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The Product of the Retinoblastoma Susceptibility Gene Has Properties of a Cell Cycle Regulatory Element

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Summary

The retinoblastoma susceptibility gene product, Rb, is suspected to supress cell growth. Rb is a 110–114 kd nuclear phosphoprotein. We have previously demonstrated that SV40 T antigen binds only to unphosphorylated Rb, and not pp112–114^{Rb}, the family of phosphorylated Rb. Here we demonstrate the cell cycle–dependent phosphorylation of Rb. In G0/G1 cells, virtually all the Rb is unphosphorylated. In contrast, during S and G2, it is largely, if not exclusively, phosphorylated. Rb phosphorylation occurs at the G1/S boundary in several cell types tested. A 14 residue peptide, corresponding to the SV40 T domain required for transformation, is able to compete effectively with SV40 T for binding to p110^{Rb}. We propose a model to explain how Rb may suppress cell growth by acting as a cell cycle regulatory element.

Introduction

In familial retinoblastoma, young children develop bilateral, multifocal tumors of the retina. Sporadic retinoblastoma is typically characterized by unifocal, unilateral tumors that develop in slightly older children. Knudson (1971) and, later, Comings (1973) statistically analyzed these observations and suggested that in the familial form, one allele of a relevant autosomal gene was inherited in a defective form in all somatic cells, and that retinal cells acquired a neoplastic phenotype upon losing the function of the other allele. In the sporadic form, it was predicted that both allelic mutations were acquired somatically. Cytogenic and molecular analyses have now shown that retinoblastoma cells often bear two defective allelic copies of a single gene, *RB1*, located on chromosome 13q14 (Cavenee et al., 1983; Dryja et al., 1986; Friend et al., 1986; Fung et al., 1987; Lee et al., 1987a; McGee et al., 1989; T'Ang et al., 1989). Given these clinical observations, the loss of *RB1* function has been linked to the development of retinal tumors and, therefore, the loss of growth regulation in the affected cells. It follows, then, that the product of this gene, a differentially phosphorylated 110 kd nuclear protein (Rb), may be able, directly or indirectly, to contribute to growth suppression (Lee et al., 1987b; Ludlow et al., 1989). How it performs this function is unknown.

Survivors of familial retinoblastoma have an increased risk of developing a second neoplasm, which is typically mesenchymal in origin (Hansen et al., 1985; Hawkins et al., 1987). These tumor cells are also defective in *RB1* function. Moreover, several laboratories have reported that a significant proportion of small cell lung and breast cancer lines and primary tumors carry a defect in the *RB1* gene (Bookstein et al., 1989; Harbour et al., 1988; Lee et al., 1988; Varley et al., 1989; Yokota et al., 1988). *RB1* defects have also been observed in bladder carcinoma cell lines (Horowitz et al., 1989) and in cells from sporadic osteogenic sarcomas (Toguchida et al., 1989). In short, while *RB1* function is required for the prevention of a specialized tumor of the eye, it may also have neoplastic growth suppression properties in a wide variety of cell types.

Further indication that Rb functions in growth suppression of mammalian cells has come from the study of DNA tumor viruses. In three separate cases, a major viral transforming product has been shown to form a specific complex with Rb (DeCaprio et al., 1988; Dyson et al., 1989a; Whyte et al., 1988). Where analyzed, the genetics of complex formation has suggested a link between the ability of the viral protein to complex with Rb and its ability to serve one or more aspects of its transforming function (DeCaprio et al., 1988; Egan et al., 1989; Horowitz et al., 1989). These findings have reinforced the thinking that would attribute growth suppression activity to Rb, since it can be argued that one aspect of the transforming function of SV40 large T antigen (SV40 T), adenovirus E1A, and human papilloma virus E7 (HPV-E7) is the modulation (?inhibition) of Rb growth suppression function.

One property of SV40 T that might prove to be relevant to how Rb functions is its ability to stimulate G1-arrested cells to enter the cell cycle (Dulbecco et al., 1965; Galanti et al., 1981; Hatanaka and Dulbecco, 1966; Henry et al., 1966; Mueller et al., 1978; Soprano et al., 1983; Tjian et al., 1978; Todaro and Green, 1967). In considering possible mechanisms to explain this phenomenon, one should note two facts: first, SV40 T complexes specifically with Rb and, second, it can bind only to un- or underphosphorylated Rb, that is p110^{Rb}. It cannot bind to pp112–114^{Rb}, the complex family of differentially phosphorylated *RB1* products (Ludlow et al., 1989). Thus, it has been proposed that p110^{Rb}, and not pp112–114^{Rb}, can perform those aspects of the Rb growth suppression function that SV40 T can perturb (Ludlow et al., 1989). Given these possibilities, it seemed reasonable to ask whether, within a given cell, a link exists between the state of Rb phosphorylation and its position in the cell cycle.

Results

Dynamics of Rb Phosphorylation in Primary Human Cells

To test for the relationship between cell cycle position and the state of Rb phosphorylation, we first studied primary human umbilical vein endothelial cells (EC). These cells

