545 [D. M. G., E. M. B.]

ABSTRACT

Staurosporine (ST), a protein kinase inhibitor, at a concentration of 20 nM arrests normal diploid fibroblasts 3 h into G_1 (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 G_1 arrest point at 6 h into G_1. 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 $p33^{CDK2}$. 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 G_1 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 G_1-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 G_1 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[3] cause cell cycle arrest in G_1 of normal diploid fibroblasts (3, 4). These cells arrest in both G_1 and G_2 at the higher concentration of 100–150 nM (3–5). Consequently, cell cycle arrest points in G_1 were mapped for more specific kinase inhibitors that are structurally related to ST (6, 7). These arrest points were found at different times in G_1, suggesting that several protein kinases regulate the progression of normal cells through G_1. Interestingly, in a number of transformed cell lines the G_1 arrest was not induced by low concentrations of ST, although the G_2 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 G_1 (8). Rb becomes inactivated by phosphorylation in mid- to late G_1 to allow progression through G_1 and remains hyperphosphorylated during S and G_2-M (9–13). Rb is dephosphorylated when cells enter G_0–G_1 (9–11). One of the enzymes, which phosphorylates the Rb protein, is CDK2 (14). The catalytic subunit $p33^{CDK2}$ 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 G_1 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 G_1 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% CO_2 in α-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-piperazinepropanesulfonic acid-buffered RPMI 1640 supplied with 10% calf serum (JRH Biosciences) or 10% fetal calf serum (Sigma). All cells were determined to be free of *Mycoplasma* contamination (16).

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 FCM. For release experiments, cells were first treated with ST for 18 h to allow cells to accumulate in G_1. ST-containing medium was removed and monolayers were washed twice with phosphate-buffered saline (130 mM NaCl-10 mM Na_2HPO_4/NaH_2PO_4, pH 7.2) before adding ST-free medium. Samples were collected at intervals after release by trypsinization and analyzed using FCM. 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 FCM 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 $p33^{CDK2}$ 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. The

Received 6/14/94; accepted 9/19/94.

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] This work was performed under the auspices of the Office of Health and Environmental Research of the Department of Energy (Contract W-7405-ENG-36) and also supported by National Institutes of Health GM-45890.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: ST, staurosporine; Rb, retinoblastoma; CDK2, cyclin-dependent kinase; FCM, flow cytometric analysis.

polyclonal antibodies against $p33^{CDK2}$ 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 supernatants was determined. Forty μl protein A-Sepharose 4B (Pharmacia) were incubated with 2 μg anti-$p33^{CDK2}$ 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 in ice. 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 $MgCl_2$, 10 mM β-glycerophosphate, 10 mM β-mercaptoethanol, 1 mM EDTA, 10 μCi [α-^{32}P]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 μl were 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.

