Okadaic acid suppresses TPA-induced differentiation by stimulating G₁/S transition in human myeloblastic leukaemia ML-1 cells

P. S. Reinach, T. Li* and L. Lu*

*Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, OH and
Department of Biological Sciences, State University of New York, OPT, New York, NY, USA

(Received 30 March 1999; revision accepted 27 September 1999)

Abstract. The association between the phosphorylation status of the retinoblastoma protein, pRb and changes in cell cycle control caused by either protein kinase C (PKC) or protein kinase A (PKA) stimulation was evaluated in human myeloblastic leukaemia ML-1 cells. TPA-induced PKC activation resulted in dephosphorylation of pRb and subsequently induced ML-1 differentiation based on morphological changes and CD14 expression. In the present study, we showed that inhibition of protein phosphatases (PP-1 and PP-2a) prevented the TPA-induced differentiation in ML-1 cells. Preinhibition of PP-1 and PP-2a activities with 1–100 nM okadaic acid dose-dependently blunted the decrease in the phosphorylation status of pRb obtained with TPA and overrode cell cycle arrest. PKA stimulation with 8-chlorophenylthio-cAMP (100 μ M) decreased cell proliferation by 65% and the distribution of cells in the G₁ phase significantly increased from 38% to 83% concomitant with a 34% decline in the number of cells present in the S phase. In addition, PKA stimulation significantly decreased the pRb phosphorylation status but did not elicit CD14 expression, indicating that cAMP-induced dephosphorylation of pRb cannot by itself trigger differentiation in ML-1 cells.

INTRODUCTION

The retinoblastoma protein, pRb, is involved in the control of cell cycle progression, cell growth and differentiation (Weinberg 1995). Its control occurs at the G₁/S transition in the cell cycle and is elicited through changes in its phosphorylation status which is determined by the balance between interlinked protein kinase phosphorylating and dephosphorylating protein phosphatase reactions. Changes in the pRb phosphorylation status affect the ability of pRb to ensnare transcription factors (Bagchi, Weinmann & Raychaudhuri 1991; Chellappan *et al.* 1991; Rustgi, Dyson & Bernards 1991a; Rustgi *et al.* 1991b; Welch & Wang 1993; Park *et al.* 1994). In its hypophosphorylated state, pRb binds to transcription factors required for the activation of early response genes. As a result, cell cycle progression through the G₁/S transition is arrested (Chen *et al.* 1989; Ludlow *et al.* 1990; Dowdy *et al.* 1993), causing the cells to enter the G₀ phase or undergo differentiation. On the other hand, the

Correspondence: L. Lu, Department of Physiology & Biophysics, School of Medicine, Wright State University, Dayton, Ohio 45435, USA. Fax: +1 937 775 3769; E-mail: llu@wright.edu

hyperphosphorylation state of pRb allows that the transcription factors can instead bind to promoter sites and activate gene expression. Therefore, the control of cell growth and differentiation by pRb is linked to the interplay between numerous protein kinases and phosphatases that affect its phosphorylation status.

Protein kinase activation can either lead to a decrease or an increase in the phosphorylation status of pRb. The overall effect on the phosphorylation status of pRb depends on whether kinase activation elicits a net stimulation of pathways that dephosphorylate or phosphorylate pRb. Dephosphorylation can occur as the result of the inhibition of the activity of a group of homologous serine/threonine kinases known as cyclin-dependent kinases (CDK) which phosphorylate pRb. Inhibition of CDK activity causes dephosphorylation of pRb and leads to cell cycle arrest. This inhibition can also occur through activation of proteins such as p16, p21 and p27 (Hunter & Pines 1994; Morgan 1995). Alternatively, stimulation of protein phosphatases (PP) designated PP-1 and PP-2a by protein kinase C may also dephosphorylate pRb (Livneh & Fishman 1997). Recent studies reveal that the possible involvement of protein phosphatase in hexamethylenebisacetamide-induced erythroleukaemia cell differentiation is through the control of pRb phosphorylation (Zhuo *et al.*, 1995; Nelson, Krucher & Ludlow 1997). But, the role of phosphatase activity in TPA-induced myeloblastic leukaemia cell differentiation is largely unknown. The regulatory role by phosphatase activity has been proposed involving that the induction of pRb phosphatase activity by anticancer agents accompanies p53 independent G₁ cell arrest and apoptosis (Dou, An & Will 1995; Yatsunami *et al.*, 1993).