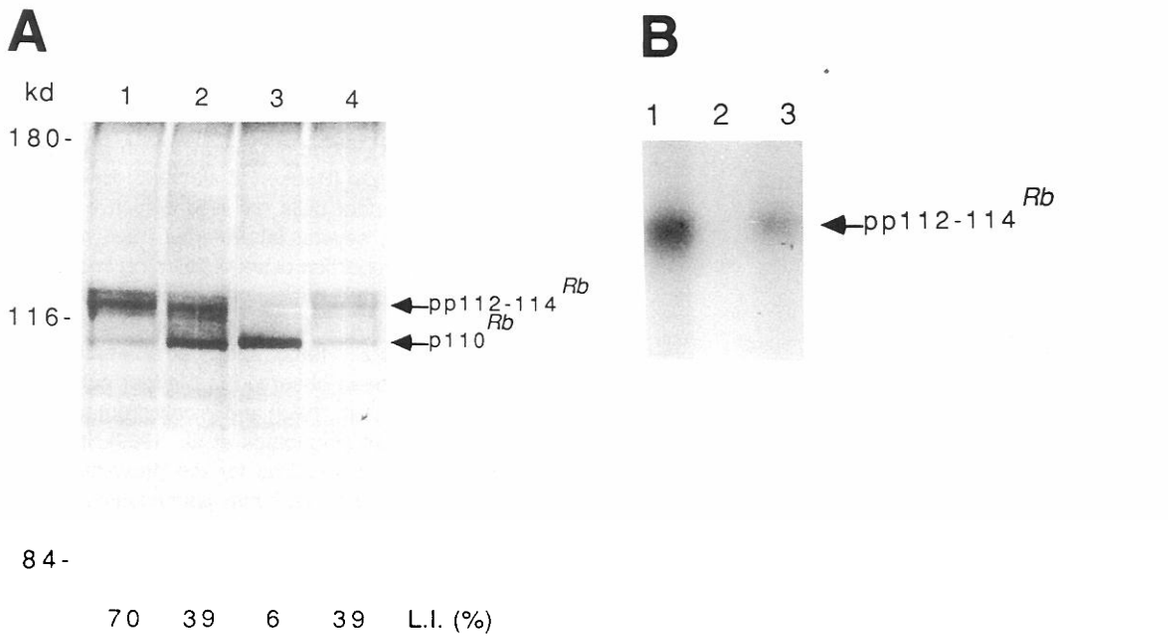

Figure 1. Rb in Growth-Arrested and Restimulated Endothelial Cells

(A) Replicate plates of subconfluent EC cells were cultured for 36 hr in the absence of ECGF. Some were destined for subsequent Western blot analysis (A), others for ^{3}[H]thymidine labeling (A), and yet others for ^{32}P-orthophosphate labeling (B) in the presence (A), lane 4, or absence of ECGF (A), lane 3. Two replicates of the ECGF-depleted culture were fed with ECGF for 36 hr (lanes 1 and 2). In one of them (lane 1), hydroxyurea was added to the ECGF-containing medium 20 hr after adding the factor. This culture was then incubated for an additional 16 hr in the presence of the drug. At that point, the drug was removed, and all cultures were incubated in ECGF-containing medium for 30 min and lysed. Equal quantities of crude extract protein (140 μg) were loaded in each lane of a 7.5% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose and then exposed to a monoclonal antibody to Rb followed by the immunostaining steps described earlier (DeCaprio et al., 1988; Ludlow et al., 1989). ^{3}[H]thymidine was added to duplicate plates of each of the variously treated cultures for the terminal 30 min period, and then exposed to radiographic emulsion. 500–700 cells/plate were analyzed to determine the labeling index (L.I.).

(B) Rb immunoprecipitation from variously treated EC cells. ^{32}P-orthophosphate (0.8 mCi/ml) was added to phosphate-free DMEM containing 1% dialyzed calf serum for 3 hr prior to harvesting a member of each of the variously treated EC cultures described above. Lane 1 contains an anti-Rb immunoprecipitate generated with an extract of cells treated identically to those shown in (A), lane 2. The immunoprecipitate in lane 2 was of an extract similar to that evaluated in (A), lane 3, and that in lane 3 was of an extract similar to that evaluated in (A), lane 4.

can grow for approximately 25 passages when endothelial cell growth factor (ECGF) is present in the medium (Maciag et al., 1979; Maciag et al., 1981; Thornton et al., 1983). Without ECGF, these cells can attach, but are unable to proliferate (Maciag et al., 1981). In a pilot study, we cultured EC cells for at least 36 hr without ECGF and found that there was no longer any cell growth, and only 6% incorporated ^{3}[H]thymidine after a 30 min pulse. We interpreted this to mean that this population was likely to be in G0/G1. We cultured EC cells at subconfluency to avoid the possible influence of contact inhibition on the growth properties of the cell. When ECGF was added back to cells that had been factor-depleted for 36 hr, a small increase above the baseline ^{3}[H]thymidine labeling index was first noted at 16 hr. A major increment in radioactive thymidine uptake into DNA was noted at 36 hr. Following these observations, we prepared a lysate of EC cells growing in medium containing ECGF, separated the proteins by SDS gel electrophoresis, blotted them onto nitrocellulose, and probed the blot for Rb with a monoclonal antibody (Ludlow et al., 1989). When the EC lysate was compared with the lysate of a continuous line of monkey kidney cells, CV-1P (DeCaprio et al., 1988; Ludlow et al., 1989), the Rb band pattern was identical (data not shown). Specifically, a series of Rb bands extending from 110 to 114 kd was observed (Figure 1a, lane 4). Given this similarity, we again prepared a lysate from a subconfluent culture that had been factor-depleted, and this revealed only p110^{Rb} (Figure 1a, lane 3). In a lysate from a culture which had been previously factor-depleted and to which ECGF had been added back for 36 hr, pp112–114^{Rb} was now readily detected (Figure 1A, lane 2). p110^{Rb} was also present in this extract. In addition, hydroxyurea, a specific inhibitor of DNA replication initiation (Adams and Lindsay, 1967), was added to the medium 20 hr after ECGF repletion, and the culture was incubated for 16 hr more. At that point, it was incubated for 30 min in drug-free medium. The goal here was to enrich the relevant cultures for S phase cells. Indeed, a greater proportion of these cells incorporated ^{3}[H]thymidine than was observed in the 36 hour refed culture not exposed to hydroxyurea (70% vs. 39%), and there was a proportional increase in the relative abundance of pp112–114^{Rb} and decrease in that of p110^{Rb} (Figure 1A; compare lanes 1 and 2). In summary, it appears that in endothelial cells, arrested in G0/G1 by ECGF depletion, only p110^{Rb}, the unphosphorylated species, was present. However when 39% to 70% of the cells had entered S, as defined by ^{3}[H]thymidine labeling, a

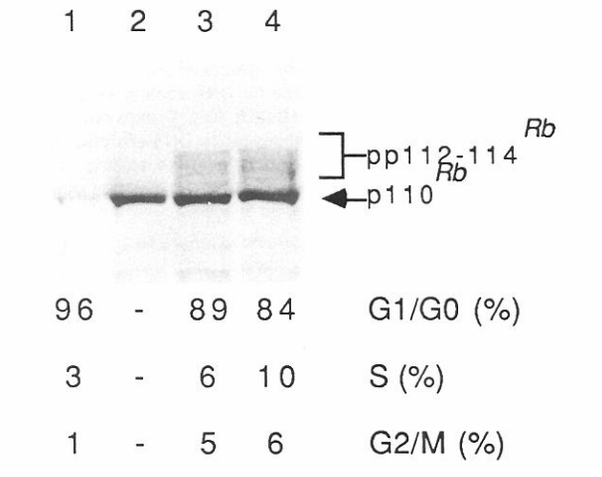

Figure 2. Western Blot for Rb in Resting and Activated T Lymphocytes

Lysates of an enriched population of T lymphocytes were prepared as described in Experimental Procedures. Aliquots of the culture to be analyzed were incubated in the presence of the two specific CD2 activating antibodies described in Experimental Procedures. Aliquots (10^6 cells) were removed before the addition of the antibodies (lane 1), and 24 (lane 2), 48 (lane 3), and 72 hr (lane 4) after addition for analysis of DNA content by cytofluorimetry and of Rb protein by Western blotting.

complex family of phosphorylated Rb bands was readily detected.

