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Transformed mammalian cells are deficient in kinase-mediated control of progression through the G_1 phase of the cell cycle

(staurosporine/cell proliferation/growth inhibition/flow cytometry)

HARRY A. CRISSMAN*†, DONNA M. GADBOIS*‡, ROBERT A. TOBEY*, AND E. MORTON BRADBURY*‡

*Life Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545; and ‡Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616

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ABSTRACT **To investigate the role of kinase-mediated mechanisms in regulating mammalian cell proliferation, we determined the effects of the general protein kinase inhibitor staurosporine on the proliferation of a series of nontransformed and transformed cultured rodent and human cells. Levels of staurosporine as low as 1 ng/ml prevented nontransformed cells from entering S phase (i.e., induced G_1 arrest), indicating that kinase-mediated processes are essential for commitment to DNA replication in normal cells. At higher concentrations of staurosporine (50–75 ng/ml), nontransformed mammalian cells were arrested in both G_1 and G_2. The period of sensitivity of nontransformed human diploid fibroblasts to low levels of the drug commenced 3 hr later than the G_0/G_1 boundary and extended through the G_1/S boundary. Interference with activity of the G_1-essential kinase(s) caused nontransformed human cells traversing mid-to-late G_1 at the time of staurosporine addition to be "set back" to the initial staurosporine block point, suggesting the existence of a kinase-dependent "G_1 clock" mechanism that must function continuously throughout the early cycle in normal cells. The initial staurosporine block point at 3 hr into G_1 corresponds to neither the serum nor the amino acid restriction point. In marked contrast to the behavior of nontransformed cells, neither low nor high concentrations of staurosporine affected G_1 progression in transformed cultures; high drug concentrations caused transformed cells to be arrested solely in G_2. These results indicate that kinase-mediated regulation of DNA replication is lost as the result of neoplastic transformation, but the G_2-arrest mechanism remains intact.**

A large body of evidence suggests that protein phosphorylation plays a central role in the regulation of cell growth, differentiation, and proliferation. Control of cell proliferation involves the temporal activation of a series of interrelated primary and secondary kinases that phosphorylate an array of essential cellular proteins. Cell cycle studies (1–8) indicate that basic cellular processes such as the commitment to DNA replication and the initiation of mitosis are regulated by kinase-mediated mechanisms. However, whereas eukaryotes ranging in complexity from yeasts to primates employ a highly homologous $p34^{cdc2}$ kinase-dependent mechanism for regulating the onset of mitosis (9–12), there is less compelling evidence for the existence in mammalian cells of a similar kinase-specific mechanism for regulating commitment to genome replication.

To obtain information on the role of kinase-mediated mechanisms in commitment to mammalian DNA replication, we examined the effects on cell proliferation of inhibition of protein kinase activity by the drug staurosporine. This drug was selected for these studies because it exhibits inhibitory activity against a wide range of protein kinases (13–16). Our results show that kinase-mediated processes are essential both for progression through most of G_1 and for initiation of DNA synthesis, but only in nontransformed mammalian cells. Staurosporine was without effect on G_1 progression and initiation of genome replication in transformed cultures, indicating that an important feature of neoplastic transformation is the loss of kinase-mediated control of these processes.

MATERIALS AND METHODS

Nontransformed Cells. Human diploid fibroblast strains 43 (HSF-43; ref. 17) and 55 (HSF-55; ref. 18) were derived from neonatal foreskin samples and cultured in alpha minimum essential medium containing 10% bovine calf serum (αMEM/10% BCS). HFL-1 diploid fibroblasts (19) from human fetal lung (CCL 153) were obtained from the American Type Culture Collection and maintained in αMEM supplemented with 10% bovine fetal serum (αMEM/10% BFS). Human cells from passages 9 to 14 were used in these studies. Early-passage (passages 3–5) cells initiated from a Chinese hamster embryo as described previously (20) were grown in αMEM/10% BFS. Fibroblasts (passage 3) isolated from the lungs of adult athymic BALB/c (nude) mice (Harlan–Sprague–Dawley), as described previously for isolation of rat lung fibroblasts (19), were grown in αMEM/10% BFS.

