Retinoblastoma Protein is Rapidly Dephosphorylated by Elevated Cyclic Adenosine Monophosphate Levels in Human B-Lymphoid Cells

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Abstract

Elevated cyclic AMP levels induce a rapid block in the mid-G1 phase of the cell cycle in B-lymphoid Reh cells, accompanied by a transient block in G2. The retinoblastoma (Rb) gene product has been implicated as a key regulator of eukaryotic cell growth. The Rb protein enforces its growth-suppressive effect in early G1, where it is underphosphorylated and firmly bound to the nucleus. A possible link between the cyclic AMP-mediated growth arrest and regulation of Rb protein phosphorylation was explored by Western blot analysis. We found that both forskolin and 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate induced a rapid (within 3 h) dephosphorylation of Rb protein. These data were confirmed by flow-cytometric analysis of isolated nuclei costained with anti-Rb antibodies and propidium iodide. The percentage of cells containing underphosphorylated Rb protein (i.e., G1 nuclei with bound Rb protein) increased from 9% to 87% after 4 h of forskolin treatment.

During the first 4 h of forskolin treatment, the cells were transiently blocked in the G2 phase of the cell cycle, and virtually no cells had passed through mitosis. The increased level of dephosphorylated Rb protein at 4 h was therefore not due to an accumulation in early G1 of cells containing underphosphorylated Rb protein. Instead, our data indicated that dephosphorylation of Rb protein occurred in cells that had already passed the point in G1 of Rb protein phosphorylation.

Dephosphorylation of Rb protein was prevented by high concentrations of the protein phosphatase inhibitor okadaic acid, indicating that activation of a phosphatase is involved in the cyclic AMP-mediated dephosphorylation of Rb protein. We suggest that the dephosphorylation of Rb protein is required for the forskolin-mediated arrest of the Reh cells in mid-G1.

Introduction

Growth inhibition as well as stimulation are a result of a complex cascade of events, neatly regulated to ensure that these fundamental cellular processes are not disturbed. One of the most important gains in our knowledge in this field of research has come from the identification of genes encoding key regulators of growth. Among such genes we find the protooncogenes and the tumor suppressor genes, which upon change in activation status or level of the protein products may lead to dysregulated, and finally to malignant, cell growth (1, 2). The Rb gene is defined as a tumor suppressor gene, and its protein product (pRb) is known to be a repressor of growth (12, 13). The activity of pRb is thought to be regulated during the cell cycle by changes in its phosphorylation state (14, 15), and the degree of phosphorylation is reflected by changes in electrophoretic mobility (14). In resting cells (G0), pRb is in the underphosphorylated state (14, 16, 17), and it is this form that binds firmly to the nucleus (18). It is also this form of pRb that prevents cell proliferation (16, 19, 20), for instance by sequestration of the transcription factor E2F (21–23).

Stokke et al. (24) recently showed that in cycling cells pRb is in its underphosphorylated state only in the beginning of G1; the dephosphorylation occurs immediately after mitosis (24). Furthermore, they showed that the fraction of pRb in G1 nuclei in continuously cycling cells increased with increased length of the G1 phase. Later in G1, the more phosphorylated forms of pRb appear, and pRb remains phosphorylated throughout the rest of the cell cycle (14, 16, 17, 20). Phosphorylation ceases before the cells complete mitosis (14, 16, 17, 20). From in vivo data, it appears that p34cdc2 kinase is able to phosphorylate pRb (25, 26), whereas it is not yet established which kinase(s) is involved in pRb phosphorylation in vivo. The dephosphorylation of pRb that occurs after mitosis is thought to be mediated by protein phosphatase 1 (27).