As a further test of the contention that phosphorylated Rb is absent in ECGF-depleted EC cells, the latter were metabolically labeled with ^{32}P-orthophosphate in an experiment that was carried out in parallel with the one described above. Lysates, from the various labeled cultures, were immunoprecipitated with monoclonal Rb antibody. As shown in Figure 1B, phosphorylated Rb was detected in the lysates of continously growing (lane 3) and ECGF restimulated cells (lane 1). It was not found in the ECGF depleted cell lysate, even though the parallel culture demonstrated p110^{Rb} by Western blotting (compare Figure 1A, lane 3, with Figure 1B, lane 2). Taken together, it would appear that in primary human endothelial cells, Rb is largely, if not exclusively, unphosphorylated in G0/G1, and phosphorylated in S.

To test whether the same relationship holds between position in the cell cycle and the state of Rb phosphorylation in previously uncultured, primary cells, we studied fresh human T lymphocytes. Nonadherent, peripheral blood mononuclear cells were isolated from a healthy adult volunteer. When assayed by immunofluorescence/flow cytofluorimetry, approximately 85% of them contained the T cell–specific antigen, CD2. Less than 5% of the population were B cells or monocytes, as defined by reactivity with specific antibodies. In a healthy adult, such peripheral T cells are growth-arrested, and will only proliferate when appropriately activated (Crabtree, 1989). To study the pattern of Rb phosphorylation in growth arrested and activated T cells, purified lymphocytes were cultured with two mitogenic, anti-CD2 monoclonal antibodies, known to promote the specific proliferation of CD2-expressing T cells and not that of monocytes or B cells (Meuer et al., 1984). Indeed, simultaneous binding of the CD2 and $CD2_R$ epitopes by these monoclonal antibodies is known to induce peripheral T lymphocytes to proliferate and become activated in the absence of antigen or antigen-presenting cells (Meuer et al., 1984). In the past, maximum T cell proliferation has been found to occur on day 4 after stimulation, with cells first beginning to enter S phase by 36 hr (Meuer et al., 1984).

The T lymphocyte–enriched population, noted above, was incubated with the two CD2 antibodies, and aliquots were removed from the culture at 24-hour intervals for cell cycle analysis by flow cytofluorimetry and for Rb protein analysis by Western blotting. As indicated in Figure 2, before the addition of the activating antibodies, 96% of the freshly prepared T lymphocytes had a 2N DNA content and were in G0/G1 (Figure 2, lane 1). A Western blot for Rb performed on an aliquot of this extract contained only p110^{Rb} (Figure 2, lane 1). Twenty-four hours after activation was initiated by antibody addition, there was no change in the size distribution of Rb bands, although more p110^{Rb} was present in the extract (lane 2). On the other hand, from 48 to 72 hr after adding the activating antibodies, 11% to 16% of the cells present were in S and G2/M as defined by cytofluorometric analysis. Along with p110^{Rb}, pp112–114^{Rb} was now readily detected in these extracts (Figure 2, lanes 3 and 4). Therefore, just as was noted with EC, p110^{Rb} was the only species present in the growth-arrested population, and pp112–114^{Rb} appeared coincident with the onset of DNA replication.

RB Phosphorylation in Continuous Cell Lines

Two questions arise from the results of the aforementioned experiments: Is p110^{Rb} the sole Rb species present during the G1 phase of the cell cycle in logarithmically growing cells, and does it disappear in S and G2/M? We addressed these questions using HeLa cells. These cells were grown in spinner culture and separated by centrifugal elutriation into fractions enriched in the various phases of the cell cycle (Draetta and Beach, 1988; Draetta et al., 1988). Shown in Figure 3 is a Western blot for Rb along with the corresponding percentage of cells of each fraction that were in G1, S, and G2/M as defined by flow cytometric analysis. As can be seen, there is a dramatic shift in the mobility of the Rb species detected as cells progress from G1 to S and G2/M. Specifically, only p110^{Rb} was present in the peak G1 fraction (lane 8), while in the peak S (lane 4) and G2/M (lane 2) fractions, the major, if not exclusive, Rb species present was pp112–114^{Rb}. Moreover, the Rb signal was more intense in the S and G2/M populations than in G1 cells. These data, taken together with the EC and T lymphocyte results, are consistent with a model in which Rb is phosphorylated in S and G2 phases of the cell cycle. In contrast, Rb is predominantly, if not wholly, unphosphorylated in G0/G1.

Because of the likelihood that HeLa cells contain the HPV-18 E7 protein encoded by genomically integrated copies of the viral genome (Schwarz et al., 1985), and because the highly conserved HPV-16 E7 protein can bind to Rb (Dyson et al., 1989), it was important to determine whether this characteristic could alter the cell cycle rela-

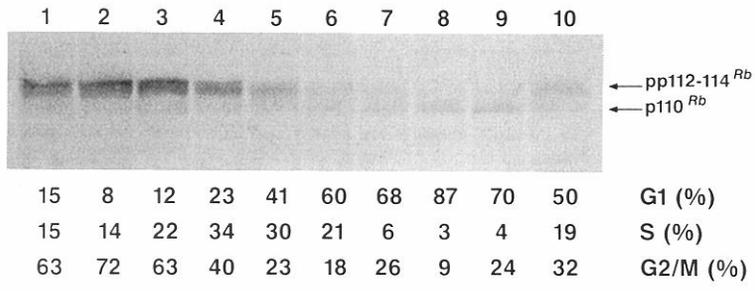

Figure 3. Western Blot for Rb in Elutriated HeLa cells

HeLa cells were elutriated and cytofluorimetrically analyzed for DNA content as descibed (Draetta and Beach, 1988; Draetta et al., 1988). Cells were aliquotted for DNA analysis, protein determination, and Western blotting. A non-elutriated population of HeLa cells is shown in lane 10.

tionships that characterize the Rb phosphorylation process. In an effort to confirm the results obtained with extracts of elutriated HeLa cells, we elected, initially, to study synchronized populations of virus-free primate cells. Extracts of continuously growing cultures of the primate line CV-1P have been shown to contain multiple Rb species that migrate as a 110 to 114 kd family (Ludlow et al., 1989). The results of alkaline and acid phosphatase digestion and of in vivo ^{32}P-labeling of Rb from CV-1P cells demonstrated that differential phosphorylation accounted for all of the gel migrational heterogeneity (Ludlow et al., 1989). Indeed, as noted earlier, the fastest migrating species, p110^{Rb} appeared to be unphosphorylated, while the entire set of 112–114 kd bands were overtly phosphorylated.