In human myeloblastic ML-1 cells, stimulation of PKC activity with the active phorbol ester analogue, TPA, elicits differentiation of these cells into monocytes/macrophages (Craig *et al.*, 1984). The differentiation induced by TPA in ML-1 cells is thought to be an irreversible process. There is a little information about the effect of TPA on stimulation of protein phosphatase activity and/or inhibition of CDK activity, and dephosphorylation of pRb. In addition to PKC stimulation, there is evidence that an increase in protein kinase A activity can also trigger differentiation through inhibition of CDK activity which results in dephosphorylation of pRb. However, there is no information on whether PKA stimulation triggers differentiation in ML-1 cells and whether it occurs through dephosphorylation of pRb. Therefore, in ML-1 cells the association with changes in the pRb phosphorylation status and growth and differentiation needs to be examined.

It is reported here that differentiation induction in ML-1 cells by TPA is associated with dephosphorylation of pRb and can be prevented by inhibition of phosphatase activity. A mechanism that could account for this event is that PKC stimulation could lead to increases in PP-1 and PP-2a activity since inhibition of their activity by okadaic acid blocked this response to TPA. On the other hand, the effect of PKA stimulation on cell cycle control is not identical to that of PKC stimulation. Even though PKA stimulation also resulted in pRb dephosphorylation and cell cycle arrest, the cells did not fully differentiate based on differences in cell marker expression.

MATERIALS AND METHODS

Cell culture

ML-1 cells, originally isolated from an acute myeloblastic leukaemia patient, were received as a generous gift from Dr R. W. Craig's laboratory, Dartmouth Medical School, NH, USA. Cells were maintained in suspension culture as described previously (Xu & Lu 1994). Briefly,

culture medium RPMI 1640 containing 25 mM HEPES buffer was supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) (Gibco/BRL). Cells were grown in a humidified incubator with 5% CO₂ at 37 °C and passed at a seeding density of 3×10^5 /ml. ML-1 cells were induced to differentiate with treatment of 1 nM TPA for 24 h.

Cell growth assays

Proliferation of ML-1 cells was determined by counting cell numbers using a haemocytometer and measuring [³H]thymidine incorporation into the host DNA. ML-1 cells from suspension culture were plated in triplicate into 35 mm culture dishes at a density of 3×10^5 /ml. After incubation with 8-chlorophenylthio (cpt)-cAMP or okadaic acid for 24 h, all cultured cells were pulsed with 1 µCi/ml of [³H]thymidine for 2 h. Cells were then harvested and washed twice with phosphate-buffered saline (PBS). Nucleic acids were precipitated with 10% trichloroacetic acid (TCA), and radioactivity of samples was quantified by liquid scintillation counting. For cell-cycle analysis experiments, ML-1 cells were plated as described above and collected after incubation of cells with various reagents for 24 h. The cells were fixed by slowly adding 1 ml ice-cold 50 mM glycine/70% ethanol (pH 2.0) and kept at -20 °C overnight. After a single wash with PBS solution, cells were suspended in 0.5 ml PBS and 0.5 ml propidium iodide stain (0.1% sodium citrate, pH 7.2, 0.5% NP-40 and 5 mg/ml propidium iodide) and 10 µl RNase (10 mg/ml, DNase-free, USB) for 30 min at 37 °C (Strobl *et al.*, 1990). Cell-cycle analysis was performed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) and cell-cycle distribution was analysed based on DNA content by a FACScan using CellFIT software (Becton Dickinson).

