dren's Hospital Research Foundation. The Centre d'Etude du Polymorphisme Humain provided DNA samples and computer software. The cell lines described in this paper and the DNA probe VK21 (including VK21A and VK21C) are available on a collaborative basis.

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Cell Cycle–Dependent Regulation of Phosphorylation of the Human Retinoblastoma Gene Product

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The human retinoblastoma gene (*RB1*) encodes a protein (Rb) of 105 kilodaltons that can be phosphorylated. Analysis of Rb metabolism has shown that the protein has a half-life of more than 10 hours and is synthesized at all phases of the cell cycle. Newly synthesized Rb is not extensively phosphorylated (it is "underphosphorylated") in cells in the G_0 and G_1 phases but is phosphorylated at multiple sites at the G_1/S boundary and in S phase. HL-60 cells that were induced to terminally differentiate by various chemicals lost their ability to phosphorylate newly synthesized Rb at multiple sites when cell growth was arrested. These findings suggest that underphosphorylated Rb may restrict cell proliferation.

WHEN ACTIVELY PROLIFERATING cells are deprived of growth factors, they become arrested at the G_0 phase of the cell cycle. Conversely, quiescent cells are induced to proliferate when provided with the appropriate growth factors. Exposure of quiescent cells to growth stimuli leads to quantitative and qualitative changes in the expression of many specific genes and their products. For example, exposure of resting cells to platelet-derived growth factor (PDGF) (*1*) or epidermal growth factor (EGF) (*2*) leads to the activation of the respective receptor tyrosine kinases, which may then directly phosphorylate phospholipase C. This, in turn, may result in other biochemical responses such as the hydrolysis of phosphoinositides, the elevation of intracellular Ca^{2+}, the modulation of protein phosphorylation, and the induction of synthesis of new mRNA [reviewed in (*3*)]. Because some of the transcriptionally activated genes are proto-oncogenes, studies on the control of cell proliferation have mainly focused on the isolation and characterization of similar genes, the expression of which is activated in the early hours of the proliferative response (*4*). A number of genes that are expressed specifically in the quiescent state have also been isolated (*5*). The expression of these genes is repressed when a cell is exposed to growth stimuli. Therefore, downregulation of specific genes may also be important for the mitogenic response, by removing blocks to cell proliferation.

The human retinoblastoma gene, *RB1*, is the prototype of a class of genes in which the inactivation of both alleles is thought to be essential for neoplastic growth. The isolation of *RB1* has allowed a confirmation at the molecular level of the loss of the gene in retinoblastoma and clinically related tumors, such as osteosarcomas (*6*). Moreover, analyses using the *RB1* cDNA probe and antibodies to its gene product (Rb) have revealed the loss of *RB1* in a large number of tumors seen in adults, such as small cell lung carcinoma (*7*) and breast tumors (*8*). That *RB1* may have an important function in the control of neoplastic growth is supported by the fact that Rb forms a physical association with the transformation proteins of several viruses: E1A of adenovirus (*9*), large T antigen of simian virus 40 (SV40) (*10*), and the E7 protein of human papillomavirus type 16 (*11*). As the inactivation of *RB1* is associated with tumorigenesis, it is possible that the function of Rb is to restrict cell proliferation. We have examined the regulation of Rb expression in cells under various growth conditions.

Peptides representing the most hydrophilic regions of Rb were synthesized and used to immunize rabbits and BALB/c mice for the production of polyclonal and monoclonal antibodies (*12*). Multiple forms of Rb, with apparent molecular masses from 105 to 115 kD, could be detected by immunoprecipitation from the human bladder tumor cell line VM-CUB-3 labeled with [^{35}S]methionine, followed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A, lane 1). No such protein could be precipitated when the antibodies were preabsorbed with the corresponding Rb peptides (Fig. 1A, lane 2). Metabolic labeling with [^{32}P]orthophosphate demonstrated that the Rb polypeptides with higher apparent molecular mass were more heavily phosphorylated (Fig. 1A, lane 3). Treatment of the phosphoproteins with potato acid phosphatase before immunoprecipitation resulted in the disappearance of these Rb species (*13*). Immunohistochemical staining of cells with these antibodies revealed the nuclear localization of Rb as has been described (*14*) and is predicted from the presence of a sequence of amino acids, VRSPKKK (residues 609 to 615) (*15*), reminiscent of the nuclear translocation signal found in SV40 large T antigen.

