

Uncoupled Cell Cycle without Mitosis Induced by a Protein Kinase Inhibitor, K-252a

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Abstract. The staurosporine analogues, K-252a and RK-286C, were found to cause DNA re-replication in rat diploid fibroblasts (3Y1) without an intervening mitosis, producing tetraploid cells. Analysis of cells synchronized in early S phase in the presence of K-252a revealed that initiation of the second S phase required a lag period of 8 h after completion of the previous S phase. Reinitiation of DNA synthesis was inhibited by cycloheximide, actinomycin D, and serum deprivation, but not by Colcemid, suggesting that a functional G1 phase dependent on de novo synthesis of protein and RNA is essential for entry into the next S phase. In a *src*-transformed 3Y1 cell line, as well as other cell lines, giant cells containing polyploid nuclei with DNA contents of 16C to 32C were produced by con-

tinuous treatment with K-252a, indicating that the agent induced several rounds of the incomplete cell cycle without mitosis. Although the effective concentration of K-252a did not cause significant inhibition of affinity-purified p34^{cdc2} protein kinase activity in vitro, in vivo the full activation of p34^{cdc2} kinase during the G2/M was blocked by K-252a. On the other hand, the cyclic fluctuation of partially activated p34^{cdc2} kinase activity peaking in S phase still continued. These results suggest that a putative protein kinase(s) sensitive to K-252a plays an important role in the mechanism for preventing over-replication after completion of previous DNA synthesis. They also suggest that a periodic activation of p34^{cdc2} is required for S phases in the cell cycle without mitosis.

Two major events in the normal cell cycle, nuclear DNA replication in S phase and nuclear division in M phase, are tightly coupled with each other. Completion of one is an essential requirement for initiation of the other (Hartwell et al., 1974; Hartwell, 1978). For example, mitosis is dependent on the completion of S phase; blocking DNA replication prevents the initiation of mitosis in somatic cells. The molecular mechanism of this coupling has been studied using early embryos (Dasso and Newport, 1990), several mutant cell lines (Nishimoto et al., 1978; Osmani et al., 1988; Weinert and Hartwell, 1988; Enoch and Nurse, 1990), and drugs which cause premature mitosis in the absence of chromosome replication (Schlegel and Pardee, 1986; Schlegel et al., 1990).

DNA replication is reciprocally dependent on previous mitosis and is controlled so as to occur precisely once in each cell cycle. An important clue to understanding the mechanism underlying this regulation was provided by Rao and Johnson (1970), who fused G2-phase cells with S-phase cells and demonstrated that a nondiffusible factor is responsible for blocking DNA re-replication in the G2 nucleus. Recently, Blow and Laskey (1988) proposed a model based on observations of chromosome replication in *Xenopus* egg extracts. They assumed that an essential factor for DNA re-replication is supplied from the cytoplasm to the chromosomes through breakdown of the nuclear envelope in mito-

sis. However, very little is known about the molecular basis of DNA re-replication control because of the paucity of suitable mutants or drugs for induction of chromosome replication without mitosis.

We have demonstrated re-replication of chromosomes uncoupled from mitosis using trichostatin A and leptomycin B, which cause specific arrest of the cell cycle at both G1 and G2 phases (Yoshida and Beppu, 1988; Yoshida et al., 1990b). Cultured rat fibroblasts arrested by these agents at G2 phase were directly introduced into G0 phase. When the agents were removed, most of the arrested cells reinitiated their growth through G1-S, producing proliferative tetraploid cells. Thus release from arrest of the cell cycle at some stage of G2 phase may induce a single round of DNA re-replication without mitosis. Recently, Broek et al. (1991) reported similar re-replication in G2-arrested *cdc2*^{ts} mutants of fission yeast after heat treatment.

On the other hand, numerous studies on the regulation of the eukaryotic cell cycle have indicated the importance of a variety of protein kinases in controlling the G0/G1 (reviewed by Hunter and Cooper, 1985; Nishizuka, 1984), G1/S (reviewed by Pardee, 1989), and G2/M transitions (reviewed by Nurse, 1990), respectively. Our recent study (Abe et al., 1991) using staurosporine, a potent inhibitor of protein kinase C (Tamaoki et al., 1986) and other protein kinases (Davis et al., 1989), also suggested the existence of essential

protein kinases acting on the G0/G1 and G2/M transitions. To obtain further clues to the role of protein kinases in cell cycle regulation, we tested the effect of several protein kinase inhibitors with different inhibitory specificities. The two staurosporine analogues, K-252a (Kase et al., 1986; Kase et al., 1987) and RK-286C (Osada et al., 1990), were found to induce another type of DNA re-replication in various cultured cell lines. In contrast to the tetraploidy induced by trichostatin A or leptomycin B, successive DNA replication cycles uncoupled from mitosis proceeded in the presence of the staurosporine analogues, and longer incubation with the agents produced highly polyploid cells with a single giant nucleus. During these processes, the periodicity of DNA replication was still maintained. This paper deals with the DNA re-replication induced by K-252a and RK-286C, which is assumed to represent a cell cycle without mitosis. Involvement of p34^{cdc2} protein kinase (Lee and Nurse, 1988; Nurse, 1990) and some other protein kinase(s) in coupling the progression of S and M phases is also discussed.