Western Blotting

ML-1 cells treated with different reagents were washed twice with PBS and harvested in lysis buffer: 50 mM TrisHCl, pH 7.4, 250 mM NaCl, 1% (v/v) nonidet P-40, 0.1% (wt/vol) SDS, 5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride. Equal amounts (20 µg) of protein lysates were fractionated on an 8% SDS-polyacrylamide gel and electrotransferred onto a PVDF membrane (Millipore, MA, USA). The protein blot was first incubated with the mouse antihuman pRB monoclonal antibody G3-245 (Pharmingen, San Diego, CA, USA) or by antihypophosphorylated pRb monoclonal antibody G99-2005 (Pharmingen) at 1 µg/ml final concentration in Tris-buffered saline overnight at 4 °C. The membranes were then incubated with goat antirabbit IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were detected with a Phototope-Star Western Blot Detection Kit (New England Biolabs, MA, USA). Data shown are from three independent experiments.

Northern Analysis

Total cellular RNA was extracted using the guanidinium thiocyanate method (Sambrook, Fritsch & Maniatis 1989). The cDNA probes for CD14 and β-actin were made by reverse transcriptase-polymerase chain reaction (RT-PCR), as previously described (Xu, Wilson & Lu 1996). Total RNA (20 µg) isolated from cells pretreated with different reagents was loaded on 1% agarose gel containing formaldehyde. The contents of the gel were transferred to nylon membranes using a Turboblotter (Schleicher & Schuell, Keene, NH, USA). Nylon membranes were hybridized with ³²P-labelled cDNA probes using a random primer labelling kit (NEBiolab). Blots were washed after hybridization and RNA bands were visualized by autoradiographic exposure on film for 24–48 h at -80 °C. Prehybridization, hybridization, and wash conditions were according to standard protocols (Sambrook *et al.*, 1989). The

membrane was stripped with $0.1 \times$ SSC and 0.5% SDS at 80 C for 30 min between hybridizations using different probes.

Microscopy

ML-1 cells were cultured in P-35 dishes and treated with different reagents. At indicated times, cells were viewed using a Nikon Optiphot II microscope equipped with Hoffman Modulation Optics ($40 \times$ water immersion, 15 ocular). Photo images of the cells were collected using an analogue camera (Hammamatsu), and digitized using Computer Eyes software.

RESULTS

There is substantive evidence in ML-1 cells that stimulation of protein kinase C activity by the phorbol ester, TPA, induces differentiation (Craig *et al.*, 1984). To assess the relationship between this effect of TPA and changes in the phosphorylation status of pRb, Western blot analysis was performed. The effects of exposure to 1 nM TPA on the pRb phosphorylation status following a time course were detected by using a monoclonal antihuman pRb antibody (Figure 1a) or by a specific antihuman hypophosphorylated pRb antibody (Figure 1b). A progressive decrease occurred in the phosphorylation status of pRb was already observable at 2 h and at 24 h phosphorylation of pRb had been essentially completed. Associated with pRb dephosphorylation after 24 h of exposure to TPA, morphological analysis (Figure 1c) and assessment of CD14 expression revealed that differentiation induction had occurred (data not shown). The rapid onset of pRb dephosphorylation by TPA is consistent with previous reports that the dephosphorylation of pRb is the consequence of stimulation of protein kinase and phosphatase activity rather than stimulation of pRb gene expression (Xu *et al.*, 1996).