As the phosphorylation state of pRb is of vital importance for its activity, it has been the interest of several investigators to study the possibility of suppression of pRb phosphorylation as part of the antiproliferative machinery (28–30). In the present paper, we have used the B-precursor cell line Reh to show that rapid dephosphorylation of pRb occurs when B-lymphocytes are treated with agents that increase the intracellular level of cAMP. The advantage of using this cell system is that, due to a cAMP-induced transient block in the G2 phase of the cell cycle, we are able to discriminate between 2 alternative models for explaining the observed increased level of underphosphorylated pRb. The 2 models are, in short: (a) cAMP-mediated dephosphorylation of pRb occurs in cells that have passed the point in G1, where pRb becomes phosphorylated, in other words cAMP induce receptor-ligand interactions is cAMP. cAMP has been implicated in negative growth control in a variety of cell types (6). The role of cAMP in growth regulation of lymphoid cells has been controversial (7), but more recent research seems to agree on cAMP as a growth inhibitor for normal lymphocytes (8, 9).

Using stimulators of the cAMP-generating enzyme adenylate cyclase or cAMP analogues, one can study the postreceptor events in a more simplified system than using physiological growth-inhibiting polypeptides. We have studied the mechanisms involved in cAMP-mediated growth inhibition of normal B-lymphocytes and cell lines by using the adenylate cyclase stimulator forskolin (9). Enhanced cAMP levels transiently blocked the cells in the G2 phase of the cell cycle before they accumulated in G1 (10). Recently we have shown that the transient MYC suppression associated with forskolin-mediated growth arrest (8) was not a prerequisite for the growth inhibition of the Reh cells (11). We therefore decided to look for cAMP-mediated regulation of genes representing the repressors of growth, the hypothesis being that enhanced levels of cAMP would lead to a higher activity or level of proteins derived from such genes.

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3 The abbreviations used are: TGF-β, transforming growth factor-β; 8-CPT, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; pRb, protein product of the retinoblastoma gene Rb; Rb, retinoblastoma; cAMP, cyclic AMP; PBS, phosphate-buffered saline; FL3-A, propidium iodide fluorescence integrated intensity; PKA, protein kinase A; IL-6, interleukin-6.
cAMP INDUCES DEPHOSPHORYLATION OF THE RETINOBLASTOMA PROTEIN

dephosphorylation of already phosphorylated pRb; and (b) cAMP prevents pRb phosphorylation by blocking the cells in early G1, leading to accumulation of cells containing underphosphorylated pRb. As presented in this paper, our data favor the first model.

MATERIALS AND METHODS

Chemicals and Antibodies

Forskolin and 8-CPT were purchased from Boehringer and Sigma, respectively. Okadaic acid was obtained from Moana Bioproducts, Inc. The antibody used for detection of Rb protein in both Western blots and flow cytometric analysis was the monoclonal antibody PMG3-245 from Pharmingen.

Cells

The B-precursor cell line Reh was originally derived from a patient with acute lymphoblastic leukemia (31), and was kindly provided by M. F. Greaves, Imperial Cancer Research Fund Laboratories. The cells were grown at a cell density between 0.1 and 1.5 X 10^6 cells/ml in RPMI supplemented with 10% fetal calf serum, penicillin, and streptomycin at 5% CO_2 in a humidified atmosphere.

Cell Growth

Cell growth was determined by Coulter counting of cell numbers, or by measuring DNA synthesis by [3H]thymidine uptake. In the [3H]thymidine incorporation assay, the cells were grown in 96-well microtiter plates (Costar), at 0.15 x 10^6 cells/ml as an initial density. The experiments were run in triplicate, and the cells were pulsed with 5 x 10^4 Bq [methyl-3H]thymidine (Radichemical Centre, Amersham) for 21 h at 37°C before being harvested on a cell harvester (Titrak, Skatron). Cell cycle progression was also estimated by measuring the mitotic index and distribution of cells in the different phases of the cell cycle by flow cytometric analysis (see below).

Gel Electrophoresis

Reh cells were washed twice in PBS and the cells (10^7/ml) were boiled in Laemli sample buffer (32) for 7 min. The proteins were separated by elec trophoresis in 7.5% sodium dodecyl sulfate-polyacrylamide gels (32) and blotted onto nitrocellulose filters (Schleicher & Schuell). The filters were pretreated with 5% dry milk in 0.1% Tween-20 for 1 h followed by incubation with the primary anti-Rb antibody (0.5 μg/ml) for 1 h. Rb protein was detected using a detection kit for mouse antibodies (RPN 22; Amersham) according to the manufacturer's protocol.