Materials and Methods

Materials

K-252a, K-252b, and staurosporine were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). RK-286C was isolated from culture broth of *Streptomyces* sp. RK-286 as described previously (Osada et al., 1990). Sangivamycin was obtained from a *Streptomyces* strain as described previously (Osada et al., 1989). H7 and genistein were obtained from Seikagaku Kogyo Co., Ltd. (Tokyo) and Funakoshi Pharmaceutical Co., Ltd. (Tokyo), respectively. Cycloheximide, actinomycin D, and Colcemid were purchased from Sigma Chemical Co. (St. Louis, MO). [methyl-³H]Thymidine (85 Ci/mmol) and [γ -³²P]ATP (5,000 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). All the other chemicals were of reagent grade.

Cells and Cell Synchronization

SR-3Y1, HR-3Y1 (Zaitu et al., 1988) and BHK21 were supplied by JCRB Cell Bank. The cells were cultured in a humidified atmosphere of 5% CO₂, 95% air at 37°C in ES medium supplemented with 2% calf serum for FM3A or MEM supplemented with 12% FBS for the other cell lines. The rat embryonic fibroblast cell line 3Y1-B (Kimura et al., 1975) was grown and synchronized at early S phase as described previously (Yoshida and Beppu, 1988). Briefly, resting 3Y1 cells grown to confluence in MEM supplemented with 12% FBS were transferred to medium containing 0.5% serum but lacking glutamine. Most of the cells were introduced into G0 phase after 36 h of incubation in this medium. To obtain synchronization at early S phase, the G0 cells were trypsinized and seeded at 2×10^5 cells/5 ml per 60-mm dish (for flow cytometry), 5×10^4 cells/0.5 ml per 16-mm well (for DNA synthesis), or 5×10^5 cells/10 ml per 100-mm dish (for kinase assay) in MEM supplemented with 12% serum, and then cultivated for 10 h without drugs and for a further 14 h with 1 mM hydroxyurea (Sigma Chemical Co.).

Flow Cytometry

Flow cytometry was used to analyze the distribution of DNA content in the cell populations. To avoid confusing binucleated diploid G2 cells with tetraploid G2 cells, measurements were performed with isolated nuclei stained with propidium iodide (Sigma Chemical Co.) according to the method of Noguchi and Browne (1978). Total fluorescence intensities were determined by quantitative flow cytometry using an Epics C System (Coulter Electronics Inc., Hialeah, FL) equipped with a 5-W argon-ion laser operated at 200 mW at a wavelength of 488 nm. For highly polyploid nuclei, the fluorescence intensity was determined on a log scale (see Fig. 6) instead of a linear scale (see Figs. 2, 3, and 5).

Assay of DNA Synthesis Activity

Cells were incubated in medium containing 1 μ Ci/ml [methyl-³H]thymi-

dine for 1 h. After washing with cold PBS, the cells were lysed with 0.2 ml of 1% SDS for 15 min at room temperature. The lysate was mixed with 1 ml of 10% TCA. The precipitates obtained by TCA treatment at 4°C for 1 h were collected on glass fiber filters, and the levels of radioactivity on the dried filters were measured using a liquid scintillation spectrometer. Data were expressed as means of quadruplicate assays.

Preparation of Cell Extracts

After starting the synchronous cultivation, the cells were harvested intermittently using a rubber policeman. After washing with PBS, the cells were homogenized in 100 μ l of homogenization buffer (60 mM β -glycerophosphate, 15 mM nitrophenylphosphate, 25 mM MOPS, pH 7.2, 15 mM EGTA, 15 mM MgCl₂, 2 mM DTT, 0.1 mM vanadate, 0.1 mM NaF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml soybean trypsin inhibitor, 100 μ M benzamide). The homogenates were centrifuged for 5 min at 4°C (full speed in an Eppendorf-type centrifuge) Kubota Co., Ltd. (Tokyo), then the supernatant was recovered and immediately loaded on p13^{suc1}-Sepharose beads.

p34^{cdc2} Kinase Assay

p34^{cdc2} kinase activity was assayed according to the method of Arion et al. (1988) using p13^{suc1}-Sepharose beads. p13 is the 13-kD product of the fission yeast *suc1* gene (Hindley et al., 1987), which was identified as a plasmid-born sequence that rescues some temperature-sensitive *cdc2* mutants (Hayles et al., 1986). This small peptide has been shown to be a subunit of the *cdc2* protein kinase in both yeast (Brizuela et al., 1987) and human cells (Draetta et al., 1987; Draetta and Beach, 1988). p13^{suc1}-bound Sepharose can be used as a reagent for rapid extraction of p34^{cdc2} and assay for its kinase activity since it absorbs not only p34^{cdc2} but also p34^{cdc2}-associated proteins such as cyclins as the fully active complex (Poudaven et al., 1990). The p13^{suc1}-Sepharose beads were kept at 4°C as a 20% (vol/vol) suspension in bead buffer (50 mM Tris, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml soybean trypsin inhibitor, and 100 μ g/ml benzamide). Just before use, 5 μ l of packed p13^{suc1} beads was washed with 0.5 ml of bead buffer and resuspended in 50 μ l of bead buffer. The supernatant of the cell extract (50 μ l) was added to the beads, and the mixture was stirred with constant rotation at 4°C for 30 min. The supernatant was removed by brief centrifugation at 10,000 g, and the beads were washed three times with 0.5 ml of bead buffer. The p34^{cdc2}-bound beads thus obtained were used immediately for determination of p34^{cdc2} kinase activity. All kinase assays contained 500 nM synthetic peptide with a structure corresponding to the inhibitory site of cAMP-dependent protein kinase inhibitor (Sigma Chemical Co.), 1 mg/ml histone H1 (type III-S, Sigma Chemical Co.) 1 μ Ci [γ -³²P]ATP, and the p13^{suc1}-Sepharose in a final volume of 25 μ l of assay buffer (60 mM β -glycerophosphate, 30 mM nitrophenylphosphate, 25 mM MOPS, pH 7.0, 5 mM EGTA, 15 mM MgCl₂, 1 mM DTT). Kinase reaction mixtures were incubated at 30°C for 10 min and the reaction was terminated by adding 8 μ l of PAGE sample buffer and boiling for 3 min. The H1 kinase activity was quantified by autoradiography after SDS-PAGE.

