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Advances in Brief

Staurosporine Blocks Cell Progression through G_1 between the Cyclin D and Cyclin E Restriction Points[1]

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Abstract

The protein kinase inhibitor staurosporine (SSP) stops progression of normal nontransformed cells in the G_1 phase of the cell cycle. This implies that at least one of the cell cycle associated kinases, essential for cell transit through G_1, is sensitive to SSP. Using multivariate flow cytometry to correlate the expression of cyclin E or cyclin D with cellular DNA content (*i.e.*, cell cycle position), we have presently characterized the point of action of SSP in relation to the expression of these cyclins. During stimulation of normal human lymphocytes by phytohemagglutinin, cyclin D was expressed early, peaking at 8–14 h, while cyclin E appeared later, reaching a maximum at the time of cell entrance to S phase (24 h). Addition of SSP at the time of cell stimulation, while markedly suppressing the expression of cyclin E, had a rather modest effect on the expression of cyclin D. The data indicate that the SSP sensitive kinase(s) involved in cell progression through G_1 operate beyond the restriction point of cyclin D but prior to that of cyclin E. Thus, the target(s) of SSP is (are) either the $p33^{cdk}$/cyclin E complex itself or other protein kinase(s), activated subsequent to the cyclin D but prior to the cyclin E restriction point, the activity of which is essential for cell transit through G_1.

Introduction

It has been reported that the nonspecific protein kinase inhibitor SSP[3] halts progression of normal, nontransformed cells in the G_1 phase of the cell cycle (1, 2). This finding implies that one of the cell cycle associated kinases, which is essential for the transit of cells through G_1, is sensitive to SSP. In contrast to normal cells, the progression through G_1 of cells from many transformed tumor cell lines was not perturbed by SSP (1–4). The point of cell arrest in G_1 induced by SSP was characterized by Gadbois *et al.* (5), who measured the time interval between removal of SSP (following cell arrest in G_1 by this inhibitor) and cell entrance to S phase in human diploid fibroblsts. These authors have observed that the SSP sensitive point, in these cells, is about 10 h prior to entrance to S phase, and it is also prior to the points sensitive to other protein kineses inhibitors, such as KT 5823, KT 5720, KT 5926, and K252b.

Characterization of the point of action of any drug or inhibitor by its temporal mapping with respect to the onset of S phase suffers shortcomings. First of all, temporal mapping is only descriptive and has no relationship to the mechanism of drug action. Furthermore, since different cell types have inherently different durations of G_1, the temporal markers are relative and therefore cannot be used to compare the cell types with different lengths of G_1 duration. In addition, cell entrance to S phase is asynchronous (stochastic) which makes the estimate of a defined time interval difficult. Finally, cell arrest, especially late in G_1 or in S, generally results in unbalanced growth, the degree of which varies depending on the mechanism of action of the arresting agent, cell type, and the duration of the arrest (6, 7). Upon release from the arrest, the rate of cell progression through the cell cycle is affected by the degree of growth imbalance (7). Temporal mapping, therefore, has drawbacks when used as a yardstick to characterize the point of action of a particular drug, especially in G_1.

For the purpose of marking the point of action of a particular drug or blocking agent we have recently proposed additional subdivision of the cell cycle by using the restriction points of G_1 and G_2 cyclins (8–10). Cyclin proteins are components of the cell cycle clockwork essential for transition from one cell cycle phase to another (for reviews, see Refs. 11–15). The onset of synthesis of each particular cyclin, therefore, may be considered as an additional landmark in cell cycle progression. Thus, in addition to the initiation and end of DNA replication, as well as mitosis, which subdivide the cell cycle into four major phases, one can include the restriction points of individual cyclins as markers along the cell cycle progression pathway. Expression of particular cyclins in relation to cell cycle position can be conveniently estimated by multivariate flow cytometry, as described previously (8–10).

In the present study, we have used this approach to characterize the G_1 point of arrest by SSP. The point of arrest by SSP was related to the onset of synthesis of cyclin E and D, members of the G_1 cyclin family.

