Multiple kinase arrest points in the G₁ phase of nontransformed mammalian cells are absent in transformed cells

(kinase inhibitors/cell proliferation/growth inhibition/flow cytometry)

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Communicated by Theodore T. Puck, June 18, 1992

ABSTRACT We have shown that nontransformed mammalian cells arrest early in the G₁ phase of the cell cycle when treated with exceedingly low concentrations of the nonspecific kinase inhibitor staurosporine, whereas transformed cells continue to progress through the cell cycle. We have now treated normal or transformed human skin fibroblasts with four other kinase inhibitors. Three of these inhibitors are highly specific: KT5720 inhibits cAMP-dependent protein kinase, KT5823 inhibits cGMP-dependent protein kinase, and KT5926 inhibits myosin light-chain kinase. The fourth inhibitor K252b has a moderate specificity for protein kinase C but also inhibits the three kinases just mentioned. We have found that these inhibitors reversibly arrest normal human skin fibroblasts at different times in the G₁ phase but do not affect the cell cycle progression of transformed cells. The times of arrest within the G₁ phase can be divided into two categories. Two of the inhibitors, KT5926 and K252b, act at an early time that is ≈4 h after the transition from G₀ to G₁. The cAMP- and cGMP-dependent protein kinase inhibitors KT5720 and KT5823 arrest cells at a later time that is ≈6 h after the G₀/G₁ boundary. These data indicate that there are multiple kinase-mediated phosphorylations of different substrates that are essential for the progression of normal cells, but not transformed cells, through the G₁ phase. These inhibitors provide us with a set of biochemical probes that should be invaluable in the study of the function of kinases during G₁ phase progression of normal cells.

It has been postulated that normal cells can shift between a proliferating and a quiescent state at a single time point, called the restriction point, in the G₁ phase of the cell cycle (1). One of the consequences of neoplastic transformation is the loss of the ability to exit from the cell cycle at the restriction point. However, it has now been shown that several events in the G₁ phase are required for a cell to progress through the G₁ phase and to commit to DNA replication (2, 3). These temporal events are defined by serum requirements. The platelet-derived growth factor-induced competence point occurs at the G₀/G₁ border, ≈12 h before initiation of DNA replication (2, 3). Progression factors (e.g., somatomedin C or epidermal growth factor) function at a point, called the V point, 6 h before the G₁/S boundary (2–4). Finally, the W point is at the G₁/S boundary (2, 3). Although the original work on competence and progression factors involved rodent fibroblasts, the same mechanisms are thought to apply to human fibroblasts (5). We have shown that staurosporine, at the extremely low concentration of 2–20 nM, arrests normal, but not nontransformed, HSF43 cells ≈3 h after the G₀/G₁ boundary (6). At higher concentrations of staurosporine (100–150 nM), normal cells arrest in both the G₁ and G₂ phases, whereas transformed cells arrest

only in the G₂ phase. The staurosporine G₁ phase arrest point is unique not only because it is different from the serum restriction time points but also because the arrest is caused by the inhibition of a kinase(s) for the period from 3 h after the G₀/G₁ transition to the G₁/S boundary (6). Staurosporine in the nanomolar range inhibits many kinases (7–11). Therefore, although the arrest of normal cells early in the G₁ phase by low concentrations of staurosporine shows that a kinase-mediated mechanism(s) is required for the progression through the G₁ phase of the cell cycle, the broad specificity of staurosporine makes it difficult to identify target kinases. A major question is whether there is a key kinase or a cascade of crucial kinases that acts throughout the staurosporine-sensitive time period. Other, more specific, kinase inhibitors have been isolated (Table 1): KT5720 preferentially inhibits cAMP-dependent protein kinase (cAPK) (12), KT5823 inhibits cGMP-dependent protein kinase (cGPK) (12), and KT5926 inhibits myosin light-chain kinase (MLCK) (12, 13). Finally, although K252b has a moderate specificity for protein kinase C, it also inhibits MLCK, cAPK, and cGPK (12). In this study, we report on the effects of these more specific kinase inhibitors on the cell cycle progression of normal or transformed human skin fibroblasts. We find that these kinase inhibitors all block normal human fibroblasts in the G₁ phase of the cell cycle whereas transformed human fibroblasts continue cycling. However, the points at which the inhibitors arrest cells in G₁ are different from the staurosporine arrest point. The inhibitors KT5720 and KT5823 cause cells to arrest ≈6 h after the G₀/G₁ boundary whereas KT5926 and K252b block cells 4 h after the G₀/G₁ transition. These data show that there are multiple kinase-mediated events that are essential for the progression of nontransformed cells through the G₁ phase of the cell cycle. All of these mechanisms are absent in transformed cells. The findings that these more highly specific kinase inhibitors arrest normal cells at unique G₁ time points have identified a set of biochemical tools to probe the cell cycle kinases and substrates that are needed for progression through the G₁ phase.