Results

Effect of Protein Kinase Inhibitors on the Cell Cycle of Rat Diploid Fibroblast Cells

We first tested the effects of various protein kinase inhibitors on the distribution of cellular DNA contents in exponentially growing rat 3Y1 fibroblasts using quantitative flow cytometry. The results obtained 24 h after addition of the agents are summarized in Table I. Sangivamycin and H7, both reported to be inhibitors of protein kinase C (Loomis and Bell, 1988; Hidaka et al., 1984) caused arrest at G1. Genistein, an inhibitor of tyrosine-specific protein kinases (Akiyama et al., 1987), caused rather nonspecific arrest, but partial accumulation of cells in G2 phase. As we showed previously (Abe et al., 1991), a low concentration of staurosporine (Fig. 1) blocked the cell cycle at G1 phase, whereas a higher concentration of the drug caused G2 arrest (Table I and Fig. 2, A

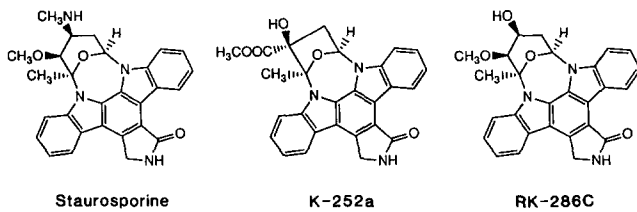


Figure 1. Structures of staurosporine, K-252a, and RK-286C.

and B). In contrast, the two indolocarbazole derivatives closely resembling staurosporine, K-252a and RK-286C (Fig. 1), characteristically induced the formation of tetraploid cells with an 8C DNA content as well as accumulation of G2 cells with a 4C DNA content (Table I and Fig. 2 C). The possibility that the 8C cells were binucleated 4C cells was ruled out, since flow cytometry was performed with isolated nuclei obtained by treating the cells with NP-40. The absence of binucleated cells was also confirmed microscopically. When K-252a was removed from the culture by washing, cell growth resumed and proliferative tetraploid clones with a DNA content of 4C to 8C (Fig. 2 E) were easily isolated. The effective concentration range of K-252a for producing the tetraploid cells was 0.2–2.0 μM , whereas concentrations $>5 \mu\text{M}$ caused G2 arrest (data not shown).

Effect of K-252a on Cells Synchronized in Early S Phase

To analyze how diploid cells were converted to tetraploid cells in the presence of K-252a, we examined the process in cultures of 3Y1 cells previously synchronized at early S phase with hydroxyurea. Fig. 3 A shows the distribution patterns of cellular DNA content in the control culture. After removal of the hydroxyurea, transition of the peak from 2C to 4C was observed within 6 h, indicating initiation of the cell cycle from early S to G2. Reappearance of the 2C peak later than 6 h indicated further progression of the cycle from G2/M to G1. Finally, the cells in the next S phase appeared after 12 h. The presence of 1 μM K-252a in the synchronous culture slightly delayed, but did not inhibit, the initial progression from early S to G2 (Fig. 3 B). The process from G2/M to G1 was completely blocked, and a second unusual transition of the DNA peak from 4C to 8C was observed after 12 h in the presence of K-252a. Analysis of DNA synthesis activity assayed by pulse incorporation of [^3H]thymidine indicated that the distinct second round of DNA synthesis started at 14 h (Fig. 4 A). During these processes, no mitotic cell or chromosome condensation was evident microscopically (Fig. 4 B). These results strongly suggested that K-252a did not inhibit either the initial or second round of DNA synthesis, but interfered with entry of the G2 cells into mitosis. As a consequence, we may assume that the cells were converted to proliferative tetraploid cells. However, it also seemed possible that asymmetric segregation of chromosome during mitosis might have produced both tetraploid and anucleated cells as progeny, as observed in fission yeast *cut* mutants (Hirano et al., 1986). To check whether such a mitotic process is necessary for tetraploid formation by K-252a, we added Colcemid to the above synchronous culture in the presence of K-252a. As shown in Fig. 5 B, Colcemid did not block the formation of tetraploid cells, indicating that mi-

Table I. Distribution of DNA Content in Asynchronous Culture of 3Y1 Cells Treated with Various Protein Kinase Inhibitors

Inhibitor	Concentration μM	Percent cell population				Relative cell number %
		2C	2-4C	4C	>4C	
None	—	56	23	21	0	267
H7	100.0	73	9	18	0	91
Sanguivamycin	3.2	76	8	16	0	177
Genistein	37.0	47	18	35	0	101
Staurosporine	0.2	14	2	79	5	69
	0.02	87	5	8	0	168
K-252a	1.0	2	4	32	62	126
K-252b	20.0	64	18	18	0	213
RK-286C	0.5	3	5	42	50	64

Exponentially growing 3Y1 cells were treated with various protein kinase inhibitors for 24 h and the distribution of DNA content and relative cell numbers (cell number at 24 h per initial cell number at 0 h \times 100) were determined.

toxis was not required for the tetraploidization. In addition, no mitotic cells were observed after Colcemid addition, indicating that K-252a itself inhibited entry into mitosis (data not shown).

