Uncoupling of the order of the S and M phases: effects of staurosporine on human cell cycle kinases

T. Stokke, L. Smedshammer, T. S. Jonassen, H. K. Blomhoff,*
K. Skarstad and H. B. Steen

*Departments of Biophysics and *Immunology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway*

(Received 18 August 1996; revision accepted 12 August 1997)

Abstract. The protein kinase inhibitor staurosporine (SSP) was employed to study the involvement of kinases in human cell cycle progression. Thirty to 100 ng/ml SSP blocks entry into S phase and M phase. Lack of entry into S phase is due to impaired activity of the retinoblastoma protein kinase. The requirement for any of the SSP-sensitive kinases for cell cycle progression can be abrogated in tumour cells. Therefore, these kinases act in a checkpoint network negatively controlling the initiation of S phase, M phase and cytokinesis, rather than being inherent parts of a substrate-product chain required for the initiation of the cell cycle phases. As a consequence of the lack of certain checkpoint effectors, tumour cells may endoreduplicate or binucleate in the presence of SSP. The latter processes, as well as meiosis, are naturally occurring in specialized cell types, leading to the idea that this checkpoint network controls the order of the cell cycle phases in normal cells. A model is presented where the cell cycle is envisioned as two independently running cycles, the S and the M cycle, which are controlled by intra and intercycle-dependent checkpoints in human somatic cells. The model accounts for the dependency of S and M phase initiation on the successful completion of the previous M and S phase, respectively, as well as entry into a resting state.

The cell cycle of somatic cells is characterized by the presence of checkpoints ensuring that an earlier event is successfully finished before a later event is initiated (Hartwell & Weinert 1989). Checkpoints may employ positively or negatively acting effectors. In the latter case, they are not part of the substrate-product chains required for the initiation of the cell cycle phases. Some of the checkpoints are silent in embryonic cells, which could in principle lead to, e.g. initiation of M phase before S phase was completed. This situation, referred to as the 'completion problem' (Murray 1992), may be avoided because initiation of, e.g. M phase may require time-dependent processes to occur, such that S phase is traversed before M phase is initiated. Candidates for gene products performing negative checkpoint-functions in mammals, i.e. gene products which are not strictly required for progression through the early embryonic cell cycles, have been suggested from experiments with transgenic mice. These candidates include the retinoblastoma gene product (pRB, Lee *et al.* 1992, Jacks *et al.* 1992, Clarke *et al.* 1992) and p53 (Donehower *et al.* 1992).

Correspondence: Dr T. Stokke, Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway.

Progression of proliferating cells through the cell cycle requires cyclin-dependent protein kinase activities at several distinct points. Active cdc2 is required (for reviews, see: Murray 1989, Nurse 1990), but is, at least in *Aspergillus nidulans* (Osmani *et al.* 1991), and rodents (Lamb *et al.* 1991, Planas-Silva & Means 1992), not sufficient for M phase entry. The checkpoint defined by controlled cdc2 activation is employed by germline cells for control of meiotic maturation (Pickham *et al.* 1992), and by somatic cells to check for completion of DNA replication (Nishitani *et al.* 1991, Smythe & Newport 1992). cdc2 activity declines in anaphase as a result of cyclin B degradation and dephosphorylation of cdc2 on threonine 161 (Lorca *et al.* 1992). cdc2 activity is only required for entry into M phase and not S phase in mammalian cells (Hamaguchi *et al.* 1992, van den Heuvel & Harlow, 1993).

Mammalian cells express several protein kinases with large similarities to the M phase kinase cdc2 (termed cdk2-6 in humans; Meyerson *et al.* 1992). Some of these kinases may commit cells for S phase entry (Fang & Newport 1991, van den Heuvel & Harlow, 1993). One of the possible targets of phosphorylation by cdks in the G₁ phase of the cell cycle is pRB. pRB functions as a growth suppressor protein, acting in the G₁ phase (Goodrich *et al.* 1991, Alberts *et al.* 1993). pRB is dephosphorylated during, or immediately after, completion of the M phase (Buchkovich *et al.* 1989, Chen *et al.* 1989, Stokke *et al.* 1993, Ludlow *et al.* 1993). Type 1 serine/threonine phosphatase (PP1) is a good candidate for the pRB phosphatase (Fernandez *et al.* 1992, Alberts *et al.* 1993, Ludlow *et al.* 1993, Durfee *et al.* 1993). The underphosphorylated form of pRB binds in the nucleus during early G₁ phase, and is phosphorylated and released from the nuclear binding sites several hours before S phase entry (Stokke *et al.* 1993). cdk4 and cdk6, in association with D-type cyclins, have been suggested to be physiological pRB kinases (Matsushime *et al.* 1992, Kato *et al.* 1993, Ewen *et al.* 1993a, Ewen *et al.* 1993b, Dowdy *et al.* 1993, Bates *et al.* 1994, Lukas *et al.* 1994).

In vitro, staurosporine (SSP), inhibits the serine/threonine kinases PKC, PKA and cdc2 at nanomolar concentrations (Ruegg & Burgess 1989, Rialet & Meijer 1991, Gadbois *et al.* 1992). In spite of the apparent plethora of inhibitory effects *in vitro*, SSP has been shown to impair cell cycle progression in specific ways. Abe *et al.* (1991) found that low concentrations (≤ 10 ng/ml) of SSP arrested normal fibroblasts specifically in early G₁. Four transformed cell lines studied by Crissman *et al.* (1991) lacked this SSP-sensitive step. However, it was later shown that other transformed cell lines were blocked in the G₁ phase by low SSP concentrations (Lin *et al.* 1992a). The latter study also gave strong evidence that PKC was the target at low SSP concentrations. Normal fibroblasts arrested in G₁ by SSP contain partially dephosphorylated pRB (Schnier *et al.* 1994). An additional block in G₂ was observed in all cell types at high concentrations ($10 \text{ ng/ml} < (\text{SSP}) \leq 100 \text{ ng/ml}$; Abe *et al.* 1991, Crissman *et al.* 1991). However, leukaemic MOLT-4 cells entered M phase, but skipped cytokinesis (C phase), and resumed DNA replication as binucleated cells in the presence of high concentrations of SSP (Traganos *et al.* 1994). The SSP-induced block in G₂ can also be overridden by caffeine (Crompton *et al.* 1993), suggesting that the SSP-sensitive kinase(s) acts in checkpoint control rather than being an essential component in a chain of substrate-product reactions leading to initiation of mitosis. Suppression of DNA replication, as well as induction of apoptosis, requires SSP concentrations above 100 ng/ml (Gekeler *et al.* 1993, Meikrantz *et al.* 1994).

In this work, we have verified the existence of the previously defined kinase-mediated steps in the G₁ phase (inhibited at low SSP concentrations, target: PKC), and in the G₂ phase (high SSP concentration). Additionally, we characterize a kinase-dependent step required for progression through G₁, which is inhibited at high concentrations of SSP. The results indicate that targets at high SSP concentrations include a kinase acting at the G₂/M border, and either the pRB kinase or a kinase acting immediately upstream of the pRB kinase (in G₁). We

present a novel model for regulation of initiation of the S, M and C phases which describes the observed effects of SSP on human cell cycle progression.

