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Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells

(cell cycle/differentiation)

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ABSTRACT **The product of the retinoblastoma susceptibility gene *RB1* (Rb) is likely to function as an inhibitor of cell growth. Previous studies have suggested that certain growth-suppressing effects of Rb are exerted in G_0/G_1 phase and that phosphorylation can inactivate these functions. We tested this hypothesis by examining the expression and state of phosphorylation of Rb in several lineages of primary hematopoietic cells that spontaneously arrest in G_0 phase. Resting lymphocytes were found to express only unphosphorylated Rb, but phosphorylation of Rb occurred as the cells entered S phase in response to mitogens. In contrast, although monocytes and granulocytes also expressed high levels of unphosphorylated Rb, these terminally differentiated cells did not phosphorylate Rb, nor could they exit from G_1 phase in response to growth factors. Thus, Rb phosphorylation appears linked to the ability of a cell to synthesize DNA. In T and B lymphocytes, Rb protein increased 8-fold after stimulation, while RB1 RNA levels increased 2- to 4-fold. Nuclear run-on assays and measurement of RB1 RNA half-life in T cells suggested that the increased RNA abundance was, at least in part, due to increased RNA stability. By contrast, Rb protein levels did not increase in either monocytes or granulocytes after stimulation, although RB1 RNA levels did increase in monocytes. Thus, there are lineage-specific differences in both the regulation of Rb phosphorylation and *RB1* gene expression in lymphoid and myeloid cells.**

Mutational inactivation of the retinoblastoma susceptibility gene (*RB1*) predisposes to the development of hereditary and sporadic retinoblastoma (1–7). Moreover, *RB1* defects have been observed in cell lines and fresh tumor tissue derived from patients with small cell lung carcinoma (8), breast cancer (9), osteogenic sarcomas (4), and bladder carcinoma (10), suggesting that the loss of *RB1* gene function can contribute to the loss of growth regulation in several different cell lineages. In retinoblastoma and osteosarcoma cell lines, the neoplastic phenotype can be suppressed by replacement of the *RB1* gene by retroviral-mediated gene transfer (11). *RB1* is located on chromosome 13q14 (3) and encodes a series of differentially phosphorylated nuclear proteins ($p110–114^{Rb}$) (12). Further evidence that the product of the *RB1* gene (Rb) is involved in growth regulation comes from observations that transforming proteins of three different DNA tumor viruses form specific complexes with Rb, including simian virus 40 (SV40) large tumor (T) antigen (13), adenovirus E1A (14), and human papilloma virus E7 (15). For T antigen, it appears that complex formation with Rb contributes to its transforming action, possibly by inactivating one or more aspects of Rb growth-suppressing activity.

The mechanism by which Rb regulates cell proliferation is not yet understood, but recent evidence suggests that phosphorylation of Rb may be linked to control of Rb function. T antigen binds preferentially to unphosphorylated Rb, $p110^{Rb}$, but not to phosphorylated Rb, $pp112–114^{Rb}$ (16). Further, T antigen does not alter the relative abundance of these species, suggesting that $p110^{Rb}$, and not $pp112–114^{Rb}$, can perform those elements of Rb growth-suppression function that T antigen can perturb (16). In various primary cells and cell lines, $p110^{Rb}$ was detected only in G_0/G_1 phase, whereas in S and G_2 phases, $pp112–114^{Rb}$ was the predominant species (17–19). These results suggest that Rb is specifically phosphorylated at the G_1/S boundary by a kinase that is either not present or inactive during G_0/G_1 phase. Thus, $p110^{Rb}$ may act as a growth suppressor in some cell lineages by blocking exit from G_1 phase. Phosphorylation of $p110^{Rb}$ (or complex formation with T antigen, adenovirus E1A, or human papilloma virus E7) can, in turn, be viewed as inactivating Rb and allowing cell cycle progression to occur.

In an effort to clarify the possible role of Rb in cell cycle control, we have investigated the abundance and state of phosphorylation of Rb in primary hematopoietic cells that have spontaneously arrested in G_0 phase during differentiation. Mature B and T lymphocytes (lymphoid cells) can be induced to reenter S phase by exposure to mitogens. In contrast, although human granulocytes and monocytes (myeloid cells) can be activated by growth factors (20, 21), they cannot be induced to synthesize DNA or to undergo mitosis (22) and, thus, are terminally differentiated. We present data here consistent with a role for Rb in regulating G_1 exit and also evidence of lineage-specific posttranscriptional regulation of the *RB1* gene.

