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The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression

JAMES A. DECAPRIO, YUSUKE FURUKAWA, FLORENCE AJCHENBAUM, JAMES D. GRIFFIN, AND DAVID M. LIVINGSTON

Dana–Farber Cancer Institute, 44 Binney Street, Boston, MA 02115

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ABSTRACT **The retinoblastoma-susceptibility gene product (RB) undergoes cell cycle-dependent phosphorylation and dephosphorylation. We characterized RB phosphorylation after mitogenic stimulation of primary human T lymphocytes, initially arrested in the G_0 state. RB is phosphorylated in at least three steps when T cells are driven into the cell cycle. The first event occurs during mid G_1 phase, the second during S phase, and the third in G_2/M. Tryptic phosphopeptide mapping indicates that the different phosphorylation events occur, at least in part, on different residues in RB. Given the known relationship of the RB phosphorylation state to function, it is possible that RB regulates growth at multiple points in the cell cycle.**

RB undergoes cell cycle-dependent phosphorylation on serine and threonine residues (1–4). During the G_0 phase of serum-starved, senescent, and terminally differentiated cells, or in the G_1 phase of cycling cells, RB is underphosphorylated (1, 2, 5–10). At, or very near, the G_1/S transition in cycling cells, RB becomes phosphorylated and remains so through S and G_2. Dephosphorylation of RB occurs in mitosis, most probably due to the action of a type 1 phosphoprotein phosphatase (ref. 11; J.A.D., J. W. Ludlow, and D.M.L., unpublished observations). Thus, in cycling cells, RB undergoes specific phosphorylation and dephosphorylation at defined points in the cell cycle.

The precise relationship of RB phosphorylation to particular events between exit from G_0 and entry into the cell cycle has not been studied in detail. Thus, we analyzed RB phosphorylation in freshly isolated, primary human T lymphocytes, a cell population that is uniformly and spontaneously arrested in G_0. After treatment with a mitogen, they enter the cycle slowly and synchronously, displaying a series of coordinated biochemical events (12, 13). T cells were investigated in an effort to evaluate the process of RB phosphorylation during this period of G_0 exit and cell cycle progression.

PROCEDURES

T-Lymphocyte Preparation. Human peripheral blood mononuclear cells were isolated by Ficoll/Hypaque gradient centrifugation (Pharmacia). Enriched lymphocyte populations were prepared by depletion of adherent cells on plastic dishes for 2 hr. These nonadherent cell preparations contained ≈85% T lymphocytes, as determined by flow cytometric analysis after staining with fluorescein-conjugated T3 monoclonal antibody (Coulter) (14). For elutriation, T lymphocytes were further purified by sheep erythrocyte rosetting (>95% T lymphocytes). Cells were cultured at a density of 2 $\times 10^6$ per ml in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and were incubated at 37°C in an atmosphere containing 5% CO_2. When indicated, purified phytohemagglutinin (PHA; Wellcome) was added at a concentration of 2 μg/ml. The rosette-positive fraction was stimulated with PHA (1 μg/ml), phorbol 12-myristate 13-acetate (0.3 ng/ml; Calbiochem), and the calcium ionophore A23187 (0.12 μg/ml; Calbiochem) (15) and then harvested for elutriation 48 hr after stimulation. Elutriation was performed in a Beckman JE-5.0 rotor (2200 rpm, 23°C) with 100-ml fractions collected at a pump speed from 14 to 30 ml/min.

DNA synthesis was determined by [^{3}H]thymidine incorporation, as described (16). For this, 2×10^5 cells were incubated with 10 μCi (370 kBq) of [^{3}H]thymidine (6.7 Ci/mmol; NEN) in 0.1 ml for 6 hr. All assays at each time point were performed in triplicate, and the results were averaged. Cell cycle analysis was performed by staining DNA with propidium iodide in preparation for flow cytometry (2).