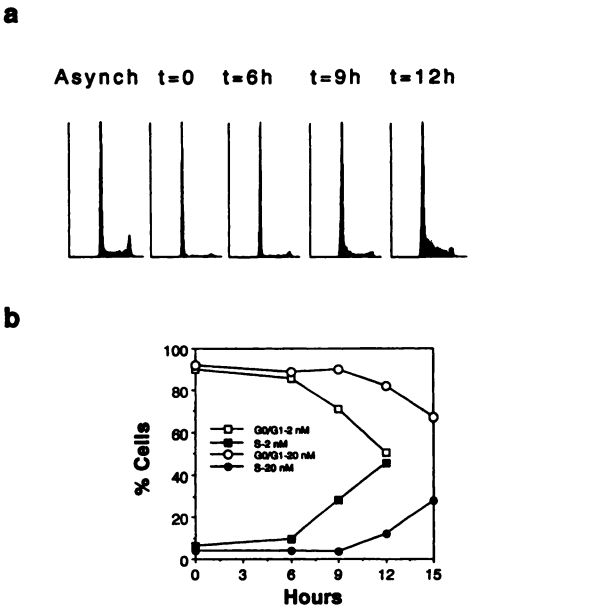


Fig. 1. Determination of the time of the G_1 arrest point in HSF55 cells after treatment with 2 nM ST. HSF55 cells were treated with ST (2 or 20 nM) for 18 h. The time of entry into S phase after release from inhibitor was monitored using FCM of mithramycin-stained cells. In *a*, flow histograms of an untreated sample (*Asynch*) and of samples taken at 0, 6, 9, and 12 h after release from inhibitor are shown for the 2 nM treatment. *Y axis*, number of cells; *X axis*, DNA content. In *b*, the percentage of cells in S phase (●) or in G_1 (○) after release from 20 nM ST and in S (■) or in G_1 (□) after release from 2 nM ST was calculated and is plotted against the time after release from inhibitor treatment.

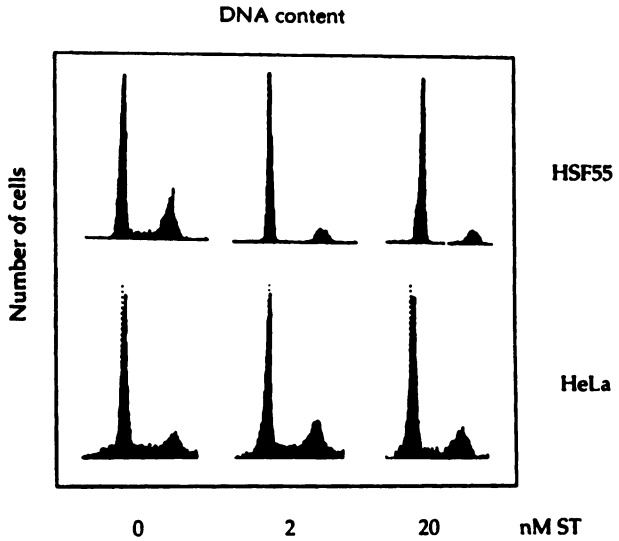
RESULTS

G_1 Arrest Times Induced by 2 and 20 nM ST. It has been shown previously that 20 nM ST treatment of normal fibroblasts induces a G_1 arrest from G_0–G_1 plus about 3 h through to the G_1–S boundary (3, 4). However, concentrations of ST as low as 1 or 2 nM also induce cell cycle arrest in G_1. 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. 1*a*), the percentages of cells in G_0–G_1 and S were calculated and plotted against the time since release from the indicated block (Fig. 1*b*). 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 G_0–G_1 boundary. This is about 3 h later than induced by 20 nM ST (Fig. 1*b;* Refs. 3 and 6). Thus depending on its concentration, ST arrests normal cells at two points in G_1.

Rb Protein Phosphorylation at the ST Arrest Points. The Rb protein is an important checkpoint protein in G_1 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 G_1 phase.

Fig. 2*a* shows the cell cycle response of normal cells HSF55 and HeLa cells to 2 and 20 nM ST. Whereas HSF55 cells arrest in G_1 at both concentrations of ST, the HeLa cell cycle progression is unaffected. HeLa cells, however, do show the typical G_2 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. 2*b*). In contrast, the Rb protein from the 20 nM ST G_1-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 G_1, 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 G_2 without affecting G_1 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 G_1 (24). However, the reduction or absence of Rb protein kinase activity cannot be explained by the cyclin levels alone.



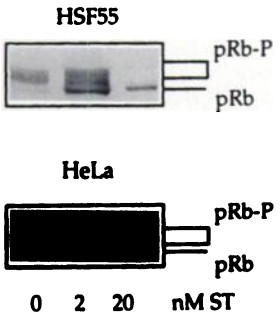


Fig. 2. *a*, flow cytometry analysis of HSF55 and HeLa cells. Exponentially growing cells were incubated in the absence (0) or presence of 2 and 20 nM ST for 20 h, harvested, and analyzed for their DNA content using FCM. *b*, effect of ST on Rb phosphorylation. Cells were grown as described in *a*. Immunoblot was performed with anti-Rb protein antibodies. *pRb*, unphosphorylated Rb protein; *pRb-P*, phosphorylated Rb protein.