Flow Cytometric Analysis

Estimation of pRb^* Nuclei. The procedure described by Stokke et al. (24) was used. Reh cells were washed in PBS, and nuclei were isolated by incubating the cells for 5 min on ice in 750 μl low salt detergent buffer (0.1% Nonidet P-40, 10 mM NaCl, 5 mM MgCl_2, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM phosphate buffer, pH 7.4). The cells were vortexed and 250 μl of 4% paraformaldehyde were added. The nuclei were fixed for 1 h, permeabilized in 0.1% Triton X-100 in PBS, followed by a 3-layer procedure for staining of pRb. The nuclei were re suspended in 100 μl of 10 μg/ml of anti-Rb antibody. After incubation for 30 min, the cells were washed with PBS, incubated in 100 μl biotinylated horse anti-mouse IgG (Vector) diluted 1/50 in PBS for 30 min, washed, and then incubated in 100 μl streptavidin-fluorescein isothiocyanate (Amersham) diluted 1/50. After 30 min, the cells were washed and resuspended in 500 μl PBS containing 0.1% Triton X-100, 200 μg/ml RNase A (Pharmacia), and 12.5 μg/ml propidium iodide (Calbiochem).

The stained nuclei were analyzed in a Facsscan laser flow cytometer as described by Stokke et al. (24), measuring the following parameters: forward light scatter, side scatter, fluorescein isothiocyanate fluorescence intensity, FL3-A, and propidium iodide fluorescence pulse width. The data were gated on forward light scatter versus side scatter, and FL3-A versus propidium iodide fluorescence pulse width dual parameter histograms to exclude dead cells and aggregates of nuclei, respectively.

Estimation of the Mitosis Index

The cells were washed in PBS and were permeabilized in 300 μl detergent buffer (0.15 M NaCl; 10 mM phosphate buffer, pH 7.4; 0.5 mM EDTA; 0.1% Nonidet P-40) for 10 min on ice (33). The cells were fixed by adding 100 μl 4% paraformaldehyde, and they were kept at 4°C. Before analysis in a FACScan laser flow cytometer, the cells were pelleted and resuspended in detergent buffer with 100 μg/ml RNase A and 10 μg/ml propidium iodide. Aggregates and nuclei of dead cells were gated as described above. With this preparation, mitotic nuclei can be separated from G_1 nuclei due to their higher fluorescence and lower forward angle light scatter (33).

RESULTS

cAMP-mediated Inhibition of Cell Growth. Forskolin inhibited the proliferation of Reh cells in a dose dependent manner. As shown in Fig. 1, 100 μM forskolin inhibited the [3H]thymidine uptake by approximately 78% after 3 days of incubation. The viability was higher than 90%. For comparison, we used 150 μM 8-CPT, which inhibited the DNA synthesis by 87%. We have previously shown that forskolin-mediated inhibition of DNA synthesis in Reh cells is associated with an accumulation of cells in the mid-G_1 phase of the cell cycle (34).

Forskolin Increases the Fraction of Underphosphorylated versus Phosphorylated Rb Protein. In continuously cycling Reh cells, most of the pRb is in the phosphorylated state, although the extent of phosphorylation varies. The Western blots in Fig. 2 show that, after 4 h of forskolin or 8-CPT treatment, a major band of faster electrophoretic mobility, consisting of underphosphorylated pRb, appears (approximately M_1, 110,000), reducing the fraction of phosphorylated pRb. The shift in phosphorylation state of pRb to the underphosphoryl ated form was transient, as the lower band disappeared after 24 h of forskolin treatment.