To determine whether the synthesis and degradation of Rb is regulated in a cell cycle–dependent manner, we measured the steady-state amount of Rb as a function of the cell cycle. Because the rabbit polyclonal antibodies Rb 1-AB 20, Rb 1-AB 16, and Rb 1-AB A1 detect the various phosphorylated forms of Rb, it was possible to measure the total amount of Rb in cells. Exponentially proliferating wild-type HL-60 cells were fixed and stained with Rb 1-AB 20 and a secondary fluorescein-conjugated antibody to rabbit immunoglobulin G (IgG). The cells were then treated with ribonuclease A (RNase A), and stained with propidium iodide for quantitation of DNA. Because fluorescein emission is green and propidium iodide emission is red, when the cells are irradiated at 488 nm, it is possible to simultaneously measure the emission intensities, and therefore the Rb and DNA content, by flow cytometry (Fig. 1B). The amount of Rb per cell increased as the cells progressively matured through the G_1, S, and G_2 + M phases of the cell cycle. Cells in the G_2 + M phases contain approximately twice as much Rb as cells entering G_1. Therefore, the Rb content of a proliferating cell is always maintained at or above the threshold level in G_0 and G_1. This would be expected if the rate of Rb synthesis exceeds that of its degradation. These results were reproduced when the experiments were performed with Rb 1-AB A1 (*13*).

To understand the dynamics of Rb turnover, we studied the synthesis and degradation of Rb with immunoprecipitation techniques. The human breast tumor cell line SW613 was pulse-labeled for 90 min with [^{35}S]methionine, and the degradation rate of labeled Rb was monitored (Fig. 1C).

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Densitometric scanning of the intensities of the various forms of Rb showed the half-life of the protein to be at least 10 hours. A similar Rb half-life profile has been observed by others (*16*).

We analyzed the synthesis of Rb as a function of the cell cycle in SW613 and VM-CUB-3 cells. Two to 3 days after the withdrawal of serum, the exponentially growing cells ceased to proliferate. Cell numbers remained constant, and no DNA synthesis occurred during the next 5 days. When the cells were released into the proliferative phase by the addition of either serum or of EGF together with insulin and transferrin, they synchronously traversed the cell cycle. Pulse-labeling experiments with [^{35}S]-methionine showed that Rb was synthesized in both the quiescent state (Fig. 2A; Fig. 2B, lane 1) and in the proliferative phases (Fig. 2B, lanes 3 to 13). The fact that Rb is stable and is constantly being synthesized is consistent with the observed steady-state accumulation of the protein as the cell volume increases.

The inability of a cell to decrease its Rb content suggests that it must have other ways of regulating the activity of this protein. In the quiescent state, the cells had only a single species of underphosphorylated Rb, with an apparent molecular mass of 105 kD (Fig. 2A, lanes 3 to 5; Fig. 2B, lane 1). On reintroduction of serum, synchronized cells traversing the cell cycle still exhibited only the underphosphorylated form of Rb at the early G_1 phase (Fig. 2B, lanes 3 and 4). However, cells at the G_1/S boundary and in the S phase (Fig. 2B, lanes 5 to 10) regained the ability to produce multiply phosphorylated, newly synthesized Rb. Ongoing DNA synthesis is not required for the cells to produce multiply phosphorylated Rb. Cells