Western Blotting. Lysates were prepared as described (11), and 140 μg of protein was electrophoresed in each lane of an SDS/polyacrylamide gel. After electrophoresis, the proteins were transferred to Immobilon-P (Millipore). Blots were incubated with blocking buffer [20 mM Tris·HCl, pH 8.0/120 mM NaCl/5% bovine serum albumin (United States Biochemical)] for 15 min and then incubated with various primary antibodies diluted in blocking buffer. Purified RB monoclonal antibody, RB-PMG3-245 (PharMingen, San Diego), was incubated overnight at a dilution of 1 μg/ml. Rabbit anti-human cdc2 serum, a gift of Helen Piwnica-Worms (Tufts University School of Medicine), was incubated for 2 hr at a dilution of 1:10,000. Monoclonal antibody to human cyclin A, C160, a gift of Ed Harlow (Massachusetts General Hospital Cancer Center), was incubated overnight at a dilution of 1:3. Rabbit anti-human cyclin B serum, a gift of Jonathon Pines (The Salk Institute for Biological Studies), was incubated for 2 hr at a dilution of 1:4000. After incubation with the primary antibody, the blots were washed in Tris-buffered saline containing 0.5% albumin, six times for 10 min each, and then incubated for an additional 30 min with either goat anti-mouse IgG-alkaline phosphatase conjugate (BRL) or goat anti-rabbit IgG-alkaline phosphatase conjugate (BRL) at a dilution of 1:7500 in blocking buffer, washed six times for 10 min each, and then developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega).

^{32}P Labeling and Tryptic Phosphopeptide Mapping. T cells ($1–2 \times 10^8$ per ml), either unstimulated or at appropriate intervals after the addition of PHA, were starved in phosphate-free RPMI 1640 (Flow Laboratories) for 30 min and then incubated in phosphate-free RPMI 1640 with [^{32}P]orthophosphate (9000 Ci/mmol; NEN) at 10 mCi/ml for 3 hr.

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Abbreviation: PHA, phytohemagglutinin.

Lysates were prepared in 0.5% Nonidet P-40/120 mM NaCl/20 mM Tris·HCl, pH 8.0/100 μM $NaVO_4$/4 mM NaF, containing aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and glycerol 2-phosphate, each at 10 μg/ml. Immunoprecipitation was performed with RB-PMG3-245 (PharMingen). Samples were separated by electrophoresis in SDS/6% polyacrylamide gels, which were then dried, fixed, and autoradiographed in the presence of an intensifying screen. Appropriate gel slices were excised from the dried gels and then incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) in (at 0.1 mg/ml) 50 mM ammonium bicarbonate buffer for 40 hr at 37°C. Samples were lyophilized and then resuspended in distilled water and lyophilized three times. Radioactivity was measured by Cerenkov counting. Samples were resuspended in distilled water containing 0.3 mM 2-mercaptoethanol (1000–3000 cpm/μl), spotted on thin-layer cellulose (EM Science), and electrophoresed at 1000 V for 40–50 min in acetic acid/formic acid/water (1.5:0.5:8, vol/vol) at pH 1.9. The samples were then chromatographed in the second dimension in 1-butanol/pyridine/acetic acid/water (5:3.3:1:4). Autoradiographs were obtained with intensifying screens.

RESULTS

RB Phosphorylation During T-Cell Mitogenic Activation. As shown in Fig. 1, the RB gel migration pattern changed significantly at two specific intervals during exit from G_0. Initially, in G_0-arrested cells, RB migrated as a single band. At 20 hr after PHA stimulation, a slower migrating doublet of RB appeared, and this species persisted through the 29-hr point. In this preparation, S phase, as determined by [^{3}H]thymidine incorporation, did not begin until 30 hr after PHA addition. In other experiments (data not shown), the earliest this doublet appeared was 12 hr after PHA stimulation. At 30 hr after stimulation, two additional RB bands appeared. Thus, during mitogenic stimulation of T cells, there are at least two points when new RB species appear. One event occurs during the G_0/G_1 transition, and another at or after the initiation of S phase.