Two experimental approaches were employed to assess the effects of the cell cycle on Rb phosphorylation in CV-1P. The results of pilot experiments designed to test different synchronization methods suggested that a modified double-block synchrony method was best (Gurley et al., 1975; Woodford and Pardee, 1986). First, amino acid (methionine) starvation for 48 hr led to the evolution of growth-arrested cells. Methionine refeeding led to the relatively rapid progress of the growth-arrested population into S, as defined by the ^{3}[H]thymidine labeling index. Moreover, methionine refeeding in the presence of hydroxyurea, followed by removal of the drug 16 hr later, led to synchronous passage of approximately 60%–70% of the cells into S (Adams and Lindsay, 1967) (Figure 4A). As also shown in Figure 4A, an Rb Western blot shows that in the G0/G1-arrested population, p110^{Rb} was the major, if not the exclusive, member of the Rb family present in the steady-state population. In contrast, pp112–114^{Rb} was the predominent species present when the labeling index was 69%. D2C2, a subclone of CV-1P transformed by an SV40 early region-encoding plasmid (Ludlow et al., 1989), was also effectively synchronized by the methionine starvation/hydroxyurea method, and approximately the same temporal pattern of Rb transition from p110^{Rb} to pp112–114^{Rb} was seen as noted in CV-1P. Again, in both the G2-enriched population (lanes 1 and 2) and S (lanes 3 and 4), pp112–114^{Rb} was the predominent Rb species present. In contrast, in G1 (lanes 7 and 8), p110^{Rb} was the predominant, if not the only, species noted. Therefore, in keeping with the aforementioned HeLa result, whether or not SV40 T was present, Rb phosphorylation first occurs at the G1/S boundary. Indeed, the fact that pp112–114^{Rb} was readily detected in these cells during active exposure to hydroxyurea (data not shown) suggests that Rb phosphorylation was synchronous with, or occurred shortly before, the onset of chromosomal replication. Additionally, there were no detectable differences in the gross gel migration behavior of pp112–114^{Rb} between S and G2 cells. Hence, there is, as yet, no clear evidence of a change in Rb phosphorylation state between these two cell cycle phases.

We were also able to synchronize CV-1P cells by serum starvation. Cells were plated at a density of 5 × 10^{5} cells per 10 cm plastic dish in DMEM containing 0.2% newborn calf serum. Within 72 hr, cell growth had stopped, and only 5% of the cells labeled with ^{3}[H]thymidine, as measured

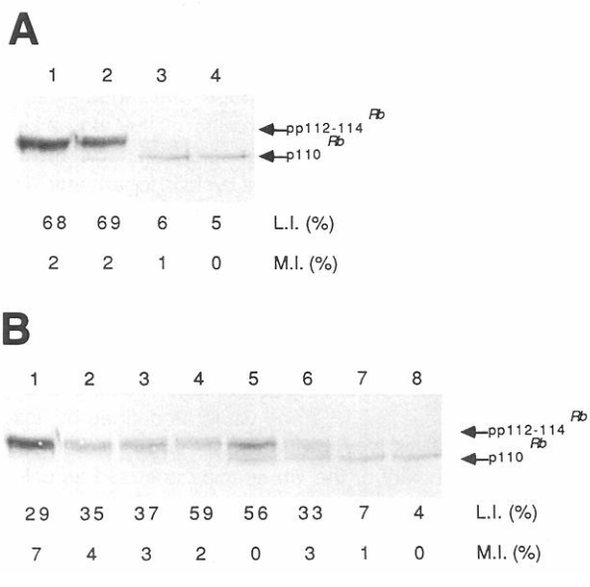

Figure 4. Western Blot for Rb in Synchronized Monkey Cells

CV-1P (4A) and D2C2 (4B) cells were synchronized by methionine-starvation/hydroxyurea treatment as described in Experimental Procedures. Cells were starved in methionine-free DMEM containing 2% dialyzed calf serum for 48 hr. They were then fed with complete DMEM containing 5% fetal calf serum for 30 min (A, lane 4; B, lane 8), 2 hr (B, lane 7), and 6 hr (A, lane 3; B, lane 6). Other cells were replenished with complete DMEM containing 5% fetal calf serum and 0.5 mM hydroxyurea for 18 hr, washed three times, and refed with medium lacking drug for 30 min (B, lane 5), 2 hr (A, lane 2; B, lane 4), 5 hr (A, lane 1; B lane 3), 8 hr (B, lane 2), or 13 hr (B, lane 1). At the time points indicated, lysates (140 μg) were prepared for Rb blotting analysis. Parallel cultures, treated in an identical manner, were incubated in ^{3}[H]thymidine-containing DMEM for 30 min prior to fixation. Labeling (L.I.) and mitotic (M.I.) indices were determined as described earlier.

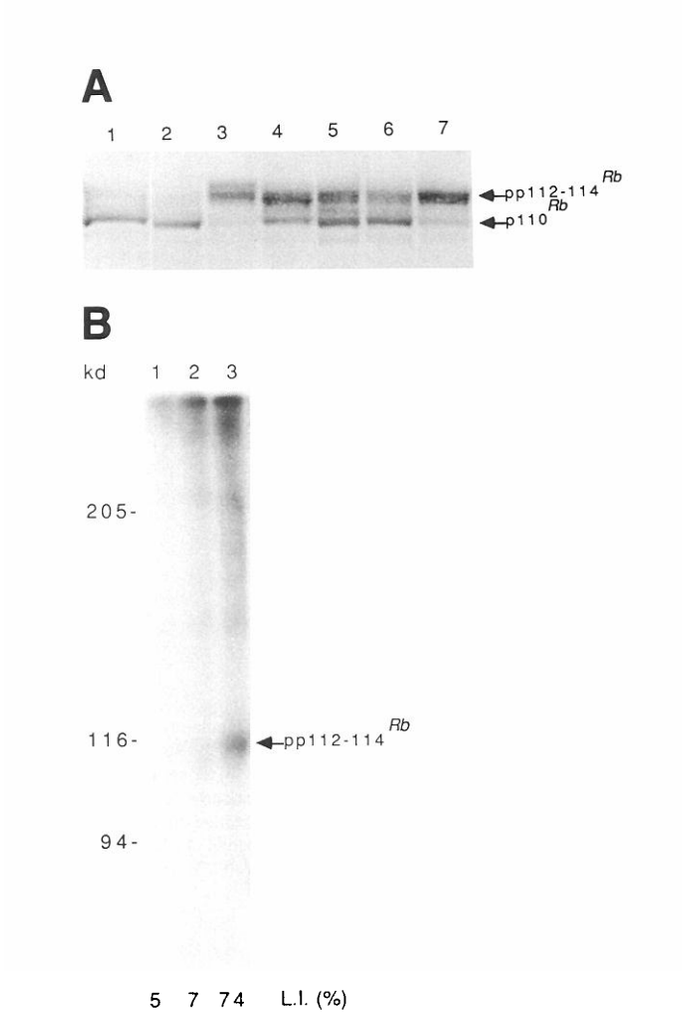

Figure 5. Rb in Serum-Starved CV-1P and D2C2 Cells

(A) CV-1P (lanes 1–4) and D2C2 cells (lanes 5–8) were cultured at subconfluency in DMEM containing 0.2% or 0.1% newborn calf serum, respectively, for 96 hr and then refed with DMEM containing 10% newborn calf serum for 30 min (lanes 1 and 5), 90 min (lanes 2 and 6), or 16 hr (lanes 3 and 7). CV-1P cells not serum-starved, and continuously growing in DMEM and 10% NCS (lane 4), served as a control. At the time points indicated, lysates (140 μg) were prepared for Rb blotting analysis.