There are a number of mechanisms that could lead to TPA-induced dephosphorylation of pRb. They include: (1) stimulation of protein phosphatase activity; (2) and inhibition of CDK phosphorylating activity due to stimulation of a cyclin inhibitory protein. To examine the first possibility, we inhibited protein phosphatase activity using okadaic acid, a protein phosphatase inhibitor, for 30 min prior to exposing the cells to TPA. Upon application of okadaic acid, in a concentration range from 1 to 100 nM, protein phosphatase (PP) 1 and 2a are inhibited. As can be seen the morphological change in Figure 2a, a pretreatment with okadaic acid blocked ML-1 differentiation. Associated with this inhibitory effect, TPA-stimulated increase in the ratio of hypo-phosphorylation vs. hyper-phosphorylation of pRb was markedly reduced by okadaic acid (Figure 2b). Measurement of hypo-phosphorylated pRb using an antihypo-phosphorylated pRb specific antibody confirmed the effect of okadaic acid that TPA-stimulated increase in of hypo-phosphorylated pRb was diminished (Figure 2c).

These effects of pretreatment with 1–100 nM okadaic acid followed by exposure to TPA suggest that the checkpoint function of pRb at the G_1/S transition was altered. If this occurred, the cell cycle population profile of the okadaic acid treated cells may have been changed from the cells not treated with okadaic acid. To assess this possibility, cell cycle analysis was performed. The results shown in Figure 2d indicate that the distribution was shifted. In the control (TPA-treated) group, 95% of the cells were in the G_1 phase whereas in the okadaic acid pretreated group only 38% remained in the G_1 phase. Moreover, another indication that dephosphorylation of pRb is associated with an increase in the proportion of cells going through cell cycle progression is indicated by our finding that a larger number of cells reached the S phase (Figure 2d). To further confirm that okadaic acid pretreatment

(a)

(b)

Figure 1 continued overleaf

Figure 1. Time course of pRb dephosphorylation and morphological changes induced by TPA in ML-1 cells. Human ML-1 cells were cultured to a density of $3 \times 10^5/\text{ml}$ and induced to differentiate by addition of 1 nM TPA. (a) Western blot analysis using monoclonal antihuman pRb antibody. The dephosphorylated form of pRb migrated faster than its phosphorylated form on SDS-PAGE gel. The ratio of hypo-/hyper-phosphorylated pRb was plotted as a function of time. Protein size markers (M) are indicated by arrows. Lane 1, time 0; lanes 2, 3, 4, 5, are 2 h, 4 h, 8 h and 24 h after treatment with TPA, respectively. (b) Western blot analysis using monoclonal antihuman hypophosphorylated pRb antibody. (c) Differentiation was assessed based on morphology. Cells were photographed using a microscope based video capture system prior to and following addition of TPA: (i) control; (ii) 2 h; (iii) 4 h; (iv) 8 h; (v) 16 h; and (vi) 24 h.

blocked TPA-induced differentiation, we determined whether the expression of a differentiation marker, CD 14, was blocked. As shown in Figure 3, pretreatment of ML-1 cells with 100 nM okadaic acid completely blocked the expression of CD 14 suggesting that TPA-induced differentiation is associated with dephosphorylation of pRb and G_1 cell cycle arrest.

(a)

(b)

Figure 2 continued overleaf

The possible role was further evaluated of protein kinase A activation in mediating dephosphorylation of pRb and ML-1 cell differentiation. This was done by determining whether an increase in intracellular cAMP levels, which induces differentiation in some other cells, had the same effect in ML-1 cells and also altered the phosphorylation status of pRb. Several lines of evidence that cAMP predisposes ML-1 to undergo differentiation were obtained. The present findings are that after a 24-h exposure to 100 μ M 8-chlorophenylthio-cAMP [3 H]-thymidine uptake by ML-1 cells decreased by 65% (Figure 4a). In addition, there was 95% decrease in [3 H]thymidine uptake caused by TPA stimulation (Figure 4a). Furthermore this treatment also increased the population profile of cells in the G₁ phase. The distribution of cells in the G₁ phase significantly increased from 38% to 83% concomitant with a decrease in the S phase population from 47% to 13% (Figure 4b). Associated with these changes, the ratio of hypo-/hyper-phosphorylation status of pRb and the amount of hypo-phosphorylated pRb were significantly increased by cAMP stimulation (Figure 4c, d). Application of 100 μ M okadaic acid reduced cAMP-induced G₁ population from 83% to