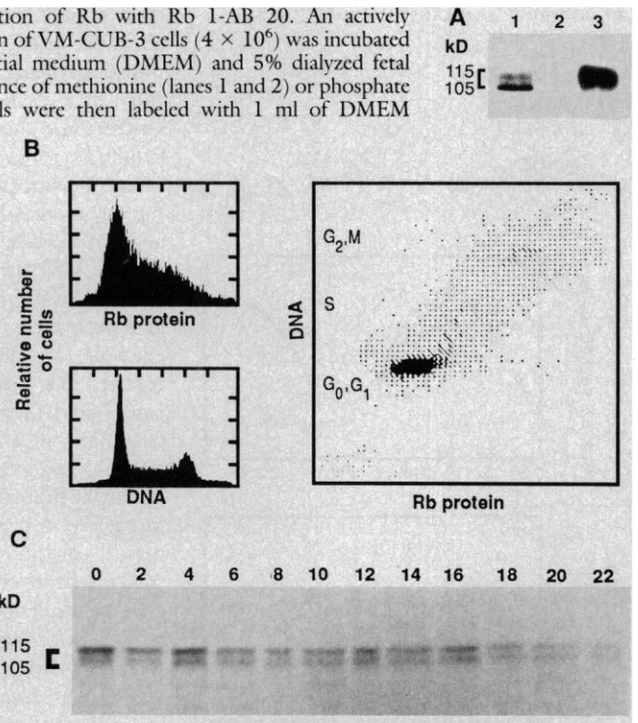

Fig. 1. (**A**) Immunoprecipitation of Rb with Rb 1-AB 20. An actively proliferating random population of VM-CUB-3 cells (4×10^6) was incubated in Dulbecco's minimum essential medium (DMEM) and 5% dialyzed fetal bovine serum (FBS) in the absence of methionine (lanes 1 and 2) or phosphate (lane 3) for 1 hour. The cells were then labeled with 1 ml of DMEM containing 5% dialyzed FBS and either 300 μCi of [^{35}S]methionine (1000 Ci/mmol) (lanes 1 and 2) or 300 μCi of [^{32}P]orthophosphate (9120 Ci/mmol) (lane 3) for 2 hours at 37°C. Cells were then washed twice with phosphate-buffered saline (PBS) and proteins were extracted with 0.5 ml of EBC [40 m*M* tris-HCl (*p*H 8.0), 120 m*M* NaCl, 0.5% NP-40, aprotinine (2 μg/ml), pepstatin (2 μg/ml), leupeptin (2 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml)] at 0°C for 1 hour. The cell lysates were clarified by centrifugation at 15,000 rpm for 10 min at 4°C. The supernatant was incubated for 2 hours at 4°C with either 5 to 10 μl of Rb 1-AB 20 antiserum (lanes 1 and 3) or Rb 1-AB 20 that had been incubated with 100 μg of the peptide P5 for 2 hours at 4°C (lane 2). The immunocomplex was collected on protein A–agarose (Calbiochem) and washed 5 times with EBC. The immunoprecipitated proteins were eluted by heating for 1 min at 90°C in sample buffer [62.5 m*M* tris-HCl (pH 6.8), 5% β-mercaptoethanol, 2.3% SDS, 10% glycerol, and 0.001% bromophenol blue] and separated by SDS-PAGE. The gel was fixed and exposed to x-ray film. (**B**) Analysis of the steady-state concentration of Rb by flow cytometry. Exponentially proliferating HL-60 cells (2×10^6) were suspended in 0.1 ml of PBS at 4°C and fixed by adding 0.9 ml of cold (−80°C) methanol. The fixed cells were resuspended at 4°C in 120 μl of PBS-NGS (50% PBS, 50% normal goat serum, and 0.002% Triton X-100) and 10 μl of Rb 1-AB 20. The suspension was incubated for 1.5 hours at 37°C and periodically mixed. The suspension was cooled on ice for 2 min, and then 150 μl of PBS was added. The cells were incubated for 30 min at 4°C and periodically mixed. For secondary staining, the cells were resuspended in 195 μl of PBS-NGS and 5 μl of FITC-GAR (fluorescein isothiocyanate–conjugated goat antibody to rabbit IgG). The suspension was incubated for 1 hour at 37°C, cooled for 2 min on ice, and 150 μl of PBS-NGS was then added. This washing step was repeated once, and the cells were then treated with RNase A (5 μg/ml) for 30 min at 37°C. The cells were finally stained with propidium iodide (5 μg/μl), and analyzed by flow cytometry. Flow cytometry was performed with an argon ion laser flow cytometer (Profile Model, Coulter Electronics). Excitation was at 488 nm. Green emission was collected with a photomultiplier masked with a 525-nm band pass filter for measuring Rb content, and red fluorescence was collected with a photomultiplier masked with a 610-nm long pass filter. Events (2×10^4) were collected at a sample flow rate of 10 μl/min. The Rb content versus the DNA content is shown as a dot density plot. Cell-cycle phases are shown in the plot. (**C**) Measurement of Rb degradation by pulse-chase experiments. SW613 cells were pulse-labeled as described in (A) with 300 μCi of [^{35}S]methionine (1000 Ci/mmol) for 90 min, and chased in RPMI 1640 containing 10% FBS for 22 hours. Rb protein was extracted, immunoprecipitated, and separated by SDS-PAGE as described in (A) at 2-hour intervals during the chase period. Numbers above lanes represent time of chase in hours.