Since these preparations of T cells can lose cell cycle synchrony after several hours of stimulation, we elutriated sheep erythrocyte rosette-positive T cells 48 hr after stimulation. This method allowed evaluation of RB throughout the cell cycle. The gel migration pattern of RB from nonelutriated cells, before or after stimulation, was compared with that of RB from elutriated cells by Western blotting (Fig. 2). Again, the fastest migrating species RB band predominated in unstimulated cells, with faint, new band(s) appearing 20 hr after

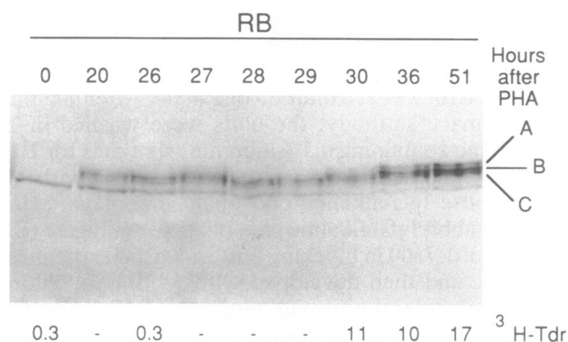

FIG. 1. RB Western blot of lysates prepared from unstimulated T cells (lane 0) or at various intervals after addition of PHA (20–51 hr). Proteins were separated in an SDS/6% polyacrylamide gel and then blotted and probed for RB. Three RB species, A–C, are indicated at right. Below each lane, [^{3}H]thymidine (^{3}H-Tdr) incorporation is expressed as cpm × 10^{-3} per 2 × 10^5 cells (dash indicates not performed).

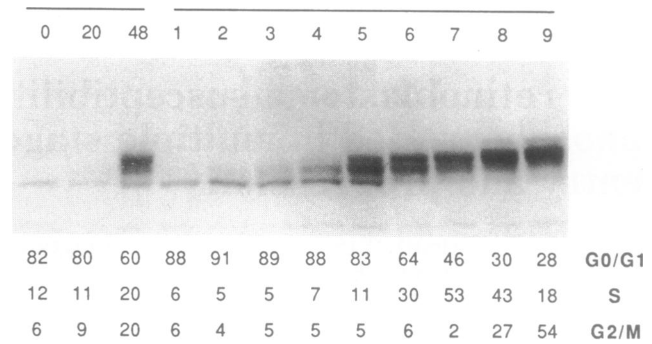

FIG. 2. RB Western blot of lysates prepared from sheep erythrocyte rosette-positive T cells before (lane 0) or 20 and 48 hr after mitogenic stimulation and from elutriated cells (fractions 1–9) prepared 48 hr after mitogenic stimulation. Proteins were separated in an SDS/6% polyacrylamide gel and then blotted and probed for RB. Percentages of cells in G_0/G_1, S, and G_2/M, as determined by flow cytometric analysis, are listed in columns below each lane.

mitogen addition. The 48-hr sample, by contrast, contained multiple RB species, including the bands that comigrated with those present at 0 and 20 hr. When these cells were elutriated, the fastest band and the next fastest migrating bands were both present in the most heavily G_1-enriched fractions (Fig. 2, lanes 1–4), similar to those present at 20 hr after stimulation (Figs. 1 and 2). In fractions enriched for S-phase cells (Fig. 2, lanes 5–7), a new slower migrating band appeared. In addition, paralleling the decrease in proportion of G_0/G_1 cells, the relative intensity of the G_1-specific bands diminished in fractions 6–9. In fractions 8 and 9, which were enriched for G_2/M cells, one, and possibly more, discrete RB species, not observed in the earlier fractions, were noted. Thus, after mitogen stimulation, at least three changes in RB banding pattern were noted in elutriated T cells: in G_1, in S, and in G_2/M. Finally, there was a dramatic shift in gel migration as the cells moved from G_2/M to the next G_1.

RB Gel Migration Shift Is Due to a Change in Phosphorylation. To confirm that the shifts in RB gel migration, observed in Figs. 1 and 2, were due to differences in phosphorylation, several experiments were performed. First, RB was immunoprecipitated from ^{32}P-labeled T cells at specific intervals after stimulation with PHA. Phosphorylated RB, not detectable in unstimulated T cells, was observed at 20 hr after PHA addition, and at 40 hr with the appearance of more slowly migrating species (data not shown). Second, in a Western blotting experiment, exposure of one aliquot of RB immunoprecipitated from stimulated T cells to calf intestine alkaline phosphatase and another to potato acid phosphatase led, in both cases, to full collapse of all intermediate and slow migrating bands (data not shown). Therefore, in keeping with the results of earlier analyses (3, 11), the RB gel migrational differences are solely due to phosphorylation.