(c)

(d)

Figure 2. Effect of okadaic acid on TPA-induced dephosphorylation of pRb and ML-1 cell differentiation. (a) Inhibition of pRb phosphatase with okadaic acid blocks TPA-induced differentiation: (i) undifferentiated cells; (ii) differentiation induction following exposure to 1 nM TPA for 24 h; (iii) blockage of differentiation caused by a 10-min treatment with 100 nM okadaic acid before and during a 24-h exposure to 1 nM TPA ML-1. (b) Western blot analysis of pRb. Data were plotted as the ratio of hypo-/hyper-phosphorylated pRb. (c) Western blot analysis of hypo-phosphorylated pRb. The hypo-phosphorylated pRb was detected by antihuman hypo-phosphorylated pRb antibody. (d) Flow cytometry analysis of cell cycle distribution changes associated with exposure to TPA in the presence and absence of okadaic acid. ML-1 cells were plated at a density of 3×10^5 /ml; (left panel) control; (middle panel) 1 nM TPA for 24 h (right panel) 100 nM okadaic acid and 1 nM TPA for 24 h. Cell cycle distributions were analysed as described in Materials and Method. The first peak of the curve represents cells in the G₁ phase and the second peak represents cells in the G₂/M phases of the cycle. The area between the G₁ and G₂/M peaks represents cells in the S phase.

Figure 3. Northern blot analysis of CD 14 expression in ML-1 cells. Total RNA (20 μ g) isolated from ML-1 cells exposed to the below indicated conditions was separated by electrophoresis on a 1% agarose gel, and then transferred to a nylon membrane. Hybridization experiments were performed with a 32 P labelled CD14 probe at high stringency (6 $\times$ SSC, 5 $\times$ Denhart's solution, 0.5% SDS and 100 μ g/ml denatured salmon sperm DNA, at 60 $^{\circ}$ C overnight). The membrane was washed at the same stringency and exposed to X-ray film for 48–96 h. Upper panel: lane 1, 100 nM OA + 1 nM TPA; lane 2, 1 nM OA + 1 nM TPA; lane 3, 1 nM TPA; and lane 4, untreated control. Lower panel, b-actin expression serves as an internal control.

47% (Figure 4b) and markedly decreased both the ratio of hypo-/hyper-phosphorylation status of pRb and the amount of hypo-phosphorylated pRb in the presence of cAMP stimulation (Figure 4c, d). Despite these changes, which are consistent with those seen with TPA, cAMP did not induce the expression of any of the ML-1 morphological correlates of differentiation. The cells remained spherical instead of becoming elongated and failed to express CD14 (Figure 5).

DISCUSSION

This study shows that increases in the phosphorylation status of pRb are associated with the inhibition of ML-1 proliferation, cell cycle arrest at the G₁/S transition and differentiation induction (Decaprio *et al.*, 1989; Goodrich *et al.*, 1991). Stimulation of ML-1 cells with exogenous cAMP or TPA resulted in hypo-phosphorylation of pRb and cell cycle arrest, however, only TPA stimulated cells were undergone differentiation. Both of these changes are the results of either protein kinase A or C stimulation caused by exposure to either 8-chlorophenylthio-cAMP or TPA. In addition, inhibition of protein phosphatase 1 and 2a activity with okadaic acid blocked the differentiation induction effect of TPA indicated by CD14 expression, suggesting a role of dephosphorylation of pRb in the process of TPA-induced ML-1 cell differentiation. The further evidence come from morphological changes of ML-1 cells during TPA induction in the presence and absence of okadaic acid. Conversely, an increase in the phosphorylation status of pRb is associated with an increased propensity by these cells to pass through the G₁/S checkpoint and to proliferate.