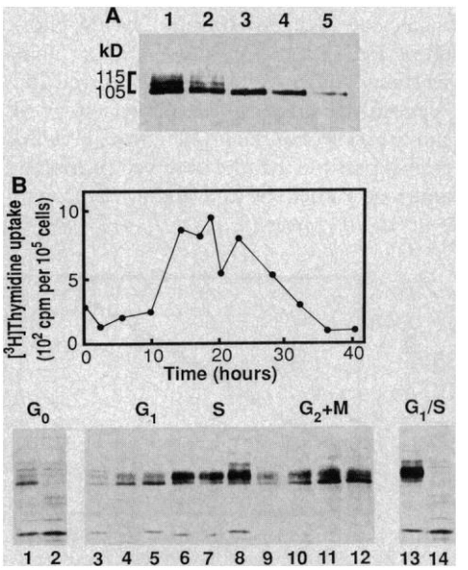

Fig. 2. Regulation of Rb phosphorylation in cells under different growth conditions. (**A**) An actively growing, random population of VM-CUB-3 cells was grown in the absence of serum in RPMI 1640 medium for up to 4 days. At time points of 0, 24, 48, 72, and 96 hours (lanes 1 to 5, respectively) during these 4 days, batches of cells were labeled with 300 μCi of [^{35}S]methionine (1000 Ci/mmol) for a 2-hour period, and Rb was extracted, immunoprecipitated, and analyzed as described in Fig. 1A. (**B**) SW613 cells made quiescent by serum-starvation for 2 days were stimulated to grow by replenishing the medium with 10% FBS. At regular intervals after serum was added to the cells, DNA synthesis was measured by [^{3}H]thymidine uptake (*19*). Similar results were obtained in six independent experiments. Duplicate cultures of cells were labeled with [^{35}S]methionine for 2 hours at various times after serum addition (lanes 1 to 12), as described in Fig. 1A. Rb protein was then extracted, immunoprecipitated, and analyzed as described in Fig. 1A. To analyze more precisely the timing of onset of multiple phosphorylation, cells synchronized by serum-starvation were arrested at the G_1/S boundary by treatment with aphidicolin (5 ng/ml) for 24 hours (lanes 13 and 14). These cells were then labeled for 2 hours with 300 μCi of [^{35}S]methionine (1000 Ci/mmol) in the continued presence of aphidicolin, and Rb was analyzed as described in Fig. 1A. Lane 1, G_0 phase; lane 2, same as lane 1 except that the antiserum Rb 1-AB 20 had been incubated at 4°C for 2 hours with 100 μg of peptide P5; lanes 3 and 4, early G_1 phase; lane 5, late G_1, early S phase; lanes 6 to 10, S phase; lanes 11 and 12, G_2 + M phases; lane 13, G_1/S boundary; lane 14, same as lane 13 except that Rb 1-AB 20 had been incubated at 4°C for 2 hours with 100 μg of peptide P5.

arrested at the G_1/S boundary with aphidicolin for as long as 24 hours were fully competent in producing and maintaining the newly synthesized Rb in its multiply phosphorylated state (Fig. 2B, lane 13). At the G_2 + M phase, the underphosphorylated forms of Rb again became more prominent (Fig. 2B, lanes 11 and 12). However, cells arrested at the M phase with 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (WAKO Chemicals U.S.A.) still exhibited more than one form of Rb (*13*).

The fact that Rb became heavily phosphorylated just before the onset of DNA synthesis suggested a possible correlation between the state of Rb phosphorylation and the control of cell proliferation. However, it is possible that the observed phenomenon was caused by the abnormal growth conditions imposed by serum starvation. We therefore examined the phosphorylation profile of Rb in cells induced to undergo terminal differentiation. HL-60 cells can be induced to terminally differentiate along several different lineages by treatment with various chemicals. Treatment with retinoic acid (RA) or dimethyl sulfoxide (DMSO) induces myelocytic differentiation, whereas 1,25-dihydroxyvitamin D_3 and 12-O-tetradecanoyl phorbol-13-acetate (TPA) induce monocytic differentiation. The induction process typically occurs over a period of time corresponding to two cell cycles (*17*). In the first cell cycle, cells exposed to inducers develop a precommitment state. The cells at this stage are not committed to either growth arrest or differentiation, and they can continue to proliferate indefinitely.

