cAMP-mediated Inhibition of DNA Replication and S Phase Progression: Involvement of Rb, p21^{Cip1}, and PCNA

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cAMP exerts an antiproliferative effect on a number of cell types including lymphocytes. This effect of cAMP is proposed to be mediated by its ability to inhibit G1/S transition. In this report, we provide evidence for a new mechanism whereby cAMP might inhibit cellular proliferation. We show that elevation of intracellular levels of cAMP inhibits DNA replication and arrests the cells in S phase. The cAMP-induced inhibition of DNA synthesis was associated with the increased binding of p21^{Cip1} to Cdk2-cyclin complexes, inhibition of Cdk2 kinase activity, dephosphorylation of Rb, and dissociation of PCNA from chromatin in S phase cells. The ability of cAMP to inhibit DNA replication and trigger release of PCNA from chromatin required Rb and p21^{Cip1} proteins, since both processes were only marginally affected by increased levels of cAMP in $Rb^{-/-}$ and $p21^{Cip1-/-}$ 3T3 fibroblasts. Importantly, the implications of cAMP-induced inhibition of DNA synthesis in cancer treatment was demonstrated by the ability of cAMP to reduce apoptosis induced by S phase–specific cytotoxic drugs. Taken together, these results demonstrate a novel role for cAMP in regulation of DNA synthesis and support a model in which activation of cAMP-dependent signaling protects cells from the effect of S phase–specific antitumor agents.

INTRODUCTION

The second messenger cyclical AMP (cAMP) plays an important role in regulation of numerous cellular functions. Elevation of intracellular cAMP levels either stimulates or inhibits proliferation of cells depending on the cell type (Pastan *et al.,* 1975; Dumont *et al.,* 1989). Generally, cAMP has an antiproliferative effect on most cells of mesenchymal origin (Blomhoff *et al.,* 1988; Dugan *et al.,* 1999). We have observed that increase in intracellular cAMP leads to accumulation of lymphoid cells in the G1 phase and correlates with rapid dephosphorylation of the retinoblastoma tumor suppressor protein (Rb; Blomhoff *et al.,* 1987, 1988; Christoffersen *et al.,* 1994; Naderi and Blomhoff, 1999; Gutzkow *et al.,* 2002). Furthermore, this antiproliferative effect of cAMP in

Abbreviations used: cAMP, cyclic AMP; Rb, retinoblastoma protein; Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; pol δ, DNA polymerase δ; CPT, camptothecin; 5-FU, 5-fluorouracil; HU, hydroxyurea; PAC, paclitaxel; VB, vinblastine; PI, propidium iodide; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; MEFs, mouse embryo fibroblasts; Aph, aphidicolin; IBMX, 3-isobutyl-1-methylxantine; PGE$_2$, prostaglandin E$_2$; CTX, cholera toxin; MNase, micrococcal nuclease.

lymphocytes has been shown to be mediated through protein kinase A type I (Skalhegg *et al.,* 1992; Tasken *et al.,* 1994).

Rb, is an important inhibitor of cell cycle progression (Wang *et al.,* 1994; Harbour and Dean, 2000). In its hypophosphorylated active form, Rb inhibits transition of cells from G1 into S phase of the cell cycle. In addition, Rb has also been shown to negatively regulate DNA replication and block S phase progression (Chew *et al.,* 1998; Knudsen *et al.,* 1998). The negative effect of Rb on cell cycle progression is countered by its phosphorylation by cyclin-dependent kinases (Cdks). When complexed to their regulatory cyclin subunits, Cdks phosphorylate and thereby inactivate the antiproliferative function of Rb (Mittnacht, 1998). Specifically, inactivation of Rb in G1 is achieved through its sequential phosphorylation by Cdk4/6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A complexes (Zarkowska and Mittnacht, 1997; Lundberg and Weinberg, 1998). Later in S phase, phosphorylation of Rb is maintained by the combined actions of Cdk2-cyclin E and Cdk2-cyclin A (Mittnacht, 1998). The activity of Cdk-cyclin complexes is in turn regulated by a number of different mechanisms. Specifically, Cdk-cyclin kinase activity is blocked by its association with small inhibitory proteins referred to as cyclin-dependent kinase inhibitors (CKIs; Morgan, 1995; Sherr and Roberts, 1995). CKIs fall into two families; INK4 proteins that inhibit Cdk4 and Cdk6 (Serrano *et al.,* 1993; Hannon and Beach, 1994; Hirai *et al.,* 1995), and Cip1/Kip1 proteins such as p21^{Cip1} and p27^{Kip1} that inhibit a broad range of Cdks including Cdk2-cyclin E and Cdk2-cyclin A complexes (el Deiry *et al.,* 1993; Harper *et al.,* 1993; Xiong *et al.,* 1993; Polyak *et al.,* 1994; Toyoshima and Hunter, 1994; Resnitzky *et al.,* 1995).

