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Original Research Article

Phosphorylation–Dephosphorylation of Retinoblastoma Protein Not Necessary for Passage through the Mammalian Cell Division Cycle

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Summary

Phosphorylation of the retinoblastoma protein (Rb) during the G1-phase of the mammalian cell division cycle is currently believed to be a controlling element regulating the passage of cells into S-phase. We find, however, that the suspension-grown cell lines U937, L1210, and MOLT-4 contain exclusively hyperphosphorylated Rb. Furthermore, when adherent NIH3T3 cells are grown at very low densities to avoid overgrowth and contact inhibition, they also contain only hyperphosphorylated Rb. NIH3T3 cells exhibit hypophosphorylation when the cells are grown at moderate to high cell densities. We propose that cultures of adherent cells such as NIH3T3, when grown to moderate cell densities, are made up of two populations of cells: (*a*) cells that are relatively isolated and therefore growing exponentially without contact inhibition, and (*b*) cells that are growth-inhibited by local cell density or contact inhibition. The common observation in adherent cell lines, that Rb is both hyper- and hypophosphorylated in the G1-phase and only hyperphosphorylated in the S- and G2-phases, is explained by the effects of cell density and contact inhibition. Thus, phosphorylation–dephosphorylation of Rb protein during the G1 phase is not a necessary process during the NIH3T3, L1210, MOLT-4, and U937 division cycles. We propose that phosphorylation–dephosphorylation of Rb is independent of the division cycle and is primarily determined by growth conditions throughout the division cycle.
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Keywords Cell cycle; G1-phase; phosphorylation; retinoblastoma protein; Western blotting.

INTRODUCTION

It is generally and widely believed that the retinoblastoma protein (Rb) is phosphorylated and dephosphorylated in a cell-cycle–dependent fashion in mammalian cells (*1–8*). Cells entering the G1-phase are proposed to have a dephosphorylated Rb protein. The Rb protein is then phosphorylated in mid-G1-phase, allowing passage of cells from the G1-phase to the S-phase. This phosphorylation–dephosphorylation cycle is believed to be one of the key cell-cycle control events in mammalian cells.

According to the current paradigm (*9*, *10*), the dephosphorylated form of Rb is a negative control element. Upon phosphorylation of the Rb protein at a particular point in the G1-phase, the negative action of the Rb protein is reversed and the cell is allowed to move from the G1-phase into the S-phase. One biochemical mechanism explaining cell-cycle control by the Rb protein is that the hypophosphorylated form sequesters the E2F transcription factor. Upon Rb phosphorylation, E2F is released. The newly released and activated E2F transcription factor then induces the expression of various genes associated with the initiation of DNA replication.

The variation of Rb phosphorylation during the division cycle, as currently widely held, has been succinctly summarized by Mittnacht (*8*): ". . . the pRB phosphorylation state fluctuates as the cell passes through the division cycle. In cycling cells pRB is found in its active underphosphorylated form only during the early period of the G1 phase." And further: ". . . pRB phosphorylation in late G1 and its dephosphorylation in late M phase are considered the two critical events regulating pRB's growth-restraining activities."

We now present experimental results demonstrating that the Rb phosphorylation–dephosphorylation cycle is not present in cells undergoing exponential, balanced growth. Rb in these cells is hyperphosphorylated in all phases of the division cycle. We also show that when NIH3T3 cells are grown under conditions of low density, avoiding contact or other forms of inhibition, Rb protein is found only in the hyperphosphorylated form. No cell-cycle–associated variation in phosphorylation is observed.

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In particular, no variation in phosphorylation in the G1-phase is observed. We conclude that the Rb phosphorylation pattern commonly observed is due to the growth conditions and not to the cell position in the division cycle.