We, therefore, investigated the effects of ST on the states of phosphorylation of $p33^{CDK2}$. Thr160 phosphorylation is required for CDK2 activity (15). Human $p33^{CDK2}$ migrates more slowly in PAGE than its phosphorylated form. Fig. 4*a* shows the effect of 0, 2, and 20 nM ST on the states of phosphorylation of $p33^{CDK2}$ in HSF55 and HeLa cells. For the control HSF55 cells, $p33^{CDK2}$ is predominantly in its active phosphorylated form. For HSF55 cells treated with 20 nM, ST $p33^{CDK2}$ is predominantly in its inactive unphosphorylated state. For the 2 nM ST-treated HSF55 cells, most of the $p33^{CDK2}$ is found in its unphosphorylated inactive state. The HeLa cell $p33^{CDK2}$ 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. 4*b*). 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 $p33^{CDK2}$ is demonstrated by the high level of H1 phosphorylation. The level of H1 phosphorylation is much reduced for the $p33^{CDK2}$ from the 2 nM ST-treated cells and totally absent for the $p33^{CDK2}$ from the 20 nM ST-treated cells. The levels of H1 phosphorylation parallel the states of phosphorylation of $p33^{CDK2}$ shown in Fig. 4*a*. For HeLa cells there is little or no effect of 2 or 20 nM ST on the activity of $p33^{CDK2}$ 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. 2*b*).

DISCUSSION

We have found that 2 nM ST arrests normal cells about 6 h into G_1, *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 are also reduced at the 20 nM arrest point as compared with the 2 nM arrest point. Therefore in normal cells, the G_1 arrest points are defined by the activity of CDK2, the amount of phosphorylated Rb protein, and the level of 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 G_1 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 G_1, but the partial inhibition caused by 2 nM ST allows cells to progress to the ST concentration-dependent arrest point about 6 h into G_1. 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 G_1 arrest points have been mapped using ST derivatives (6). Although it appears that the G_1 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 G_1 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 $p33^{CDK2}$ 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

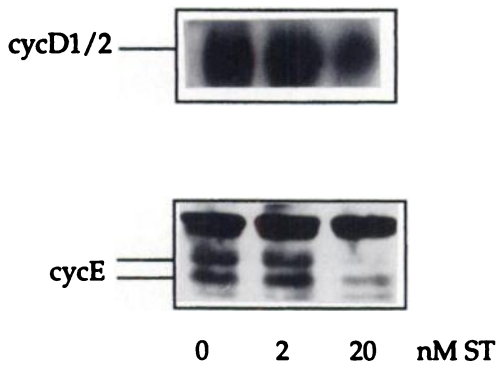


Fig. 3. Effect of ST on cyclins D1/2 and cyclin E level. Cells were treated as in Fig. 2. Immunoblots with anti-cyclin D and anti-cyclin E antibodies were performed.