MATERIALS AND METHODS

Cell Culture. Human diploid fibroblast strain 55 (HSF55) was kindly provided by D. Chen (Los Alamos National Laboratory) and was derived from neonatal foreskin samples. Cells from passages 6 to 10 were used in these studies. The CT10-2C-T1 cell line was kindly provided by F. A. Ray (Los Alamos National Laboratory) and was derived from nontransformed HSF43 human diploid fibroblasts by introducing a plasmid containing the gene for the simian virus 40 large tumor antigen linked to a Rous sarcoma virus promoter

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Abbreviations: cAPK, cAMP-dependent protein kinase; cGPK, cGMP-dependent protein kinase; MLCK, myosin light-chain kinase; C, haploid DNA content.

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Table 1. Inhibition constants of kinase inhibitors

Inhibitor	K_i , μM			
	MLCK	cAPK	cGPK	PKC
Staurosporine*	0.0013	0.0070	0.0085	0.0007
KT5926†	0.018	1.2	0.158	0.723
K252b‡	0.147	0.090	0.1	0.020
KT5823‡	>10.0	>10.0	0.234	4.0
KT5720‡	>2.0	0.060	>2.0	>2.0

PKC, protein kinase C. *, Ref. 7; †, ref. 13; ‡, ref. 12.

(14). The resultant CT10-2C-T1 transformed cells were recovered from rapidly growing tumors in nude mice. All cells were cultured at 37°C and 5% CO₂/95% air in α -modified minimum essential medium (α MEM; GIBCO/BRL) containing 10% (vol/vol) bovine calf serum (HyClone). Cells were determined to be free of mycoplasma (15).

Kinase Inhibitor Studies. Stock solutions of staurosporine, KT5720, KT5823, KT5926, and K252b (Kamiya Biomedical, Thousand Oaks, CA) were prepared in dimethyl sulfoxide and stored at 4°C. The concentration of dimethyl sulfoxide used (<0.75%) did not induce cell cycle arrest. Cells were plated in 75-mm flasks (24 h before the addition of an inhibitor) at a density that allowed cells to grow logarithmically throughout the experiment. For the arrest studies, an inhibitor was added and the cells were collected 18 h later for flow cytometric analysis. For release experiments, cells were first treated with an inhibitor for 18 h to allow cells to accumulate in the G₁ phase of the cell cycle. Inhibitor-containing medium was removed, monolayers were washed two times with phosphate-buffered saline, and inhibitor-free medium was added. Samples were collected at intervals after release and analyzed using flow cytometry.

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 (16). The percentage of cells in each phase of the cell cycle was calculated by computer-fit analysis (17).