Materials and Methods

Cells. Human peripheral blood lymphocytes, obtained from healthy volunteers by venipuncture, were isolated by density gradient centrifugation as described (2, 8, 16). The cells were washed twice with buffered saline, resuspended in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics at a density of 10^6 cells/ml. All media, supplements, and sera were obtained from GIBCO (Grand Island, NY). The lymphocytes were mitogenically stimulated with PHA (Sigma Chemical Co. St. Louis, MO) at a final concentration of 10 μg/ml. SSP (Kamiya Biomedical Co., Thousands Oaks, CA), dissolved in dimethyl sulfoxide at a concentration of 0.1 mg/ml was stored at −20°C, and used in the cultures at a final concentration of 10 ng/ml. Lymphocytes stimulation was measured by multiparameter flow cytometry as described before (17), using the metachromatic dye acridine orange, which differentially stains DNA and RNA. Cell viability was estimated using the trypan blue exclusion test; there were fewer than 1% trypan blue positive cells at the onset of cultures and their number did not exceed 10%, up to 48 h following stimulation with PHA, in either the presence or the absence of SSP.

Immunocytochemistry; Flow Cytometry. Aliquots of cells fixed in 80% ethanol were centrifuged, washed with PBS, and treated with 0.25% Triton X-100 in PBS for 5 min on ice. After addition of 5 ml of PBS and centrifugation, the cells were incubated overnight at 4°C in the presence of the mouse monoclonal antibody to human cyclin E (PharMingen, San Diego, CA), which was diluted 1:100 in PBS containing 1% bovine serum albumin. Cells were then washed and incubated for 30 min with an fluorescein 5-isothiocyanate-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:40 in PBS containing 1% bovine serum albumin, at room temperature. The cells were washed again, resuspended in 10 μg/ml of propidium iodide and 0.1% RNase A in

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[3] The abbreviations used are: SSP, staurosporine; PBS, phosphate buffered saline; PHA, phytohemagglutinin.

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PBS, and incubated at room temperature in the dark for 20 min prior to measurement (9, 10). The control was prepared as described above, except that an isotype-specific antibody (mouse IgG1; Sigma) was used instead of the cyclin E or cyclin D antibody. The threshold discriminating cyclin D or E positive from negative cells was based on the mean value of fluorescence of the isotypic control plus 3 SD of that value. All incubations in which fluorochromes were present were done in the dark. Cellular fluorescence was measured using the FACScan flow cytometer (Becton Dickinson, San Jose, CA). The red (propidium) and green (fluorescein 5-isothiocyanate) emission from each cell were separated and quantitated using the standard optics of this instrument. Cell doublets were excluded from the analysis based on the fluorescence pulse width and pulse height values of the cell. Other details of the procedure are provided in our earlier publications (8, 9) and in the "Methods" chapter on cyclin protein detection (10). The experiment was repeated twice, using blood of different donors, with essentially the same results.

Results

Nonstimulated human lymphocytes had no measureable amounts of either cyclin E or cyclin D (Figs. 1 and 2). During stimulation, the appearance of cyclin D preceded the appearance of cyclin E. Thus, a significant number of lymphocytes expressing cyclin D were already present 8 h after addition of PHA, and maximum expression of cyclin D was seen after 14 h (Fig. 1). At 24 h the level of cyclin D decreased, and it remained low at 48 and 72 h (not shown). In contrast, there was no evidence of the presence of cyclin E in lymphocytes 8 h after stimulation, maximal expression of this protein was at 24 h (Fig. 2), and it remained high (expressed by a fraction of G_1 and early S phase cells) at 48 and 72 h (not shown).

Confirming our earlier observation (17), it was noticed that SSP included into the lymphocyte cultures at the time of addition of PHA precluded cell entrance to S phase for the next 3 days, as measured by differential staining of cellular RNA and DNA with acridine orange (not shown). The presence of SSP in the cultures had a very different effect on the expression of cyclin E compared with that of cyclin D (Figs. 1–3). Specifically, the expression of cyclin E was considerably more suppressed in the SSP treated cultures, compared with the expression of cyclin D, both in relation to the cells growing with PHA alone. Thus, for example, in SSP treated cultures the number of cyclin D positive cells was diminished by only 24% at 8 h and by 21% at 16 h, whereas the number of cyclin E expressing cells was decreased by as much as 76% at 16 h and 71% at 24 h. The same trend was apparent when, instead of the percentage of cells expressing cyclin D or E, the data were presented as the mean value of cyclin antibody fluorescence of the G_1 cell population (Fig. 3).

Discussion

The induction of cyclin E expression in human lymphocytes stimulated by PHA is markedly suppressed by SSP. This is not the case with cyclin D, the expression of which still remains high in the presence of SSP. These data indicate that the SSP sensitive kinase involved in cell progression through G_1 is beyond the restriction point of cyclin D but prior to that of cyclin E.