MATERIALS AND METHODS

Cell Preparation and Culture. Blood mononuclear cells (MNC) were isolated by Ficoll/Hypaque gradient centrifugation (Pharmacia). T lymphocytes were prepared by erythrocyte rosetting and depletion of adherent cells on plastic dishes for 1 hr. T cells contained <5% monocytes or B lymphocytes by flow cytometric analysis. B lymphocytes were prepared from human spleen by erythrocyte rosetting and plastic adherence to remove T cells and monocytes.

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Abbreviations: FBS, fetal bovine serum; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipopolysaccharide; MNC, mononuclear cells; PHA, phytohemagglutinin; *RB1*, retinoblastoma susceptibility gene; Rb, retinoblastoma susceptibility gene protein product; SV40, simian virus 40; T antigen, SV40 large tumor antigen; SAC, Cowan strain *Staphylococcus aureus*.

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Monocytes were purified from erythrocyte rosette-depleted MNC by plastic adherence and contained >90% $CD14^+$ cells and <10% of either $CD2^+$ or $CD20^+$ cells. In some experiments, an erythrocyte rosette negative fraction was used without plastic adherence to avoid activation of monocytes (nonadhered monocytes) and typically contained 70–80% $CD14^+$ cells, which were further cultured in polypropylene tubes (Falcon) to avoid activation of cells by plastic (23). Granulocytes were prepared by dextran sedimentation (24). An interleukin 2-dependent human T-cell clone was obtained from Chikao Morimoto (Dana–Farber Cancer Institute). Thymocytes were obtained from thymectomy specimens (provided by Michael Caligiuri, Dana–Farber Cancer Institute). All samples were obtained after informed consent of donors and under institutional review board-approved protocols.

Cells were cultured at $2–5 \times 10^6$ cells per ml in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) at 37°C with 5% CO_2. Endotoxin content of fetal bovine serum was <5 pg/ml by limulus amoebocyte assay. The following agents were used as stimulants: phytohemagglutinin P (PHA) (Wellcome) at 2 μg/ml; mitogenic anti-CD2 monoclonal antibodies anti-$T11_2$ and -$T11_3$ (Stuart Schlossman, Dana–Farber Cancer Institute) (25) at a 1:1000 dilution of ascites; Cowan strain *Staphylococcus aureus* (SAC, Calbiochem) at 1:10,000; lipopolysaccharide (LPS, *Escherichia coli* 0111:B4, Sigma) at 100 ng/ml; purified recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) (Genetics Institute, Cambridge, MA) at 250 ng/ml; and recombinant granulocyte colony-stimulating factor (G-CSF) (Genetics Institute) at a 1:1000 dilution of G-CSF cDNA-transfected Chinese hamster ovary cell line supernatant.

Cell Cycle Analysis. Cell cycle analysis was performed by staining DNA with propidium iodide and flow cytometric analysis (17). [^{3}H]Thymidine incorporation was measured after incubating cultures with 0.2 μCi of [^{3}H]thymidine (2 Ci/mmol, 1 Ci = 37 GBq; New England Nuclear) for 16 hr.

Northern (RNA) Blot Analysis, RNA Half-Life Analysis, and Nuclear Run-on Assays. Northern blotting by using total cellular RNA was performed as described (26). *RB1* gene expression was detected by using a 3.5-kilobase (kb) *Eco*RI fragment of RB1 cDNA (4) labeled with [^{32}P]dCTP (27). RNA half-life and nuclear run-on assays were performed as described (26, 28). Human CD2 cDNA (29) in the CDM8 vector was obtained from Brain Seed (Massachusetts General Hospital, Boston); interleukin 1β cDNA in the pSP64 vector was obtained from Gordon Wong (Genetics Institute, Cambridge, MA). A mouse β-actin cDNA was used to reprobe blots, and the pUR290 expression vector (30) was used as a negative control.