The correlation has been described in other cells that changes in pRb phosphorylation status affect cell cycle progression through the G₁/S checkpoint. In the dephosphorylated state, a preponderant number of cells are phase arrested and can undergo changes leading to differentiation whereas in a more phosphorylated state a majority of the cells proliferate (Szekely *et al.*, 1992; Kiyokawa *et al.*, 1993; Zhuo *et al.*, 1995). It has been explained that the ability of pRb to modulate the events which lead to growth and differentiation is related to the binding capacity of pRb to various transcription factors including E2F (Chen *et al.*, 1989; Buchkovich, Duffy & Harlow 1989; Mihara *et al.*, 1989; Hiebert *et al.*, 1992; Shirodkar *et al.*, 1992). In a phosphorylated state, binding to pRb is less likely, which frees the transcription

Figure 4 continued overleaf

factors to bind to promoter sites which regulate the expression of genes needed for proliferation (Johnson *et al.*, 1993; Nevins 1992; Szekely *et al.*, 1992; Kiyokawa *et al.*, 1993; Zhuo *et al.*, 1995). Alternatively, in its dephosphorylated form, pRb binding to transcription factors is preferred, which results in cell cycle arrest at the G₁/S checkpoint in the cell cycle. This halting effect predisposes the cells to enter G₀ and start to differentiate (Hamel *et al.*, 1993; Wiman 1993).

The effects of TPA, okadaic acid and cAMP on differentiation in ML-1 cells are consistent with the cell-cycle dependent changes that occur in response to changes in the phosphorylation status of pRb; namely dephosphorylation resulting from exposure to either

Figure 4. Effect of okadaic acid on cAMP-induced ML-1 cell G_1 arrest and dephosphorylation of pRb. (a) Inhibition of proliferation resulting from stimulation of PKA and PKC. ML-1 cells were plated at a density of 3×10^5 /ml in 35 mm culture dishes. Cell proliferation was monitored based on [3 H]-thymidine incorporation into DNA. Data are presented as the mean $\pm$ SE from 4 to 6 independent experiments and where significant denoted with (*) as determined by the Student's *t*-test (paired data). (b) Effect of cAMP-stimulation on the cell cycle in ML-1 cells. Analysis was performed as described in the legend for Figure 2d (left panel), control; (middle panel) following 24 h exposure to 100 μ M 8-chlorophenylthio-cAMP; (right panel) following 24 h combined exposure to 100 μ M 8-chlorophenylthio-cAMP and 100 nM okadaic acid. (c) cAMP-mediated changes in pRb phosphorylation status in the presence and absence of 100 nM okadaic acid: Western blot analysis was used as described in the Figure 1a legend. (d) Effect of okadaic acid on cAMP-stimulated hypo-phosphorylation of pRb. Western analysis was performed using specific antihypo-phosphorylated pRb antibody in the presence and absence of 100 nM okadaic acid.

Figure 5. Northern blot analysis of the CD14 differentiation marker. CD14 probe was used as described in the legend for Figure 3. ML-1 cells were either exposed to 100 μ M 8-chlorophenylthio-cAMP for either 24 or 72 h. Upper panel: lane 1, 1 nM TPA for 24 h; lane 2, control; lane 3, 8-cpt-cAMP for 24 h; and lane 4, 8-cpt-cAMP for 72 h. Lower panel: β -actin expression serves as an internal control.

TPA or cAMP leads to phase cycle arrest whereas an increase in its phosphorylation caused by inhibition of protein phosphatase activity promotes cell cycle progression and proliferation. Dephosphorylation resulted from net stimulation of protein phosphatase activity whereas phosphorylation resulted from a net stimulation of protein kinase activity. In this context, the induction of differentiation by TPA may be explained by its stimulation of protein kinase C activity that in turn phosphorylates protein phosphatases resulting in the stimulation of their activity. Such stimulation leads to a decrease in the phosphorylation status of pRb (Fernandez, Brautigan & Lamb 1992; Alberts *et al.*, 1993; Dou *et al.*, 1995). The interpretation explains our finding that inhibition of protein phosphatase activity forestalls the differentiation response to TPA.