MATERIALS AND METHODS

Cell culture

Normal human fibroblasts, obtained from mouth biopsies, were grown in RPMI 1640 medium (Gibco) with 10% fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine. The transformed cell lines Reh, KG1 A, MOLT-4 (leukaemias), BEX-c (melanoma), U698, Daudi (lymphomas), T47D, and MDA-MB-231 (breast carcinomas) were grown in the same medium, but with addition of 200 units/litre of insulin for T47D and MDA-MB-231. NHIK 3025 cells (cervix carcinoma) were grown in MEM medium with 10% fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine.

Stock solutions of staurosporine (SSP, Sigma) were prepared in 50% ethanol. Cell growth in the presence of different concentrations of SSP was assessed by Coulter counting.

Cell cycle distribution

All steps were done at 0 °C. Harvested cells were washed once in PBS. Cells to be analysed for cell cycle distribution were extracted in 750 µl 0.1% Nonidet P40, 150 mM NaCl, 0.5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM phosphate buffer (pH=7.4). After 5 min, cells were vortexed and 250 µl 4% paraformaldehyde was added. Cells were fixed for at least 1 h. The fixed cells were stained for DNA directly in the fixative by addition of mithramycin (Pfizer, New York, NY) and MgCl₂ to final concentrations of 50 µg/ml and 25 mM, respectively. Alternatively, cells were spun down after fixation, and stained with 12.5 µg/ml propidium iodide (Calbiochem, La Jolla, CA) in the presence of 100 µg/ml RNase A (Pharmacia, Uppsala, Sweden) in 0.1% Nonidet P40, 150 mM NaCl, 0.5 mM EDTA, 10 mM phosphate buffer (pH=7.4). The fluorescence and forward angle light scatter of mithramycin-stained cells were measured in an Argus flow cytometer (Skatron, Lier, Norway) with excitation at 405/436 nm. The simultaneous measurement of fluorescence and light scattering allows discrimination of G₁, S, G₂, and M phase cells, mitotic cells bind more dye and scatter less light than G₂ cells (Larsen *et al.* 1986). Propidium iodide stained cells were measured in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The data were gated on pulse width vs. area of the fluorescence signals to eliminate aggregates of cells.

Simultaneous analysis of bound pRB/PCNA and DNA content

All steps were done at 0 °C. Harvested cells were washed once in PBS. Detergent-extracted fixed cells were prepared by resuspending cells in 750 µl 0.1% Nonidet P40, 10 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM phosphate buffer (pH 7.4). After 5 min, cells were vortexed and 250 µl 4% paraformaldehyde was added. Cells were fixed for 1 h. Cells to be stained for PCNA were denatured in 6 M urea for 10 min. A three-layer procedure for staining of pRB or PCNA was employed; $\approx 1 \times 10^6$ cells or nuclei were resuspended in 100 µl of either 10 µg/ml purified PMG3-245 anti-pRB monoclonal antibody (Pharmingen, San Diego, CA) or 20 µg/ml 19F4 anti-PCNA monoclonal antibody (Boehringer Mannheim, Mannheim, Germany), or PBS (controls). After incubation with the first layer for 30 min, cells were washed with PBS, incubated in 100 µl biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) diluted 1/50 in PBS for 30 min, washed, and then

incubated in 100 μ l streptavidin-FITC (Amersham, Buckinghamshire, UK) diluted 1/50 in PBS for 30 min, and washed. The antibodies and the streptavidin-FITC were present in saturating amounts. The pellets were resuspended in 500 μ l PBS containing 0.1% Triton X-100, 100 μ g/ml RNase A, and 12.5 μ g/ml propidium iodide. Stained cells were analysed in a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA). The following parameters were measured: FITC fluorescence intensity, propidium iodide fluorescence pulse area (DNA content) and propidium iodide fluorescence pulse width. The data were gated on propidium iodide fluorescence area vs. width dual parameter histograms to exclude aggregates of cells.

Immunoblotting

Proteins were separated by electrophoresis in 10% SDS-polyacrylamide gels (Laemmli 1970), and blotted over to Immobilon PVDF transfer membranes (Towbin, Staehelin & Gordon 1979). The filters were stained using blotting detection kits for mouse or rabbit antibodies (Amersham, Buckinghamshire, UK) according to the manufacturers protocol. The concentrations of the primary antibodies were 0.6 μ g/ml (antiPRB, clone PMG3-245, Pharmingen, San Diego, CA), 0.125 μ g/ml (anti53, clone 1801, Oncogene Science, Uniondale, NY).

Table 1. Cell growth and cell cycle arrest after staurosporine treatment. Cell growth was considered to take place (+) if the number of cells increased as in the control cultures (50% to 100%) after 24 h. The cell numbers increased by less than 10% in the other cases (—). Cell cycle arrest was determined from flow cytometry experiments as those shown in Figures 1 and 2 after 24 h

Cell type	Concentration of staurosporine						pRB	TP53 mutation	p53
	0 ng/ml (control)		1, 3 and 10 ng/ml		30 and 100 ng/ml				
	Cell growth	Cycle arrest	Cell growth	Cycle arrest	Cell growth	Cycle arrest ¹			
Normal fibroblasts	+	—	—	G ₁	—	G ₁ G ₂	+	—	—
BEX-c	+	—	—	G ₁	—	G ₁ G ₂	+	—	—
KG1 A	+	—	+	—	—	G ₁ G ₂	+	—	—
T47D	+	—	+	—	—	G ₁ G ₂	+	+	+
Daudi	+	—	+	—	—	G ₁ G ₂	+	+	+
MDA-MB-231	+	—	+	—	—	G ₁ G ₂	+	+	+
Reh	+	—	+	—	—	G ₁ G ₂	+	—	—
U698	+	—	+	—	—	G ₂ ²	—	—	—
MOLT-4	+	—	+	—	—	G ₂ ³	+ ⁴	+	ND
NHIK 3025	+	—	+	—	+	—	—	—	—

¹See Figure 5 for the fractions of Reh, MDA-MB-231, Daudi, T47D, and KG1 A cells arrested in G₁ after 24 h treatment with 30 ng/ml SSP.

²The accumulation of U698 cells in G₂ was only transient. These cells polyploidized as mononuclear cells upon continued SSP exposure.

³The accumulation of MOLT-4 cells in G₂ was probably also transient. It has been reported that these cells polyploidize as binucleated cells upon continued SSP exposure (Traganos *et al.* 1994).

⁴The pRB in MOLT-4 cells is nonfunctional, because no G₁ cells are found with bound pRB in the nucleus in exponentially growing cultures.

Analysis for mutations in the TP53 gene

Constant denaturation gel electrophoresis was used to screen for mutations within exons 5, 6, 7, and 8 in the TP53 gene after PCR amplification with appropriate primers (Lothe *et al.* 1992). In cases where TP53 gene mutations were found, the PCR products were directly sequenced with standard dideoxy sequencing reactions.