In the normal somatic cell cycle, progression of G1 phase depends on the presence of growth factors and is fairly sensitive to inhibitors of both RNA and protein synthesis. During tetraploid induction by K-252a, there was an interval of ~ 8 h between completion of the first DNA replication and initiation of the second S phase (Fig. 4). To examine whether this interval corresponds to the normal G1 phase, we analyzed the effect of serum deprivation on the formation of tetraploid cells. Cells released from hydroxyurea arrest were exposed to serum-free medium in the presence or absence of K-252a. In the control cultures without K-252a, most of the cells were introduced into G0/G1 phase with a 2C DNA content; through S, G2, and M phases, within 24 h (without K-252a; Fig. 5 C). On the other hand, the presence of K-252a arrested the cells in a 4C DNA state and inhibited the appearance of cells with an 8C DNA content (with K-252a; Fig. 5 C). Readdition of serum to the K-252a-treated culture caused reinitiation of S phase without passage through mitosis (data not shown).

The effects of metabolic inhibitors on tetraploidy formation were also examined using actinomycin D and cycloheximide. The minimal effective concentrations of actinomycin D and cycloheximide for G1 arrest were determined to be 0.01 $\mu\text{g}/\text{ml}$ and 0.2 $\mu\text{g}/\text{ml}$, respectively (Fig. 5 D and Fig. 5 E [without K-252a]). Each drug was then added with K-252a to the early S-synchronous culture at 0 h. As shown in Fig. 5 D and E, both drugs failed to inhibit the first DNA synthesis but prevented initiation of the subsequent second replication in the presence of K-252a. All these results indicate that a functional G1 phase, which is dependent on serum factors and RNA/protein synthesis, intervenes between the two rounds of DNA replication seen in the presence of K-252a.

Induction of Polyploid Giant Cells by K-252a

The above results indicate that K-252a induces an incomplete cell cycle composed of S, G2, and G1. If such a cycle is

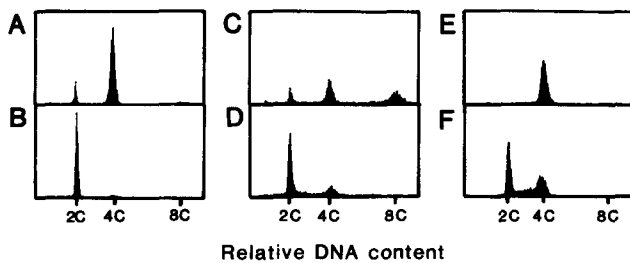


Figure 2. Formation of proliferative tetraploid cells during treatment with K-252a. Asynchronous 3Y1 cultures were treated with staurosporine (A and B) or K-252a (C and D) at various concentrations for 24 h. The treated cells were collected and the distribution patterns of DNA content in their isolated nuclei were determined by flow cytometry. (A) 0.2 μ M staurosporine. (B) 0.02 μ M staurosporine. (C) 2 μ M K-252a. (D) 0.2 μ M K-252a. (E) Confluent grown clonal tetraploid cells isolated from the culture shown in C. (F) Control diploid 3Y1 cells.

repeated during the treatment, higher polyploid cells will be formed. Although prolonged treatment of 3Y1 cells with K-252a did not produce cells with a DNA content higher than 8C, highly polyploid cells were produced by various other cell lines. These cell lines were: SV-3Y1, an SV40-transformed 3Y1 cell line; SR-3Y1, an RSV-transformed 3Y1 cell line; HR-3Y1, a v-H-ras-transformed 3Y1 cell line; Balb/c-3T3, an embryonic fibroblast cell line from Balb/c mouse; BHK21, a fibroblast cell line from the kidney of a Syrian hamster; HeLa-S3, an epithelium-like carcinoma cell line from the human cervix; MEL, a murine Friend erythroleukemia cell line; and FM3A, a murine mammary gland tumor cell line. Exponentially growing cells in random culture were challenged with 1 μ M K-252a and then cultivated for 48 h. The distribution profiles of cellular DNA contents were determined by flow cytometry (Table II). As expected, some cell lines, especially SR-3Y1, produced fairly large numbers of cells with extremely high DNA contents of more than 16C. Cytofluorograms obtained from cultures of SR-3Y1 treated with K-252a are shown in Fig. 6. Five sharp peaks corresponding to 2C, 4C, 8C, 16C, and 32C were observed