To verify that the Rb protein became associated with the nucleus, i.e., that the apparent dephosphorylation of Rb was of biological significance, we isolated nuclei from forskolin-treated Reh cells, and the fixed and permeabilized nuclei were stained with antibody against pRb. As shown in Fig. 3B, the percentage of G_1 nuclei positive for pRb increased from 9 to 87% in Reh cells treated with forskolin for 4 h, i.e., pRb became underphosphorylated. Judged by the flow cytograms of Rb staining versus cell cycle distribution (fluorescein isothiocyanate fluorescence intensity versus FL3-A; data not shown), dephosphorylation of pRb occurred in G_1 only. Furthermore, pRb lost its ability to bind to the nuclei within the next 24 h of forskolin treatment.

![Fig. 1. Dose dependent effect of forskolin on DNA synthesis. Reh cells (0.15 X 10^7/ml) were treated with different doses of forskolin or by 8-CPT (150 μM). DNA synthesis measured as uptake of [3H]thymidine was analyzed after 3 days, as described in "Materials and Methods." Vertical bars, SD of the mean of 3 experiments.](https://example.com/fig1.png)
The cAMP-mediated Shift in the pRb Phosphorylation State Is Due to Dephosphorylation of pRb. As explained in the "Introduction," the change in the phosphorylation state of pRb could be due to block of cell cycle progression in early G1, leading to accumulation of cells with underphosphorylated pRb. Alternatively, it could be the result of dephosphorylation of pRb occurring in cells that have already passed the point of pRb phosphorylation in G1. To distinguish between these 2 possibilities, we took advantage of our previous findings that forskolin induced a transient cell cycle block in G1 for 10 h (10). We showed that there was no increase in cell number during 4-h treatment with forskolin (Table 1), i.e., none or very few cells went through mitosis. This was verified by a completely different method estimating the percentage of cells in mitosis (mitotic index). The mitotic index dropped from 1.01 to 0.25% after 4 h of forskolin treatment (Table 2). Finally, analysis of the cell cycle distribution (Fig. 3A) showed that the fraction of cells in G1 after 4 h of forskolin treatment was reduced simultaneously with an increased percentage of cells in G2. Thus, as no or insignificant numbers of cells entered G2 during the first 4 h of forskolin treatment, the increased fraction of pRb+ nuclei at this timepoint could not be due to accumulation of G1 cells with underphosphorylated pRb. Furthermore, we observed a reduction in the percentage of G1 cells from 45 to 37% after 4 h of forskolin treatment (Fig. 3A), which could only account for an increase in the fraction of pRb+ G1 nuclei from 9% to approximately 11%. Hence, the observed increase in pRb+ G1 nuclei from 9 to 87% seemed to be due to dephosphorylation of pRb occurring in cells that have passed the pRb phosphorylation point in early G1. This latter model also fit with our results presented in previous publications (34) that forskolin induces a cell cycle block in mid- rather than early G1.

### Table 1: Effect of forskolin on cell numbers

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Cells (×10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td>Forskolin</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>4 h</td>
<td>1.38 ± 0.06</td>
</tr>
<tr>
<td>10 h</td>
<td>1.66 ± 0.07</td>
</tr>
<tr>
<td>15 h</td>
<td>2.15 ± 0.20</td>
</tr>
<tr>
<td>24 h</td>
<td>1.00 ± 0.02</td>
</tr>
</tbody>
</table>

### Table 2: Effect of forskolin on the mitotic index

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.01 ± 0.23</td>
</tr>
<tr>
<td>Forskolin</td>
<td>0.25 ± 0.19</td>
</tr>
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Fig. 2. Effect of cAMP on the phosphorylation state of pRb. Reh cells (0.5 × 10^6/ml) were incubated with forskolin (100 μM) (A) or with 8-CPT (150 μM) (B) for the number of hours (parentheses). Cell lysates were made and Western blot analysis performed as described in "Materials and Methods." Rb, phosphorylated pRb; Rb, unphosphorylated pRb. A, Western blot is from 1 of 6 reproducible experiments; B, 1 of 3 experiments. C, control; F, forskolin.