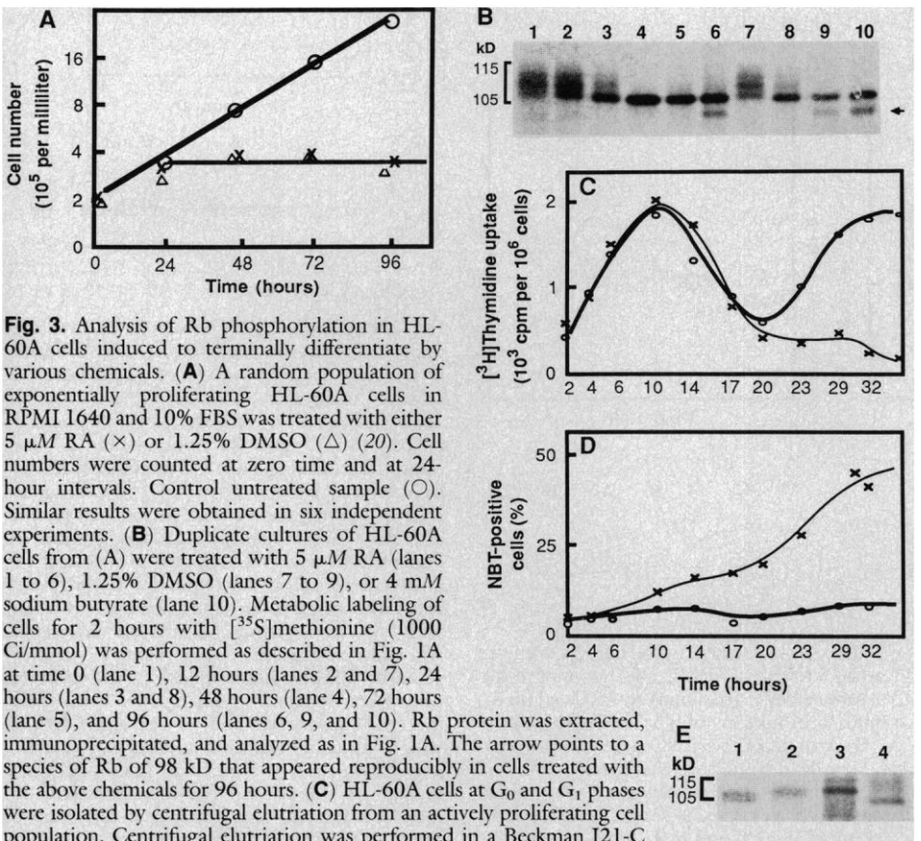

Fig. 3. Analysis of Rb phosphorylation in HL-60A cells induced to terminally differentiate by various chemicals. (**A**) A random population of exponentially proliferating HL-60A cells in RPMI 1640 and 10% FBS was treated with either 5 μM RA (×) or 1.25% DMSO (△) (*20*). Cell numbers were counted at zero time and at 24-hour intervals. Control untreated sample (○). Similar results were obtained in six independent experiments. (**B**) Duplicate cultures of HL-60A cells from (A) were treated with 5 μM RA (lanes 1 to 6), 1.25% DMSO (lanes 7 to 9), or 4 mM sodium butyrate (lane 10). Metabolic labeling of cells for 2 hours with [^{35}S]methionine (1000 Ci/mmol) was performed as described in Fig. 1A at time 0 (lane 1), 12 hours (lanes 2 and 7), 24 hours (lanes 3 and 8), 48 hours (lane 4), 72 hours (lane 5), and 96 hours (lanes 6, 9, and 10). Rb protein was extracted, immunoprecipitated, and analyzed as in Fig. 1A. The arrow points to a species of Rb of 98 kD that appeared reproducibly in cells treated with the above chemicals for 96 hours. (**C**) HL-60A cells at G_0 and G_1 phases were isolated by centrifugal elutriation from an actively proliferating cell population. Centrifugal elutriation was performed in a Beckman J21-C centrifuge with a JE-6 rotor containing a 40-ml chamber, and 10^9 cells were loaded into the chamber. The rotor speed was kept at 2000 rpm, and the flow rate was increased from an initial rate of 11.5 ml/min to a final rate of 29 ml/min in 12 increments. G_0 and G_1 cells were collected at 14.3 ml/min, and their size was monitored under the microscope. Duplicate cultures of the collected samples were grown in RPMI 1640 and 10% FBS in the presence (×) or absence (○) of 5 μM RA for 48 hours. DNA synthesis was measured by [^{3}H]thymidine uptake as described (*18*). This experiment has been repeated three times with similar results. (**D**) From the cultures described in (C), a small sample was taken at each time point to analyze for cell differentiation by uptake of NBT as described (*21*). NBT-positive cells were expressed as a percentage of the total number of cells counted; (×) RA-treated cells; (○) control. Similar results were obtained in three independent experiments. (**E**) Samples of G_0 and G_1 HL-60A cells from the cultures described in (C) were used for analysis of Rb expression. Harvested cells were treated with RA at time 0, and Rb analysis after labeling with [^{35}S]methionine and immunoprecipitation was performed at 0, 4, 8, and 18 hours as described in Fig. 1A. Because of a time lapse of 2.5 hours, the cells were finally harvested at 2.5 hours (lane 1), 6.5 hours (lane 2), 10.5 hours (lane 3), and 20.5 hours (lane 4) after collection. As it is not possible to separate the early and the late G_1 cells, some cells traversed the G_1/S boundary during the period of labeling.