Therefore, not only do we show that the phosphorylation–dephosphorylation cycle is not a necessary part of the normal division cycle of growing mammalian cells, but we also provide an explanation for the results that have led to the current view that such a phosphorylation cycle is regulatory feature of the mammalian cell division cycle.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture. NIH3T3 cells (an adherent mouse cell line) were grown in Dulbecco's modified essential medium (high glucose, and 10% calf serum; Gibco) at 37 °C in an atmosphere of 5% CO_2. L1210 cells, a murine lymphoma cell line, and U937 and MOLT-4, two human leukemic cell lines, were grown in Liebovitz's L-15 medium (Gibco) at 37 °C. These 3 leukemic lines grow in suspension without adhering to the flask surface. All growth media were supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Cell-Cycle Analysis by Flow Cytometry. NIH3T3 cells were harvested by trypsinization in the presence of EDTA and resuspended in phosphate-buffered saline. L1210, MOLT-4, and U937 cell lines were harvested directly by centrifugation without trypsinization. The cells were suspended in 50% ethanol and before analysis were suspended in a solution of 50 μg/ml propidium iodide and 100 μg/ml RNase in phosphate-buffered saline. Flow-cytometric analysis was performed with a Coulter Epics analyzer, and the results were then subjected to the Multicycle program (Phoenix Flow Systems) to determine the relative fraction of cells with either a G1-, S-, or G2/M-phase amount of DNA.

Sorting of Cells by Flow Cytometry. Cells were prepared for flow-cytometric analysis by fixing cells in 50% ethanol and then treating the cells with propidium iodide and RNase. Such fixed cells were sorted with a Coulter Epics sorting instrument. After a preliminary analysis of the total DNA pattern, the instrument was successively gated to select either G1, S, and G2 fractions or, for simplicity, G1 and S+ G2 fractions. About 10^6 cells were collected for an analysis of Rb protein. After collection, the cell fractions were analyzed for the purity of the selected fractions. An example of such a sorting purity check is illustrated in Fig. 1a. Each of the sorted fractions (G1, S, and G2) was pure and did not contain any significant number of cells with DNA contents outside of the selected content.

Isolation of Newborn L1210 Cells. Newborn L1210 cells were obtained by using the classical "baby machine" approach, a method developed by Charles Helmstetter (*11*). Lysates from newborn L1210 cells were kindly provided by Charles Helmstetter and Maureen Thornton (from unpublished experiments). In the experiment described in Fig. 1b, cells were collected for 30 min, therefore, these cells represent only the first