(B) ^{32}P-labeled Rb immunoprecipitation of serum-starved CV-1P cell extracts. Parallel cultures of CV-1P cells treated identically to those described in (A), lanes 1–3, were incubated in phosphate-free DMEM containing 10% dialyzed calf serum and 0.8 mCi/ml of ^{32}P-orthophosphate for the last 60 min prior to lysis. Another plate was incubated in DMEM containing 10% newborn calf serum and ^{3}[H]thymidine (10 μCi/ml; 5 Ci/mM) for 30 min before preparation for determination of the labeling index (L.I.). After lysis, each extract (300 μg) was subjected to monoclonal anti-Rb immunoprecipitation.

by autoradiography. The media was then replaced with fresh DMEM containing 10% serum. One hour later, there was no significant increase in ^{3}[H]thymidine uptake. Sixteen hours later, approximately 74% of the cells were in S, as defined by ^{3}[H]thymidine labeling. As shown in Figure 5A, we found that p110^{Rb} was the predominant Rb species in serum-deprived cells (Figure 5A, lane 1), and that pp112–114^{Rb}, was the predominant, if not the sole, species detected in the 16 hour serum-repleted population (Figure 5A, lane 3). Figure 5B shows the results of a parallel experiment, in which serum starved and refed cultures were metabolically labeled with ^{32}P-orthophosphate and then immunoprecipitated with monoclonal Rb antibody. In serum-starved cells (Figure 5B, lane 1) and in those refed with serum for 1 hr (Figure 5B, lane 2), there was little detectable DNA synthesis and no detectable phosphorylated Rb. However, there was readily apparent phosphorylated Rb 16 hr after serum refeeding (Figure 5B, lane 3).

We attempted to apply the serum-starvation protocol to D2C2. Incubation of these cells in medium containing as little as 0.1% serum for 4 days did not lead to cell growth arrest. In turn, as shown in Figure 5A, the pattern of Rb phosphorylation varied little between the serum-starved and serum-fed D2C2 and contained both p110^{Rb} and the phosphorylated Rb band set. When compared in a parallel experiment with its immortal but untransformed parent, CV-1P (Figure 5A, lanes 1–4), the SV40 T–containing derivative D2C2 (Figure 5A, lanes 5–7) could not be growth-arrested by severe serum deprivation.

Peptide Blocking of SV40 Large T and RB

Previously, by genetic means, we identified a discrete SV40 T sequence which is essential to the T-Rb and T-p120 binding reactions (DeCaprio et al., 1988; Ewen et al., 1989). It extends from Asn 102 to Glu 114. Close homologs of this sequence exist in each of the other DNA tumor viral proteins with Rb binding activity, and, where tested, these sequences have also been found to be important to Rb binding (Dyson et al., 1989a; Egan et al., 1989; Horowitz et al., 1989; Kimelman et al., 1985; Stabel et al., 1985). A consensus sequence of minimal degeneracy has been drawn from these various viral protein segments (Figge et al., 1988). Thus far, it has not been possible to know whether this sequence directly or indirectly governs the interaction with Rb and, therefore, how it might participate in the proposed effects of the relevant transforming protein on Rb function, e.g., during the cell cycle.

To approach the question of whether or not this sequence in SV40 T directly contacts Rb and provides a measurable fraction of the specificity of the SV40 T–Rb interaction, we synthesized a peptide containing the wild-type sequence, Asn 102–Glu 115. A second peptide containing the same sequence, but with a Lys for Glu substitution at position 107, was also generated. The latter is a replica of this segment in the SV40 T mutant, K1, a stable but transformation-, and Rb binding–, defective derivative (DeCaprio et al., 1988; Kalderon and Smith, 1984; Chen and Paucha, unpublished data). Each peptide was mixed at two different concentrations, in parallel, with aliquots of [^{35}S]methionine-labeled CV-1P cell extracts prior to adding a source of crude, nonradioactive SV40 T. Control mixtures lacking either peptide or SV40 T were also generated. As can be seen from the data shown in Figure 6A (compare lane 4 with lane 2), both Rb and p120 were coimmunoprecipitated with the anti-SV40 T antibody from the mixture when SV40 T was present and not when it was absent. In contrast, increasing concentrations of the wild-type peptide led to progressively less coprecipitation of both proteins (Figure 6A, compare lanes 5 and 6 with lane