RESULTS

Treatment of Normal Fibroblasts with Kinase Inhibitors. Treatment of normal HSF55 cells with KT5720 (11 μM), KT5823 (15 μM), KT5926 (3 μM), or K252b (11 μM) for 18 h causes them to arrest with a 2C DNA content (where C is the haploid DNA content), showing that these cells have arrested in the G₀ or G₁ phase of the cell cycle (Fig. 1). From repeat experiments, we determined the average percentage of cells that accumulated in each phase of the cell cycle after treatment with the inhibitors (Table 2). Inhibitors KT5720, KT5823, and K252b caused an average 84–91% of the cells to arrest in the G₀ or G₁ phase of the cell cycle. Treatment with KT5926, however, resulted in a lower average accumulation (75.3 $\pm$ 11.2%) of cells in the G₀ or G₁ phase. These fluctuations in the percentages of cells arrested were due to leakiness in the block (D.M.G. and R.A.T., unpublished data). Although different concentrations of KT5926 were explored, we could not find conditions that induced more cells to arrest in the G₀ or G₁ phase. Increasing the concentration of KT5926 (9.5 μM) led not to an increase in the percentage of cells arrested in the G₀ or G₁ phase but to a second arrest point in the G₂ or M phase (Fig. 2). In contrast, increasing the concentrations of the three other drugs KT5720, KT5823, or K252b to levels as high as 30 μM did not induce a second G₂ or M phase arrest in HSF55 cells in addition to the G₀ or G₁ phase arrest (data not shown). Thus these three drugs inhibit kinases required only in the G₀ or G₁ phase of the normal cell cycle, whereas KT5926 inhibits

FIG. 1. Treatment of normal or transformed cells with kinase inhibitors. Normal (HSF55) or transformed (CT10-2C-T1) human skin fibroblasts were treated with 3 μM KT5926, 11 μM K252b, 11 μM KT5720, or 15 μM KT5823 for 18 h. DNA content of mithramycin-stained cells was determined using flow cytometry. The number of cells analyzed is represented on the y axis and the amount of DNA in each cell (fluorescence intensity) is shown on the x axis.

kinases required both in the G₀ or G₁ phase and in the G₂ or M phase.

Treatment of Transformed Fibroblasts with Kinase Inhibitors. We next determined the effects of the kinase inhibitors KT5720, KT5823, KT5926, and K252b on the cell cycle progression of simian virus 40 large tumor antigen-transformed human skin fibroblasts (CT10-2C-T1). Three of these inhibitors KT5720, KT5823, or K252b at low (11, 15, or 11 μM) or high (30 μM) concentrations had no effect on the cell cycle distribution of the transformed cells (Fig. 1). Although the fourth inhibitor KT5926 at low concentrations (3 μM) had no effect on transformed cell cycle progression, at high concentrations (9.5 μM) it induced only an arrest point

Table 2. Percentage of HSF55 cells in each phase of the cell cycle after treatment with low concentration of kinase inhibitor

Treatment	% cells		
	G ₁	S	G ₂ /M
None*	63.3 $\pm$ 6.2	24.8 $\pm$ 5.3	11.9 $\pm$ 3.0
KT5926†	75.3 $\pm$ 11.2	13.8 $\pm$ 7.1	10.9 $\pm$ 5.2
K252b‡	91.1 $\pm$ 3.4	4.5 $\pm$ 2.1	4.5 $\pm$ 1.4
KT5720‡	83.9 $\pm$ 8.0	9.7 $\pm$ 5.2	6.3 $\pm$ 2.9
KT5823‡	84.5 $\pm$ 7.0	11.8 $\pm$ 5.5	5.3 $\pm$ 2.7

HSF55 cells were treated with 3 μM KT5926, 11 μM K252b, 11 μM KT5720, or 15 μM KT5823 for 18 h. The cell cycle distribution of mithramycin-stained cells was determined using flow cytometry. Data are the mean $\pm$ SEM. *, $n = 9$ experiments; †, $n = 6$; ‡, $n = 7$.