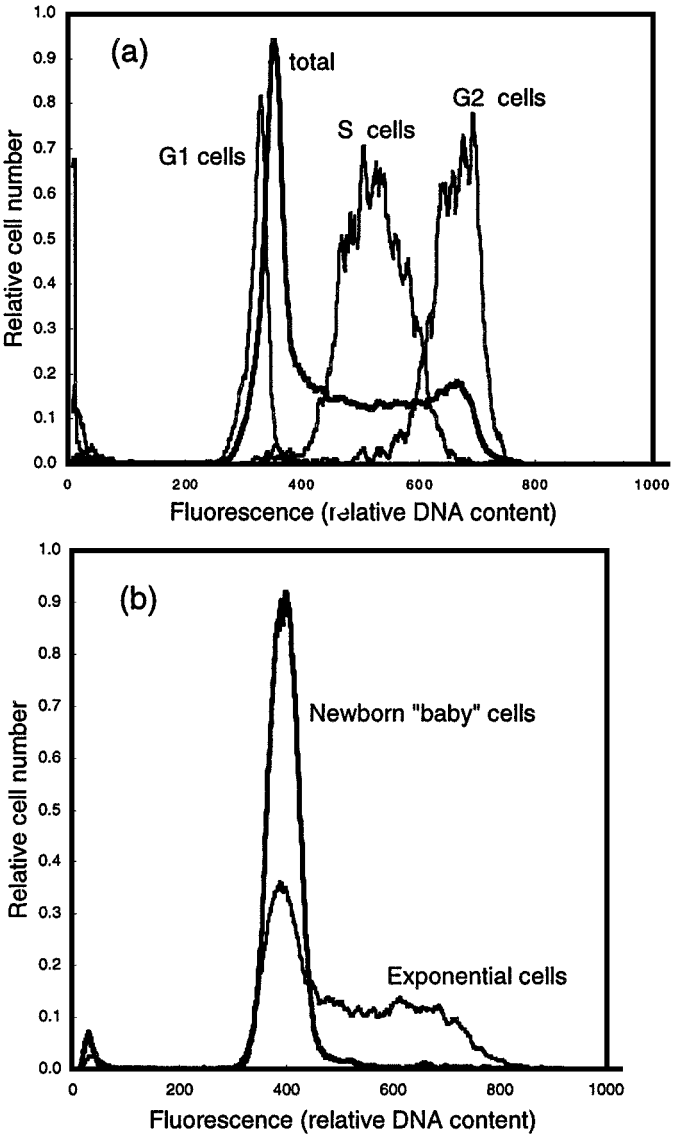

Figure 1. DNA contents of cells analyzed for Rb phosphorylation pattern. (a) Flow-cytometric patterns obtained from exponentially grown cells are compared with those for purified G1-, S-, and G2-phase cells separated by flow cytometry. (b) DNA content of newborn L1210 cells released from the "baby machine" and the DNA content of the exponential L1210 culture placed on the apparatus. In both graphs, the lines show the running average (20 points running average) of the data.

30 min of the L1210 division cycle, which takes approximately 12 hours in all.

Western Blot Assay of Rb Protein and Phosphorylation Pattern. Cells were collected by centrifugation, resuspended in PBS, and lysed (50 mM Tris-HCl, pH 8.0, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodiumdeoxycholate, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride). The lysate was separated on a 6% polyacrylamide gel and after transfer of the protein to a membrane

(High Bond, Life Science) mouse anti-human Rb (Pharmingen) was added at a dilution of 1/1000. After 1 h at room temperature, the membrane was washed with Tris-buffered saline and probed with goat anti-mouse antibody conjugated with horseradish peroxidase (Bio-Rad). The Rb protein bands were detected with ECL reagent (Amersham). Control bands were included to identify the hyper- and hypophosphorylated Rb. Lysates of serum-starved NIH3T3 cells gave the location of the hypophosphorylated Rb. Hyperphosphorylated Rb was revealed by lysates from restimulated (with IGF-1, 10 nM) serum-starved NIH3T3 cells.

RESULTS

To determine whether Rb phosphorylation changed during the division cycle of exponentially growing cells in suspension, we studied Rb protein in extracts of three different cell lines that do not grow in an adherent state. Western blot analysis of the Rb protein in total cell extracts of cell lines L1210, MOLT-4, and U937 revealed that all the Rb protein was present in the hyperphosphorylated form (Fig. 2a). This result shows that it may not be necessary to have a change in phosphorylation state during the division cycle of cells grown in suspension.

To support the conclusion that there was no hypophosphorylated Rb in these cells, we separated the L1210 cells by flow-cytometric sorting (see Fig. 1a for an indication of the purity of the sorted subfractions) and the subfractions from G1-, S-, and G2-phase cells were analyzed for Rb protein by Western blotting. Only one band, representing hyperphosphorylated Rb protein, was observed in the G1-, S-, and G2-phase fractions (Fig. 2b). This confirms the results obtained with total cell extracts (Fig. 2a), that only the hyperphosphorylated form was present in these cells.

The flow-cytometric sorting experiment (Fig. 2b) leads to a slight improvement in the ability to detect a minor band of hypophosphorylated Rb. If the dephosphorylated form is a small fraction of the total protein, and is present only in the G1-phase of the division cycle, then looking only at the G1-phase cells increases the sensitivity of the assay for this minor band. For example, consider that one-third of the total cell population has a G1-phase DNA content. When the G1-phase subfraction is analyzed, there will be a 3-fold improvement in the ability to observe a minor band such as the hypophosphorylated form. The improved sensitivity results from excluding the S- and G2-phase cells, which contain only the hyperphosphorylated Rb protein.