Fig. 3. Cell cycle analysis and estimation of pRb+ nuclei. Reh cells (0.5 × 10^6/ml) were incubated with forskolin (100 μM) for the indicated number of hours. Cell cycle distribution (A) was measured by propidium staining of cells analyzed by flow cytometry as described in "Materials and Methods." The percentage of pRb+ G1 nuclei (B) was measured by flow cytometric analysis of isolated nuclei fixed, permeabilized, and stained for pRb as described in "Materials and Methods." B, percentage of pRb+ G1 nuclei. Data are from 1 of 3 reproducible experiments.

Cell Cycle Arrest in G1. In agreement with our previous results (34), the flow-cytometric analysis of the Reh cells indeed revealed that the cells became arrested in G1 by forskolin treatment. Due to the G2 block, the percentage of cells in G1 after 4 h of treatment with forskolin would theoretically be reduced from 45 to 22%, given that no cells accumulated in G1. We, however, observed a reduction only from 45 to 37% (Fig. 3A), indicating that some cells actually became arrested in G1. Furthermore, it was evident from the flow diagrams in Fig. 3A that the percentage of S-phase cells in forskolin-treated cells was very low after 10 to 15 h as compared with that of the control. The emptying of the early S-phase also suggested a block of cells in G1. As previously mentioned, the forskolin-mediated dephosphorylation of pRb occurred in G1 only.
A reasonable hypothesis would be that the cell cycle block in G_1 was caused by dephosphorylation of pRb, whereas the transient G_2 block was independent of pRb dephosphorylation.

**Dephosphorylation of pRb Is Prevented by Okadaic Acid.** P34^{cdC2} kinase can phosphorylate pRb at least in vitro, whereas dephosphorylation possibly is mediated by phosphatase 1 (25–27). Activation of p34 kinase is dependent on cyclin interactions (35–38), and is regulated by phosphorylations (49). Thus, a complex cascade of phosphorylation/dephosphorylation reactions is involved in pRb phosphoprotein regulation. Forskolin acts via cAMP to activate PKA (39), and the appearance of underphosphorylated pRb in the presence of forskolin could therefore be due to PKA phosphorylation—and thereby activate a phosphatase and/or inhibiting a kinase in the pRb phosphorylation cascade. The involvement of a phosphatase was assessed by treating the cells with okadaic acid, an inhibitor of protein phosphatases 1 and 2A (40–42). As shown in Fig. 4, okadaic acid prevented the PKA-mediated dephosphorylation of pRb observed at 3 h of forskolin treatment. These experiments were done on whole cells, and a relatively high concentration (1 µM) of okadaic acid was used to obtain the described effects. Whether phosphatase 1 or 2A was involved could therefore not be assessed. Our attempts to verify the cAMP-mediated dephosphorylation of pRb by using homogenates of Reh cells and purified catalytic subunit of PKA were inconclusive due to spontaneous dephosphorylation of pRb occurring in the homogenates at 37°C.

Not surprisingly, okadaic acid at 1 µM inhibited the DNA synthesis measured at day 3 by 80–90%. We could therefore not perform the obvious experiment to assess whether okadaic acid would prevent cAMP-mediated inhibition of proliferation. The reason why okadaic acid alone inhibits DNA synthesis is presumably due to its general inhibition of phosphatase 1/2A activity, which is important in many events leading to DNA synthesis and proliferation.

**DISCUSSION**

We have previously shown that elevated levels of cAMP mediate a pronounced growth inhibition of Reh cells (8, 10, 34). In the present report, we have shown that this growth inhibition is associated with a shift in the phosphorylation state of pRb to more dephosphorylated forms, and that this shift is due to an active dephosphorylation process. This conclusion was based on the fact that the Reh cells were transiently blocked in the G_2 phase of the cell cycle for the first 10 to 15 h of forskolin treatment. We did not observe any entrance of cells into the G_1 phase from mitosis after 4 h of forskolin treatment, at a time when a pronounced shift to underphosphorylated pRb was notable. Thus, in our cell system such a shift could not be due to accumulation of cells prevented from pRb phosphorylation by a block in the early G_1 phase of the cell cycle. Rather, our results indicated a cAMP-mediated dephosphorylation of pRb occurring in cells already phosphorylated in later parts of G_1. There have been other reports on the effects of growth inhibitors on the state of pRb phosphorylation (28–30). In neither of these reports, however, were the different models for pRb dephosphorylation elucidated.