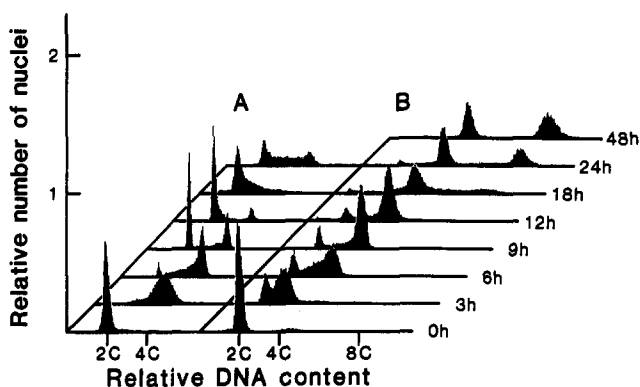


Figure 3. Effect of K-252a on distribution of DNA content in synchronous cultures released from hydroxyurea block. Early S-synchronous cultures were obtained by treatment with 1 mM hydroxyurea as described in Materials and Methods, and the cells were released and exposed to normal growth medium (A), or medium containing 1 μ M K-252a (B). At the indicated times, cells were collected and their isolated nuclei were analyzed by flow cytometry.

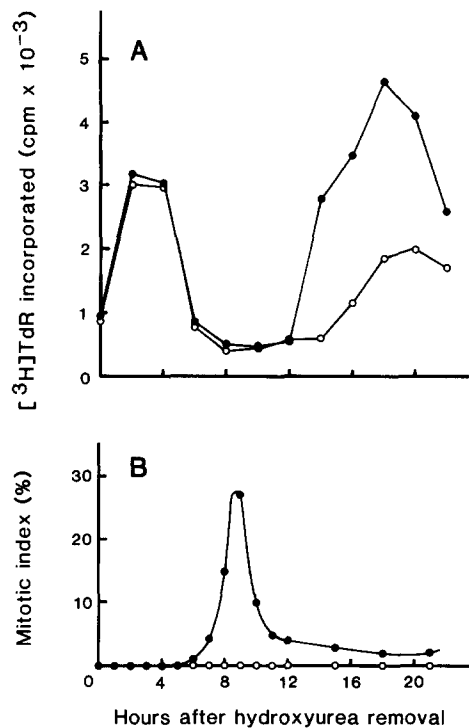


Figure 4. Effect of K-252a on DNA synthesis activity and entry into mitosis in early S-starting synchronous culture. Early S-synchronous cultures were released by washing away the hydroxyurea, and then exposed to growth medium (●) or medium containing 1 μ M K-252a (○). At the indicated times, incorporation of [3 H]thymidine into the acid-insoluble fraction for 1 h (A) and mitotic indices (B) were determined.

in the SR-3Y1 culture treated for 48 h. 16C was the main peak, suggesting that at least three rounds of DNA re-replication occurred without mitosis. The sharp profiles of all the peaks implies that a considerably long gap phase is present in each replication cycle. These cells with extremely high DNA contents were distinctly larger than the control cells

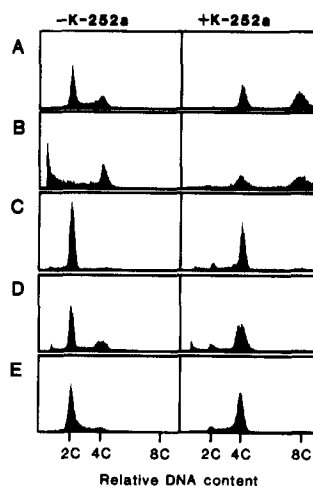


Figure 5. Effects of culture conditions on K-252a-induced tetraploidization. Early S-starting synchronous cultures were exposed to growth medium (A) or several growth inhibition media (B-E) with (right column) or without (left column) 1 μ M K-252a at time 0, and the distribution patterns of DNA content were determined 24 h after removal of hydroxyurea. The growth inhibition medium for blocking of M phase contained 1 μ g/ml Colcemid (B). For inhibition of G1 progression, the cells were transferred to serum-free medium (C), medium containing 0.01 μ g/ml actinomycin D (D), or that containing 0.2 μ g/ml cycloheximide (E). Since analysis was performed with isolated nuclei, metaphase cells in the absence of a nuclear envelope showed a broad peak lower than 4C (B, left column).

Table II. Distribution of DNA Content of Various Cell Lines Treated with K-252a

Cell line	Percent cell population					
	2C	2-4C	4C	>4C	>8C	>16C
3Y1	5	7	43	45	0	0
SV-3Y1	6	8	1	78	48	6
SR-3Y1	2	1	4	93	82	45
HR-3Y1	4	6	8	82	66	23
Balb/c-3T3	7	9	7	77	49	19
BHK21	11	10	40	39	8	4
HeLa-S3	3	8	15	74	10	0
MEL	8	9	33	50	13	0
FM3A	12	10	17	61	22	4

Exponentially growing cells of each cell line were treated with K-252a for 48 h and the distribution of their DNA content was determined. To avoid confusing multinucleated cells with polyploid cells, flow cytometry was performed with isolated nuclei. All cell lines except FM3A were treated with 1 μ M K-252a. A lower concentration of 0.4 μ M was used for FM3A due to deleterious effects at a concentration of 1 μ M.

and possessed a single large nucleus (Fig. 7). Treatment of SR-3Y1 cells with K-252a for 72 h produced a main peak at 32C and a small population of cells with a 64C DNA content, but no further polyploidization was observed even after 72 h (data not shown).