A still greater increase in sensitivity can be obtained by looking specifically at the cells in the earliest part of the G1-phase. For example, for a collection of cells less than 30 minutes old, obtained from cells with a 12-h doubling time, searching for a minor hypophosphorylated Rb band present only in the youngest cells would provide a 24-fold increase in sensitivity. Just such an assay was performed by isolating newborn cells produced with use of a eukaryotic baby-machine (*11*). As shown in Fig. 1b, one can obtain a pure collection of cells with a G1-phase DNA. Although these newborn cells have a G1-DNA content similar

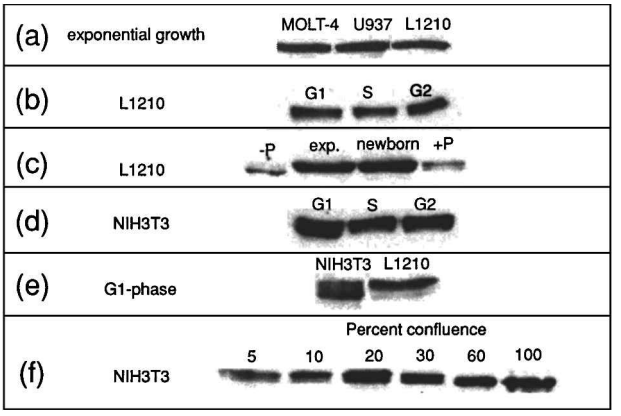

Figure 2. Western blot analysis of Rb protein. (a) Rb phosphorylation pattern of extracts from different suspension-grown cell lines. The Rb phosphorylation pattern was analyzed by Western blotting of extracts prepared from different cell lines. The different lanes contain extracts from exponentially growing L1210, MOLT-4, and U937. The three suspension-grown cell lines exhibit a single band of hyperphosphorylated Rb protein. (b) Analysis of the phosphorylation pattern of Rb in cells from different cell-cycle phases of strain L1210. Cells were sorted by flow cytometry and ~ 10^6 cells were analyzed for Rb pattern by Western analysis. There is only one species—hyperphosphorylated—of Rb protein in all phases of strain L1210. (c) Rb protein analysis of newborn cells obtained from a mammalian cell "baby machine." No hypophosphorylated Rb is seen in the newborn cells nor in the exponential culture. The + P and - P bands beside the two experimental bands indicate control samples with hyperphosphorylated (+ P) and hypophosphorylated (- P) Rb protein. (d) Rb phosphorylation pattern in cells isolated from different phases of the NIH3T3 cell cycle. One hyperphosphorylated Rb species is seen in the S and G2 fractions of cells, whereas in the NIH3T3 G1-phase cells, two bands of Rb protein, hypo- and hyperphosphorylated, are observed. (e) G1-phase cells were isolated from L1210 and NIH3T3 cells and their Rb phosphorylation patterns were compared. The L1210 cells show one hyperphosphorylated band and the NIH3T3 cells show the characteristic two bands. The preparations in this experiment are independent of other experiments; that is, these bands are not merely reruns of the G1-phase cells of (b) and (d) above. (f) Phosphorylation pattern of Rb in NIH3T3 cells grown to different cell densities. Strain NIH3T3 cells were inoculated into 15-cm petri dishes as follows: (i) 1.5×10^4 cells per plate, 8 plates; (ii) 3×10^4 cells per plate, 4 plates; (iii) 6×10^4 cells per plate, 2 plates; (iv) 1.25×10^5 cells per plate, 1 plate; (v) 2.5×10^5 cells per plate, 1 plate; and (vi) 5×10^5 cells per plate, 1 plate. The cells were allowed to grow 5 days, and the degrees of confluence estimated by microscopic examination were (i) 5% confluence; (ii) 10% confluence; (iii) 20% confluence; (iv) 30% confluence; (v) 60% confluence; and (vi) 100% confluence (overgrown). Cells were harvested, lysed, and subjected to Western analysis for Rb protein.

to the cells obtained by flow cytometry, it is important to realize that the two populations of cells with an amount of G1-DNA are very different. The flow-cytometric population is a selection of cells from all parts of the G1-phase, whereas the selected newborn cells are from only the very first part of the G1-phase.