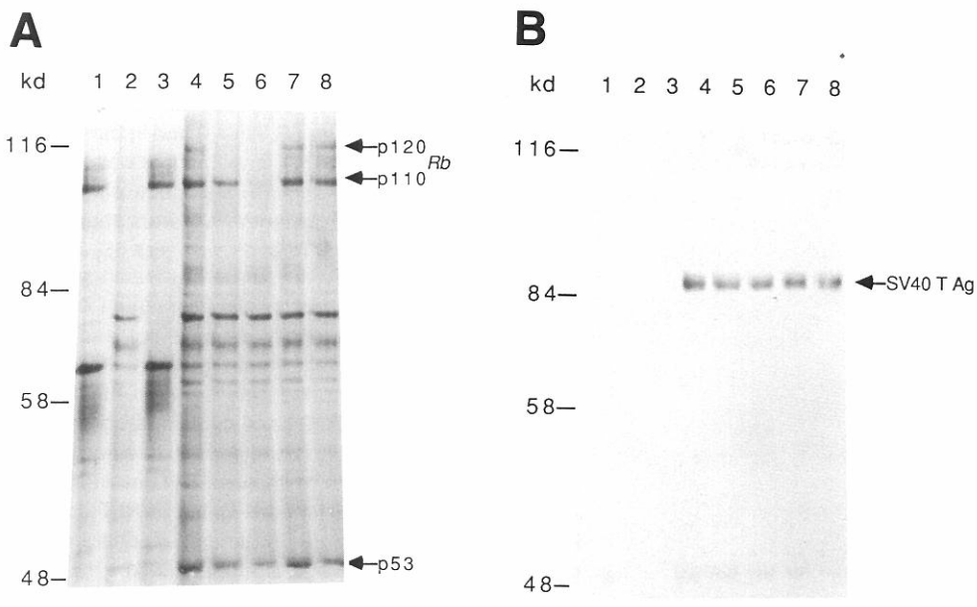

Figure 6. Peptide Mixing Experiment

CV-1P cells were labeled with [^{35}S]methionine (200 μCi/ml) for 3 hr, and lysed as described (DeCaprio et al., 1988). Aliquots of labeled lysate were mixed with wild-type peptide (NLFCSEEMPSSDDE) at 3 μM (lane 5) and 54 μM (lane 6) or mutant peptide (NLFCSKEMPSSDDE) at 3 μM (lane 7) and 54 μM (lane 8) for 60 min at 4°C. Then 100 μl of unlabeled SV80 cell extract (10 μg/μl) was added to each mixture (lanes 3–8) for an additional 60 min. Lysates were immunoprecipitated with an Rb monoclonal antibody (lanes 1 and 3), or with an SV40 T monoclonal antibody (lanes 2 and 4–8). Immunoprecipitates were eluted, and separated by electrophoresis through a 7.5% SDS–polyacrylamide gel. The gel was blotted onto nitrocellulose, and the paper immunostained for SV40 T (B). An autoradiogram was also obtained of this blot (A). The migration positions of p120 (Ewen et al., 1989), p110^{Rb} (DeCaprio et al., 1988; Ludlow et al., 1989), and p53 are indicated along the righthand border of (A). SV40 T is noted along the righthand border of (B). Molecular weight standards are indicated along the lefthand border of each panel.

4), while identical quantities of the mutant peptide (Figure 6A, compare lanes 7 and 8 with lane 4) were inert. In this experiment, addition of the wild-type peptide seemed to reduce slightly the amount of coprecipitating p53. However, this effect was not enhanced by increasing the concentration of the peptide, and was not reproducible from experiment to experiment. These data suggest that the wild-type peptide can form a specific complex with Rb and with p120 and, therefore, that the role of this sequence in the SV40 T–Rb and the SV40 T–p120 interaction is direct. Analogous results have been obtained in parallel by Harlow and co-workers using peptide replicas of the Rb binding control sequences of E1A (personal communication). The possible significance of this observation, i.e., a role for Rb in cell cycle regulation, will be discussed below.

Discussion

The data presented here indicate that in primate cells, both primary and established, epithelial and mesenchymal, and suspended and attached, Rb phosphorylation is tied to specific events in the cell cycle. In G0/G1, virtually all of the detectable Rb exists in an un- or underphosphorylated state. In contrast, in S and G2, it is largely, if not exclusively, phosphorylated and can be identified after SDS gel electrophoresis as pp112–114^{Rb}. Similar results have been obtained, in parallel, by Buchkovich and Harlow (1989, see accompanying paper). The state of Rb phosphorylation during mitosis is not yet known. Nevertheless, given the existing results, it would appear that a specific kinase, inactive or absent in G1, is active at the G1/S boundary and, perhaps, thereafter in G2. At least one of its targets is Rb. Alternatively, the kinase could be constitutively active throughout the cell cycle, and a specific phosphatase, active only in G0/G1, could yield the same results. In the past, we and others have shown that Rb is phosphorylated on serine and threonine. Thus far, no phosphotyrosine has been detected under the analytic conditions used (Ludlow et al., 1989; Shew et al., 1989; Buchkovich and Harlow, 1989). Therefore, one could assume that the Rb kinase is of the serine/threonine variety. Moreover, given the wide spectrum of cell types in which this cyclical Rb phosphorylation process was observed, one might also predict that this type of biochemical behavior is a general phenomenon in mammalian cells.

That Rb is phosphorylated in G2 and not in the ensuing G1 period might mean that the same molecules which were phosphorylated in G2 become unphosphorylated in the next G1. If that were true, a specific phosphatase would have to be invoked. The alternative possibility is that the population of pp112–114^{Rb} in G2 is degraded before the beginning of the next G1 phase, leaving newly synthesized Rb molecules to populate the cell. Without an active kinase, these molecules would constitute the G1-associated p110^{Rb} noted in the results described here. We are investigating which of these two possibilities is relevant.

Activation of the Rb kinase in the cells studied is likely

to occur just before, or possibly coincident with, chromosomal DNA replication. In the experiments described here, the timing of the $p110^{Rb}$ to $pp112\text{-}114^{Rb}$ transition and of the first measureable uptake of radioactive thymidine were indistinguishable. During exposure to hydroxyurea, a specific inhibitor of DNA synthesis, extracts of CV-1P and D2C2 cells contained both $p110^{Rb}$ and some phosphorylated Rb (data not shown); and, within one hour of drug removal, virtually all Rb became phosphorylated. Thus, the blockade of DNA synthesis by hydroxyurea did not prevent Rb phosphorylation. This result is consistent with the notion that the first Rb phosphorylation event preceded the initiation of DNA synthesis. Clearly, more direct analysis is needed to test this hypothesis.

The presence of SV40 T had no apparent effect on the Rb phosphorylation. Specifically, we found that the same temporal and biochemical transition from $p110^{Rb}$ to $pp112\text{-}114^{Rb}$ occurred in CV-1P, D2C2, COS-1, and SV80 cells (data not shown for last two lines). D2C2 contains enough SV40 T to bind all of the $p110^{Rb}$ present in a crude extract of these cells (Ludlow et al., 1989; Ludlow, unpublished observations). The same could be said of SV80, an SV40 transformed human fibroblast line, which contains at least 10^6 molecules of SV40 T per cell (Kilton et al., 1981), and COS-1, an SV40 transformed monkey cell line (Gluzman, 1981). That SV40 T does not appear to affect the transition quantitatively or qualitatively is consistent with observations reported previously, which led to the conclusion that SV40 T could bind $p110^{Rb}$ without grossly altering the process of Rb phosphorylation (Ludlow et al., 1989). The results of genetic analyses strongly suggest that SV40 T operates, in part, as a dominant transforming element by modulating (?inhibiting) one or more aspects of the Rb growth suppression function. Thus, it was further reasoned that $p110^{Rb}$, and not $pp112\text{-}114^{Rb}$, could perform those aspects of the Rb growth suppression function that SV40 T can perturb (DeCaprio et al., 1988; Ludlow et al., 1989).

What might those functions be? The data presented here offer a clue to their generic identity. First, the "active" form of the Rb family seems to be present in G0/G1 and not in S or G2, where "active" means able to carry out those aspects of Rb function which SV40 T can perturb. Second, from what is known of hereditary retinoblastoma and the relationship between the genotype and growth behavior, it can be predicted that at least one Rb function is to suppress cell growth (see Introduction). Third, SV40 T is a potent mitogen, well known to be able to drive G0/G1-arrested cells into S (Dulbecco et al., 1965; Galanti et al., 1981; Hatanaka and Dulbecco, 1966; Henry et al., 1966; Mueller et al., 1978; Soprano et al., 1983; Tjian et al., 1978; Todaro and Green, 1967). Putting these observations together, one might conclude, first, that active Rb is present in G1 and not in S and that there is a transition from "active" to "inactive" at the G1/S boundary. "Inactive" here means unable to perform its SV40 T–modulated growth suppression function(s). This would suggest that $p110^{Rb}$ normally contributes to the interposition of a block to exit from G1.