To determine whether the differently migrating forms of phosphorylated RB were likely to be phosphorylated on the same or different residues, phosphotryptic maps of the entire collection of phosphorylated RB bands were generated at two time points. The tryptic phosphopeptide maps from the entire ^{32}P-labeled RB signal apparent at 20 hr and 40 hr after PHA addition are shown in Fig. 3. The maps are remarkably similar, but as noted by the large arrowheads, there is at least one phosphopeptide at 40 hr that was not detected at 20 hr after stimulation. There are several other phosphopeptides whose relative intensities increased or decreased markedly between the two time points.

We also mapped three individual ^{32}P-labeled RB gel species (equivalent to the A, B, and C species identified in Fig. 1, but taken from another experiment) that were apparent at 40 hr after PHA addition. These bands were separated from one

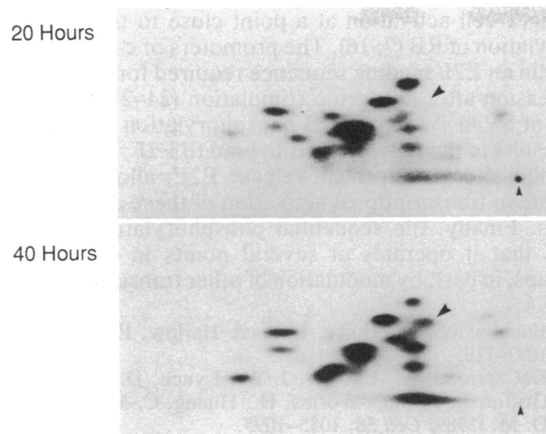

FIG. 3. Tryptic phosphopeptide maps of RB isolated at 20 and 40 hr after PHA addition. All detectable ^{32}P-labeled RB was isolated by immunoprecipitation and SDS/polyacrylamide gel electrophoresis and was subjected to tryptic phosphopeptide mapping. The map of RB (300 cpm) at 20 hr (*Upper*) was exposed to film for 2.5 weeks in the presence of an intensifying screen. The map of RB (800 cpm) isolated at 40 hr (*Lower*) was exposed for 6 days in the presence of a screen. The origin is marked by the small upward-pointing arrowhead at the bottom right of each panel. The cathode was on the right, and chromatography proceeded in the vertical dimension. The larger arrowhead indicates a spot present at 40 hr but not detectable at 20 hr.

another by electrophoresis in a long gel, identified by autoradiography, excised, and subjected to phosphopeptide mapping. There were discrete differences in the maps of all three bands (Fig. 4 *Left*), which were confirmed by the maps of the variously mixed digests (Fig. 4 *Right*). Taken together, these results indicate that the state of RB phosphorylation changes quantitatively as well as qualitatively, at least three times during passage from G_0 to M phase in T cells.