Comparing the Rb phosphorylation pattern in these newborn G1-phase cells with that of the exponential, unsorted culture (lysates prepared and supplied by Helmstetter) reveals that no hypophosphorylated Rb protein is to be found in strain L1210 in even the very earliest part of the division cycle (Fig. 2c). This eliminates the possibility that the phosphorylation step occurs so early in the G1-phase that it would not be seen by analysis of exponentially growing cells.

Because no hypophosphorylated Rb protein was observed in suspension-grown cells, we reexamined the effect of growth conditions on the Rb phosphorylation pattern in NIH3T3 cells, a widely studied line of adherent cells. We first observed that the Rb protein composition in NIH3T3 cells grown by standard methods—that is, grown to moderate cell densities—produced the classic finding that G1-phase cells had both hyper- and hypophosphorylated Rb protein, whereas the S and G2 cells had only the hyperphosphorylated Rb protein (Fig. 2d). This confirms the observation that led to the model holding that there is a conversion, in mid-G1-phase, of the hypophosphorylated Rb to a hyperphosphorylated form; in S- and G2-phases, only the hyperphosphorylated form is present. In a separate experiment, illustrated in Fig. 2e, G1-phase cells isolated from L1210 cells and NIH3T3 cells were compared. Only hyperphosphorylated Rb protein was observed in the G1-phase cells obtained from the suspension-grown L1210 cells, whereas hyper- and hypophosphorylated Rb protein were observed in the NIH3T3 G1-phase cells. In Fig. 2d and e, the NIH3T3 cells were obtained from subconfluent but relatively densely grown cultures.

A major difference between the NIH3T3 cells and the suspension-grown cells is that when NIH3T3 cells are grown on plates to moderately high but still subconfluent cell densities, areas of higher and lower cell densities are obtained: Some cells are in a low-density area, and others are more crowded. To check whether the differences found in Rb phosphorylation were cell-density–dependent, we grew NIH3T3 cells over a range of cell densities (from 5% to 100% confluence) and analyzed the Rb protein for the phosphorylation pattern (Fig. 2f). Decreasing densities of final growth were associated with an increase in Rb protein phosphorylation. That is, the confluent cells exhibited a significant amount of hypophosphorylated Rb protein; with decreasing densities, however, we found there was a decrease in the hypophosphorylated Rb as well as an increase in the hyperphosphorylated form. This result has been repeated in many experiments, which show that NIH3T3 cells grown to low densities (1–5% confluence) have only hyperphosphorylated Rb protein.

DISCUSSION

Nonadherent, suspension–grown cells can be maintained in continuous, exponential, balanced growth conditions by merely ensuring that there is enough medium to prevent cells from overgrowing and altering the growth conditions (*12*). In contrast, cells growing to a higher density may alter the medium (by removing or decreasing various growth factors, excreting catabolites, or changing pH) or may affect each other (e.g., by contact inhibition). Depending on the local environment of each cell, a spectrum of cell types may exist.

In contrast to suspension-grown cells, ensuring balanced growth in adherent cell lines such as NIH3T3 cells is more difficult, because the spatial arrangement of cells on plates is not uniform. For example, when cells are 50% confluent—a density used for many experiments—there are some areas of confluence, in which the cells are quite dense, and other areas with cells at lower densities. The overall cell density does not reflect the variation in density over the entire plate. From this basic consideration of cell growth conditions, we can now suggest a model to explain the results we obtained with Rb protein in different cell lines.

That cells growing exponentially in suspension culture do not exhibit an Rb phosphorylation–dephosphorylation cycle is explained by a simple proposal that cells growing in unlimited, exponential, balanced, and normal growth do not have a cell-cycle–dependent Rb phosphorylation–dephosphorylation cycle.