We were unable to establish polyploid clones from such cultures because they died rapidly after the removal of K-252a. Abnormalities of mitosis, such as disruption of the giant nucleus into multiple nuclei and degradation of the fragmented cells, were frequently observed during release (data not shown), suggesting that cells containing polyploid nuclei higher than tetraploid may lose their reproductive integrity.

Effect of K-252a on Activity of p34^{cdc2} Kinase

The *cdc2* gene product, p34^{cdc2}, has been revealed to be the key protein kinase component of the M phase-promoting factor (MPF) and a universal controller of mitosis (Nurse, 1990). In yeast, p34^{cdc2} is also assumed to play a role in G1 in the processes immediately preceding the initiation of DNA replication (Nurse and Bissett, 1981; Piggott et al., 1982). Although solid evidence for the requirement of p34^{cdc2} in G1/S has not been obtained in higher eukaryotes, involvement of a p34^{cdc2}-like protein in DNA replication has

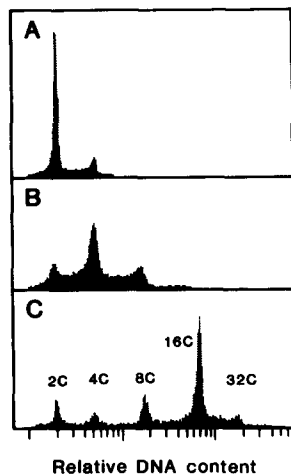


Figure 6. Effect of K-252a on distribution of DNA content in culture of SR-3Y1 cells. An asynchronous culture of SR-3Y1 cells was treated with 1 μ M K-252a. The distribution patterns of DNA content in the control culture without K-252a treatment (A), and in cultures treated with K-252a for 24 (B) or 48 (C) h, were determined as described in Materials and Methods.

been suggested (Blow and Nurse, 1990), and weak kinase activity has been detected during interphase (Giordano et al., 1989).

We examined the effects of K-252a on the in vitro activity of the p34^{cdc2} kinase, as well as the in vivo activity of p34^{cdc2} kinase during DNA re-replication without mitosis. The most active p34^{cdc2} preparation was obtained from the 3Y1 cells arrested at metaphase with Colcemid, using the affinity of p13^{suc1}-bound Sepharose (Arion et al., 1988). The effect of K-252a on the kinase activity in vitro was investigated using histone H1 as a substrate (Fig. 8). The IC₅₀ value was estimated to be 6 μ M, which was appreciably higher than the effective concentration for production of tetraploid cells in vivo (0.2–2.0 μ M). Although direct inhibition of the p34^{cdc2} kinase activity by K-252a seems insufficient to explain its in vivo induction of DNA re-replication, this cannot be concluded with certainty, since intracellular accumulation of the agent may also occur.

Next we examined changes in the intracellular activity of p34^{cdc2} kinase in cells synchronized in early S phase in the presence or absence of K-252a. The p34^{cdc2} kinase was extracted from cells in each culture and assayed for H1 kinase activity in the absence of drugs. In the control cultures without the agent, kinase activity increased to reach a peak at M phase and then decreased (Fig. 9 A). The presence of Colcemid prevented the decrease after M phase due to metaphase arrest (Fig. 9 B). In the culture with K-252a, weak kinase activity was detected during S phase, but no further activation of the kinase to the maximum at M phase was observed. Thereafter, the activity was more suppressed during the period corresponding to the G1 phase of tetraploid cells (Fig. 9 C). Another interesting observation was that the weak activity in the cells treated with K-252a appeared again after 18–24 h, coinciding with initiation of the second S phase in the treated cells. A similar but longer cultivation until 36 h after exposure to K-252a clearly showed oscillation of the kinase activity (Fig. 9 D). The results show that DNA re-replication in the presence of K-252a is accompanied by partial activation of p34^{cdc2} kinase activity.

Discussion

The effects of several protein kinase inhibitors on the cell cycle summarized in Table I may reflect the involvement of a variety of protein kinases in cell cycle control in either G1 or G2 phase. Staurosporine, sangivamycin, and H7, all described as inhibitors of protein kinase C, caused G1 block, suggesting the importance of the kinase in G1 phase. In addition, staurosporine causes G2 block at higher concentrations (Abe et al., 1991), probably due to inhibition of a kinase different from protein kinase C. In fact, various protein kinases are inhibited by staurosporine to varying degrees (Davis et al., 1989). Although the two staurosporine analogues, K-252a and RK-286C, were originally reported to be protein kinase C inhibitors, we observed here that they induced distinctly different changes in the cell cycle; i.e., marked DNA re-replication uncoupled from mitosis. Preferential inhibition of some specific protein kinase by these analogues may cause these differences in their mode of action.