Several lines of evidence have linked the state of pRb phosphorylation to its function as an inhibitor of cell cycle progression (16, 19, 20). Due to the increased phosphorylation (and loss of growth inhibiting ability) of pRb late in G_1, pRb has been suggested to function as a growth regulator at the G_1/S boundary (16). Recent reports, however, have indicated that pRb also might regulate the cell cycle progression in early G_1. In T-cells, for instance, the first step in the phosphorylation of pRb occurs several hours prior to the G_1/S boundary (15). Furthermore, Stokke et al. (24) have shown that cycling cells contain underphosphorylated pRb in the early parts of G_1, and that the fraction of underphosphorylated pRb increases with the length of the G_1 phase. As it has been shown that pRb in its underphosphorylated and active state in G_1 is tightly bound to nuclear structures (18, 24), we found it important to establish that the dephosphorylation we observed was physiological, i.e., that this form of pRb actually bound to the nucleus. We found that at 4 h of forskolin treatment the fraction of pRb* nuclei increased from 9 to 87%.

It is tempting to suggest that the increased fraction of underphosphorylated Rb protein is responsible for the cAMP-mediated growth arrest. We could exclude that the initial transient block of cells in G_2 was due to dephosphorylation of pRb in G_2, as after forskolin treatment pRb* nuclei were found in G_1 only. The inhibition of cell cycle progression of cells in G_1 could, on the other hand, be caused by dephosphorylation of pRb. At 4 h of forskolin treatment, the G_2 block and dephosphorylation of pRb was notable. The cell cycle block in G_1 was also detectable at this time point, but became more evident at 10 to 15 h of forskolin treatment when almost no cells were found in S-phase. The G_2 block was released in accordance with our previous observations (10) after 10–15 h, whereas the accumulation of cells in G_1 continued. We have previously documented that the cells eventually accumulate in mid-G_1 upon forskolin treatment (34). Taken together, our data indicate that dephosphorylation of pRb precedes, and could be responsible for, the inhibition of cell cycle progression in G_1. Recent support for this interpretation of our results was provided by Goodrich et al. (43). They showed that microinjecting purified pRb blocked the cell cycle only when pRb was applied in early G_1.

The transient nature of the cAMP-mediated dephosphorylation of pRb has interesting implications. If pRb has a vital role in cAMP-mediated growth inhibition, it implies that the initial dephosphorylation of pRb initiates a cascade of events leading to a G_1 arrest. In other words, the cells become committed to growth arrest, and a release of this signal (observed as rephosphorylation of pRb) would not release the G_1 block. This is in agreement with the observation that the cells are still blocked in G_1, after 3 days of forskolin treatment, despite the fact that the phosphorylation state of pRb is as in the control cells. An alternative explanation could be that only one or at least very few of the pRb phosphorylation sites are essential for mediating the antiproliferative effect, and that these sites could remain dephosphorylated without being detected in our electrophoresis system. However, if we assume that the nuclei-associated form of pRb is the active one, the latter alternative seems more unlikely in light of the flow-cytometric data. Interestingly, we have recently obtained results indicating that the forskolin-mediated growth inhibition is reversible. Washing the cells after 3 days of forskolin treatment reverses the growth rate of Reh cells to normal (data not shown). Thus, the transient nature of cAMP-mediated pRb dephosphorylation may lead to a reversible growth inhibition signal.