We then explain our results for NIH3T3 cells—an absence of hypophosphorylated Rb in cells grown at low densities compared with cells grown at high densities—by the model shown in Fig. 3. We propose that cells that have left ideal growth conditions are primarily cells with a G1-amount of DNA (*13*). Because of poor growth conditions (either local or systemic), these cells now accumulate hypophosphorylated Rb protein. We do not know whether the growth cessation is a cause or a result of Rb dephosphorylation. It may even be that there is no relationship between growth and phosphorylation state of Rb; the two events may be merely coincidentally associated with cell growth conditions.

We propose that these "arrested" cells with a G1-amount of DNA are not necessarily arrested at a point in the G1-phase. This analysis has been presented previously (*12*, *14–17*). According to this view, cells arrested with a G1-phase amount of DNA are not representative of G1-phase cells in the normal division cycle. We will not review the arguments for this proposal here; instead, we merely point out that a distinction should be made between nongrowing or slow-growing cells with a G1-amount of DNA and cells with a G1-amount of DNA that are in the normal G1-phase of the division cycle. The cells arrested with a G1-amount of DNA are not equivalent to exponentially growing cells with a G1-amount of DNA.

According to the model presented in Fig. 3, the cells in the S- and G2-phases of NIH3T3 cultures grown to moderate and high cell densities define the growing subpopulation not arrested with a G1-phase amount of DNA. If the nongrowing cell subpopulation exhibits G1 arrest, then the S- and G2-phase cells must come from the growing fraction. Fig. 3 suggests that analyzing cells not in balanced growth can give a misleading result because

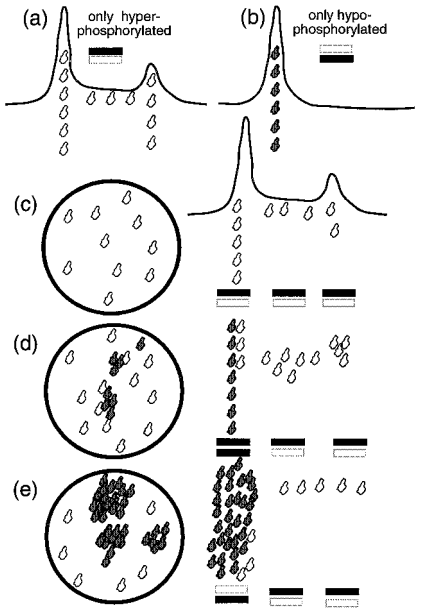

Figure 3. Explanation of how growth conditions can affect the G1-phase phosphorylation pattern even though in growing cells there is no cell-cycle variation in Rb phosphorylation. (a) An idealized representation of growing cells: There is a normal cell-cycle flow-cytometric pattern with peaks for G1 and G2 and an S-phase band between the two peaks. The unshaded cells in this diagram are proposed to all have the hyperphosphorylated form of Rb with no cell-cycle–specific variation in phosphorylation pattern. (b) The shading indicates cells that have been arrested in their growth (by either medium conditions, contact inhibition, or other unknown factors) and have accumulated with a G1-phase amount of DNA. The growth conditions that lead to cell-growth arrest are presumed to also give the dephosphorylated form of Rb. Although the cause–effect relationship of growth cessation and phosphorylation state is not explicitly an issue here, one may consider that the phosphorylation state is merely a correlate of growth cessation, and may or may not be causative. (c, d, e) The proposed outcomes for cells grown at low cell densities (c), intermediate cell densities (d), and high cell densities (e). Under conditions of low cell density, because the cells are growing in balanced, exponential growth, all cells are proposed to have only the phosphorylated form and there is no difference between the Rb phosphorylation pattern in the different phases of the cell cycle. At higher concentrations of cell growth (d), the adherent types of cells are a mixed population, with some cells being arrested (the shaded cells) and others growing vigorously and oblivious to the other cells in the medium. When such a mixture is analyzed, the S- and G2-phases give the same pattern as in the dilute case, whereas the cells with a G1-phase amount of DNA show both bands: one band from the growing cells (a) and the other band from the growth-arrested or inhibited cell fraction (b). At the highest cell densities (e), one may get a very large fraction of arrested cells and thus have a situation where the cells with a G1-phase DNA content dominate; thus we see a pattern where the Rb protein from cells with a G1-phase amount of DNA has essentially all dephosphorylated Rb protein.

the G1-phase cells may include cells that are arrested or slowed in their growth.