Accurate and timely duplication of the genome once each cell cycle ensures transmission of genetic information from one cell generation to the next (Kelly and Brown, 2000). In eukaryotes, this process begins in G1 when a preinitiation complex, consisting of ORC, MCM, and Cdc6 proteins is formed at the origins of replication (Dimitrova and Gilbert, 2000; Kelly and Brown, 2000). On activation of Cdk2-cyclin and Cdc7-Dbf4 kinases at the G1/S transition, DNA replication starts from discrete origins of replication (Diffley, 1996; Dutta and Bell, 1997). Initiation of replication is accompanied by unwinding of the replication origins and displacement of MCM and Cdc6 from the origin (Todorov *et al.*, 1995; Dimitrova *et al.*, 1999; Jiang *et al.*, 1999; Petersen *et al.*, 1999; Dimitrova and Gilbert, 2000). Elongation of replication then begins when proliferating cell nuclear antigen (PCNA) recruits DNA polymerase δ (pol δ) onto chromatin (Waga and Stillman, 1998). PCNA serves as DNA sliding clamp for pol δ, whose function is essential for chromosomal replication (Kelman, 1997). Interruption of either initiation or elongation of replication during S phase leads to block of DNA replication.

In this article, we present evidence for the involvement of cAMP in regulation of S phase progression. We show that elevation of intracellular cAMP levels in S phase leads to inhibition of DNA synthesis and arrest of cells in S phase. The inhibitory effect of cAMP on DNA synthesis is dependent on Rb and $p21^{Cip1}$ proteins, because both Rb- and $p21^{Cip1}$-deficient cells were unable to halt DNA synthesis in response to treatment by the cAMP-elevating agent, forskolin. Furthermore, we show that cAMP diminishes the cytotoxic effect of S phase–specific antitumor drugs, suggesting that intracellular cAMP levels could affect the ability of this class of drugs to treat cancer.

MATERIALS AND METHODS

Reagents and Antibodies

Forskolin, aphidicolin (Aph), thymidine, roscovitine, cholera toxin (CTX), prostaglandin E_2 (PGE_2), camptothecin (CPT), 5-fluorouracil (5-FU), hydroxyurea (HU), paclitaxel (PAC), and vinblastine (VB) were purchased from Calbiochem (La Jolla, CA). 3-isobutyl-1-methylxanthine (IBMX), 5-bromo-2-deoxyuridine (BrdU), propidium iodide (PI), RNase A, Hoechst 33342, and micrococcal nuclease (Mnase) were obtained from Sigma (St. Louis, MO). 8-CPT-cAMP was from Biolog (Hayward, CA). Antibodies against cyclin A (C-19), cyclin E (HE12), Cdk2 (M2-G), $p21^{Cip1}$ (C-19), $p27^{Kip1}$ (C-19), and PCNA (PC10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MCM2 antibody (610700) was from BD Transduction Laboratories (Lexington, KY). Anti-Rb (G3–245) and FITC-conjugated anti-BrdU (556028) antibodies were obtained from PharMingen (San Diego, CA).