We have previously shown that the cAMP-mediated cell cycle inhibition of Reh cells also is accompanied by a transient reduction in
the level of MYC at both the RNA and protein levels (8). The level was at its minimum at 3–4 h, before it was restored to normal within 24 h of forskolin treatment. The underphosphorylated form of pRb (the active form) has been shown to be able to sequester the transcription factor E2F (21–23). As E2F binding sites are found in the MYC promoter, it has been suggested that pRb has a role in the regulation of MYC transcription (44). Furthermore, Pieterpol et al. (45) presented evidence that TGF-β-induced down-regulation of MYC could involve pRb. The expression of the transforming proteins of DNA tumor viruses blocked the TGF-β-mediated MYC down-regulation, and these transforming proteins have been shown to compete with E2F for binding to pRb (21). It was therefore interesting to examine the effect of forskolin on pRb phosphorylation within the same time period that MYC was down-regulated. We found that dephosphorylation of pRb paralleled in time, but apparently did not precede the MYC down-regulation process. It is not known which or how many phosphorylation sites have to be dephosphorylated in order for pRb to gain its growth suppressive effect. Still, the delayed kinetics of pRb dephosphorylation as compared to MYC down-regulation do not indicate a causative link. We are however in the process of further elucidating this possibility by expressing Rb antisense RNA in Reh cells.

The opposite situation, namely, that MYC is involved in Rb regulation, has also been raised as a possibility (30). Thus, down-regulation of MYC could be responsible for the cAMP-mediated dephosphorylation of pRb. This question was addressed by transfecting the Reh cells with constructs expressing c-myc constitutively (11). The results, however, did not indicate that MYC down-regulation was involved in pRb dephosphorylation, as dephosphorylation of pRb occurred to the same extent and with the same kinetics in MYC-transfected as in the control cells (data not shown). Similar conclusions were drawn by Resnitzky et al. (30) working with IL-6-induced G0/G1 cell cycle arrest. They showed that stable MYC transfectants abrogated the G0/G1 arrest without preventing the IL-6-mediated dephosphorylation of pRb.

The turnover of phosphate groups on pRb is presumably more rapid than the turnover of the protein itself, and therefore the cAMP-mediated dephosphorylation of pRb could be due to either a prevention of a kinase or an activation of a phosphatase. In vitro p34\(^{\text{cdc2}}\) was shown to phosphorylate all the known phosphorylation sites on pRb based on phosphopeptide maps (26), and also other lines of evidence link this kinase to phosphorylation of Rb (25). Lately, however, it has been questioned whether cdc2-kinase can be responsible for phosphorylation of pRb in early G1, as the kinase is not yet detectable in this part of the cell cycle (15), and due to the fact that inhibition of cdc2 synthesis in peripheral blood lymphocytes with antisense oligodeoxynucleotides still allows for partial phosphorylation of Rb (26). In stead, recent data indicate that the complex of cyclin D and cdk4 might be responsible for pRb phosphorylation (47). Mitosis-associated dephosphorylation of pRb has been linked to the action of protein phosphatase 1 (27). Treatment of Reh cells with the phosphatase inhibitor okadaic acid prevented the forskolin-mediated dephosphorylation of pRb at 4 hours, indicating that a phosphatase is involved in the process. Whether or not this phosphatase is identical to the phosphatase responsible for pRb dephosphorylation after mitosis, remains to be clarified. It was not possible in our experiments to distinguish between protein phosphatase 1 and 2A, because high concentrations of okadaic acid are needed for inhibition of phosphatase activity in intact cells (47). By blocking the cells in the S-phase by hydroxyurea, Resnitzky et al. (30) concluded that neither IL-6, interferon, nor TGF-β activated a cell cycle-independent phosphatase, but speculated that dephosphorylation of pRb could involve a cell cycle specific phosphatase or inhibitor of the Rb kinase.

Support for a direct link between PKA and p34\(^{\text{cdc2}}\) kinase in human fibroblasts was recently presented by Tournier et al. (48), demonstrating a complex between these 2 kinases through the RIα subunit of PKA.

A complex cascade of phosphorylation/dephosphorylation events is involved in the regulation of pRb phosphorylation. We are currently addressing the involvement of the putative CDK-kinases and their associated cyclins in the cAMP-mediated events in Reh cells.

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