Transformed Cells. The FT210 mouse mammary carcinoma line (21) was provided by Masa-atsu Yamada (Faculty of Pharmaceutical Sciences, University of Tokyo) and Hideyo Yasuda (Faculty of Pharmaceutical Science, Kanazawa University); the cells were grown at 32°C in RPMI-1640/10% BFS. The tumorigenic WCHE/5 clone 23 T3 Chinese hamster cells (22) were grown in αMEM/10% BFS. HL-60 human leukemia cells (23) were maintained in RPMI/10% BFS. The CT10-2C-T1 cell line (17) was obtained by introducing into nontransformed HSF-43 human diploid fibroblasts a plasmid containing the gene for the simian virus 40 large tumor (T) antigen linked to a Rous sarcoma virus promoter. The resultant CT10-2C-T1 transformed cells were recovered from rapidly growing tumors in nude mice (17) and grown in αMEM/10% BCS.

Flow Cytometric Measurements and Preparation of Stock Solutions. For determination of DNA distributions, mithramycin-stained cells were analyzed in a flow cytometer at 457 nm (24). The 5-bromodeoxyuridine (BrdUrd)–flow cytometric procedure (25, 26) was used to distinguish between noncycling and cycling G_1 cells. Stock solutions of staurosporine (Kamiya Biomedical Company, Thousand Oaks, CA) and BrdUrd (Sigma) were prepared in dimethyl sulfoxide and water, respectively. The amount of dimethyl sulfoxide added

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Abbreviations: BCS, bovine calf serum; BFS, bovine fetal serum; αMEM, alpha minimum essential medium.

†To whom reprint requests should be addressed at: Los Alamos National Laboratory, LS-1, MS-M888, Los Alamos, NM 87545.

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to cell cultures was ≤0.1% (vol/vol) and did not affect cell growth rate.

RESULTS

Effect of Staurosporine on Nontransformed Cells. Our experiments, designed to determine the role of protein kinase activity in mammalian cell proliferation, were carried out with cultures of early-passage, nonimmortalized, nontransformed cells that were treated with various concentrations of staurosporine. Exposure of normal cells to the drug elicited two types of proliferative response. One response was obtained when normal cells were treated with low concentrations of staurosporine, ranging from 1 to 10 ng/ml (2.2–22 nM) for periods approximating the culture doubling time. This caused an emptying of S, G_2, and M phases and accumulation of cells with a 2*C* (G_0/G_1) DNA content (Fig. 1). (Because these cultures were maintained at $<3 \times 10^6$ cells per 75-cm^2 flask, arrest in G_0/G_1 is attributable to staurosporine effects rather than confluency inhibition of growth.) Continued exposure of these nontransformed cultures to staurosporine at 1–10 ng/ml yielded no further changes in cell number or DNA distribution. A second type of behavior was observed when these nontransformed cultures were exposed to high levels of staurosporine (≥50 ng/ml). At high drug concentrations, cells accumulated in both G_0/G_1 and G_2 (Fig. 1), and cell number failed to increase. Microscopic examination of cells arrested in late interphase revealed that the cells were nonmitotic. These results show that staurosporine produces two effects on the proliferation of normal mammalian cells. At low drug levels, normal cells were arrested solely in G_0/G_1, whereas at high drug concentrations, cells were arrested in both G_0/G_1 and G_2.