RESULTS**Staurosporine inhibits three steps in cell cycle progression which require distinct kinase activities**

Cell growth and cell cycle distribution were measured at different times after addition of different concentrations of SSP. Normal human fibroblasts were arrested in G₁ after treatment with low concentrations of SSP (1–10 ng/ml, Table 1). Cell cycle traverse was not significantly inhibited in any other phases. We also tested several transformed cell lines for this property; highly malignant melanoma BEX-c cells (Rofstad *et al.* 1991) were arrested in G₁ at ≥ 1 ng/ml SSP (Figure 1, Table 1). None of the other cell lines demonstrated impaired cell cycle progression at ≤ 10 ng/ml, as cell growth was not inhibited (Table 1), and no changes in the cell cycle distribution were observed (Figure 1, Table 1).

At high concentrations of SSP (>10 ng/ml, ≤ 100 ng/ml), the cell number did not increase by more than 10% for normal fibroblasts and any of the cell lines, except for NHIK 3025

Figure 1. The distribution of cells in the G₀/G₁, S, and G₂/M phases after 24 h treatment with SSP at different concentrations. BEX-c, Daudi, Reh, and U698 cells were treated with 0, 10, or 30 ng/ml SSP for 24 h, and DNA content was measured by flow cytometry.

cells (Table 1). This result, and the accumulation of cells with a G_2/M phase DNA content (Figure 1), suggested the presence of another SSP-sensitive kinase-mediated step close before cytokinesis. However, U698 cells were not permanently arrested with a G_2/M DNA content, which will be discussed later. The method of Larsen *et al.* (1986), which differentiates between G_1 , S, G_2 and M phase cells, was used to determine whether the cells were arrested in G_2 or M phase. Initial experiments demonstrated the absence of M phase cells and an increase in the number of G_2 cells at times later than 3 h after SSP addition (Figure 2, Table 1). Cells arrested in metaphase by nocodazole are included in Figure 2 as a control for the specificity of the assay.

The position of the block-point in the G_2 phase could in principle be determined from the fractional increase in cell number after addition of SSP. However, the uncertainties in the cell counts obtained by Coulter counting, $\approx 5\%$ (data not shown), precluded accurate determination of the time of action of SSP in the G_2 phase. For this purpose, the kinetics of

Figure 2. Accumulation of Reh cells in the G_2 phase after treatment with SSP at high concentrations. Reh cells were treated with 0, 10, or 30 ng/ml SSP, or with 100 ng/ml nocodazole for 5, 9, or 24 h. After harvest, the cells were permeabilized, fixed with paraformaldehyde, stained with mithramycin, and measured by flow cytometry to distinguish between cells in the G_1 , S, G_2 and M phases of the cell cycle. M phase cells have more fluorescence and less light scatter than G_2 cells under these conditions. The same volume was run in each case, hence, this figure also gives an impression of changes in cell number in the cultures

the depletion of M phase cells was investigated in more detail by flow cytometry and fluorescence microscopy. A step immediately (<20 min) before entry into M phase was inhibited by high concentrations of SSP (Figure 3a, data not shown for KG1 A and Daudi). The M phase fraction disappeared more slowly than theoretically predicted if the block had been absolute and at the G₂/M border (broken lines), indicating that progression through M phase was slightly inhibited by SSP. Mitotic figures were counted for the cell line T47D which grows as monolayer cultures (Figure 3b). The fraction of cells in prophase started to decrease before the fraction of metaphase cells and eventually the fraction of anaphases/telophases (Figure 3c), confirming that the cells progressed through M phase. The data in Figure 3b were used to calculate the range of fractional decreases in transition rates between the mitotic compartments (Figure 3d), assuming that exit from telophase was not inhibited by high concentrations of SSP (closed symbols), or that entry into prophase was completely blocked (open symbols). Independent of assumptions, entry into prophase was inhibited more than the prophase/prometaphase transition. The metaphase/anaphase and the telophase/interphase transitions were impaired to a smaller degree. No binucleated cells were observed.

The transformed cells Reh, KG1 A, Daudi, MDA-MB-231 and T47D were arrested also in G₁ after 24 h of treatment with high concentrations of SSP (Figure 1, Table 1). No further change in the fraction of cells in G₁ occurred after 24 h (data not shown), demonstrating that this was a specific G₁ block, and not simply the result of a slowed progression through G₁. The SSP concentration dependence of the G₁ block was similar to that of the G₂ block in these cell lines. As the G₂ block was induced at similar SSP concentrations in all the cell types reported here, the large difference in sensitivity between the G₁ target in normal fibroblasts/BEX-c and Reh/Daudi/KG1 A/T47D/MDA-MB-231 cells cannot be explained by differences in the intracellular concentrations of SSP.

NHIK 3025 cells grown in the presence of 30 or 100 ng/ml SSP multiplied at the same rate as in control cultures, and there was no change in the cell cycle distribution after 24 h of SSP treatment (Table 1).

Staurosporine inhibits retinoblastoma protein kinase activity in G₁ at high concentrations

In addition to the impaired entry into mitosis at high concentrations of SSP, S phase entry was also inhibited in five of the eight cell lines which were not blocked at low SSP concentrations (Figures 1 & 2, Table 1). This block may also be present in normal fibroblasts and BEX-c cells, but would in these cell types be masked by the block at low concentrations of SSP. We will therefore in this section discuss only the cell types which are not inhibited at low concentrations of SSP. One notable difference between U698 and NHIK 3025 cells, which are not arrested in G₁ at high concentrations of SSP, and Reh/MDA-MB-231/Daudi/T47D/KG1 A cells, is that the former cells do not express pRB (determined by immunoblotting, data not shown). MOLT-4 cells did not arrest in G₁ at high SSP concentrations, in agreement with the results of Traganos *et al.* (1994). MOLT-4 cells expressed high levels of pRB, however, no cells in G₁ contained bound and dephosphorylated pRB in exponentially growing cultures (data not shown). Hence, pRB is functionally inactive in MOLT-4 cells, which may be due to constitutive expression of a pRB kinase, lack of pRB phosphatase activity, or expression of a mutant pRB. Assuming any, or both, of the two latter explanations to be correct, a possible explanation for the lack of a G₁ arrest in U698, NHIK 3025 and MOLT-4 cells is that the pRB kinase activity is the G₁ target at high concentrations of SSP, i.e. that the latter cells do not depend on an active pRB kinase for progression through G₁.

Figure 3. Depletion of mitotic cells after treatment with high concentrations of SSP. (a) The fraction of mitotic cells in cultures of Reh ($\times$; four experiments), U698 (Δ ; five experiments), and T47D ($\circ$; two experiments) cells treated with SSP (30 ng/ml) for different times was determined from cytograms as those shown in Figure 2 (Reh, U698; bars represent standard deviations) or by fluorescence microscopy counting (T47D; bars represent ranges). (b) The fraction of T47D cells in prophase ($\blacktriangle$; PP), prometaphase/metaphase ($\bullet$; PMP/MP), and anaphase/telophase ($\blacksquare$; AP/TP) was determined by fluorescence microscopy counting (two experiments; bars represent ranges) of at least 2000 cells per experiment. The cells were fixed with methanol in the culture flasks and stained with Hoechst 33258. The broken lines, intercepting the time axis at the length of mitosis in (a), and at the end of the respective mitotic phase in (b), show the theoretical evolution of the number of mitotic cells if a complete block of the entry into prophase had been the only effect of SSP (in G_2/M). The mitotic times (and the lengths of the different mitotic phases in T47D) were obtained from the fraction of mitotic cells and the culture doubling time taking into account the exponential distribution of the number of cells with age in the cell cycle. (c) Normalized representation of the data in (b). (d) Fractional decrease in transition rates between the different mitotic compartments in T47D cells in the presence of 30 ng/ml SSP. The transition rates between the compartments were calculated from the data in (b). Since we could not measure the cell number with high enough precision, it was assumed either that entry into prophase was completely inhibited (∇), or that exit from telophase was not inhibited at all ($\blacktriangledown$). The abscissa in (d) represents progression through mitosis in control cultures (entry into prophase defined as time 0), and not time after SSP addition (as in (a), (b) and (c)).