The block to exit from G1 may prove to be distinquishable from that preventing exit from G0. Cells that are not continuously dividing are said to be out of the cell cycle and in a phase called G0 (Pardee et al., 1978). From three different experiments shown here, one has the impression that the state of Rb phosphorylation does not change immediately after the addition of the stimulus to the growth-arrested cells, but rather when the cells eventually enter S (see Figures 1, 2, and 5). Specifically, there was a lag of up to 36 hr in the various experiments described here between introduction of a stimulus to growth and the first appearance of $pp112\text{–}114^{Rb}$. There was, however, a close temporal link between the onset of Rb phosphorylation and of cellular DNA synthesis.

If $p110^{Rb}$ contributes to the development of a G1 exit block, how might such a boundary be eliminated? First, it could be reasonably argued that specific phosphorylation could bring about such an effect in a physiological manner. Alternatively, SV40 T binding might constitute a pathological route to overcoming such a hurdle (see Figure 7). In this regard, SV40 T is an established mitogen (Dulbecco et al., 1965; Galanti et al., 1981; Hatanaka and Dulbecco, 1966; Henry et al., 1966; Mueller et al., 1978; Soprano et al., 1983; Tjian et al., 1978; Todaro and Green, 1967) capable of stimulating G1-arrested permissive and non-permissive cells to enter the cycle. Although not yet formally tested, it would not be surprising to find that Rb binding represents an important step in the mechanism underlying the G1 to S promotion effect of this viral protein. Given that E1A is also a mitogen with activity analogous to that of SV40 T (Kaczmarek et al., 1986; Moran and Zerler, 1988; Stabel et al., 1985), one could draw a similar model accounting for at least one aspect of the mechanism underlying its mitogenic action.

It could also be argued that $p110^{Rb}$ facilitates entry into the quiescent state, G0. It seems likely that both SV40 T and polyoma can prevent cells from entering this growth-arrested state (see Figure 5; Smith et al., 1971; Baserga et al., 1973; Burstin and Basilico, 1975; Martin and Stein, 1976; Bikel et al., 1987). Indeed while certain untransformed, but immortal, fibroblast lines, deprived of relevant growth factors, can become quiescent, their SV40 transformed derivatives do not (Scher et al., 1979). Viewed in the context of Rb function, the untransformed parental cells can perhaps enter the quiescent state only when

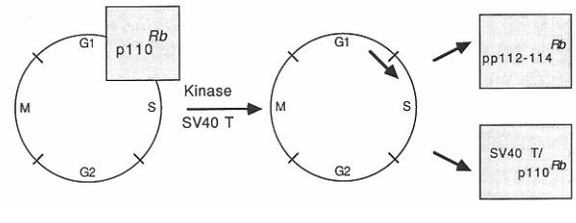

Figure 7. Model for the Growth Suppression Function of $p110^{Rb}$

It is proposed that $p110^{Rb}$ helps to provide a block to exit from G1 and, thereby, prevents the cell from progressing to S. The block can be removed by specific phosphorylation of $p110^{Rb}$ to yield $pp112\text{–}114^{Rb}$ or by binding to SV40 T. Once $p110^{Rb}$ is inactivated by phosphorylation or binding to SV40 T, the cell can advance to the DNA replication phase of the cycle.

$p110^{Rb}$ is present, while SV40 T binding to $p110^{Rb}$ would, in theory, block this effect. In addition, if $p110^{Rb}$ normally facilitates entry into G0, it is conceivable that it, thereby, facilitates the appearance of certain differentiated behavior, given the tendence of terminally differentiated cells to demonstrate G0/G1 arrest.

Another observation that may be relevant is that, in several experiments reported here, there was an apparent increase in the total amount of Rb present in G1 compared with S and G2/M cell extracts (Figure 3, compare lanes 3 and 9; Figure 4A, compare lanes 1 and 2 with lanes 3 and 4; Figure 4B, compare lanes 4 and 8). Hence, the Rb protein concentration might change in the cell at the G1/S boundary. This observation needs to be validated in experiments employing several Rb antibodies, as we cannot rule out the possibility that the Rb monoclonal antibody used here has a greater affinity for $pp112\text{-}114^{Rb}$ than for $p110^{Rb}$. If the observation is valid, it will then be interesting to determine whether it results from alterations in *RB1* transcription or from posttranscriptional effects.

Hence, from data such as that presented here, and from analogous results obtained in parallel by Buchkovich and Harlow (1989), one might be led to construct a model in which at least one aspect of Rb growth suppression function is a contribution to normal cell cycle regulation (Figure 7). These findings in no way imply that $pp112\text{–}114^{Rb}$ is biologically or biochemically inert. Indeed, it seems possible that this complex family of differentially phosphorylated proteins plays other growth regulatory functions, perhaps even in the cell cycle.

That the peptide representing the 102–115 residues of SV40 T competed effectively with SV40 T for Rb binding is consistent with the prediction that Rb has cell cycle regulatory behavior. From the results shown in Figure 6, one could argue that this sequence binds directly to Rb and to p120, another SV40 T binding protein (Ewen et al., 1989; Dyson et al., 1989b), and that it likely contributes a significant measure of the free energy of SV40 T–Rb complex formation. In short, it plays a direct and essential role in the reaction. The data presented here strongly suggest that, in at least one case, it may also be modular and that other SV40 T sequences are not essential to Rb binding. This conclusion is consistent with earlier results of Moran (1988). Given these considerations, it seems logical to ask whether any cellular gene products have a similar sequence. Underlying this question is the suspicion that one or more cellular proteins physiologically interacts with Rb by an analogous mechanism to that employed by the family of Rb-binding viral proteins. Indeed, Figge and Smith (1988) have concluded, from a computer-assisted search, that the cdc25 product of S. pombe contains a sequence which fits the rather tight consensus sequence drawn from an analysis of the related viral protein Rb binding sequences noted above. S. pombe cdc25 plays a specific role in regulating cell cycle behavior, in concert with cdc2 (Fantes, 1979; Russell and Nurse, 1986). cdc2 is a serine/threonine kinase known to promote the overriding of two physiological blocks to cell cycle traverse—one at the G1/S boundary, the other at the G2/M boundary (Nurse and Bissett, 1981; Nurse et al., 1976; Riabowol et al., 1989; Simanis and Nurse, 1986). Thus far, cdc25 has been shown to be active in the latter case. Whether it is also active at the G1/S boundary is not yet clear. Nevertheless, a cell product bearing a small, colinear segment which is, structurally, a homolog of a modular Rb binding domain of SV40 T, is also a positive cell cycle regulatory element. That SV40 T and cdc25 both have discrete mitogenic effects, and that the former may operate, at least in part, by perturbing the function of Rb, could be viewed as being consistent with the hypothesis that $p110^{Rb}$ has a cell cycle blocking effect.