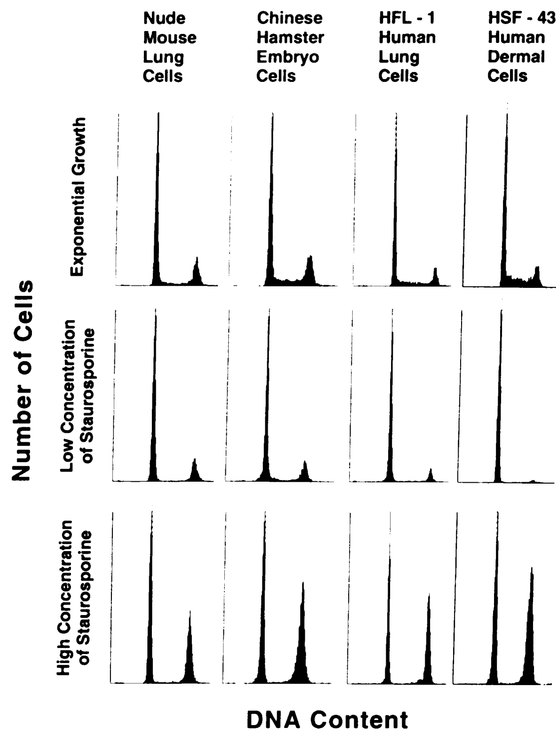

FIG. 1. Effect of staurosporine on cell cycle progression of nontransformed mammalian cells. Staurosporine concentrations and exposure periods were varied in preliminary studies to optimize the redistribution of cells around the division cycle and to minimize drug-induced cytotoxicity. Conditions for the low and high concentrations of staurosporine were as follows: 1 ng/ml and 50 ng/ml for 18 hr for nude mouse lung cells; 7.5 ng/ml and 50 ng/ml for 16 hr for Chinese hamster embryo cells; 10 ng/ml and 50 ng/ml for 18 hr for HFL-1 cells; and 10 ng/ml and 75 ng/ml for 18 hr for HSF-43 cells. Population DNA distributions were determined by flow cytometry (20). Cells comprising the peak on the left side of the DNA histograms represent cells with a 2*C* (G_1) DNA content. Cells in the peak on the right side of the histogram represent cells with a 4*C* (G_2/M) DNA content, and cells in between the two peaks represent cells in various stages of S phase.

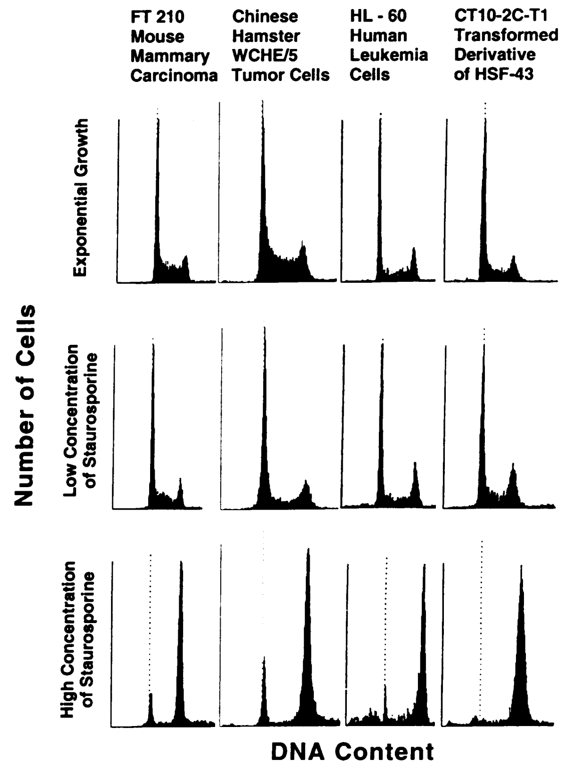

FIG. 2. Effect of staurosporine on cell cycle progression of transformed mammalian cells. Drug exposure conditions were optimized for each cell type as described in Fig. 1. Conditions for the low and high concentrations of staurosporine were as follows: 10 ng/ml and 75 ng/ml for 18 hr for FT210 cells; 10 ng/ml and 50 ng/ml for 18 hr for WCHE/5 cells; 10 ng/ml and 75 ng/ml for 18 hr for HL-60 cells; and 10 ng/ml and 75 ng/ml for 18 hr for CT10-2C-T1 cells. Population DNA distributions were obtained as described in Fig. 1.