Cyclin D forms a complex with $p33^{cdk2}$ which has protein kinase activity. The peak of cyclin D expression is prior to cyclin E, early in G_1 or even possibly at the G_0 to G_1 transition (18–20). Its expression is triggered during cell stimulation by growth factors, but its content does not change significantly during the cell cycle once the cells proliferate continuously (15, 19). It is presumed, therefore, that D type cyclins are induced in response to growth factors, and, unlike the periodically expressed E, A, and B cyclins, D type cyclins may not be integral parts of the cell cycle machinery (15, 18–20). The present data indicate that the kinase activity of neither the cyclin D -$p33^{cdk2}$ complex nor any other protein kinases with activity that precedes that of this complex and are essential for cell transit through G_1, is sensitive to SSP.

Cyclin E is synthesized late in G_1 and its maximal accumulation in the cell coincides with the moment of cell entrance into S phase

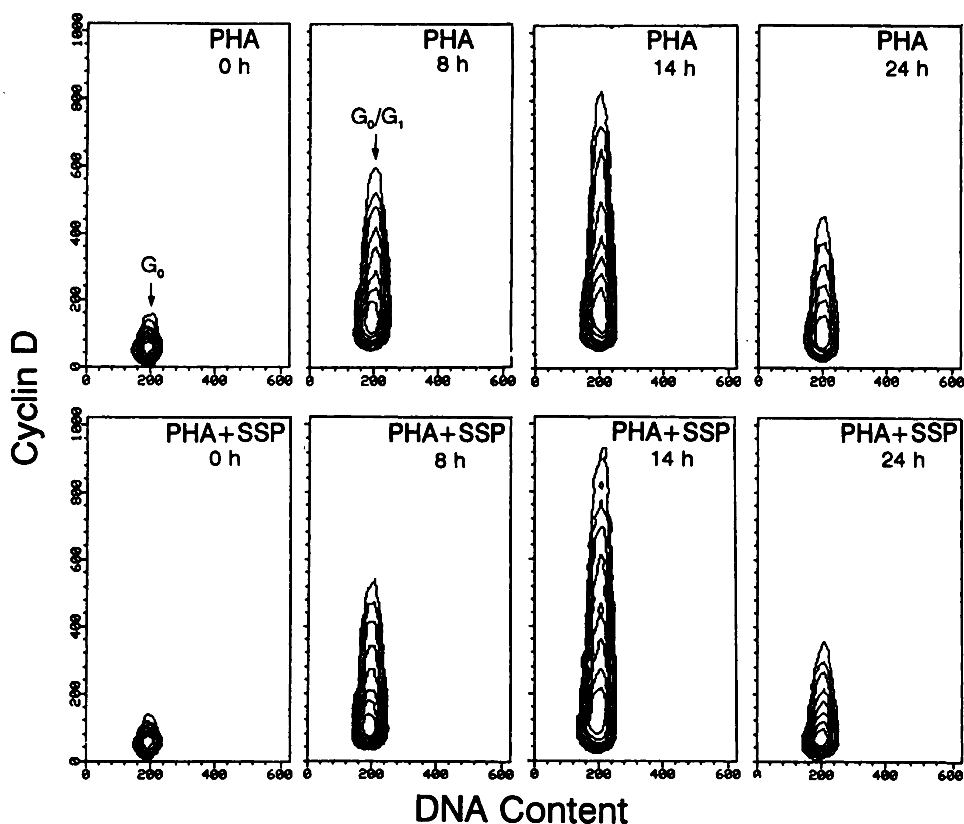

Fig. 1. Induction of cyclin D expression during stimulation of normal human lymphocytes with PHA in the absence (*top*) and presence (*bottom*) of SSP. Bivariate DNA content/cyclin D expression displays (*contour maps*) representing populations of lymphocytes (10^4 cells/sample), unstimulated and stimulated with PHA for 8, 14, and 24 h. Nonstimulated lymphocytes are cyclin D negative. The increased cyclin D expression is already apparent 8 h after addition of PHA and this protein is maximally expressed at 14 h. Thereafter the cyclin D level decreases. Note no significant changes in cyclin D expression in cells growing the presence of SSP compared to cells growing in the absence of this inhibitor.