FIG. 2. Treatment of normal or transformed cells with high concentrations of KT5926. Normal (HSF55) or transformed (CT10-2C-T1) human skin fibroblasts were treated with $9.5 \mu\text{M}$ KT5926 for 18 h and the DNA content of the cells was determined using flow cytometry as in Fig. 1.

in the G₂ or M phase with a 4C DNA content (Fig. 2). It can be concluded from these kinase inhibitor studies that the multipoint kinase arrests of normal G₁ cell cycle progression are deficient in the transformed cells. The arrest at the G₂ or M phase induced by KT5926 in normal cells is also found in the transformed cell.

Time of G₁ Arrest Points. Cells that arrest with a 2C DNA content may be in the G₀ or G₁ phase of the cell cycle. We distinguished between these possibilities by releasing HSF55 cells from an 18-h treatment with 20 nM staurosporine, 11 μM KT5720, 15 μM KT5823, 3 μM KT5926, or 11 μM K252b and measuring the time from release to the onset of DNA synthesis. Normal HSF55 cells were treated with these kinase inhibitors for 18 h to accumulate cells with a 2C DNA content. Inhibitor-containing medium was removed, the monolayer was washed twice with phosphate-buffered saline, and then inhibitor-free medium was added to the culture. Cells were harvested at intervals beginning at 5.5 h and ending 15.5 h after release. The release from treatment with staurosporine, KT5720, KT5823, KT5926, or K252b was compared to the release from a low serum-induced G₀ arrest. The data from a representative experiment are shown in Fig. 3. Fig. 3A shows flow histograms from three of the six time points at 0, 11, and 13.5 h after release from the G₁ phase cell cycle arrest induced by the indicated kinase inhibitors. From the flow histograms, the percentages of cells in the G₀ or G₁ phase and in S phase were calculated and plotted against the time since release from the indicated block (Fig. 3B). From these plots the times taken for the cells to progress from the block point to the onset of DNA replication, relative to the serum-depleted control, were calculated. These times were averaged for three experiments to give the mean time of release from each inhibitor (Fig. 4).

The HSF55 cells arrested by the cAPK inhibitor KT5720 and the cGPK inhibitor KT5823 were the first to enter the S phase after release from their blocks; KT5720- and KT5823-treated cells entered the S phase 6.7 ± 0.4 h and 7.2 ± 0.2 h after release, respectively. The HSF55 cells treated with the MLCK inhibitor KT5926 and the more general kinase inhibitor K252b took longer times to reach the S phase; KT5926- and K252b-treated cells entered the S phase 9.2 ± 0.4 h and 9.1 ± 0.3 h after release, respectively. In these experiments, we found that, after release from staurosporine, HSF55 cells took 10.5 ± 0.2 h to enter the S phase. These data for HSF55 cells agree quite well with the staurosporine arrest point determined in earlier experiments for HSF43 cells (6). By

FIG. 3. Determination of the time of the kinase inhibitor-induced G₁ phase arrest points in HSF55 cells. HSF55 cells were treated with staurosporine (20 nM, Stp), KT5926 (3 μM), K252b (11 μM), KT5720 (11 μM), or KT5823 (15 μM) for 18 h. The time of entry into the S phase after release from inhibitor was monitored using flow cytometry of mithramycin-stained cells. (A) Flow histograms of samples taken 0, 11, and 13.5 h after release from inhibitor are shown. (B) The percentage of cells in the S phase (■) or in the G₁ phase (○) after release from an inhibitor was calculated and is plotted against the time after release from inhibitor treatment.

knowing that the length of the G₁ phase in these cultures of HSF55 cells is 13 h (data not shown) and by assuming rapid recoveries for all inhibitors, the time points after the G₀/G₁ transition when these kinase inhibitors induce G₁ phase arrests are as follows: staurosporine, 2.5 h; KT5926 and also K252b, 3.8 h; KT5823, 5.8 h; KT5720, 6.3 h, as in Fig. 4.