Immunologic Blot Analysis. Cells were washed with Tris-buffered saline (25 mM Tris·HCl, pH 8.0/150 mM NaCl) and lysed for 30 min at 4°C with 0.25 ml of lysis buffer [50 mM Tris·HCl, pH 8/120 mM NaCl/0.5% Nonidet P-40/100 mM NaF/200 μM sodium orthovanadate containing protease inhibitors (10 μg each of aprotinin, phenylmethylsulfonyl fluoride, and leupeptin; Sigma)]. Immunoblotting was performed as described (17) by using a 1:200 dilution of the anti-Rb monoclonal antibody, RB-PMG3-245 or -340 (Pharmingen, San Diego), for 12 hr and developed with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega) (13). Alkaline phosphatase treatment of anti-Rb immune complexes was performed as described (16).

RESULTS

Phosphorylation of the *RB1* Gene Product in Normal Hematopoietic Cells. The expression and phosphorylation status of Rb were analyzed in fresh, primary hematopoietic cells by immunologic blotting using anti-Rb monoclonal antibody Rb-PMG3-245 (which reacts with both $p110^{Rb}$ and $pp112–114^{Rb}$) (13). Resting T cells were found to contain $p110^{Rb}$, but not $pp112–114^{Rb}$ (Fig. 1*A*). Stimulation of these cells with PHA for 48–96 hr induced entry into S phase as shown by flow cytometric analysis and [^{3}H]thymidine incorporation. Before stimulation, >96% of T cells were in G_0/G_1 phase and <3% were in S phase. After 72 hr of stimulation, the G_0/G_1 phase fraction decreased to 84%, and the S phase fraction increased to 10%. [^{3}H]Thymidine incorporation increased 37-, 59-, and 29-fold after stimulation for 48 hr, 72 hr, and 96 hr, respectively. In parallel with the entry of a fraction of these cells into S, the appearance of $p112–114^{Rb}$ was observed (Fig. 1*A*). Similar results were observed with anti-Rb antibody 340, which recognizes a distinct epitope (E. Huang, personal communication). The increase in Rb protein phosphorylation was also observed after stimulation with anti-CD2 monoclonal antibodies (17). In addition to the *de novo* appearance of phosphorylated Rb, the total amount of Rb protein increased 8-fold in response to either PHA or CD2 stimulation (Fig. 1*A*). To confirm that the slower migration of $pp112–114^{Rb}$ was