Cloning, Transfection, and Retroviral Infection

Human $p21^{Cip1}$ cDNA was cloned by PCR into the *BamHI* and *EcoRI* sites of the *pcDNA3.1(+)* vector (*pcDNA-$p21^{Cip1}$*; Invitrogen, Carlsbad, CA). The Cal-Phos Mammalian Transfection Kit (Clontech, Palo Alto, CA) was used to transfect $p21^{Cip1-/-}$ 3T3 cells plated at 50% confluence 12 h before transfection. Twelve hours after transfection, the cells were washed three times with phosphate-buffered saline (PBS) and fed with fresh medium before returning to the incubator. At 36 h posttransfection, the cells were treated and analyzed. Control transfection of cells with *pcDNA-EGFP* revealed a transfection efficiency of ~65%. Wt Rb cDNA was subcloned from *pCMV-Rb(WT)* plasmid (Chen and Wang, 2000) into the unique *BamHI* site of the *pBABE-puro* (Morgenstern and Land, 1990) retroviral vector to produce *pBABE-wt Rb* vector. The day before transfection, the Phoenix packaging cells were seeded at 2×10^6 cells per 6-cm plate with 5 ml of medium per plate. Cells were grown to 80% confluency and transfected with 25 μg of *pBABE-puro* or *pBABE-wt Rb* using the CalPhos Mammalian Transfection Kit. The viral supernatants were collected at 48 h after transfection and centrifuged at $250 \times g$ to remove cells and debris. For retroviral infection of cells, 3T3 cells were plated at ~10^5 cells per well on a six-well plate. After 24 h, medium was removed and 2 ml of the culture supernatant containing retroviral vectors was added to the wells together with Polybrene (Sigma) at a final concentration of 16 μg/ml. Infection was allowed to proceed for 24 h. The viral supernatant was then removed and replaced with fresh medium containing puromycin at 2 mg/ml. Cells

were selected in puromycin for 48 h at which time 100% of mock-infected 3T3 cells were killed.

Cell Culture, Synchronization, and Drug Treatment

The B-lymphoid precursor cell line Reh was originally derived from a patient with acute lymphoblastic leukemia (Rosenfeld *et al.*, 1977). Cells were cultured at a density between 0.4×10^6 and 1.0×10^6 cells/ml in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Rockville, MD), 2 mM glutamine, 125 U/ml penicillin, and 125 μg/ml streptomycin. Wt, $Rb^{-/-}$ and $p21^{Cip1-/-}$ 3T3 fibroblasts were generated from *wt*, $Rb^{-/-}$ and $p21^{Cip1-/-}$ MEFs, respectively, as previously described (Chau *et al.*, 2002). 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 125 U/ml penicillin, 62.5 μg/ml streptomycin, and 0.001% β-mercaptoethanol. To synchronize Reh cells in S phase with Aph, cells were cultured in the presence of Aph (1 μg/ml) for 24 h. Cells were released from Aph arrest by two washes with drug-free medium and then were resuspended in drug-free medium. For synchronization of Reh cells by the thymidine double-block method, cells were exposed to 2 mM thymidine for 24 h, released into thymidine-free medium for 9 h, and then reexposed to 2 mM thymidine for 16 h. Cells were released from thymidine block by two washes with drug-free medium and then resuspended in drug-free medium. For synchronization of 3T3 cells in early S phase, cells were first made quiescent by culture in medium containing 0.1% FBS for 72 h and then stimulated with 10% FBS in the presence of 2 μg/ml Aph for 24 h. Cells were released from S phase synchrony by washing once with PBS, followed by two washes with drug-free medium for 5 min.

Flow Cytometry

Cells were incubated with BrdU (10 μM). Cells were harvested by centrifugation, washed once in cold PBS, resuspended in 100 μl PBS, fixed in 4 ml of ice-cold 70% ethanol, and incubated overnight at −20°C. Cells were pelleted at $250 \times g$ for 5 min and incubated in 1 ml of a solution containing 2 M HCl and 0.3 mg/ml pepsin for 30 min at RT. The acid was then neutralized with 3 ml 0.1 M sodium tetraborate (pH 8.5). Cells were pelleted, washed once in IFA buffer (10 mM HEPES [pH 7.4], 25 mM NaCl, 4% fetal calf serum) and then incubated in 1 ml IFA containing 0.5% Tween for 10 min at RT. Cells were then washed once in IFA, pelleted, and incubated in 100 μl of IFA containing 10 μl FITC-conjugated anti-BrdU antibody for 30 min at RT. After antibody incubation, cells were washed once in IFA and incubated for 10 min on ice in 0.5 ml of PBS containing 20 μg/ml PI and 40 μg/ml RNase. Cells were then analyzed on a fluorescene-activated cell sorting (FACS) instrument to determine PI (red) and FITC (green) staining for nuclear DNA and BrdU content, respectively.