This hypothesis predicts that the cells expressing functional pRB should be arrested in the G₁ phase with underphosphorylated and bound pRB. This prediction was tested in Reh, KG1 A and Daudi cells employing flow cytometry to study cell cycle resolved binding of pRB in nuclei (Stokke *et al.* 1993). The cells were treated with 30 ng/ml of SSP for 0, 6, and 24 h. After 6 h of SSP treatment, there was a decrease in the number of G₁ nuclei without pRB (pRB- nuclei, Figure 4, data not shown for Daudi). After 24 h of treatment, almost all KG1 A cells and the majority of Reh cells being in G₁, i.e. the cells arrested in G₁, contained bound pRB. Thus, it appears that the pRB- G₁ cells selectively left G₁ after addition of SSP. The cells which progressed through S phase and entered G₂ during the treatment were pRB- (Figure 4, 6 h). Surprisingly, a fraction of the cells accumulating in G₂ were also observed to contain pRB in the bound form. This fraction was low at 6 h, but included the majority of cells at 24 h, showing that dephosphorylation of pRB occurs after the cells have spent some time in G₂. pRB+G₂ nuclei are not observed in exponentially growing cultures (Figure 4, Stokke *et al.* 1993). Immunoblotting revealed that pRB in Reh and Daudi cells migrated faster after 24 h incubation with 30 ng/ml SSP, but not after 4.5 h or less, confirming that time-dependent dephosphorylation occurred (data not shown).

We have shown previously that only a fraction of the G₁ cells of pRB expressing cell lines contain bound and underphosphorylated pRB; these cells are in early G₁ (Stokke *et al.* 1993). Assuming that cells with bound and underphosphorylated pRB cannot leave G₁, and that the

Figure 4. Binding of pRB in nuclei after treatment with SSP. KG1 A and Reh cells were incubated with SSP (30 ng/ml) for the indicated amount of time, fixed and stained for bound pRB as described in 'Materials and methods', and measured for pRB (abscissa) and DNA (ordinate) content by flow cytometry. The results of one experiment out of two are shown.

pRB kinase activity is the target of high concentrations of SSP, it is predicted that the fraction of cells arrested in G₁ after prolonged treatment is similar to the fraction of pRB+ cells in exponentially growing cultures. Figure 5 shows that this was the case.

Reh cells grown in low serum concentrations proliferate more slowly, and a larger fraction of the G₁ cells in such cultures contain underphosphorylated and bound pRB (Jonassen *et al.* 1994). We employed this model as an additional test of the correlation between the fraction of cells in G₁ with bound pRB and the fraction of cells arrested in G₁ after SSP treatment. Reh cells were grown for 48 h in the presence of different concentrations of fetal calf serum, whereafter the cells were treated with 30 ng/ml SSP for another 24 h. The cell cycle distributions revealed that the fraction of cells arrested in G₁ by SSP increased with decreasing serum concentration (Figure 6a). Again, the fraction of cells arrested in G₁ by SSP was equal to the fraction of pRB+ G₁ cells (Figure 6b).

Wild-type p53 is involved in arresting cells in the G₁ phase of the cell cycle (Diller *et al.* 1990, Lin *et al.* 1992b). To determine whether the p53 genotype and phenotype were

Figure 5. Correlation between the fraction of cells with bound pRB in control cultures and the fraction of cells arrested in the G₁ phase after incubation with SSP. The fraction of cells arrested in G₁ after 24 h of treatment with SSP (30 ng/ml) was determined from histograms like those shown in Figure 1 for Reh (x; five experiments), Daudi (■; four experiments), MDA-MB-231 (▲; two experiments), T47D (○; three experiments), and KG1 A (▼; four experiments). Horizontal bars represent standard deviation (>two experiments) or range (two experiments). The fraction of pRB+ nuclei was taken from Stokke *et al.* (1993). The latter reference also gives the cell cycle distributions and doubling times of these cell lines under conditions of exponential growth. The broken line was obtained by linear regression. The correlation coefficient r was 0.889.

correlated with the ability to arrest cells in G_1 after SSP treatment, we performed a screen for mutations in TP53 exons 5, 6, 7 and 8 using constant denaturation gel electrophoresis in Daudi, Reh, U698, KG1 A and NHIK 3025 cells. Such analysis revealed a mutation only in Daudi cells in exon 8. Direct sequencing showed the mutation to be in codon 266. Bartek *et al.* (1990) have reported TP53 mutations in codon 194 in T47D, and in codon 280 in MDA-MB-231. TP53 codon 248 is mutated in MOLT-4 (Rodrigues *et al.* 1990). Mutant p53s often have longer half-lives than wild-type p53; accordingly, we detected p53 by immunoblotting and cellular staining in Daudi, T47D and MDA-MB-231, but not in Reh, U698, KG1 A,

Figure 6. Correlation between the fraction of Reh cells with bound pRB and the fraction of cells arrested in the G_1 phase after incubation with SSP. (a) Accumulation of Reh cells grown in different serum concentrations in G_1 and G_2 after treatment with SSP. Reh cells were grown for 48 h in 10%, 2%, 0.5% and 0% fetal calf serum (FCS). SSP (30 ng/ml) was added, and the cells were incubated for another 24 h, harvested, stained for DNA content, and measured by flow cytometry for cell cycle distribution. (b) Correlation between the fraction of Reh cells with bound pRB (pRB+ nuclei) in control cultures grown in different serum concentrations and the fraction of cells arrested in the G_1 phase after incubation with SSP. The latter parameter was determined from histograms like those shown in Figure 6(a) for 10% serum (●), 2% serum (▼), 0.5% serum (▼), and 0% serum (□). Horizontal bars represent range (two experiments). The fraction of pRB+ nuclei was taken from Jonassen *et al.* (1994). The latter reference also gives the cell cycle distributions and doubling times of Reh cells grown in the different serum concentrations. The broken line was obtained by linear regression. The correlation coefficient r was 0.998.

Figure 6. continued

and NHIK 3025 (immunoblots and fluorescence images not shown, Table 1). We conclude that p53 genotype and phenotype are not correlated with the ability to arrest cells in G₁ after SSP treatment.

U698 cells, which do not express the retinoblastoma gene product, become polyploid in the presence of staurosporine

U698 cells were not arrested in the diploid G₁ phase even at high concentrations of SSP (Figure 1). These cells did not enter mitosis in the presence of SSP (Figure 3a), and there was no significant increase in cell number (Table 1). Preliminary experiments showed a considerable amount of cells with a DNA content above 4n after 24 h, indicating that a new round of DNA synthesis was initiated after a delay with tetraploid DNA content. The DNA content of SSP treated U698 cells was assessed for up to 72 h (Figure 7). The DNA content increased with time, and distinct peaks were successively observed at 4n, 8n, 16n, and in some experiments 32n. Hence, the cells stopped, and after a pause, resumed DNA replication after each round of duplication of the genome.