Recently, Kung et al. (1990) reported that agents inducing disruption of the mitotic spindle apparatus (Colcemid, nocodazole, and taxol) introduced M-phase cells into subse-

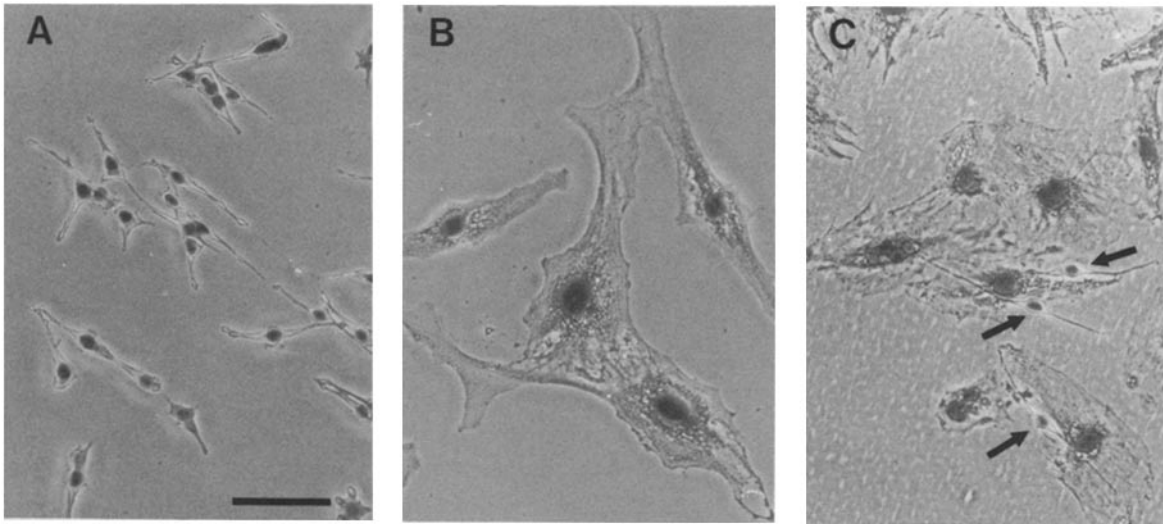


Figure 7. Polyploid giant cell formation by K-252a. *A* shows the control culture of SR-3Y1 cells without treatment. Cells were stained with Giemsa after methanol fixation. *B* shows polyploid giant cells that were generated from SR-3Y1 cells by treatment with 1 μ M K-252a for 48 h. *C*) A direct comparison between cells in *A* and *B*; diploid cells in *A* were trypsinized, seeded into the polyploid cell culture in *B*, and cocultivated for 2 h. The diploid cells are indicated by arrows. All the photographs were taken after the experiment using the same magnification. Bar, 100 μ m.

quent stages of the cell cycle without division, resulting in so-called endoreduplication to form polyploid cells. The mechanism of polyploidization induced by K-252a is clearly different from endoreduplication by these agents, since K-252a did not cause any accumulation of M-phase cells or chromosome condensation, but inversely inhibited the entry of G2 cells into mitosis. The results presented here strongly suggest that a protein kinase(s) sensitive to K-252a is involved in the blocking of DNA re-replication in the G2 nuclei of somatic cells, thus assuring the tight coupling of S phase with M phase.

Our previous studies using the G1 and G2 inhibitors, trichostatin A (Yoshida and Beppu, 1988) and leptomycin B (Yoshida et al., 1990b), have shown that tetraploid formation is induced after release of 3Y1 fibroblasts from G2 arrest by these agents. Biochemical and genetic analyses have revealed that trichostatin A is not a protein kinase inhibitor, but a potent and specific inhibitor of mammalian histone deacetylase (Yoshida et al., 1990a). Although the target molecule of

leptomycin B is still unknown, the agent did not show any inhibitory effect on histone deacetylase and protein kinases (unpublished results). Appearance of tetraploid cells was also observed upon release of some temperature-sensitive mutants of 3Y1 from arrest at G2 (Yamada and Kimura, 1985). We may assume that the G2-arrested cells gradually lose their "G2 memory" during arrest by these agents or through mutation, probably due to degradation of some factor essential for mitosis, and that the arrested cells are reprogrammed to initiate S phase without mitosis. Recent analysis of several mutants of *Schizosaccharomyces pombe* diploidized by transient heat treatment indicated that the factor responsible for such a "G2 memory" was *cdc2* protein kinase itself (Broek et al., 1991). Although these increases in ploidy are similar to the effects of K-252a, an obvious difference is that the DNA re-replication induced by K-252a proceeds in the presence of the agent. This may indicate that K-252a causes selective arrest of the G2 progression for M phase without inhibition of the "start" activity for S phase during G1. Since *cdc2* kinase is assumed to be required for the progression of both G1 and G2 phases, inhibition of some other kinase specific for G2 may be responsible for the extensive re-replication induced by K-252a.

Blow and Laskey (1988) proposed a simple model for the apparent DNA re-replication block in G2, based on experiments with a cell-free DNA replication system from *Xenopus* eggs. They observed that replicated nuclei never re-replicated unless they had passed through mitosis, whereas breakdown or permeabilization of the nuclear envelope allowed re-replication in this system. They assumed that an essential initiation factor allowing DNA replication becomes exhausted in the nucleus during DNA replication, and that this cytoplasmic "licensing factor" is supplied to chromosomes upon breakdown of the nuclear envelope during the next mitosis. This hypothesis alone cannot explain our present results, since re-replication induced by K-252a occurred without mitotic processes.

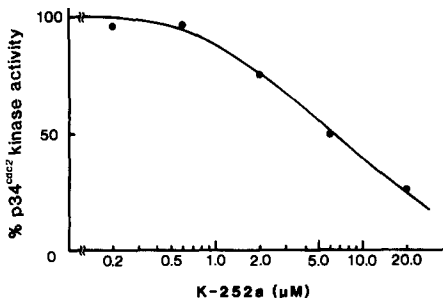


Figure 8. Effect of K-252a on the active p34^{cdc2} kinase from M-phase cells. Active p34^{cdc2} kinase was extracted from Colcemid-arrested metaphase 3Y1 cells using p13^{supcl}-Sephacryl beads. Beads-bound p34^{cdc2} kinase activity was assayed as described in Materials and Methods and the effect of K-252a on the activity was determined.