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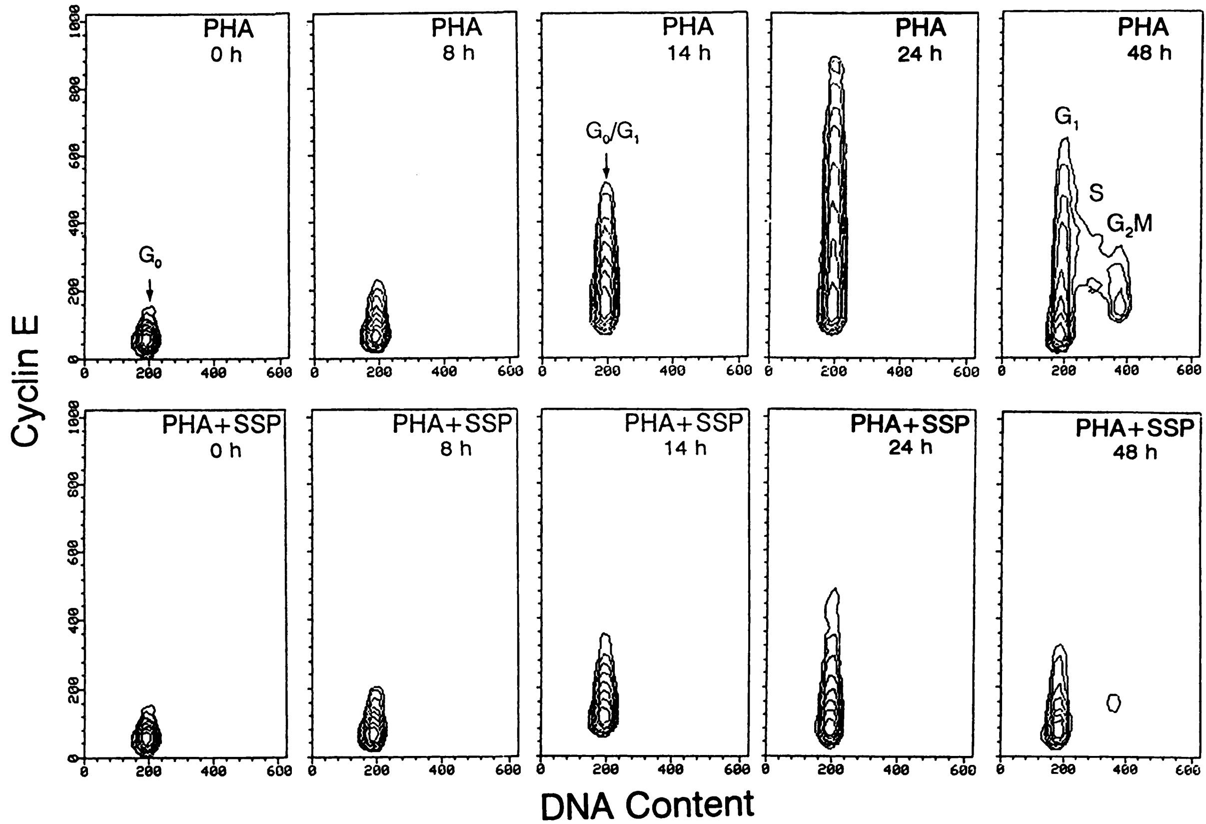

Fig. 2. Induction of cyclin E expression during stimulation of lymphocytes in the absence (*top*) and presence (*bottom*) of SSP. Bivariate DNA content/cyclin E expression contour maps representing populations of lymphocytes, unstimulated (0 h) and stimulated with PHA for 8, 14, 24, and 48 h. The increase in cyclin E, in the absence of SSP, is seen at 14 h and is maximal at 24 h. By 48 h, many cells are in S, G_2, and M. In the presence of SSP the expression of cyclin E is much lower and the cells do not enter S phase.