Binding of the DNA replication proteins DNA polymerase (Stokke *et al.* 1991), and proliferating cell nuclear antigen (PCNA, Stokke *et al.* 1993), are characteristics of S phase cells. PCNA was bound in all U698 cells with DNA contents between 4n and 8n, and between 8n and 16n (Figure 8a). Cells with 4n, 8n and 16n DNA content did not contain bound PCNA, demonstrating that these cells share at least this characteristic with G₂ and G₁ cells in exponentially growing cultures. In contrast, cells which were treated for 72 h with SSP, and then grown in fresh medium without the drug for 48 h showed a continuum of DNA contents from 2n both among the cells with bound PCNA (presumably replicating DNA), and those without bound PCNA (Figure 8b). This indicates that asymmetric mitotic divisions had taken place, and that S phase was initiated in spite of inappropriate segregation

of chromosomes during the previous mitosis. The cell number increased by a factor of 2 after removal of SSP, but did not increase any further (data not shown), indicating that the S phase cells in Figure 8b were not able to divide.

DISCUSSION

Specific targets of staurosporine

We show in this paper that the kinase inhibitor SSP blocks several distinct steps in cell cycle progression. Two of these have been characterized earlier. The first is a step in G₁ which requires a kinase-activity which is inhibited at low concentrations of SSP (Abe *et al.* 1991), later identified as PKC (Lin *et al.* 1992a). The requirement for this step is circumvented in many types of tumour cells (Crissman *et al.* 1991), but not in all types (Lin *et al.* 1992a, this work).

The second step is inhibited at 10-fold higher SSP concentrations than those required for inactivation of PKC *in vivo*, and is positioned in G₂ (Abe *et al.* 1991, Crissman *et al.* 1991, Lin *et al.* 1992a, this work). This kinase-activity is needed for prophase entry in most cell types

Figure 7. Polyploidization of U698 cells in the presence of SSP. U698 cells were grown in the presence of SSP (30 ng/ml) for the indicated amount of time, and DNA content was measured by flow cytometry after staining with propidium iodide. One experiment out of three is shown.

Figure 8. The sequential S phases of polyploidizing U698 cells are interrupted by pauses. (a) U698 cells were grown in the presence of SSP (30 ng/ml) for 48 h, mixed with control cells, extracted, fixed, stained for bound PCNA and DNA content, and measured by flow cytometry. Separate runs showed that only the cells treated with SSP had a DNA content above 4n. (b) U698 cells were grown for 72 h in the presence of SSP (30 ng/ml), transferred to fresh medium without SSP and grown for another 24 h. Staining was as under (a).

(Figure 3). *cdc2* was suggested as an obvious candidate for this kinase (Abe *et al.* 1991, Crissman *et al.* 1991). *cdc2* is activated at this point in the cell cycle (Norbury *et al.* 1991), its activity was required for entry into mitosis in the cells studied by Hamaguchi *et al.* (1992), and *cdc2* is sensitive to SSP *in vitro* (Rialet & Meijer 1991, Gadbois *et al.* 1992). The transition rate inhibition profile (Figure 3d) matches the proposed activity of *cdc2* at different stages of mitosis (Moreno *et al.* 1989).

Our experiments also revealed a kinase-activity required for G_1 progression in the cell lines expressing functional pRB, i.e. cells in which pRB can be dephosphorylated and bound in the nucleus. Several lines of evidence suggest that the pRB kinase activity is the target of SSP at high concentrations: (1) the early G_1 cells with underphosphorylated and bound pRB are arrested in G_1 by SSP, while the late G_1 cells with phosphorylated and unbound pRB enter S phase (Figure 4); (2) The temporal activation of the pRB kinase occurs at or close to the time in G_1 when SSP acts (Figures 5 & 6); (3) U698, MOLT-4, and NHIK 3025 cells, which lack functional pRB, are not arrested in G_1 by SSP (Figures 1 & 7, Table 1). *cdks*, at least *cdk4* and *cdk6*, are not required for initiation of S phase in cells lacking pRB (Tam *et al.* 1994). In view of the similar structures of the *cdks*, the *in vitro* sensitivity of *cdc2* to SSP, and the possible *in vivo* activity of SSP towards *cdc2*, it is possible that SSP inhibits *cdk4/6* directly, although we cannot exclude the possibility that SSP inhibits upstream activators of the pRB kinase. Since the negative regulator pRB appears to be necessary for SSP-induced G_1 arrest, we conclude that SSP does not inhibit any essential kinases which are absolutely required for initiation of S phase.

Dephosphorylation of pRB occurs in late M phase in exponentially growing cultures. Hence, a signal generated by the passage through mitosis activates the pRB phosphatase.

SSP induced delayed dephosphorylation and nuclear binding of pRB in G₂ arrested cells (Figure 4), showing that entry into mitosis is not required for activation of the pRB phosphatase. This activation is not directly caused by SSP, and not by transforming events, because we have observed dephosphorylation and binding of pRB in the G₂ phase of slowly growing primary human fibroblasts (unpublished results). It appears that pRB dephosphorylation, if not induced by passage through mitosis, can be induced by exit from S phase after a certain time delay.

It is possible that the lack of effects of SSP on certain tumour cell types is due to acquisition of, e.g. specific mutations in the kinases required for the initiation of S phase, M phase, or C phase. However, these cell types have not been grown in the continuous presence of intermediate SSP concentrations to select for 'gain of function' mutants, and a much more likely explanation is that SSP inactivates a kinase which inactivates a factor which negatively regulates the onset of S phase, M phase, or C phase (pRB in the case of S phase). These negatively acting factors may be lost or nonfunctional in tumour cells as a result of the tumorigenic process or general genetic instability.

Staurosporine inhibits kinases acting in a complex network of controls ensuring proper order of the cell cycle phases

The cell cycle is often considered to consist of a linear chain of events (S phase→M phase→C phase→S phase..., Figure 9A), where M phase must follow S phase, etc. Most somatic cells grow and divide in this way. However, the results with U698 and MOLT-4 cells clearly demonstrate that SSP may uncouple the order of the cell cycle phases in certain tumour cells. Cells are found in which the SSP-sensitive kinases are not required for transition through any phase. Hence, these kinases appear to act upstream of negative checkpoint effectors (Hartwell & Weinert 1989). We will in the discussion that follows describe why the traditional way of thinking about the cell cycle should be revised. Negative effectors blocking the entry into the S and M phases in the 'conventional cell cycle model' (Figure 9A) are appropriate for explaining the effects of SSP on the cell cycle progression of most cell types. (pRB is suggested from our experiments to be the S phase regulator, while the unknown factors Y and Z in Figure 9 regulate entry into M and C phase, respectively.) However, to explain the binucleation and the following DNA replication of MOLT-4 cells in the presence of SSP (Traganos *et al.* 1994), we need to introduce a model which is constructed in such a way that the C phase may be skipped (the 'optional C phase model', Figure 9B). The signal relieving the negatively acting factor Z, thereby allowing initiation of C phase, must be generated before entry into M phase, because cells that have entered M phase at the time of SSP addition proceed through M and C phase. But the 'optional C phase model' fails to explain the repeated S phases without intervening M and C phases in SSP-treated U698 cells (Figure 7). The 'optional M and C phase model' is therefore introduced (Figure 9C) as the simplest model which can explain the effects of SSP on all cell types reported here and in the literature (Abe *et al.* 1991, Crissman *et al.* 1991, Lin *et al.* 1992a, Traganos *et al.* 1994), assuming permanent functional inactivation of the negative S phase regulator pRB in U698, MOLT-4, and NHIK 3025 cells, of the M phase regulator Y in MOLT-4 and NHIK 3025 cells, and of the C phase regulator Z in NHIK 3025 cells.