Another mechanism involved in the control of the pRb phosphorylation status is the activity of CDK inhibitory proteins (Hirama & Koeffler 1995; Morgan 1995; Weinberg 1995). In other tissues, where cAMP is an inducer of differentiation, this response is associated with the stimulation of CDK inhibitory proteins. Such stimulation inhibits the phosphorylating activity of CDK that results in a decrease in the phosphorylation status of pRb. Even though we did not determine whether cAMP stimulates CDK inhibitory protein activity or induces its expression, we have some suggestive evidence that the mechanism leading to the dephosphorylation of pRb may be different from how TPA induces dephosphorylation of pRb. This could be the case because only TPA induced the expression of the morphological correlates of differentiation even though both cAMP and TPA caused G_1 phase arrest. Our suggestion that such a difference exists agrees with the results of other studies in which it was shown that cAMP induces expression of the CDK inhibitory protein p27 instead of stimulating protein phosphatase activity (Morgan 1995). It is also possible that differentiation could only occur in response to TPA because only protein kinase C stimulation results in the phosphorylation of the multitude of substrates needed for this response to occur. This dependence exists even though both protein kinase C and A stimulation support the reactions needed for the dephosphorylation of pRb and the initiation of the chain of events that lead to differentiation.

REFERENCES

- 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 [published erratum appears in *Proc. Natl. Acad. Sci. USA* (1993) **90**, 2556]. *Proc. Natl. Acad. Sci. USA* **90**, 388–392.

- BAGCHI S, WEINMANN R, RAYCHAUDHURI P. (1991) The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* **65**, 1063–1072.
- BUCHKOVICH K, DUFFY LA, HARLOW E. (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58**, 1097–1105.
- CHELLAPPAN SP, HIEBERT S, MUDRYJ M, HOROWITZ JM, NEVINS JR. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell* **65**, 1053–1061.
- CHEN PL, SCULLY P, SHEW JY, WANG JY, LEE WH. (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58**, 1193–1198.
- CRAIG RW, FRANKFURT OS, SAKAGAMI H, TAKEDA K, BLOCH A (1984) Macromolecular and cell cycle effects of different classes of agents including the maturation of human myeloblastic leukemia (ML-1) cells. *Cancer Res.* **44**, 2421–2429.
- DECAPRIO JA, LUDLOW JW, LYNCH D *et al.* (1989) The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58**, 1085–1095.
- DOU QP, AN B, WILL PL (1995) Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. *Proc. Natl. Acad. Sci. USA* **92**, 9019–9023.
- 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**, 499–511.
- FERNANDEZ A, BRAUTIGAN DL, LAMB NJ. (1992) Protein phosphatase type 1 in mammalian cell mitosis: chromosomal localization and involvement in mitotic exit. *J. Cell Biology* **116**, 1421–1430.
- GOODRICH DW, WANG NP, QIAN YW, LEE EY, LEE WH. (1991) The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**, 293–302.
- HAMEL PA, PHILLIPS RA, MUNCASTER M, GALLIE BL. (1993) Speculations on the roles of RB1 in tissue-specific differentiation, tumor initiation, and tumor progression. [Review]. *FASEB J.* **7**, 846–854.
- HIEBERT SW, CHELLAPPAN SP, HOROWITZ JM, NEVINS JR. (1992) The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes & Development* **6**, 177–185.
- HIRAMA T, KOEFFLER HP. (1995) Role of the cyclin-dependent kinase inhibitors in the development of cancer. [Review]. *Blood* **86**, 841–854.
- HUNTER T, PINES J. (1994) Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age [see comments]. [Review]. *Cell* **79**, 573–582.
- JOHNSON DG, SCHWARZ JK, CRESS WD, NEVINS JR. (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**, 349–352.
- KIYOKAWA H, RICHON VM, VENTA-PEREZ G, RIFKIND RA, MARKS PA. (1993) Hexamethylenebisacetamide-induced erythroleukemia cell differentiation involves modulation of events required for cell cycle progression through G1. *Proc. Natl. Acad. Sci. United States America* **90**, 6746–6750.
- LIVNEH E, FISHMAN DD. (1997) Linking protein kinase C to cell-cycle control. *Eur. J. Biochem.* **248**, 1–9.
- LUDLOW JW, SHON J, PIPAS JM, LIVINGSTON DM, DECAPRIO JA. (1990) The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell* **60**, 387–396.
- MIHARA K, CAO XR, YEN A *et al.* (1989) Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science* **246**, 1300–1303.
- MORGAN DO (1995) Principles of CDK regulation. [Review]. *Nature* **374**, 131–134.
- NELSON DA, KRUCHER NA, LUDLOW JW. (1997) High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *J. Biol. Chem* **272**, 4528–4535.
- NEVINS JR (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**, 424–429.
- PARK K, CHOE J, OSIFCHIN NE, TEMPLETON DJ, ROBBINS PD, KIM SJ. (1994) The human retinoblastoma susceptibility gene promoter is positively autoregulated by its own product. *J. Biol. Chemistry* **269**, 6083–6088.
- RUSTGI AK, DYSON N, BERNARDS R. (1991a) Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. *Nature* **352**, 541–544.
- RUSTGI AK, DYSON N, HILL D, BERNARDS R. (1991b) The c-myc oncoprotein forms a specific complex with the product of the retinoblastoma gene. *Cold Spring Harbor Symposia Quantitative Biol.* **56**, 163–167.
- SAMBROOK J, FRITSCH EF, MANIATIS T. (1989) *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory Press, New York.
- SHIRODKAR S, EWEN M, DECAPRIO JA, MORGAN J, LIVINGSTON DM, CHITTENDEN T. (1992) The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* **68**, 157–166.