DISCUSSION

We have shown (6) that the major difference in the response of normal and transformed cells to staurosporine is the extraordinary sensitivity of normal cells to low levels of staurosporine in the G₁ phase, a sensitivity that extends from the G₀/G₁ boundary plus 3 h through to the G₁/S phase transition, a period of ≈ 10 h. Treatment of normal human fibroblasts with staurosporine at any time in this 10-h period in G₁ sets the cells back to the initial block point at ≈ 3 h into the G₁ phase (6). A major question that arises from these observations is whether there is a single key kinase or a series of kinases involved in the G₁ phase progression of normal cells. To address this question, we have studied the effects of three more specific kinase inhibitors (KT5720, KT5823, and KT5926) and another general kinase inhibitor (K252b) on the cell cycle progression of normal and simian virus 40-

FIG. 4. Temporal location of kinase inhibitor-induced arrest points in the G₁ phase of normal human skin fibroblasts.

transformed human diploid fibroblasts. At the lower concentrations, all of these inhibitors arrest the normal fibroblasts in the G₁ phase but at different time points (Figs. 3 and 4). In contrast they have no effect on the G₁ phase progression of the simian virus 40-transformed cell (Fig. 1). The G₁ phase arrest points, induced in the normal cells by the inhibitors, are shown in Fig. 4. Staurosporine induces arrest very early in G₁ at 2.5 h, KT5926 and K252b induce arrest early in G₁ at 3.8 h, and KT5823 and KT5720 induce G₁ arrest later at 5.8 and 6.3 h. These data provide evidence that multiple kinases are required for the progression of normal cells through the G₁ phase of the cell cycle and that these kinase functions are inactivated or bypassed by the process of neoplastic transformation.

Cell Cycles of Normal and Transformed Cells. A summary of reversible arrest points in the cell cycles of normal and transformed cells is shown in Fig. 5. Both normal and transformed cells respond in very similar fashions to agents that arrest cells at points from the G₁/S phase transition through M phase. However, common G₁ phase arrest points have also been described. Group B amino acid deficiency (amino acid restriction point) causes normal human fibroblasts to arrest late in G₁ phase (18). The amino acid restriction point has not been demonstrated in transformed human fibroblasts, probably because of the difficulty in depleting human cells of endogenous pools of group B amino acids (18). However, the amino acid restriction point may be similar to the isoleucine-deficiency G₁ arrest observed in normal and transformed rodent cells (19). Recently, lovastatin has been reported to induce G₁ arrests in both normal and transformed cells (20, 21). Both aphidicholin and hydroxyurea reversibly arrest normal and transformed cells throughout S phase.

Aphidicholin inhibits DNA polymerase α , and hydroxyurea inhibits nucleoside diphosphate reductase (22). Numerous G₂ arrest points are common to both normal and transformed cells. It is well established that the activity of p34^{cdc2}/cyclin kinase complexes are required for the progression through G₂ and M phases in both normal and transformed cells; temperature-sensitive mutants for p34^{cdc2} in both yeast (23, 24) and mammalian cells (25) arrest in the G₂ phase when grown at the nonpermissive temperature. Staurosporine at 100–150 nM induces a G₂ phase arrest in both normal and transformed cells (6). We have shown that staurosporine is a potent inhibitor of p34^{cdc2}-cyclin A, p34^{cdc2}-cyclin B1, and p34^{cdc2}-like-cyclin A kinases and have suggested that the staurosporine-induced G₂ phase arrests, common to both normal and transformed cells, result from inhibition of the p34^{cdc2}-cyclin kinases (26). High levels of KT5926 (9.5 μ M) also cause a G₂ phase arrest in both normal and transformed cells, but it is unlikely that the mechanism of arrest is through inhibition of p34^{cdc2} kinases because these kinases are not inhibited appreciably by KT5926 *in vitro* (D.M.G., R. A. Swank, and E.M.B., unpublished results). Similar mechanisms are required for cell cycle progression through S, G₂, and M phases of the cell cycles of normal and transformed cells.