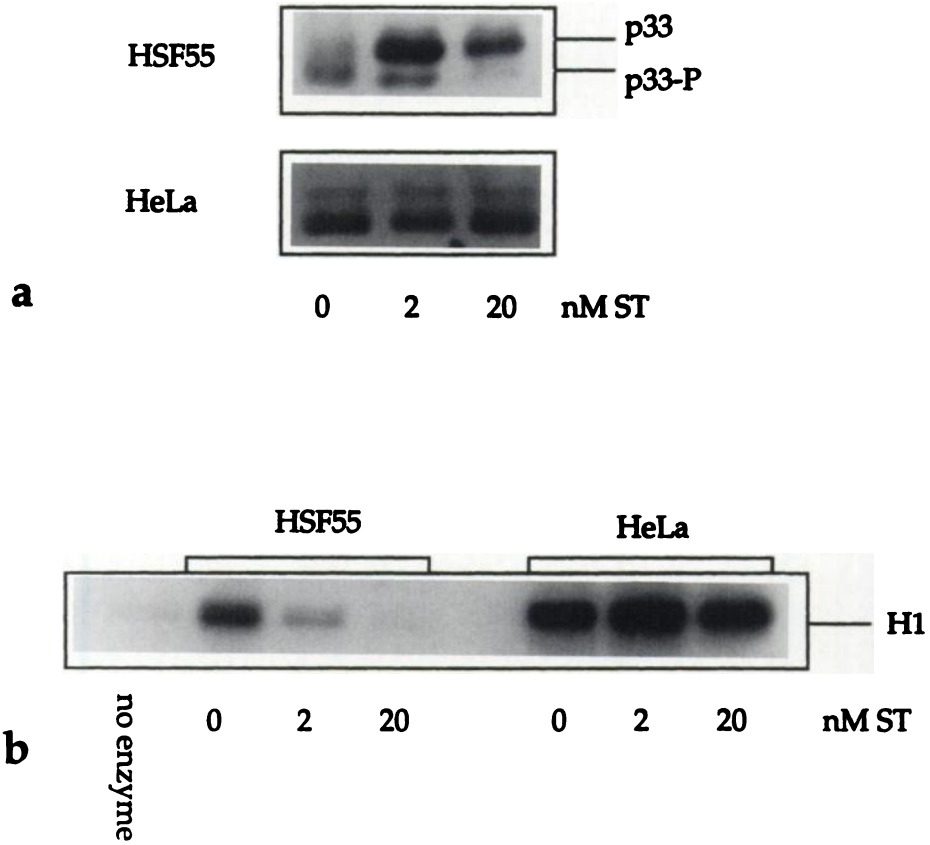


Fig. 4. Effect of ST on CDK2. Cells were grown in the absence and presence of ST as described in Fig. 2. *a*, immunoblot with anti-$p33^{CDK2}$ antibodies. In *b*, $p33^{CDK2}$ from HSF55 and HeLa cells was immunoprecipitated and the kinase activity was determined with H1 as a substrate. The amount of total HeLa cell protein that was used for immunoprecipitation was about 1.5-fold more than the HSF55 total protein. *p33*, unphosphorylated $p33^{CDK2}$; *p33-P*, $p33^{CDK2}$ phosphorylated at Thr160.

phosphorylation was decreased or absent. This correlated with a change in the ratio of the Thr160 phosphorylated to unphosphorylated $p33^{CDK2}$. 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 G_1 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 G_2 by 100 nM ST or higher. Therefore it appears that the protein kinase(s) that is inhibited in the G_1 phase of normal fibroblasts is not inhibited or is bypassed in HeLa cells by transformation.

ACKNOWLEDGMENTS

We thank Carolyne Bell-Prince at Los Alamos National Laboratories for carrying out FCM analysis. We further thank Dr. Xiowen Guo for supplying H1 protein.