Effect of Staurosporine on Transformed Cells. In striking contrast to the above results, staurosporine was unable to arrest transformed cells in G_0/G_1. At 10 ng/ml the drug had no effect on population DNA distribution (Fig. 2). Furthermore, the increase in cell number was equivalent to that of the non-drug-treated control, indicating that the cells grew and divided in the presence of the drug rather than undergoing arrest at all stages of the cell cycle. High concentrations of staurosporine, however, caused cells to be arrested solely in G_2 (Fig. 2). Although a wide range of cell growth conditions and staurosporine concentrations were investigated, we were unable to induce G_1 arrest in transformed cells. From Figs. 1 and 2 we make the following conclusions: (*i*) in nontransformed cells staurosporine-sensitive kinase-mediated controls are essential for both G_1 and G_2/M progression and (*ii*) the staurosporine-sensitive kinase-mediated controls of G_1 progression found in normal cells are lost during neoplastic transformation, leaving only the kinase-mediated control of G_2/M intact in transformed cells.

Identification of the Staurosporine Arrest Point in G_1. Because the effects of staurosporine on cycle progression are reversible, we were able to determine the cell cycle arrest point (G_0 or G_1) of nontransformed cells following exposure to low levels of the drug. Serum-deprived HSF-43 cells in G_0 were placed in complete medium to permit them to return to the cycle. The kinetics of resumption of proliferation for this

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initially G_0 population were then compared with the kinetics of recovery of HSF-43 cells after exposure to a low concentration of staurosporine (Fig. 3). Cells recovering from the drug's effects commenced synthesizing DNA 9 hr after drug removal, whereas 12 hr elapsed before the initially G_0 cells began to replicate their DNA. Assuming rapid reversal of drug effects, these results place the kinase-mediated marker for commitment to DNA synthesis in normal human cells in early G_1, ≈3 hr after the $G_0 \rightarrow G_1$ transition.

The data in Fig. 3 suggest that the cells recovering from the effects of staurosporine progressed as a quasisynchronized cohort of cells blocked in early G_1. This might result from a block in cell progression exclusively at that precise point in the cell cycle. In that event, cells in later portions of G_1 at the time of staurosporine addition should be able to move out of G_1, complete the cell cycle, and divide before arrest at the staurosporine block point in G_1 of the subsequent cell cycle. Alternatively, the staurosporine-specific block point in early G_1 might represent the earliest time within G_1 at which kinase-mediated processes are required for further progression through G_1. If kinase-dependent processes are required at multiple stages of G_1 beyond the staurosporine block, then cells that are initially located in mid-to-late G_1 at the time of staurosporine addition should be unable to complete G_1 and initiate DNA synthesis.

To determine which possibility was correct, it was necessary to distinguish (*i*) cells trapped in G_1 throughout the staurosporine-sensitive G_1 period and (*ii*) cells located in G_1 after the initial staurosporine block point that were able to escape from G_1 and be arrested in early G_1 of the subsequent cell cycle. Separation of these two distributions of cells was accomplished with the BrdUrd–flow cytometry procedure (26). In this procedure, cells that replicate their DNA before being arrested in G_1 of the subsequent cycle incorporate BrdUrd into their DNA and are readily distinguishable from non-BrdUrd-labeled cells that remained in G_1 throughout the experiment. We found that treatment of asynchronous HSF-43 cells with staurosporine at 10 ng/ml for 24 hr caused 52% of the cycling G_1 cells to remain in G_1 throughout the period of exposure to the drug (Table 1). Increasing the drug concentration to 75 ng/ml resulted in an increase to 74% in the fraction of the cycling G_1 population that failed to escape from G_1. These results indicate that nearly all of the G_1 cells located after the initial staurosporine blockade point fail to initiate DNA synthesis in the presence of the drug, and further suggest that staurosporine-sensitive processes are