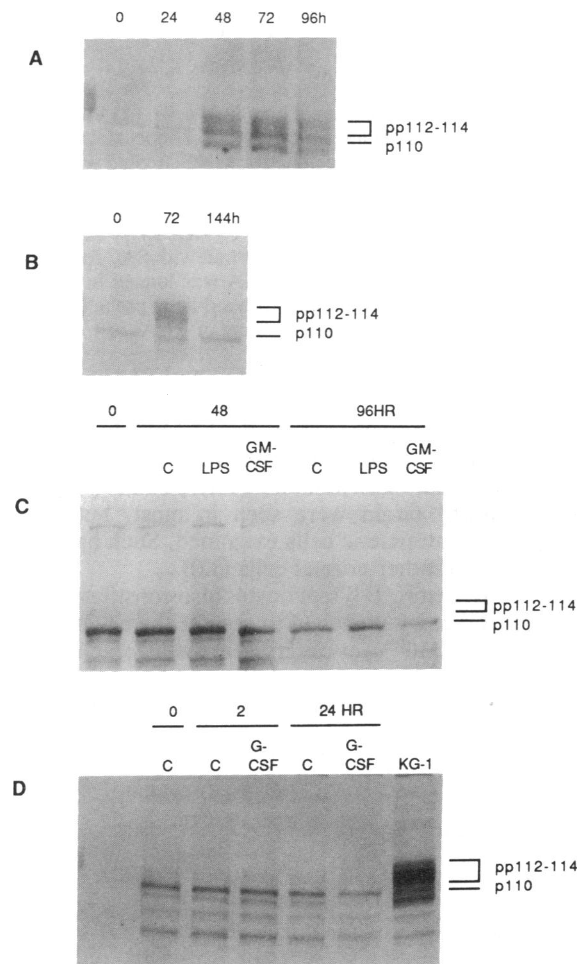

FIG. 1. Analysis of Rb protein expression in human hematopoietic cells by immunologic blot. (*A*) Purified T cells stimulated with PHA for 0–96 hr. (*B*) B cells cultured with SAC for 0–144 hr. (*C*) Monocytes cultured in serum-containing medium for 0–96 hr without (C) or with LPS (100 ng/ml) or GM-CSF (250 ng/ml). (*D*) Granulocytes cultured in serum-containing medium for 0–24 hr without (C) or with G-CSF. Each gel lane contains an equal amount of lysate protein (150 μg). Rb protein was detected with monoclonal antibody RB-PMG3-245. p110 indicates the position of unphosphorylated Rb and pp112–114 indicates the location of phosphorylated Rb. A lower molecular mass band (≈95 kDa) was often observed in T-cell immunoblots. A growing culture of the myeloid leukemic cell line KG-1 (31), which contains both phosphorylated and nonphosphorylated Rb, was used as control in *D*.

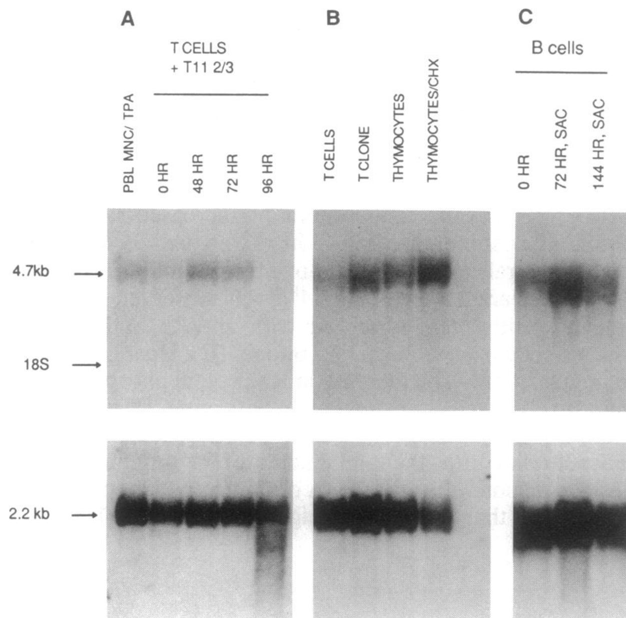

FIG. 2. RB1 RNA expression in lymphoid cells. (*A*) Blood T cells cultured in the presence of mitogenic anti-CD2 monoclonal antibodies. RNA from blood mononuclear cells (PBL MNC) treated with phorbol 12-myristate 13-acetate (TPA) (10^{-9} M) served as a positive control. (*B*) RNA from resting T cells, a T-cell clone, and thymocytes, pre- and postexposure to cycloheximide (CHX). (*C*) RB1 RNA expression in B cells. Splenic B lymphocytes were cultured with SAC for 0–144 hr. Fifteen micrograms of total cellular RNA was loaded in each lane. Blots were washed and rehybridized with a β-actin probe (2.2 kb; see bottom) to demonstrate equivalent loading of RNA.

solely due to phosphorylation (16), anti-Rb immunoprecipitates containing this species were treated with alkaline phosphatase, which eliminated the pp112–114^{Rb} bands but did not alter the migration of p110^{Rb} (data not shown).

Lower molecular mass immunoreactive bands (<110 kDa) of undetermined origin were seen in most, but not all, analyses of hematopoietic cells examined. Such bands have been reported in other normal cells (32).

Like T cells, resting B lymphocytes also constitute a G_0 cell population and can be induced to reenter the cell cycle with mitogens. Only p110^{Rb} was observed in resting splenic B cells, whereas overtly phosphorylated Rb protein appeared after stimulation with SAC for 3 days (Fig. 1*B*). The appearance of pp112–114^{Rb} correlated temporally with entry of B cells into S phase. Moreover, the intensity of the Rb bands observed in the blot increased 8-fold at 3 days. B-cell proliferation slowed after 6 days (33), and pp112–114^{Rb} disappeared.