Endoreduplication (S phase→S phase→S phase...) is a normal process occurring in, e.g. bladder of rodents (Farsund 1980). Hepatocytes transiently binucleate during polyploidizing growth (S phase→M phase→S phase→M phase→C phase, for a review, see: Brodsky & Uryvaeva 1977). Finally, in meiosis, S phase does not follow C phase (S phase→M phase→C

phase→M phase→C phase). It is therefore obvious that also the order of the cell cycle phases must be controlled in some way. This is also the case in fission yeast, where the state of the cdc2 complex controls whether the cell enters S or M phase (Broek *et al.* 1991, Hayles *et al.* 1994). The ability of SSP to uncouple the order of appearance of the cell cycle phases in some tumour cells resulting in 'binucleation' and 'endoreduplication' growth phenotypes, indicates that this network may normally, with proper external input, be employed to regulate the order of the cell cycle phases. The 'meiotic' growth phenotype, i.e. M phase→(C phase)→M phase, has not been observed after SSP treatment. Such a 'meiotic' phenotype would require inactivation of the M phase (and C phase) regulator, but not the S phase regulator, and this combination of events is probably not selected for during tumorigenesis. To also include the possibility for repeated M phases without intervening S phases, we therefore propose that the cell cycle should be described as two cycles, the S cycle and the M cycle (Figure 9D). These run independent of each other with approximately equal lengths in the 'dual cycle model' (Figure 9D), indicated by the thick, vertical arrows leading from S to S, and from M to M. We do not need to invoke a C cycle, because there are no examples where C is not preceded by M. Hence, C may optionally follow M in our model (Figure 9D). The essential features of these two cycles are their intrinsic 'clocks' and the autonomous reappearance of S and M in the absence of checkpoint controls. Embryonic cells may rely on this internal timing to solve the completion problem (Murray 1992). The terms 'G₁' and 'G₂' are not relevant in the 'dual cycle model', but we have still used these terms in this paper to describe the gaps between the M and S phases in the conventional cell cycle.

Normal cells with damaged or incompletely replicated DNA do not enter M phase (Nishitani *et al.* 1991, Smythe & Newport 1992). SSP inhibits a kinase which is downregulated after DNA damage (Crompton *et al.* 1993). NHK 3025 cells also do not arrest in G₂ after irradiation, but U698, Reh, and Daudi cells do (unpublished results). These two observations

Figure 9. The 'dual cycle model'. The approximate duration of the cell cycle phases S, M (mitosis), and C (cytokinesis) are indicated by the boxed space between the thick arrows in the vertical direction. These arrows represent time-consuming processes which eventually lead to initiation of a new cell cycle phase in the absence of checkpoint control. Processes indicated with light lines are believed to occur rapidly compared to the cycle time. Note that any positive or negative signal indicated by thin lines may still be transduced by a chain of biochemical events. Positive and negative signals end with arrows and bars, respectively. As discussed in the text, SSP inhibits kinases which relieve the effects of the negative checkpoint effectors blocking entry into S phase (pRB), M phase (Y), and C phase (Z). After successful completion of the S and M phases, signals are generated which eventually lead to relief from Y and pRB, respectively. (A) The order of the cell cycle phases is fixed in the 'conventional cell cycle model' (S→M→C→S...). This model explains the effects of SSP on normal cells and tumour cell lines like, e.g. Reh cells, which arrest in G₁ and G₂. (B) C phase may be skipped in the 'optional C phase model' (S→M→S→M). This model explains the binucleation of SSP-treated MOLT-4 cells assuming pRB and Y are nonfunctional in these cells. The negative checkpoint effector Z receives a signal which is generated by some feature of entry into M, since cells like, e.g. Reh cells do not arrest in M phase or before C phase after addition of SSP. (C) Both the M and the C phases or only the C phase may be skipped in the 'optional M and C phase model'. This model explains the endoreduplication of SSP-treated U698 cells (S→S→S→S...), since pRB is lacking in these cells. This model also explains binucleation as discussed for the 'optional C phase model'. (D) In the 'dual cycle model', the cell cycle is modelled as two independently running cycles: The S cycle and the M cycle. In the absence of checkpoints, the S and M phases are reinitiated after a certain time-lag. S phase may be skipped, which happens during meiosis (M→C→M→C). This model also explains binucleation and endoreduplication as discussed for the 'optional M and C phase model'.

suggest that the signal indicating completion of S phase and lack of DNA damage is also mediated through the M cycle regulator Y. This control is indicated as a thin line originating from the end of S phase and eventually releasing the negative effects of Y in Figure 9(A–D).

Yeast cells check for successful completion of M phase before S phase is initiated (Hoyt *et al.* 1991, Li & Murray 1991). U698 cells enter a new S phase without having passed through M phase in the presence of SSP. Moreover, after release from SSP, U698 cells go through mitosis with improper chromosome segregation resulting in nonfunctional offspring, but these initiate S phase. It is suggested that the signal indicating successful completion of M phase is mediated through the S cycle regulator pRB in human cells, which is indicated in Figure 9A–D as a thin line originating from the end of M phase and eventually releasing the negative effects of pRB.

The signals shown in Figure 9 are indicated to be of the ‘all or none’ type. This is probably an over-simplification, and we expect that many of these processes may eventually be found to be dose-dependent and possibly subject to regulation by competition. There is some evidence for dose-dependent regulation by cyclin-dependent kinase inhibitors (ckis; Nasmyth & Hunt 1993).

Binucleation, meiosis, and endoreduplication may be achieved in normal cells by manipulating the activities of pRB, Y, and Z. Since negative control of initiation of both the S and M cycles is an inherent property of the ‘dual cycle model’, establishment of a resting state is also easily envisioned.

Molecular oncology and the ‘dual cell cycle model’

Who are the actors in the network controlling the S and M cycles in the ‘dual cycle model’? Of particular interest are of course oncogene- and tumour-suppressor gene-products, since tumour cells often display aberrant cell cycle progression upon challenge with SSP. Also, endoreduplication, inaccurate DNA replication, and incorrect chromosome segregation are common events during tumorigenesis, suggesting that the network regulating the S and M cycles does not work properly in many tumour cells.