We have isolated a variant HL-60 cell line (HL-60A) that has developed this precommitment state. Exposure of HL-60A cells to an inducer for a single cell cycle is enough to trigger cell growth arrest and the onset of differentiation. To study the relation between the control of cell proliferation and the state of phosphorylation of Rb, we treated a random population of actively proliferating HL-60A cells with RA. The cell number doubled in the first 24 hours of treatment with RA (Fig. 3A). Inhibition of proliferation appears to set in at this point, as no further increase in cell number was observed. As early as 12 hours after treatment with RA or DMSO, a decrease in the phosphorylation state of Rb became apparent (Fig. 3B, lanes 2 and 7). After 24 hours of treatment with RA or DMSO, virtually all of the newly synthesized Rb was underphosphorylated (lanes 3 and 8). However, these data alone do not readily distinguish whether the change in Rb phosphorylation profile is a cause or a consequence of growth arrest. It is possible that RA treatment can directly reduce the extent of Rb phosphorylation in all cells, and thereby lead to growth arrest. Alternatively, the decrease in the phosphorylation state of Rb may occur only at a restricted point of the cell cycle, for example, at the G_0 and G_1 phases. The reduced Rb phosphorylation in the latter case would therefore be a reflection of the fact that the cells' growth is arrested at G_0 and G_1, and the observed accumulation of underphosphorylated Rb after RA treatment may represent the gradual arrival of the cells at the restriction point. To address this question, we isolated a synchronous G_0/G_1 population of HL-60A cells by centrifugal elutriation. After isolation, cells were divided into two portions, one of which was treated with RA. At regular intervals, cell samples were removed for measurement of proliferation by [^{3}H]thymidine uptake (Fig. 3C), for measurement of differentiation by nitrobluetetrazolium (NBT) staining (Fig. 3D), and for analysis of the Rb phosphorylation profile (Fig. 3E). The synchronized HL-60A cells were able to grow in the presence of RA for one cell division before the onset of growth arrest. During this cycle, the newly synthesized Rb was phosphorylated as the cells moved into the S phase, in a manner indistinguishable from that of untreated cells. At 30 hours after treatment, the growth of RA-treated cells was arrested, and they lost the ability to produce multiply phosphorylated Rb (*13*). A significant percentage of the cells became differentiated.

We conclude that RA treatment does not directly modulate the phosphorylation of the protein. Rather, the decreased phospho-

rylation state of newly synthesized Rb is a consequence of RA-induced growth arrest. This conclusion is supported by the observation that treatment of HL-60A cells with compounds such as TPA (*13*), DMSO, or sodium butyrate all resulted in a similar time course of the decrease of Rb phosphorylation (Fig. 3B, lanes 7 to 10), despite the fact that these compounds each affect a different cellular target. After 96 hours of treatment with RA, sodium butyrate, or DMSO, the cells exhibited an underphosphorylated Rb of 98 kD. As RNA blot (Northern) analysis revealed a normal sized mRNA (*13*), the origin of this truncated Rb could be due to the initiation of translation at an internal ATG codon of *RB1*. As this species of Rb is observed in cells treated with the various chemical inducers, its appearance may be related to the state of growth arrest rather than differentiation of the cells.