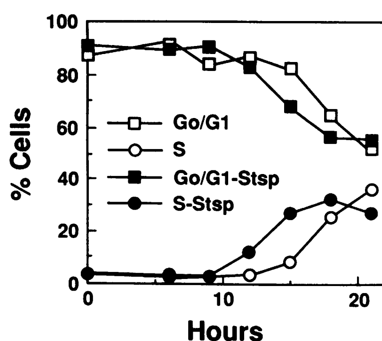

FIG. 3. Comparison of the kinetics of recovery of normal human HSF-43 fibroblasts from a serum-deprived G_0 state or from exposure for 18 hr to staurosporine (Stsp) at 10 ng/ml. Initially asynchronous cultures were maintained for 48 hr in αMEM/0.1% BCS to arrest the cells in G_0; at 0 hr, the cells were restored to αMEM/10% BCS to permit them to return to a cycling mode. Asynchronous cultures were exposed to Stsp at 10 ng/ml for 18 hr; at 0 hr, all monolayers were rinsed with phosphate-buffered saline and provided with drug-free complete medium.

Table 1. Determination of the fraction of cycling G_1 cells that failed to escape from G_1 in the presence of staurosporine (Stsp)

Culture	% unlabeled G_1 cells	% labeled G_1 cells	% cells in S	% cells in G_2 + M
Control (0 hr)	75	—	23*	2†
+ Stsp (10 ng/ml)	43	52	4	1
+ BrdUrd (5 μM, 24 hr)				
+ BrdUrd (5 μM, 36 hr)	10†	70	15	5

*Cells in S phase at the time of Stsp addition (23%) contributed to the percentage of BrdUrd-labeled cells that were arrested in G_1 after Stsp treatment. The percent cycling cells that were initially in G_1 that became labeled during the experiment is then 52% − 23% = 29%.

†Cells that failed to cycle during the experiment (10%) and cells initially in G_2 or M, which divided and entered G_1 without passing through S phase/incorporating BrdUrd (2%) contributed to the percent non-BrdUrd-labeled cells arrested in G_1 after Stsp treatment. The percent cycling cells initially in G_1 that failed to leave G_1 in the presence of Stsp is then 43% − (10% + 2%) = 31%. The percent cycling G_1 cells that failed to escape G_1 in the presence of Stsp at 10 ng/ml is then 31%/(31% + 29%) = 52%. In asynchronous HSF-43 cells exposed to Stsp at 75 ng/ml for 24 hr, cells located in S and G_2 at the time of drug addition were arrested in G_2 and the cell number remained essentially unchanged, which simplified calculations. Accordingly, the percent unlabeled cycling G_1 cells at the end of the Stsp treatment period was 34% out of a total of 46% cycling G_1 cells, or 74%.

required at multiple stages of G_1 to allow cells to commit to genome replication.

To provide further information on the approximate location of the end of the staurosporine-sensitive period, cultures of nontransformed human dermal HSF-55 fibroblasts were first treated with a low, readily releasable concentration (2 ng/ml) of staurosporine to arrest them at the initial staurosporine block point. The cells were then placed in drug-free medium for different periods to allow them to progress through different portions of G_1 before application of a second staurosporine block (50 ng/ml). Only cells past the terminal point of staurosporine sensitivity were able to continue progression through the cell cycle, where they were then arrested in G_2 in the presence of the high concentration of staurosporine. From determination of the number of cells escaping from the second staurosporine block and knowing the location of cells within G_1 at the time the second block was applied, an estimate of the duration of the drug-sensitive period in G_1 can be obtained. The time required for 50% of the cohort of escaping cells to pass the terminal staurosporine block point was ≈9.8 hr (Fig. 4). Thus, cells located in any portion of the cell cycle from the initial staurosporine block point at 3 hr past G_0/G_1 through the next 9.8 hr are sensitive to staurosporine. This terminal point of sensitivity to staurosporine, ≈12.8 hr past the G_0/G_1 transition, lies very close to the G_1/S boundary; therefore, in nontransformed cells, kinases sensitive to inhibition by staurosporine are required for cells to progress through the final 9 hr of G_1 and to initiate replication of DNA. Interference with kinase activity at any portion of this staurosporine-sensitive period within G_1 causes cells to be, in effect, "set back" to the initial staurosporine block point located 3 hr beyond the G_0/G_1 boundary.