What is the nature of the Rb kinase? Nothing reported here gives the answer. Nevertheless, there is at least one specific possibility. As noted above, cdc2 is a serine/threonine kinase, and it facilitates passage through a G1/S block. It is, therefore, a reasonable Rb kinase candidate, especially since Rb contains multiple cdc2 consensus phosphorylation sites (Shenoy et al., 1989) and is phosphorylated at the G1/S interface. These consensus sequences have been defined for $pp60^{c\text{-}src}$, which is known to be phosphorylated at mitosis (Chackalaparampil and Shalloway, 1988; Morgan et al., 1989; Shenoy et al., 1989). On the other hand, it should also be noted that there is no direct, published evidence for specific substrates that are phosphorylated by cdc2 at the G1/S interface, nor is there evidence regarding the nature of the enzyme which operates in vivo. Indeed, there could be more than one kinase. Whatever the identity of this enzyme(s), it likely plays an important role in cell cycle regulation, and would, in principle, have the properties of a proto-oncogene product.

Experimental Procedures

Cell Culture

Primary cultures of human umbilical vein (EC) endothelial cells in their fourth passage were a gift from Dr. M. Gimbrone (Brigham and Women's Hospital, Boston, MA). They were grown on a plastic substrate at 37°C in a 5% CO_2-containing humidified atmosphere in Medium 199 (Gibco) containing 20% newborn bovine serum (Flow Laboratories), penicillin (50 U/ml), streptomycin (50 μg/ml), amphotericin B (4 μg/ml), endothelial cell growth factor (250 μg/ml) (Maciag et al., 1979), and heparin (100 μg/ml; Sigma) (Thornton et al., 1983). Subconfluent cultures between passages 4 and 7 were used.

Mononuclear cells were isolated from a healthy adult volunteer by Ficoll-Hypaque gradient centrifugation. Monocytes were depleted by adherence to a plastic tissue culture dish for 1 hr. After this step, the nonadherent fraction contained greater than 90% CD2+ cells, and less than 5% of either CD14+ (B cells) or CD20+ (monocytes) cells, as measured by indirect immunofluorescence followed by flow cytometry. Further enrichment of T cells by rosette formation was not performed to avoid activation of T cells. This enriched T cell population was incubated at 37°C in 10% CO_2-containing humidified atmosphere at a density 5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco) in the presence of the anti-CD2 monoclonal antibodies, T11.2 and T11.3, each at 1:1000 dilution of ascites (Meuer et al., 1984). Aliquots were removed at various times thereafter for immunophenotype analysis, cytometric analysis to define cell cycle status, and extraction for Rb Western blotting.

HeLa cells were cultured and elutriated as described earlier (Draetta and Beach, 1988; Draetta et al., 1988) by G. Draetta and D. Beach, who kindly and generously forwarded them to us. Elutriated fractions were aliquotted for cytofluorimetric and Western blot analysis. Lysis buffer contained 10 mM Na phosphate (pH 7.2), NP40 (1%), 150 mM NaCl, glycerol (10%), 50 mM NaF, 10 mM Na pyrophosphate, 1 mM Na orthovanadate, 1 mM PMSF, 20 μM leupeptin, and aprotinin (5 μg/ml).

CV-1P and D2C2 cells were grown in DMEM containing 10% new-

born calf serum (Gibco) in a 10% CO_2-containing humidified atmosphere. For the serum starvation experiments, subconfluent cultures were trypsinized into DMEM containing 0.1%–0.2% newborn calf serum (Gibco) and incubated for 96 hr. At that time the cells were washed with serum-free DMEM and fed with DMEM containing 10% newborn calf serum.

Cell Synchronization by Methionine Starvation and Hydroxyurea Treatment

CV-1P and D2C2 cells grown in DMEM containing 5% fetal calf serum (Gibco) were washed once with methionine-free DMEM (Gibco), trypsinized, and split into methionine-free DMEM containing 2% dialyzed calf serum (Gibco) at 5×10^5 cells per 100 mm dish. Cells were incubated in this medium for 48 hr at 37°C. The plates were then washed three times with DMEM and changed into DMEM containing 5% fetal calf serum and 0.5 mM hydroxyurea (Sigma) for 16 hr. The cells were then washed three times with DMEM, and fed with DMEM containing 5% fetal calf serum. Fresh 2 mM glutamine was added to the media at each refeeding.

T Lymphocyte Cell Cycle Analysis by Cytometry

Enriched T lymphocytes (2×10^6) were resuspended in 1.0 ml of propidium iodine solution (propidium iodine [50 μg/ml], Na citrate [0.1%], and NP40 [0.1%]) and incubated for 15 min at 4°C. DNA histograms were analyzed with the help of a Coulter Para-1 computer program.

[^{3}H]Thymidine Labeling

Cells growing on plastic dishes were incubated with ^{3}[H]thymidine, (10 Ci/ml; 5 μCi/mM, Amersham) in DMEM (CV-1P and D2C2) or Medium 199 (EC) containing 10% serum for 30 min at 37°C. The dishes were then placed on ice, and the medium removed. The cells were washed once with PBS, once with a 1:1 solution of PBS and methanol/acetic acid (3:1), and then fixed on ice for 10 min with methanol(3)/acetic acid(1) (Freshney, 1983). The plates were dried at room temperature, and then exposed to autoradiographic emulsion (NTB 2; Kodak) for 7 days. The dishes were developed and fixed using the manufacturer's protocols. Each measurement of the labeling and mitotic index was made by counting 500–700 cells.

Western Blotting and Immunoprecipitation

All cells, except HeLa cells, were lysed in EBC buffer [50 mM Tris (pH 8.0), 120 mM NaCl, NP40 (0.5%), 100 mM NaF, 200 μM Na orthovanadate, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonylfluoride (10 μg/ml)] for 20 min on ice. The lysates were cleared by centrifugation at 14,000 × g for 15 min. For the Western blotting experiments, each gel slot was loaded with 140 μg of cell lysate protein. Electrophoresis was performed in 7.5% SDS–polyacrylamide gels (Bradford, 1976; Laemmli, 1970). Blotting was performed as previously described (Ludlow et al., 1989). ^{32}P-orthophosphate labeling and immunoprecipitations were also performed as described (Ludlow et al., 1989).

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