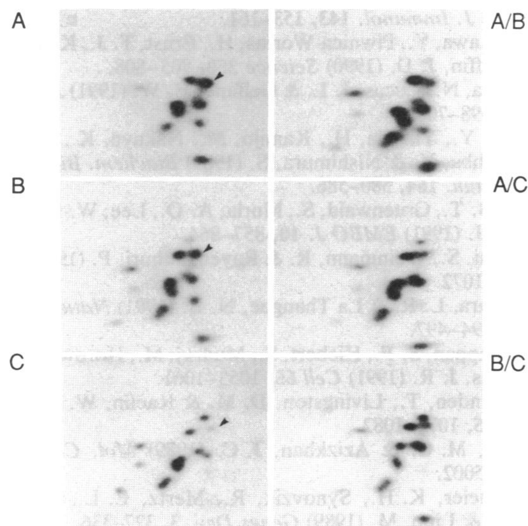

FIG. 4. Tryptic phosphopeptide maps of various RB subspecies. Individual, ^{32}P-labeled RB bands A, B, and C (similar to those noted in Fig. 1, but from a separate experiment) were isolated from labeled T cells 40 hr after PHA addition. They were excised, digested, and mapped as described in *Procedures*. The quantities of the digests used were as follows: band A (550 cpm) (*Top Left*), band B (730 cpm) (*Middle Left*), band C (540 cpm) (*Bottom Left*). Mixtures of digests of bands A and B (560 cpm and 500 cpm, respectively) (*Top Right*), A and C (370 cpm and 360 cpm, respectively) (*Middle Right*), and B and C (470 cpm and 360 cpm, respectively) (*Bottom Right*) were also mapped. All films were exposed for 50 hr in the presence of an intensifying screen. The arrowhead in maps of A, B, and C indicates the same spot identified in Fig. 3.

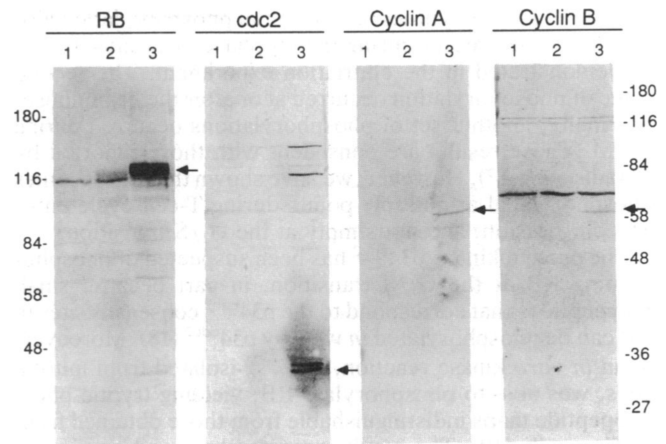

FIG. 5. Western blot for RB, cdc2, cyclin A, and cyclin B in stimulated T cells. Lysates were prepared from T cells before the addition of PHA (lanes 1) or 16 (lanes 2) and 36 hr (lanes 3) after addition. Aliquots of protein (140 μg) were electrophoresed in an SDS/7.5% (RB) or 10% (cdc2, cyclin A, cyclin B) polyacrylamide gel. Molecular weight markers ($M_r \times 10^{-3}$) at left are for the RB blot, and those at right are for the cdc2, cyclin A, and cyclin B blots. The arrows point to the respective proteins for which each blot was probed.

The First RB Phosphorylation Precedes cdc2 Expression. Since RB function has been linked to its phosphorylation state, knowledge of the identity of the kinase(s) responsible for RB modification would contribute to a better understanding of how RB function is regulated. A putative kinase responsible for RB phosphorylation is the *cdc2* gene product, $p34^{cdc2}$. Because the cdc2 protein plays a pivotal role in the control of cell cycle regulation, there has been considerable suspicion that it may phosphorylate RB. Here, we asked whether $p34^{cdc2}$ could have participated in the first of the several observed steps of RB phosphorylation.

Lysates were prepared from unstimulated cells (Fig. 5, lanes 1) and from cells stimulated for 16 hr (lanes 2) or 36 hr (lanes 3) with PHA. Aliquots were electrophoresed in SDS/polyacrylamide gels, and Western blotting was performed, in parallel, using monospecific antibodies for RB, cdc2, cyclin A, and cyclin B. No signal for cdc2, cyclin A, or cyclin B was observed at 0 or 16 hr after PHA addition, when the first phosphorylation(s) of RB was readily apparent (Fig. 5, lanes 1 and 2). However, 36 hr after PHA addition, when RB had undergone additional phosphorylation(s), cdc2, cyclin A, and cyclin B were all readily detectable (Fig. 5, lanes 3). Thus, in keeping with the previously reported demonstration that such human T cells do not express cdc2 mRNA until near the onset of S phase (16), it appears that $p34^{cdc2}$, in association with cyclin A or cyclin B, cannot be the kinase responsible for the pre-S-phase phosphorylation of RB. Hence, another enzyme(s) must be responsible for the RB phosphorylations occurring during the G_0 to G_1 transition. It remains possible that $p34^{cdc2}$ participates in the later stages of the RB phosphorylation process.