Fresh monocytes and granulocytes were found to be composed of only G_0/G_1 cells, and only p110^{Rb} was detected (Fig. 1 *C* and *D*). pp112–114^{Rb} was not seen in these cells, even after activation with LPS, GM-CSF, or G-CSF. Furthermore, the total amount of Rb protein did not change after stimulation (Fig. 1 *C* and *D*). These three stimulants were selected because they are growth factors for myeloid progenitor cells (GM-CSF and G-CSF) or because they cause cell activation, including induction of the CD11b adhesion protein, enhancement of the respiratory burst in neutrophils (LPS, GM-CSF, G-CSF) (34–39), and induction of cytokine gene expression in the monocyte (LPS and GM-CSF) (40, 41). Cell cycle analysis of both populations showed that virtually all cells remained in G_0/G_1 phase after stimulation. Cell activation was monitored in these experiments by visually detecting increased aggregation and plastic adherence and in parallel experiments by showing increased expression of CD11b (data not shown).

***RB1* Gene Expression in Hematopoietic Cells.** To investigate the mechanisms responsible for the increased Rb protein expression observed after stimulation of lymphoid cells (Fig. 1 *A*–*D*), relative RB1 RNA abundance was evaluated. Repeatedly, in purified T cells, the level of RB1 RNA increased 2- to 4-fold after stimulation with mitogenic anti-CD2 monoclonal antibodies for 48 hr (Fig. 2*A*). An interleukin 2-dependent T-cell clone and fresh normal thymocytes also contained detectably more RB1 RNA than resting T cells (Fig. 2*B*). In purified splenic B lymphocytes, RB1 RNA also increased by 1.5- to 2-fold (when compared with actin RNA by densitometry) in multiple experiments after 3 days of SAC stimulation (Fig. 2*C*). Unexpectedly, in monocytes activated by exposure to fetal bovine serum (FBS), plastic adherence, LPS, or GM-CSF, the level of RB1 RNA also increased 5- to 8-fold (Fig. 3), despite the lack of any increase in Rb protein abundance (cf. Fig. 1*C*). In contrast, the steady-state level of RB1 RNA in neutrophils was unaffected by treatment with GM-CSF or G-CSF for 2–24 hr in multiple experiments (Fig. 3).

The basis for RB1 RNA induction in T cells and monocytes was further investigated by estimating changes in transcription rate by nuclear run-on assays and measuring RNA stability in the presence of actinomycin D. No significant change in the rate of *RB1* transcription was noted after stimulation of either T cells or monocytes (Fig. 4*A*). The

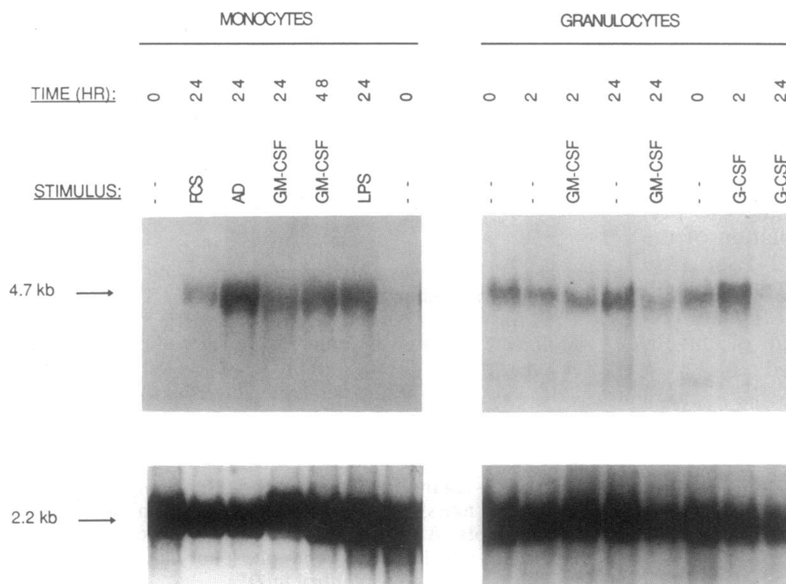

FIG. 3. *RB1* gene expression in myeloid cells. Monocytes were isolated without plastic adherence and cultured for 24–48 hr in serum-free medium containing either no additive, GM-CSF (250 ng/ml), or LPS (100 ng/ml) in polypropylene tubes to minimize adherence, or in polystyrene dishes to maximize adherence (AD). Granulocytes were cultured with or without GM-CSF or G-CSF for 2 and 24 hr in polystyrene dishes. RB1 and β-actin RNAs were detected by Northern (RNA) analysis in 15-μg samples of total cellular RNA.