Many studies have shown that pRB is a negative regulator of S phase entry, a function which is revealed also by the experiments using SSP. In contrast to pRB, p53 does not play a role in SSP-induced G₁-arrest. This does not imply that p53 is not a part of the checkpoint network, as p53 may act upstream of the SSP-sensitive kinase. We have previously shown that expression of mutant p53 is strongly correlated with gross aneuploidy in colorectal carcinomas (DeAngelis *et al.* 1993), and bladder (unpublished data). Aneuploidy is caused by one round of endoreduplication during carcinogenesis (Giaretti 1993), indicating that p53 has input to the effectors of S cycle regulation through several steps which may also be modulated by other pathways. (The endoreduplication frequency of p53 mutants is very low, but endoreduplication occurs in all SSP-treated U698 cells.) p53 has been shown to inhibit the activity of cdks through upregulation of ckis (Xiong *et al.* 1993, Slebos *et al.* 1994), which is consistent with our observations.

MAP kinases activate a cascade of events during the activation of cells for S phase entry by growth factors, and PKC works upstream of these kinases. This may explain the requirement for PKC activity for the initiation of DNA replication in normal cells and some tumour cell lines. Any transformation-associated event abrogating the requirement for PKC in the activation of the MAP kinase pathway would lead to lack of inhibition of S phase entry at low SSP concentrations, which is observed in most tumour cell lines. Modulation of cdk

activity by signals from growth factor pathways is apparently also transduced through the ckis (Polyak *et al.* 1994, Toyoshima & Hunter 1994). Growth factor signals may therefore also be necessary for downregulation of ckis and activation of the pRB kinase, in addition to the signal indicating successful completion of M phase.

ACKNOWLEDGEMENTS

Anne Lise Børresen kindly analysed the TP53 gene for mutations. Trond Stokke received support from the Norwegian Cancer Society.

REFERENCES

- ABE K, YOSHIDA M, USUI T, HORINOCHI S, BEPPU T. (1991) Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. *Exp. Cell Res.* **192**, 122.
- ALBERTS AS, THORBURN AM, SHENOLIKAR S, MUMBY MC, FERAMISCO JR. (1993) Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proc. Natl. Acad. Sci. USA* **90**, 388.
- BARTEK J, IGGO R, GANNON J, LANE DP. (1990) Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* **5**, 893.
- BATES S, PARRY D, BONETTA L, VOUSDEN K, DICKSON C, PETERS G. (1994) Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. *Oncogene* **9**, 1633.
- BRODSKA WY, URYVAEVA IV. (1977) Cell polyploidy: Its relation to tissue growth and function. *Int. Rev. Cytol.* **50**, 275.
- BROEK D, BARTLETT R, CRAWFORD K, NURSE P. (1991) Involvement of p34^{cdc2} in establishing the dependency of S phase on mitosis. *Nature* **349**, 388.
- BUCHKOVICH K, DUFFY LA, HARLOW E. (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58**, 1097.
- CHEN P-L, SCULLY P, SHEW J-Y, WANG JYJ, LEE W-H. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**, 1193.
- CLARKE AR, MAANDAG ER, VAN ROON M *et al.* (1992) Requirement for a functional Rb-1 gene in murine development. *Nature* **359**, 328.
- CRISMAN HA, GADBOIS DM, TOBEY RA, MORTON BRADBURY E. (1991) Transformed mammalian cells are deficient in kinase-mediated control of progression through the G₁ phase of the cell cycle. *Proc. Natl. Acad. Sci. USA* **88**, 7580.
- CROMPTON NEA, HAIN J, JAUSSE R, BURKART W. (1993) Staurosporine- and radiation-induced G₂-phase cell cycle blocks are equally released by caffeine. *Radiation Res.* **135**, 372.
- DEANGELIS P, STOKKE T, SMEDSHAMMER L *et al.* (1993) p53 expression is associated with a high degree of tumor DNA aneuploidy and incidence of p53 gene mutation, and is localized to the aneuploid component in colorectal carcinomas. *Int. J. Oncology* **3**, 305.
- DILLER L, KASSEL J, NELSON CE *et al.* (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* **10**, 5772.
- DONEHOWER LA, HARVEY M, SLAGLE BL *et al.* (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215.
- DOWDY SF, HINDS PW, LOUIE K, REED SI, ARNOLD A, WEINBERG RA. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**, 499.
- DURFEE T, BECHERER K, CHEN P-L *et al.* (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7**, 555.
- EWEN ME, SLUSS HK, SHERR CJ, MATSUSHIME H, KATO J-Y, LIVINGSTON DM. (1993a) Functional interactions of the retinoblastoma protein with mammalian d-type cyclins. *Cell* **73**, 487.
- EWEN ME, SLUSS HK, WHITEHOUSE LL, LIVINGSTON DM. (1993b) TGF β inhibition of cdk4 synthesis is linked to cell cycle arrest. *Cell* **74**, 1009.
- FANG F, NEWPORT JW. (1991) Evidence that the G₁-S and the G₂-M transitions are controlled by different cdc2 proteins in higher eucaryotes. *Cell* **66**, 731.

- FARSUND T. (1980) Mechanisms of proliferation and polyploidy formation in normal and regenerating mouse urinary bladder epithelium. In: Lærum OD, Lindmo T, Thorud E, eds. *Flow cytometry IV*. Oslo, Norway: Universitetsforlaget, 367.
- FERNANDEZ A, BRAUTIGAN DL, LAMB NJC. (1992) Protein phosphatase type 1 in mammalian cell mitosis: Chromosomal localization and involvement in mitotic exit. *J. Cell Biol.* **116**, 1421.
- GADBOIS DM, HAMAGUCHI JR, SWANK RA, BRADBURY EM. (1992) Staurosporine is a potent inhibitor of p34^{cdc2} and p34^{cdc2}-like kinases. *Biochem. Biophys. Res. Commun.* **184**, 80.
- GEKELER V, WILISCH A, PROBST G *et al.* (1993) Staurosporine suppresses replicon initiation in mammalian cells. *FEBS* **327**, 150.
- GIARETTI W. (1993) A model on the origin and evolution of DNA aneuploidy. *Int. J. Oncology* **2**, 165.
- GOODRICH DW, WANG NP, QIAN Y-W, LEE EY-HP, LEE W-H. (1991) The retinoblastoma gene product regulates progression through the G₁ phase of the cell cycle. *Cell* **67**, 293.
- HAMAGUCHI JR, TOBEY RA, PINES J, CRISSMAN HA, HUNTER T, MORTON BRADBURY E. (1992) Requirement for p34^{cdc2} kinase is restricted to mitosis in the mammalian cdc2 mutant FT210. *J. Cell Biol.* **117**, 1041.
- HARTWELL LH, WEINERT TA. (1989) Checkpoints: Controls that ensure the order of cell cycle events. *Science* **246**, 629.
- HAYLES J, FISHER D, WOOLARD A, NURSE P. (1994) Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34^{cdc2}-mitotic B cyclin complex. *Cell* **78**, 813.
- VAN DEN HEUVEL S, HARLOW E. (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**, 2050.
- HOYT MA, TOTIS L, ROBERTS BT. (1991) *S. cerevisia* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**, 507.
- JACKS T, FAZELI A, SCHMITT EM, BRONSON RT, GOODELL MA, WEINBERG RA. (1992) Effects of an Rb mutation in the mouse. *Nature* **359**, 295.
- JONASSEN TS, SEGLEN PO, STOKKE T. (1994) The fraction of cells in G₁ with bound retinoblastoma protein increases with the duration of the cell cycle. *Cell Prolif.* **27**, 95.
- KATO J-Y, MATSUSHIME H, HIEBERT SW, EWEN ME, SHERR CJ. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin d- dependent kinase CDK4. *Genes Dev.* **7**, 331.
- LAEMMLI UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680.
- LAMB NJC, CAVADORE J-C, LABBE J-C, MAURER RA, FERNANDEZ A. (1991) Inhibition of cAMP-dependent protein kinase plays a role in the induction of mitosis and nuclear envelope breakdown in mammalian cells. *EMBO J.* **10**, 1523.
- LARSEN JK, MUNCH-PETERSEN B, CHRISTIANSEN J, JØRGENSEN K. (1986) Flow cytometric discrimination of mitotic cells: Resolution of M, as well as G₁, S, and G₂ phase nuclei with mithramycin, propidium iodide, and ethidium bromide after fixation with formaldehyde. *Cytometry* **7**, 54.
- LEE EY-HP, CHANG C-Y, HU N *et al.* (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**, 288.
- LI R, MURRAY AW. (1991) Feedback control of mitosis in budding yeast. *Cell* **66**, 519.
- LIN Y, CHREST FJ, GABRIELSON EW. (1992a) Reversible G₁ arrest of a human lung epithelial cell line by staurosporine. *J. Cell. Phys.* **152**, 646.
- LIN D, SHIELDS MT, ULLRICH SJ, APPELLA E, MERCER WE. (1992b) Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G₁ phase. *Proc. Natl. Acad. Sci. USA* **89**, 9210.
- LORCA T, LABBE J-C, DEVAULT A *et al.* (1992) Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. *EMBO J.* **11**, 2381.
- LOTHE RA, FOSSLI T, DANIELSEN HE *et al.* (1992) Molecular genetic studies of tumor suppressor gene regions on chromosome 13 and 17 in colorectal tumors. *J. Natl. Cancer Inst.* **84**, 1100.
- LUDLOW JW, GLENDENING CL, LIVINGSTON DM, DECAPRIO JA. (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol. Cell. Biol.* **13**, 367.
- LUKAS J, MULLER H, BARTKOVA J *et al.* (1994) DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G₁. *J. Cell Biol.* **125**, 625.