DISCUSSION

Our results show that, in both normal and tumor cells of mammalian origin, a staurosporine-sensitive process is essential for cells to initiate mitosis (Figs. 1 and 2). High concentrations of staurosporine (50–75 ng/ml) were required to bring about this drug-induced arrest of cells in G_2. In view of the extensive genetic and biochemical evidence showing that *cdc2*-encoded protein kinases are essential for initiation

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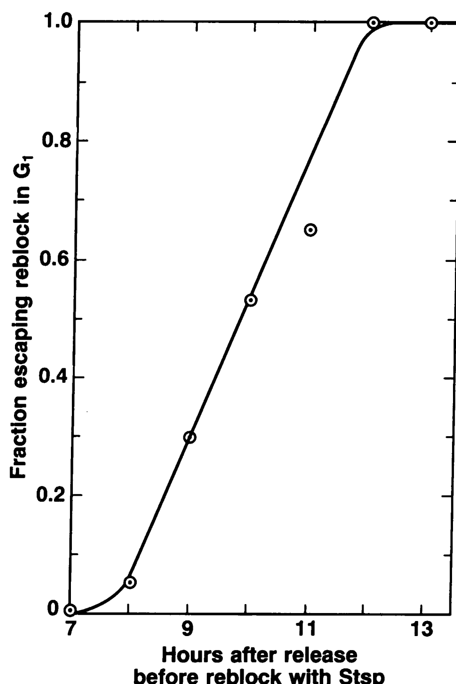

FIG. 4. Determination of the terminal location of the staurosporine (Stsp)-sensitive phase in the early cycle of nontransformed human dermal fibroblasts. Asynchronous HSF-55 cells were treated for 18 hr with Stsp at 2 ng/ml and then were returned to fresh, drug-free medium for various periods (7–13 hr) before reblock with Stsp at 50 ng/ml. All cultures were harvested 24 hr after release of cells from the initial (2 ng/ml) Stsp block. The fraction of cells progressing beyond G_1/very early S phase was determined for each culture as an estimate of the fraction of cells situated beyond the terminal stage of early-cycle Stsp sensitivity at the time of addition of the second (50 ng/ml) Stsp block.

of mitosis in a variety of species (reviewed in ref. 11) and the demonstration that staurosporine is a potent inhibitor of a range of protein kinases (9–12), including $p34^{cdc2}$ (D.M.G., J. Hamaguchi, and E.M.B., unpublished observations), it seems likely that inhibition of $p34^{cdc2}$ kinases contributes to the staurosporine-induced G_2 arrest observed in both nontransformed and transformed cells. Other protein kinases may also be involved in the induction of G_2 arrest by staurosporine.

We also provide evidence for the existence only in nontransformed mammalian cells of a staurosporine-sensitive pathway that is required for progression of cells through most of G_1 and for commitment to DNA replication. An exciting feature of this staurosporine-sensitive G_1 regulatory process is that it is absent from all tumor cells so far examined, suggesting that a mammalian cell's capacity to regulate commitment to DNA replication via this presumptive protein kinase-dependent mechanism is lost during neoplastic transformation. Another interesting feature of the G_1 regulatory mechanism in nontransformed cells is the multihour period during G_1 in which staurosporine-sensitive kinase activity is required for a cell to prepare to initiate DNA replication. This suggests the existence of a kinase-mediated "G_1 clock" mechanism whose continuous operation is essential for progression of cells out of G_1. Disruption of kinase activity causes this G_1 clock to be reset to the initial staurosporine block point. These studies show that the staurosporine-induced arrest point in early G_1 is probably a general feature of nontransformed cells. Note also that exposure of nontransformed (albeit immortalized) 3Y1-B rat fibroblasts (27) to low levels of staurosporine caused cells to be arrested in early G_1 within 2 hr after serum stimulation of G_0 cells (28).