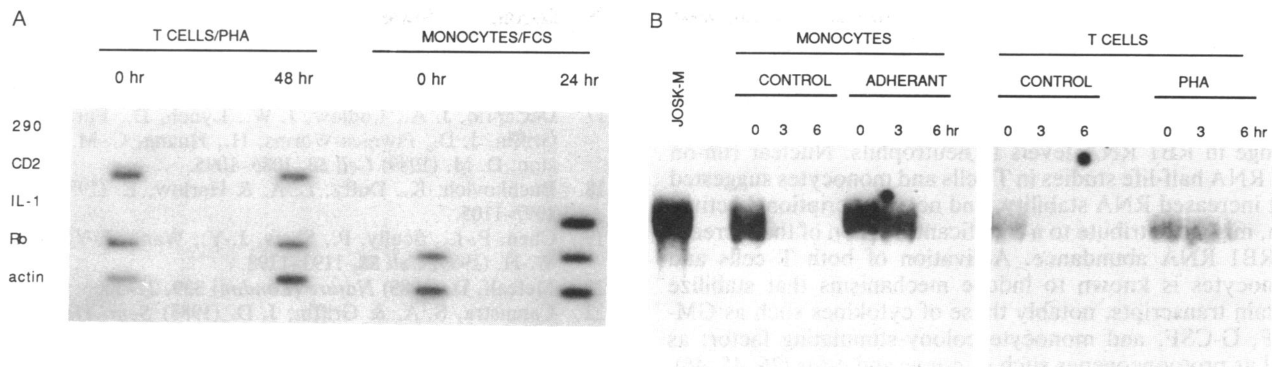

FIG. 4. Regulation of *RB1* gene expression in T cells and monocytes. (*A*) Nuclear run-on assay. T cells were stimulated with PHA (2 μg/ml) for 48 hr, and monocytes were stimulated by serum-containing media and plastic adherence for 24 hr. Nascent nuclear RNA was elongated *in vitro*, labeled with [^{32}P]UTP, isolated, and hybridized to immobilized plasmids containing cDNAs for the T-cell specific surface antigen CD2, the monocyte-associated cytokine interleukin 1β (IL-1), RB1, or actin. The autoradiogram was exposed for 96 hr. The expected induction of interleukin 1β gene transcription in monocytes, but not in T cells, is shown. (*B*) RB1 RNA half-life was estimated by treating cells before or after stimulation as indicated with actinomycin D (10 μg/ml) and removing aliquots of cells at the time points shown for extraction of RNA and Northern blotting. Control monocytes were purified by brief plastic adherence (30 min) before adding actinomycin D. Adherent monocytes were cultured for 24 hr in FCS-containing medium on plastic. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The figure shows one of two identical experiments. JOSK-M is a human myelomonocytic leukemia cell line (42) used as a positive control.

half-life of RB1 RNA in freshly isolated monocytes was 2 hr, and this increased, modestly, to 3.5 hr after plastic adherence for 24 hr (Fig. 4*B*). A more impressive increase in RB1 RNA half-life was noted in T cells, where it was found to be 2 hr before stimulation with PHA and 6 hr after PHA stimulation. Thus, the increase in RB1 RNA observed in response to stimulation of T cells and monocytes is posttranscriptional. These data, summarized in Table 1, suggest that RNA stability underlies at least part of this effect.

DISCUSSION

The data presented here support a model of Rb function in which this protein regulates cell cycle progression at G_1/S phase. Hematopoietic cells are of particular interest in the study of Rb function because they spontaneously arrest in G_0/G_1 phase during differentiation and because certain lineages (lymphoid cells) can then enter S phase in response to specific mitogens, whereas others (myeloid cells) cannot (20–22). All hematopoietic cells examined contained readily detectable levels of unphosphorylated Rb. Treatment of resting lymphocytes with mitogens induced Rb phosphorylation at the time of onset of DNA synthesis, whereas terminally differentiated granulocytes and monocytes were unable to generate phosphorylated Rb or to exit G_0/G_1 phase. Thus, myeloid cells may lack, or be unable to activate, the specific Rb kinase that is expressed in lymphoid cells and, therefore, cannot overcome the G_1 boundary block provided, in part, by the presence of unphosphorylated Rb. The available data do not exclude the possibility that Rb promotes entry into G_0 phase and that its phosphorylation promotes exit therefrom, with the latter effect facilitating entry into S phase. It is also possible that granulocytes and monocytes contain excessive phosphatase activity that neutralizes the effect of an active Rb kinase.