Immunoblotting, Immunoprecipitation, and Kinase Assay

For immunoblot analysis, cells were lysed in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, and 0.5% aprotinin) and equal amount of proteins were separated on a 7.5% (for Rb) or 10% (for other proteins) SDS-PAGE. After transfer to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ), proteins were detected by use of standard immunoblotting procedures. For detection of Rb in 3T3 cells, cell lysates containing 500 μg protein were immunoprecipitated with 2 μg anti-Rb antibodies. The immunocomplexes were then absorbed to protein G-Sepharose (Amersham Biosciences) and washed four times in RIPA buffer. Proteins were then resuspended in SDS sample buffer, boiled, and subjected to Western blot analysis with anti-Rb antibodies. For immunoprecipitation of Cdk2 from Reh cell extracts, cells were resuspended in Triton X-100 lysis buffer (20 mM Tris [pH 7.5], 250 mM NaCl, 0.1% Triton X-100, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.2 mM PMSF, 10 μg/ml leupeptin, and 0.5% aprotinin). The samples were placed on ice and vortexed at 5-min intervals for 20 min. To immunoprecipitate Cdk2 from 3T3 cell extracts, cells were resuspended in Triton X-100 lysis buffer and incubated on ice for 1 h with intermittent vortexing, followed by sonication. After removal of insoluble materials by centrifugation, 300 μg cell extracts were immunoprecipitated with 2 μg anti-Cdk2 for 2 h at 4°C. The immunocomplexes were then absorbed to 30 μl of a 1:1 slurry of protein G-Sepharose for 1 h at 4°C, collected by centrifugation, and washed twice with Triton X-100 lysis buffer. The beads were then resuspended in SDS sample buffer, boiled, and subjected