The initial staurosporine-sensitive block point appears to represent a unique arrest point within G_1. Its location at 3 hr beyond the G_0/G_1 boundary (Fig. 3) is different from the nutrient restriction points described by Pardee (29). The serum restriction point in HSF-43/HSF-55 cells occurs at the G_0/G_1 interface, while the amino acid restriction point in nontransformed human fibroblasts of dermal origin occurs at a point in late G_1 located 2–3 hr before the G_1/S boundary (30). The relationship between the staurosporine and nutrient restriction points in HSF-43/HSF-55 cells is illustrated in Fig. 5.

What types of kinase-mediated processes might regulate progression through G_1? One possible example involves cancer-suppressor gene products such as the retinoblastoma (RB) protein. In normal cells, unphosphorylated wild-type RB protein prevents initiation of DNA synthesis, but during G_1 the RB protein is inactivated by phosphorylation and cells enter S phase (31–33). Staurosporine-induced inhibition of the RB kinase(s) should prevent phosphorylation of RB protein and should cause nontransformed cells to arrest in pre-S phase. In some transformed cells, it has been postulated that kinase-mediated RB regulatory function is lost as the result of complex formation between wild-type RB protein and certain viral proteins (e.g., simian virus 40 large T antigen, adenovirus E1A; refs. 34–36) or through introduction of missense mutations into the RB protein (37). Staurosporine would have no effect on entry into S phase in such cells because initiation of DNA synthesis no longer requires phosphorylation-mediated inactivation of the RB protein. Other types of essential kinase-mediated processes may operate at different stages of G_1.

The studies described in this report merely hint at the potential usefulness of staurosporine. This drug's potent capacity to arrest normal cells, but not transformed cells, in G_1 suggests that the loss of the staurosporine-sensitive arrest point in G_1 may serve as an indicator of a specific step in the neoplastic process or may even provide a useful diagnostic marker of neoplasia.

The ability of very low levels of staurosporine to reversibly arrest normal human cells, but not their tumorigenic deriv-

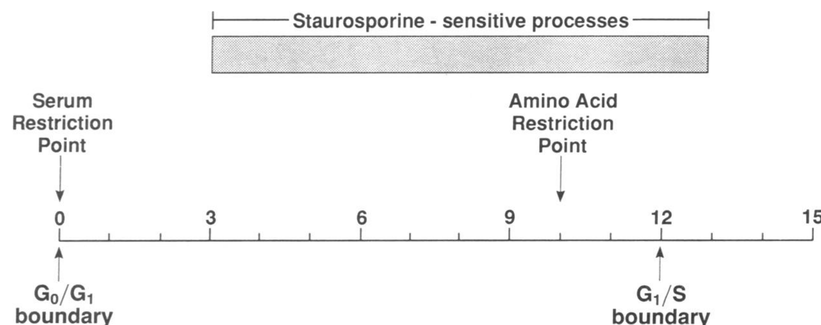

FIG. 5. Temporal characterization of the period of early-cycle staurosporine sensitivity in nontransformed human cells of dermal origin.

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atives, in G_1 suggests a potential role for this drug as an adjuvant in cancer therapy. It may be possible to arrest transiently in G_1, and thereby protect, actively proliferating cells from normal tissue in cancer patients through the use of a low level of staurosporine that has no effect on the proliferative capabilities of tumor cells. The patient could then be treated with a therapeutic regimen with maximum toxicity for actively cycling (i.e., tumor) cells. If such a strategy can be applied successfully in the clinic, far more effective therapeutic doses could be delivered to tumors while simultaneously reducing the harmful side-effects these therapeutic agents produce in normal tissue.

Finally, staurosporine might favor selective outgrowth of newly isolated tumor cells in culture. Maintenance of low levels of staurosporine in the medium during the early stages of tumor growth in culture may allow the tumor cells to proliferate actively while slowing or stopping altogether the growth of normal stromal cells, which are unavoidably isolated along with tumor material.

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