Conceivably, phosphorylation of $p110^{Rb}$ is a consequence, rather than an effector, of G_1 exit. However, the observation that SV40 T antigen binds only to $p110^{Rb}$ and not to its phosphorylated derivatives, and the strong genetic correlation between T antigen–Rb complex formation and T antigen transforming activity, suggests that the growth-suppressing function of Rb is most likely to be exerted in G_1 phase (13, 16). Thus, it seems more likely that Rb phosphorylation contributes to overcoming a G_1 block rather than simply responding to it.

If, as proposed, Rb is a regulator of the G_1/S transition in hematopoietic and other cells, it is not the only such regulator. Gewirtz *et al.* (43) have shown that the product of the c-*myb* gene is required by T lymphocytes for entry into S phase. Treatment of resting T cells with PHA leads to an increase in the abundance of c-*myb* RNA and protein. Pretreatment of T cells with a c-*myb* antisense oligomer blocked entry into S phase, but not cellular activation. Immature myeloid cells have been shown to express high levels of c-*myb* RNA, which decreases precipitously upon induction of differentiation to monocytes (44). Thus, it is likely that multiple events contribute to S phase entry in hematopoietic cells. Conceivably, the ability to phosphorylate Rb and the ability to express c-*myb* are related in some manner.

Table 1. Comparison of Rb phosphorylation, *RB1* gene regulation, and response to activating signals in primary hematopoietic cells

	T cell	B cell	Monocyte	Granulocyte
Stimulus	PHA, α-CD2	SAC	GM-CSF, LPS	GM-CSF, G-CSF, LPS
Proliferation	Increase	Increase	None	None
Activation	Increase in IL-2R	Increase in μ gene	Increase in size, adherence	Increase in viability
	Increase in cytokine production	Increase in IL-2R	Increase in cytokines	Increase in aggregation
		Increase in cytokines	Increase in O_2^- production	Increase in O_2^- production
			Increase in phagocytic ability	Increase in phagocytic ability
Rb protein level	Increase	Increase	No change	No change
Rb phosphorylation	+	+	None	None
RB1 transcription	No change	NT	No change	NT
RB1 RNA level (Northern blot)	Increase	Increase	Increase	No change
RB1 RNA half-life	Increase	NT	Increase	NT

IL-2R, interleukin 2 receptor; NT, not tested; O_2^-, superoxide anion.

We have also demonstrated that expression of the *RB1* gene is specifically and differently regulated in the various types of hematopoietic cells. In T cells, B cells, and monocytes activation of resting cells induced a 2- to 8-fold increase in steady-state RB1 RNA (Table 1). By contrast, there was no change in RB1 RNA levels in neutrophils. Nuclear run-on and RNA half-life studies in T cells and monocytes suggested that increased RNA stability, and not transcriptional activation, might contribute to a significant fraction of the increase in RB1 RNA abundance. Activation of both T cells and monocytes is known to induce mechanisms that stabilize certain transcripts, notably those of cytokines such as GM-CSF, G-CSF, and monocyte colony-stimulating factor; as well as protooncogenes such as c-*myc* and c-*fos* (26, 45, 46). Posttranscriptional regulation of RB1 RNA stability has not previously been reported, but the presence of poly(AUUUA) destabilizing sequences, similar to those observed in cytokines and oncogenes, have been noted in both the human and murine RB1 cDNAs (47, 48). RB1 RNA could be subject to a specific degradation system common to RNAs bearing this sequence (45).

The increase in RB1 RNA was associated with an increased amount of Rb protein in T cells and B cells but not in monocytes. This dichotomy suggests that there could be multiple levels of posttranscriptional control of Rb synthesis. However, these diverse control mechanisms regulate steady-state levels of Rb protein in a precise and predictable manner. It will be interesting to learn whether these lineage-associated differences in both *RB1* gene expression and Rb phosphorylation contribute to the control of terminal differentiation in human hematopoietic cells.

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