Although not all previously published work provides information on cell density at harvest, our results are consistent with previously published work on Rb phosphorylation. For example, published studies have found that in rapidly growing cells, the Rb protein is found all in the hyperphosphorylated state, and when growth is inhibited, there is a loss of phosphorylation and the appearance of the hypophosphorylated Rb (*2*). Additional studies of Daudi cells that were either grown exponentially or inhibited by serum starvation, α-interferon, or phorbol ester also indicated that growing cells had a preponderance of hyperphosphorylated Rb protein and that inhibition of growth led to a loss of phosphorylation (*7*). In this same study U937 cells, which were not inhibitable by α-interferon, did not exhibit the dephosphorylation of Rb when α-interferon was added.

It is difficult to compare previous results with those presented here. In many cases one cannot determine the precise cell densities at which cells were harvested. For this reason, we suggest there should be a reexamination of the proposal that the Rb protein is phosphorylated in a cell-cycle–specific manner.

The major difference between the experiments presented here and those proposing a G1-phase phosphorylation event is the experimental system we have used for growing and analyzing cells. We have concentrated on cells in continuous, balanced, exponential growth. Previous work analyzed a number of different systems such as lymphocytes stimulated to grow (*18*), cells synchronized by various inhibitors or starvation treatments (*6*, *19–22*), or cells that may have been grown to such densities as to introduce contact inhibition or other phenomena. Cells have been subjected to stresses to demonstrate cell-cycle regulation of Rb phosphorylation (*23*). A corollary result that supports the model proposed here is the finding that it is possible to produce conditions whereby cells in all phases of the division cycle have hypophosphorylated Rb protein (*24*), suggesting again that it is growth conditions, rather than the position in the cell cycle, that determine the phosphorylation pattern.

To summarize, the current view of the cell cycle proposes that Rb phosphorylation–dephosphorylation is a necessary process for passage through the cell division cycle. We have directly tested this hypothesis and have demonstrated that the general proposition that Rb phosphorylation–dephosphorylation is a necessary component of the division cycle of all mammalian cells is not correct. We conclude that the phosphorylation–dephosphorylation cycle for Rb protein is not a necessary process in NIH3T3 cells or any of three different suspension grown cell lines. Most interesting, it is a possible that the stronger proposition emanating from these results—that Rb phosphorylation–dephosphorylation is not a component of the division cycle of all growing mammalian cells—is correct.

Another way to formulate the conclusions presented here is to state that, although it is not necessary for cells growing at low densities in balanced growth to have a phosphorylation–dephosphorylation cycle in the G1-phase, the phosphorylation–dephosphorylation of Rb protein is primarily a function of the cellular growth state. Cells in an overgrown condition will have

a different phosphorylation pattern than the cells grown at low densities. These phosphorylations are not related to the division cycle.

The results here were anticipated in a proposal made two decades ago (*25*). One of us (S.C.) proposed that there are no G1-specific events and that the G1-phase existed when the interdivision time or mass-doubling time of a mammalian cell was greater than the sum of the S+ G2+ M-phases (*12*, *16*). According to this view of the mammalian division cycle, the G1-phase is the time when the biosynthetic processes begun at the start of the previous S-phase are completed. This viewpoint, referred to as the continuum model, has been supported by a large number of experimental observations (*12*, *26*). The results presented here are a direct support of the main prediction of the continuum model, that there are no G1-specific events occurring during the normal division cycle of mammalian cells.

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