to Western blot analysis. For Cdk2 kinase assay, immunocomplexes recovered from 300 μg lysates were washed twice with Triton X-100 lysis buffer, and once with kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 2 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4, 5 mM β-glycerophosphate). The beads were resuspended in 20 μl of kinase buffer containing 30 μM ATP, 5 μg histone H1 (Upstate Biotechnology, Lake Placid, NY), 10 μCi of $[\gamma^{-32}P]ATP$ per reaction mixture and incubated for 30 min at RT. Reactions were stopped with addition of 10 μl $3\times$ SDS sample buffer. The samples were boiled and subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie blue, dried, and subjected to autoradiography.
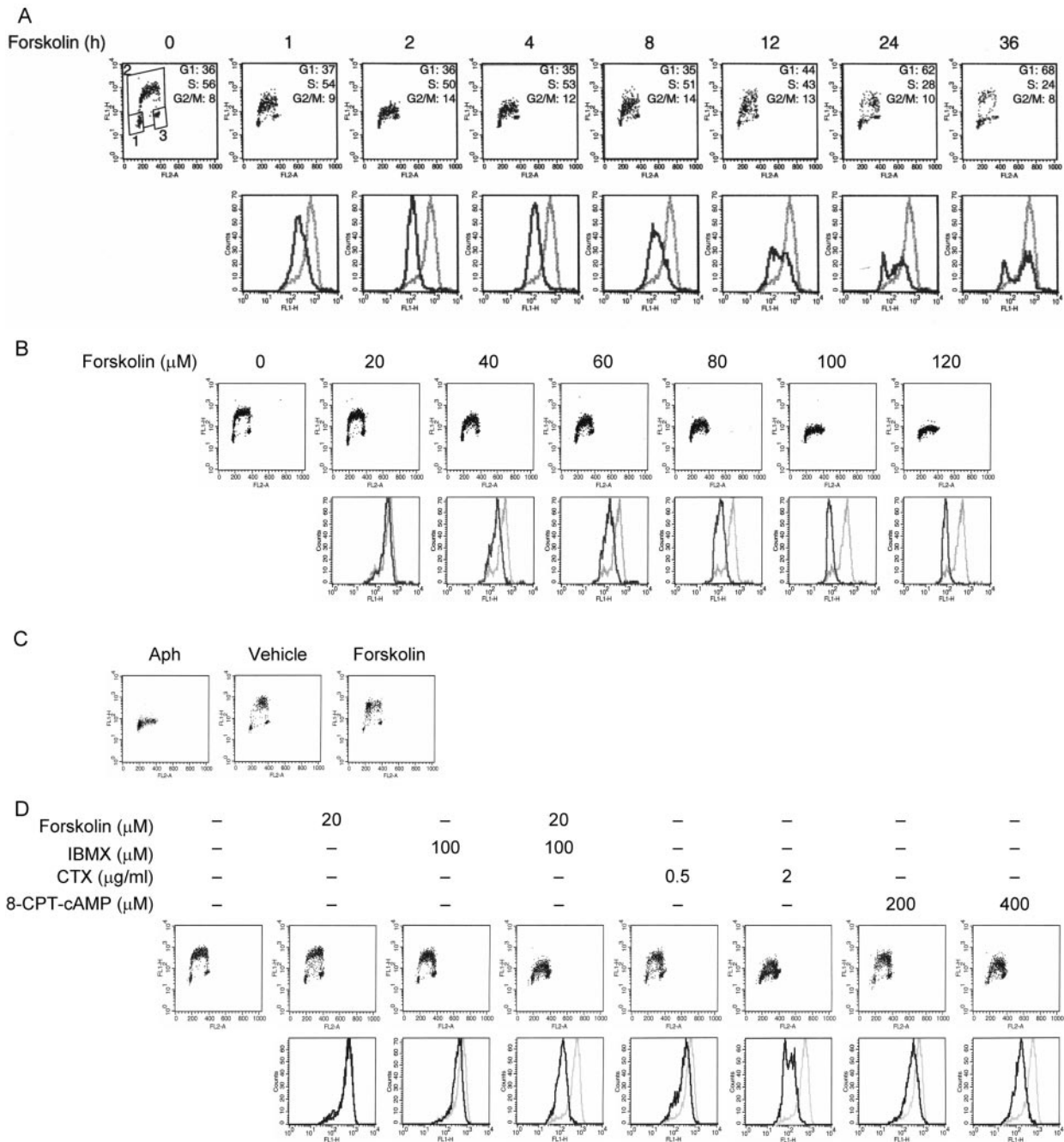
Figure 1. Inhibition of DNA replication by cAMP. (A) Asynchronously proliferating Reh cells were cultured in the absence or presence of 100 μM forskolin. Cells were pulse-labeled with BrdU during the last 30 min of treatment before harvesting at the indicated times. Cells were fixed, stained with FITC-coupled anti-BrdU antibody and PI, and analyzed by flow cytometry to determine BrdU incorporation and cell cycle distribution. Top: representative scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). Cell cycle distribution of cells was calculated using the gates shown in the leftmost plot. 1, G1 phase; 2, S phase; 3, G2/M phase. Bottom: the relative rate of BrdU incorporation was visualized by plotting FITC anti-BrdU staining in S phase cells. Gray lines, no treatment; black lines, treatment with forskolin. (B) Reh cells were treated with increasing concentrations of forskolin for 2 h and pulsed with BrdU for the final 30 