A major difference between the cell cycles of normal and transformed cells is that there are arrest points in the G₁ phase of the normal cell cycle that are absent in the transformed cell cycle (Fig. 5). As mentioned, serum factors that describe the competence V and W points act in normal but not transformed cells (2, 3). In this manuscript we present data for a set of highly specific kinase inhibitors that arrest only normal cells at unique points in the G₁ phase. These time points represent, as yet, unidentified processes that are controlled

FIG. 5. Control of cell cycle progression in normal or transformed cells. Control points that exist in the cell cycle of normal fibroblasts (A) are compared to control points that exist in the cell cycle of transformed fibroblasts (B). STSP, staurosporine; LOV, lovastatin; AA, amino acid restriction point; APC, aphidicholin; HU, hydroxyurea; HO 33342, Hoechst 33342; COL, Colcemid; NOC, nocodazole.

by kinases. Therefore, even though common kinase-mediated mechanisms are found in G₂ and M phases of both normal and transformed cells, there are multiple kinases that act only in the G₁ phase of normal cells. It is possible that normal cells respond to extracellular signals through these kinase-mediated pathways in the G₁ phase. The deficiencies of these kinase-mediated mechanisms in transformed cells may render them insensitive to extracellular growth control signals resulting in uncontrolled cell proliferation. Regardless of the specific mode of action, these data show the importance of kinases to progression through the G₁ phase of the cell cycle and to the maintenance of the nontransformed phenotype.

Use of Specific Kinase Inhibitors as Chemotherapy Adjuvants. We have suggested that staurosporine could be used to protect normal cells from the toxic effects of chemotherapeutic agents (6). If in an animal a low concentration of staurosporine could transiently arrest proliferating normal cells in the G₁ phase, then a more effective dose of an S- or G₂-phase-specific chemotherapeutic drug could be used to kill the cycling tumor cells. The data presented here, however, indicate that there are multiple G₁ phase kinases that could be targeted by these more highly specific kinase inhibitors. This extends considerably the range of kinase inhibitors that could be used as adjuvants in chemotherapy. Further, specificity could be enhanced by simply modifying the structure of the kinase inhibitor. The disadvantage of using the inhibitors described in this study compared to staurosporine is that a fairly high concentration of the inhibitor (micromolar) is required to induce G₁ phase arrest. However, by modifying the structure of the inhibitor, it should be possible to design kinase inhibitors that are both more highly sensitive and more selective.

Substrate Specificities of the Kinase Inhibitors. A question that arises from this study is whether the *in vitro* substrates of these kinase inhibitors are also the cellular substrates. If this is so, then these results indicate that cAPK, MLCK, and cGPK are required at different points in the G₁ phase for progression of normal cells. Early signals of the mitogenic response include calcium and cyclic nucleotide fluxes (for review, see refs. 27 and 28). These signals may modulate the activity of MLCK, cAPK, and cGPK in the G₁ phase. Also there is *in vitro* evidence that the activity of MLCK is regulated by cAPK and cGPK (29). However, although it is intriguing to speculate about the possible roles for MLCK, cAPK, and cGPK in the G₁ phase of the cell cycle, the possibility remains that the inhibitors are also affecting other kinases. The kinases that act at the inhibitor-induced arrest points may be members of one pathway that is required for G₁ progression. However, it would be just as interesting if the arrest points described independent kinase-mediated processes that all must occur for cells to progress through the G₁ phase. These are the types of questions that this set of biochemical tools can be used to address in cellular culture systems of transformed and normal cells. Therefore, it will now be necessary to examine alterations in gene expression and protein phosphorylation during the G₁ phase arrest induced by these kinase inhibitors. These studies should yield valuable information concerning the role of kinases during G₁

phase progression of the cell cycle in normal cells and how these mechanisms are lost during neoplastic transformation.

We thank Anita Stevenson, Paul Kraemer, and Walker Wharton for helpful discussions. This work is supported by the Office of Health and Environmental Research, the U.S. Department of Energy, the Los Alamos National Flow Cytometry and Sorting Resource (funded by National Institutes of Health Grant P41-RR01315), the American Cancer Society (CD-484F), and the National Institutes of Health (PHS GM 45890).

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