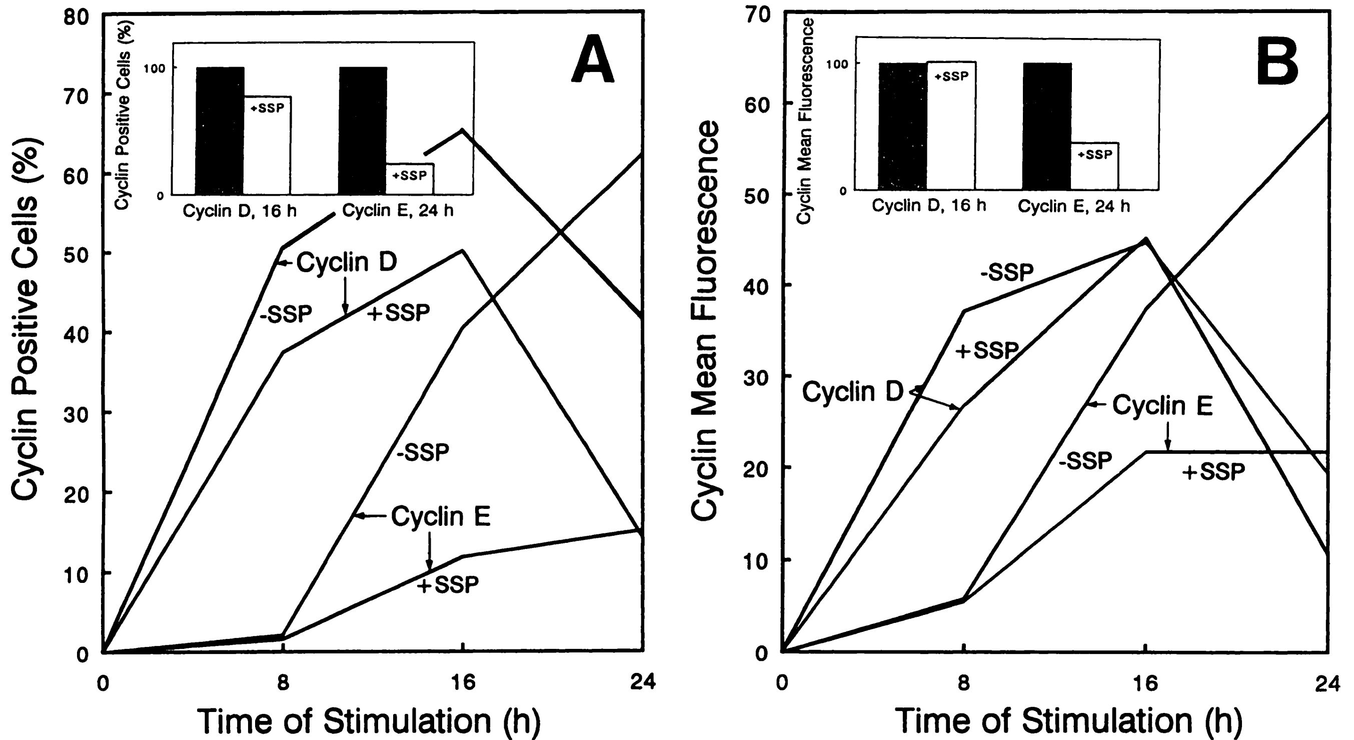

Fig. 3. Effect of SSP on the PHA induced expression of cyclin D and cyclin E in human lymphocytes. Data show the percentage of cyclin D or cyclin E positive cells (*A*) or mean cyclin D or cyclin E associated fluorescence (*B*) of the populations of cells growing in the absence and presence of SSP, at different times after stimulation with PHA. *Bars* in *insets*, percentage of change in expression of the respective cyclins in the SSP treated cultures, related to the SSP-untreated cells (■), at the time of maximal cyclin expression. Note the much stronger effect of SSP on the expression of cyclin E compared to cyclin D.

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(21, 22). Cyclin E also associates with the $p33^{cdk2}$ cyclin dependent protein kinase, resulting in activation of this enzyme. The active heterodimer phosphorylates histone H1 and several other substrates during the G_1 to S transition. Cyclin E is then degraded during cell progression through S phase (for reviews, see Refs. 11–15). The present data indicate that either $p33^{cdk2}$/cyclin E complex itself or other protein kinase(s) the activity of which is essential for cell progression through G_1 prior to cyclin E and past the cyclin D restriction points is (are) the target(s) of SSP.

Earlier observations of Crissman *et al.* (1) and ourselves (2) indicate that the sensitivity to SSP of protein kinase(s) which is (are) essential for cell progression through G_1 is lost in many transformed tumor cell lines. It is possible, therefore, that in these transformed lines, the molecular structure of these kinase(s) is changed in such a way that they become resistant to SSP. More likely, however, another protein kinase, which is not sensitive to SSP and which in normal cells is not scheduled to be active in G_1, is expressed and activated in these transformed cell lines. We have recently observed that cyclin B1, which ordinarily, in normal cells, is expressed only in G_2 and M, in several transformed cell lines was expressed in unscheduled fashion, being abundant in cells progressing through G_1 and S as well.[4] This protein kinase appears to be significantly less sensitive to SSP, since approximately 50-fold higher concentrations of SSP are needed to arrest cells in G_2 compared to G_1 (1). It is quite possible, therefore, that the unscheduled expression and activation of a G_2 type cyclin in G_1 could account for the differential sensitivity of normal *versus* transformed cells to SSP.

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