We have demonstrated that newly synthesized Rb in cells at the G_0 and G_1 phases is underphosphorylated and that newly synthesized Rb is phosphorylated at multiple sites only in cells at the G_1/S boundary and in the S phase. Our observations are consistent with the interpretation that the underphosphorylated form of Rb inhibits cell proliferation and that this form of Rb is rendered inactive by phosphorylation at one or more critical sites before the initiation of DNA synthesis. The SV40 large T antigen can only bind to, and presumably inactivate, the underphosphorylated form of Rb (*18*). Thus, the inactivation of Rb by phosphorylation may be an obligatory event for cells to traverse the G_1/S boundary. Control of cell proliferation may therefore be exerted at the level of Rb inactivation.

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12. For preparation of antibodies against Rb, peptides synthesized according to the protein sequence deduced from the *RB1* cDNA sequence were used to immunize young New Zealand rabbits. For Rb 1-AB 16, the peptide (P4) used was CEEIYLKNKDLDARLFLDHDK (amino acids 322 to 341) (*15*). For Rb 1-AB 20, the peptide (P5) used was CEGSNPPKPLKKLRFDIEGSDEAD (amino acids 864 to 886). For Rb 1-AB A1, the peptide (P2) used was CRMQKQKMNDSMDTSNKEEK (amino acids 910 to 928).
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15. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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An Inducible Endothelial Cell Surface Glycoprotein Mediates Melanoma Adhesion

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Hematogenous metastasis requires the arrest and extravasation of blood-borne tumor cells, possibly involving direct adhesive interactions with vascular endothelium. Cytokine activation of cultured human endothelium increases adhesion of melanoma and carcinoma cell lines. An inducible 110-kD endothelial cell surface glycoprotein, designated INCAM-110, appears to mediate adhesion of melanoma cells. In addition, an inducible endothelial receptor for neutrophils, ELAM-1, supports the adhesion of a human colon carcinoma cell line. Thus, activation of vascular endothelium in vivo that results in increased expression of INCAM-110 and ELAM-1 may promote tumor cell adhesion and affect the incidence and distribution of metastases.

HEMATOGENOUS METASTASIS IS AN inefficient process, with most cancer cells failing to survive in the circulation [reviewed in (*1*)]. Endothelial injury or denudation enhances metastasis formation (*2*), suggesting that the lining of blood vessels may normally act as a barrier to tumor cell extravasation. However, tumor cells adhere focally to intact endothelium and subsequently transmigrate to the basal lamina, often after many hours (*3*). Characteristic patterns of metastatic spread have led to the suggestion that tumor cells preferentially interact with microvascular endothelium in particular organs or tissue sites (*4*). Activation of endothelium by cytokines increases the adhesion of human melanoma and carcinoma cells in vitro (*5–7*). Thus, tumor cell adhesion to the vessel wall may result from focal alterations in endothelial cells, perhaps involving the expression of specific cell surface molecules.

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Endothelial cell surface properties can be altered in response to cytokines, bacterial endotoxin, and coagulation factors (*8*). Interleukin-1 (IL-1), tumor necrosis factor (TNF), and endotoxin increase leukocyte adhesion through biosynthesis and expression of two cell surface glycoproteins, endothelial leukocyte adhesion molecule 1 (ELAM-1) (*9*) and intercellular adhesion molecule 1 (ICAM-1) (*10, 11*). Monoclonal antibody (MAb) studies did not demonstrate a role for ELAM-1 (*5*) or ICAM-1 (*5–7*) in melanoma cell adhesion.

To identify endothelial surface structures involved in the adhesion of melanoma cells, MAbs were generated to TNF-stimulated human endothelial cells (HECs) from umbilical vein (*12*). The MAb E1/6 recognized an inducible HEC surface antigen whose expression paralleled adhesiveness for melanoma cells. Continuous exposure of HEC monolayers to recombinant TNF resulted in increased melanoma adhesion (Fig. 1A), and increased expression of E1/6 antigen (Fig. 1B) that was detectable at 2 hours, maximal at 6 to 8 hours (an increase of 13.6 ± 1.9 times in MAb binding, mean