DISCUSSION

Several laboratories have reported that RB is un- or underphosphorylated in G_0/G_1 and becomes phosphorylated at the G_1/S boundary in cycling cells (1, 2, 8). Cell cycle-dependent phosphorylation of RB of this type has been observed in primary cells as well as in established cell lines (2). We report here that, during the activation of human T lymphocytes, RB becomes phosphorylated in at least three stages during cell cycle entry and progression. The first evidence of phosphorylation was noted in G_1, after exit from G_0 but before the

initiation of DNA synthesis. This initial phosphorylation also appears to be present during the G_1 phase of cycling T cells as demonstrated in the elutriation experiment. The second stage of phosphorylation occurred at or after the beginning of S. Finally, another set of phosphorylations occurred during G_2/M. These results are consistent with those reported by Terada *et al.* (17). However, we have shown that RB becomes phosphorylated at multiple points during T-cell cycle entry and progression, and not simply at the G_1/S transition.

The protein kinase $p34^{cdc2}$ has been suspected of phosphorylating RB at the G_1/S transition, in part because small polypeptides that correspond to the $p34^{cdc2}$ consensus sites in RB can be phosphorylated *in vitro* by $p34^{cdc2}$ (18). Moreover, in an *in vitro* kinase reaction, $p34^{cdc2}$, isolated from mitotic cells, was able to phosphorylate RB, yielding tryptic phosphopeptide maps indistinguishable from those obtained from cycling cells (19). The results reported here and previously (16) are inconsistent with the suggestion that $p34^{cdc2}$ contributes to the first phosphorylation of RB in human T cells driven out of G_0 by mitogen stimulation. By standard Northern and Western blotting techniques, there was no detectable cdc2 mRNA or protein when the first wave of RB phosphorylations in T cells had already occurred (16). Furthermore, a cdc2 antisense oligonucleotide appeared to have little effect on the first phosphorylation step as observed in an RB Western blot and prevented the appearance of what we now observe as the second and third phases of RB phosphorylation (see figure 3 in ref. 16). Given that the RB phosphopeptide maps before and after cdc2 expression are similar, one could speculate that there are kinases with sequence recognition properties similar to those of cdc2, and one or more of these kinases participate in the first wave of RB phosphorylation observed here. Whatever the case, it remains possible that the cdc2 protein participates in the later phases of RB phosphorylation—i.e., during S and G_2/M.

Since none of the various RB phosphorylated species bound to the simian virus 40 (SV40) large tumor (T) antigen (data not shown), one could argue that each had lost one or more of its growth-regulating functions; for binding to T antigen inactivates certain RB functions, and this viral protein can bind only to un(der)phosphorylated RB (3, 11). However, since at least some of the phosphorylated species of RB can bind to adenovirus E1A protein (R. Lin and J.A.D., unpublished observations), they may have unique functions that can be modulated by E1A expression and not by SV40 large T antigen. Furthermore, if a specific change in phosphorylation state at various times in the cell cycle means that the functional state of RB also changes at each of these transitions, then one can impute to RB a role in the regulation of cell cycle progression from G_0 to G_1, from G_1 to S, or from one segment of S to another, and then within G_2/M. Following the same logic, one may conclude that it is also possible that RB dephosphorylation, occurring in mitosis, is required for proper exit from M.

Unphosphorylated RB has been demonstrated to associate with the transcription-activating factor E2F (20–23). Some have argued that RB binding results in the sequestration or inactivation of this protein. E2F may play a significant role in T-cell activation, by promoting the expression of genes that contain the cognate sequence in its promoter. For example, expression of c-*myc* and c-*myb* gene products increases during T-cell activation at a point close to the initial phosphorylation of RB (7, 16). The promoters of c-*myc* and c-*myb* contain an E2F binding sequence required for the increase in expression after mitogenic stimulation (24–27). If, as in the case of SV40 T antigen, RB phosphorylation upon exit from G_0 results in the failure of RB to bind to E2F, then the G_0/G_1 RB phosphorylation might release E2F, allowing it to participate in transcriptional activation of these as well as other genes. Finally, the sequential phosphorylation of RB suggests that it operates at several points in the cell cycle, perhaps, in part, by modulation of other transcription factors.

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