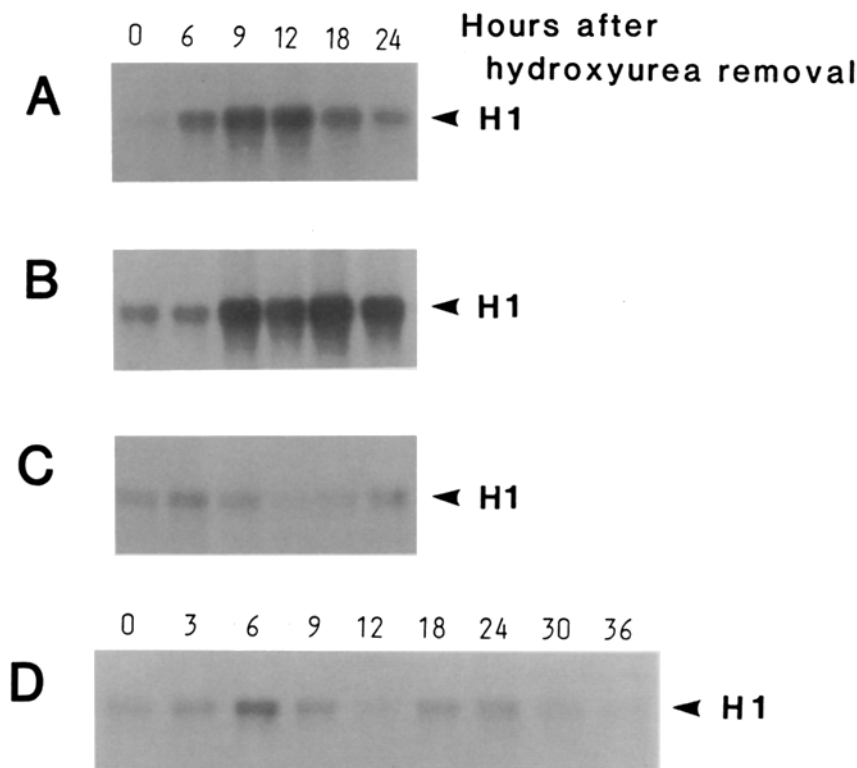


Figure 9. Change in intracellular activity of p34^{cdc2} kinase in early S-synchronous culture. (A) The p34^{cdc2} kinase was extracted from control synchronous cultures without drugs at the indicated times and assayed for H1 kinase activity. (B) Early S-synchronous cultures were challenged with 1 μ g/ml Colcemid at time 0. The p34^{cdc2} kinase was extracted from the cells at the indicated times and assayed for H1 kinase activity. (C and D) Early S-synchronous cultures were challenged with 1 μ M K-252a at time 0. The p34^{cdc2} kinase was extracted from the cells at the indicated times and assayed for H1 kinase activity.

We observed a distinct interval corresponding to G1 between the first and second DNA replications in 3Y1 cells treated with K-252a. Flow cytometric analysis of SR-3Y1 after prolonged culture with K-252a also suggested the presence of fairly long intervals between the DNA re-replication phases to produce 8C or 16C cells. These results indicate that the periodicity of DNA replication is maintained without mitotic processes in K-252a-treated cells. Thus the whole process may be considered to be an incomplete cell cycle without mitosis (Fig. 10).

It seems evident that some unknown oscillation mechanism or clock determines the timing for initiation of DNA replication in K-252a-treated cells irrespective of mitotic processes. The weak intracellular activity of the p34^{cdc2} ki-

nase in K-252a-treated cells showed an increase corresponding to the DNA replication, but full activation of the kinase normally occurring in M phase was not observed, as illustrated in Fig. 10. Thus K-252a seems useful for observing the lower activity of p34^{cdc2} kinase by diminishing the very potent activity present in M phase. The results obtained using K-252a suggest that partially active p34^{cdc2} kinase may play an essential role in DNA replication as a putative diffusible S-phase activator (Rao and Johnson, 1970) or as a start-promoting factor associated with G1 cyclins (Wittenberg et al., 1990; Murray, 1991).

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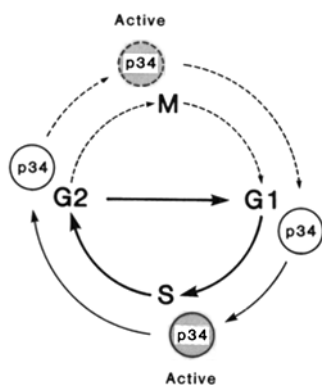


Figure 10. A model for the cell cycle without mitosis induced by K-252a. In the normal cell cycle, active p34^{cdc2} is assumed to be required for both the G1/S and G2/M transitions. None of the mitotic processes, including nuclear envelope breakdown, nuclear and cell division, and mitotic activation of the p34^{cdc2} kinase (broken lines), are observed in the presence of K-252a, but periodic activation of p34^{cdc2} in S phase and DNA replication

can still occur even in the absence of mitosis (solid lines). Consequently, continuous cell cycles without mitoses induced by K-252a result in automatic polyploidization.

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