REFERENCES

1. Pardee, A. B. G_1 events and regulation of cell proliferation. Science (Washington DC), *246:* 603–608, 1989.
2. Bishop, J. M. Molecular themes in oncogenesis. Cell, *64:* 235–248, 1991.
3. Crissman, H. A., Gadbois, D. M., Tobey, R. A., and Bradbury, E. M. Transformed mammalian cells are deficient in kinase-mediated control of progression through the G_1 phase of the cell cycle. Proc. Natl. Acad. Sci. USA, *88:* 7580–7584, 1991.
4. Abe, K., Yoshida, M., Ususi, T., Horinouchi, S., and Beppu, T. Highly synchronous culture of fibroblasts from G_2 block caused by staurosporine. Exp. Cell Res., *192:* 122–127, 1991.
5. Bruno, S., Ardelt, B., Skierski, J. S., Tragonos, F., and Darzynkiewicz, Z. Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res., *52:* 470–473, 1992.
6. Gadbois, D. M., Crissman, H. A., Tobey, R. A., and Bradbury, E. M. Multiple kinase arrest points in the G_1 phase of nontransformed mammalian cells are absent in transformed cells. Proc. Natl. Acad. Sci. USA, *89:* 8626–8630, 1992.
7. Akinaga, Sh., Nomura, K., Gomi, K., and Okabe, M. Effect of UCN-01, a selective inhibitor of protein kinase C, on the cell-cycle distribution of human epidermoid carcinoma, A431 cells. Cancer Chemother. Pharmacol., *33:* 273–280, 1994.
8. Goodrich, D. W., Wang, N. P., Qian, Y-W., Lee, E. Y.-H. P., Lee, W-H. The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. Cell, *67:* 293–302, 1991.
9. DeCaprio, J., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C-M., and Livingston, D. M. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell, *58:* 1085–1095, 1989.
10. Buchkovich, K., Duffy, L. A., and Harlow, E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell, *58:* 1097–1105, 1989.
11. Chen, P-L., Scully, P., Shew, J-Y., Wang, J. Y. J., and Lee, W-H. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell, *58:* 1193–1198, 1989.
12. Zhang, W., Hittelman, W., Van, N., Andreeff, M., and Deisseroth, A. The phosphorylation of retinoblastoma gene product in human myeloid leukemia cells during the cell cycle. Biochem. Biophys. Res. Commun., *184:* 212–216, 1992.
13. DeCaprio, J., Furukawa, Y., Aichenbaum, F., Griffin, J. D., and Livingston, D. M. The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. Proc. Natl. Acad. Sci. USA, *89:* 1795–1798, 1992.
14. Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K., and Toyoshima, K. Phosphorylation of the retinoblastoma protein by cdk2. Proc. Natl. Acad. Sci. USA, *89:* 7900–7904, 1992.
15. Gu, Y., Rosenblatt, J., and Morgan, D. O. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J., *11:* 3995–4005, 1992.
16. House, W., and Waddell, A. Detection of *Mycoplasma* in cell cultures. J. Pathol. Bacteriol., *93:* 125–132, 1967.
17. Tobey, R. A., and Crissman, H. A. Cell-cycle analysis in 20 minutes. Science (Washington DC), *184:* 1297–1298, 1974.
18. Dean, P. N., and Jett, J. H. Mathematical analysis of DNA distributions derived from flow microfluorometry. J. Cell Biol., *60:* 523–527, 1974.
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 580–685, 1970.

20. Huang, H-J. S., Yee, J-K., Shew, J-Y., Chen, P. L., Bookstein, R., Friedmann, T., Lee, E. Y-H. P., and Lee, W-H. Suppression of the neoplastic phenotype by replacement of the retinoblastoma gene product in human cancer cells. Science (Washington DC), *242:* 1563–1566, 1988.
21. Ludlow, J. W., DeCaprio, J. A., Huang, C-M., Lee, W-H., Paucha, E., and Livingston, D. M. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell, *56:* 57–65, 1989.
22. Dulic, V., Lees, E., and Reed, S. R. Association of human cyclin E with a periodic G_1-S phase protein kinase. Science (Washington DC), *257:* 1958–1961, 1992.
23. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science (Washington DC), *257:* 1689–1694, 1992.
24. Won, K.-A., Xiong, Y., Beach, D., and Gilman, M. Z. Growth-regulated expression of D-type cyclin genes in human diploid fibroblasts. Proc. Natl. Acad. Sci. USA, *89:* 9910–9914, 1992.
25. Dlugosz, A. A., and Yuspa, S. H. Staurosporine induces protein kinase C agonist effects and maturation of normal and neoplastic mouse keratinocytes *in vitro*. Cancer Res., *51:* 4677–4684, 1991.
26. Koff, A., Ohtsuki, M., Polyak, K., Roberts, J. M., and Massague, J. Negative regulation of G_1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGFβ. Science (Washington DC), *260:* 536–539, 1993.

The Kinase Inhibitor Staurosporine Induces G_1 Arrest at Two Points: Effect on Retinoblastoma Protein Phosphorylation and Cyclin-dependent Kinase 2 in Normal and Transformed Cells

Joachim B. Schnier, Donna M. Gadbois, Kayoko Nishi, et al.

Cancer Res 1994;54:5959-5963.