- STROBL JS, KIRKWOOD KL, LANTZ TK, LEWINE MA, PETERSON VA, WORLEY JFD. (1990) Inhibition of human breast cancer cell proliferation in tissue culture by the neuroleptic agents pimozide and thioridazine. *Cancer Res.* **50**, 5399–5405.
- SZEKELY L, JIANG WQ, BULIC-JAKUS F *et al.* (1992) Cell type and differentiation dependent heterogeneity in retinoblastoma protein expression in SCID mouse fetuses. *Cell Growth & Differentiation* **3**, 149–156.
- WEINBERG RA. (1995) The retinoblastoma protein and cell cycle control. [Review]. *Cell* **81**, 323–330.
- WELCH PJ, WANG JY. (1993) A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell* **75**, 779–790.
- WIMAN KG. (1993) The retinoblastoma gene: role in cell cycle control and cell differentiation. [Review]. *FASEB J.* **7**, 841–845.
- XU B, LU L. (1994) Protein kinase A-regulated Cl⁻ channel in ML-1 human hematopoietic myeloblasts. *J. Membrane Biology* **142**, 65–75.
- XU B, WILSON BA, LU L. (1996) Induction of human myeloblastic ML-1 cell G1 arrest by suppression of K⁺ channel activity. *Am. J. Physiology* **271**, C2037–C2044.
- YATSUNAMI J, KOMORI A, OHTA T, SUGANUMA M, FUJIKI H. (1993) Hyperphosphorylation of retinoblastoma protein and p53 by okadaic acid, a tumor promoter. *Cancer Res.* **53**, 239–241.
- ZHUO S, FAN S, HUANG S, KAUFMAN S. (1995) Study of the role of retinoblastoma protein in terminal differentiation of murine erythroleukemia cells. *Proc. Natl. Acad. Sci. USA* **92**, 4234–4238.