- MATSUSHIME H, EWEN ME, STROM DK *et al.* (1992) Identification and properties of an atypical catalytic subunit (p34^{PSK-J3}/cdk4) for mammalian D type G1 cyclins. *Cell* **73**, 323.
- MEIKRANTZ W, GISSELBRECHT S, TAM SW, SCHLEGEL R. (1994) Activation of cyclin A-dependent protein kinases during apoptosis. *Proc. Natl. Acad. Sci. USA* **91**, 3754.
- MEYERSON M, ENDERS GH, WU C-L *et al.* (1992) A family of human cdc2-related kinases. *EMBO J.* **11**, 2909.
- MORENO S, HAYLES J, NURSE P. (1989) Regulation of p34^{cdc2} protein kinase during mitosis. *Cell* **58**, 361.
- MURRAY AW. (1989) The cell cycle as a cdc2 cycle. *Nature* **342**, 14.
- MURRAY AW. (1992) Creative blocks: Cell-cycle checkpoints and feedback controls. *Nature* **359**, 599.
- NASMYTH K, HUNT T. (1993) Dams and sluices. *Nature* **366**, 634.
- NISHITANI H, OHTSUBO M, YAMASHITA K *et al.* (1991) Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. *EMBO J.* **10**, 1555.
- NORBURY C, BLOW J, NURSE P. (1991) Regulatory phosphorylation of the p34^{cdc2} protein kinase in vertebrates. *EMBO J.* **10**, 3321.
- NURSE P. (1990) Universal control mechanism regulating onset of M phase. *Nature* **344**, 503.
- OSMANI AH, MCGUIRE SL, OSMANI SA. (1991) Parallel activation of the NIMA and p34^{cdc2} cell cycle-regulated protein kinases is required to initiate mitosis in *A. nidulans*. *Cell* **67**, 283.
- PICKHAM KM, MEYER AN, LI J, DONOGHUE DJ. (1992) Requirement of mos^{Xc} protein kinase for meiotic maturation of *Xenopus* oocytes induced by a cdc2 mutant lacking regulatory phosphorylation sites. *Mol. Cell. Biol.* **12**, 3192.
- PLANAS-SILVA MD, MEANS AR. (1992) Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to arrest of the cell cycle in G₂. *EMBO J.* **11**, 507.
- POLYAK K, LEE M-H, ERDJUMENT-BROMAGE H *et al.* (1994) Cloning of p27^{kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59.
- RIALET V, MEIJER L. (1991) A new screening test for antimitotic compounds using the universal M phase-specific protein kinase, p34^{cdc2}/cyclin B^{cdc13}, affinity-immobilized on p13^{sac1}-coated microtitration plates. *Anticancer Res.* **11**, 1581.
- RODRIGUES NR, ROWAN A, SMITH MEF *et al.* (1990) p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. USA* **87**, 7555.
- ROFSTAD EK, WAHL A, HYSTAD ME, NESLAND JM, STOKKE T. (1991) Establishment in monolayer culture and characterization of four human melanoma cell lines. *Virchows Archiv. B. Cell Pathol.* **60**, 189.
- RUEGG UT, BURGESS GM. (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol. Sci.* **10**, 218.
- SCHNIER JB, GADBOIS DM, NISHI K, MORTON BRADBURY E. (1994) The kinase inhibitor staurosporine induces G1 arrest at two points: Effect on retinoblastoma protein phosphorylation and cyclin-dependent kinase 2 in normal and transformed cells. *Cancer Res.* **54**, 5959.
- SLEBOS RJC, LEE MH, PLUNKETT BS *et al.* (1994) p53-dependent G₁ arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 protein. *Proc. Natl. Acad. Sci. USA* **91**, 5320.
- SMYTHE C, NEWPORT JW. (1992) Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates p34^{cdc2}. *Cell* **68**, 787.
- STOKKE T, ERIKSTEIN BK, HOLTE H, FUNDERUD S, STEEN HB. (1991) Cell cycle-specific expression and nuclear binding of DNA polymerase α . *Mol. Cell. Biol.* **11**, 3384.
- STOKKE T, ERIKSTEIN BK, SMEDSHAMMER L, BOYE E, STEEN HB. (1993) The retinoblastoma gene product is bound in the nucleus in early G₁ phase. *Exp. Cell Res.* **204**, 147.
- TAM SW, THEODORAS AM, SHAY JW, DRAETTA GF, PAGANO M. (1994) Differential expression and regulation of cyclin D1 protein in normal and tumor human cells: association with Cdk4 is required for cyclin D1 function in G₁ progression. *Oncogene* **9**, 2663.
- TOWBIN H, STAEGELIN T, GORDON J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350.
- TOYOSHIMA H, HUNTER T. (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67.

- TRAGANOS F, GONG J, ARDELT B, DARZYNKIEWICZ Z. (1994) Effect of staurosporine on MOLT-4 cell progression through G₂ and on cytokinesis. *J. Cell. Phys.* **158**, 535.
- XIONG Y, HANNON GJ, ZHANG H, CASSO D, KOBAYASHI R, BEACH D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701.