min of treatment. Cells were processed as in A for detection of BrdU incorporation and cell cycle phase determination. Top: representative scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). Bottom: the relative rate of BrdU incorporation was visualized by plotting FITC anti-BrdU staining in S phase cells. Gray lines, treatment with vehicle; black lines, treatment with forskolin. (C) Reh cells were synchronized in early S phase with Aph as described in *Materials and Methods*. Cells were then washed to remove Aph, incubated in drug-free medium for 2 h, and then pulse-labeled with BrdU for 20 min. Subsequently, BrdU was removed by extensive washing, and cells were incubated in the absence or presence of 100 μM forskolin for 2 h. Cells were then harvested and processed for visualization of BrdU-positive cells to monitor progression through S phase. (D) Reh cells were treated with forskolin (20 μM), or IBMX (100 μM) alone or the combination of two, CTX (0.5 or 2 μg/ml), or 8-CPT-cAMP (200 or 400 μM) for 2 h, and pulsed with BrdU for the final 30 min of treatment. Cells were then processed as in A for detection of DNA synthesis and cell cycle phase determination. Top: representative scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). Bottom: the relative rate of DNA synthesis was visualized by plotting FITC anti-BrdU staining in S phase cells. Gray lines, no treatment; black lines, treatment with indicated agents.
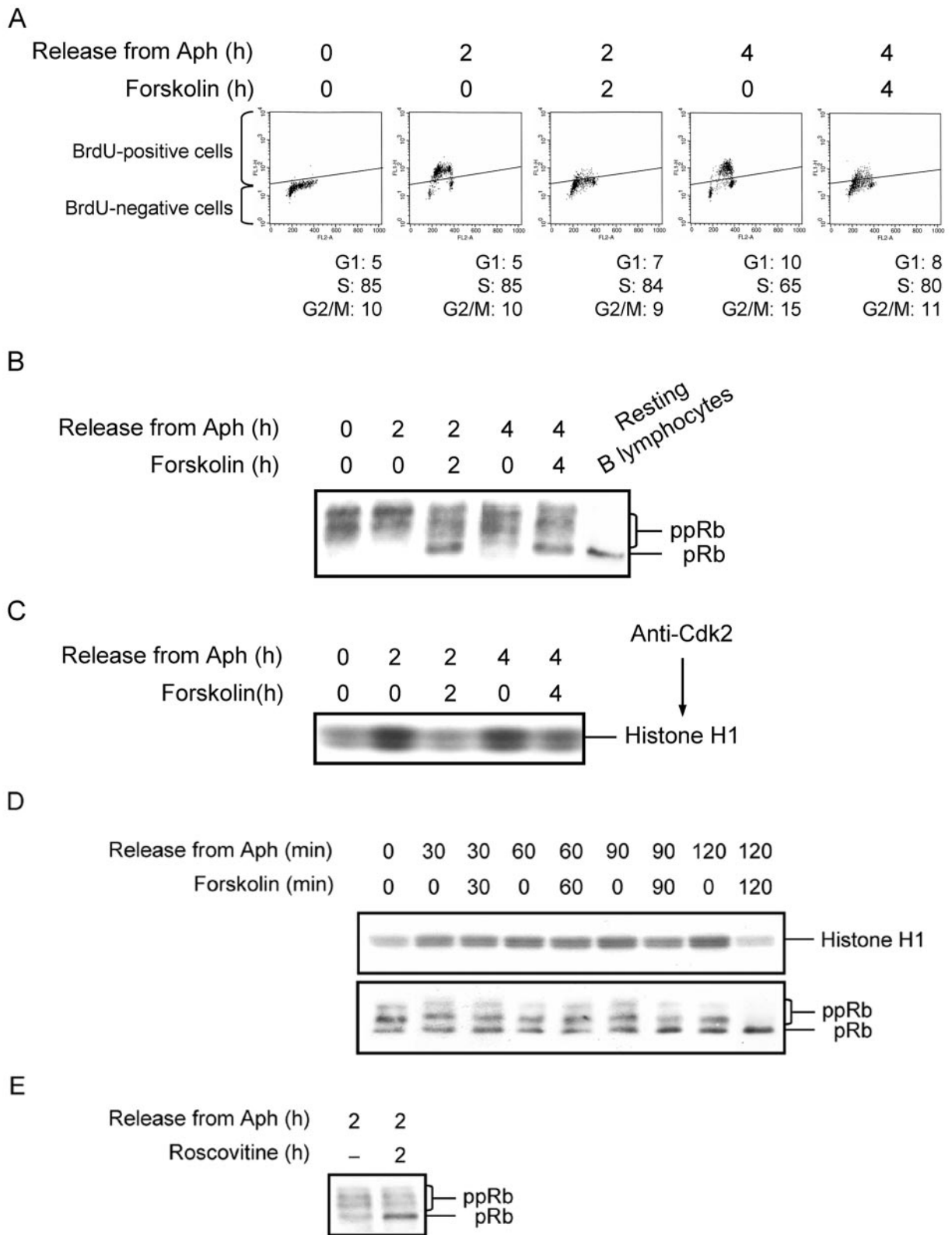
A

Release from Aph (h)   0   2   2   4   4
Forskolin (h)   0   0   2   0   4

BrdU-positive cells

BrdU-negative cells

G1: 5   G1: 5   G1: 7   G1: 10   G1: 8
S: 85   S: 85   S: 84   S: 65   S: 80
G2/M: 10   G2/M: 10   G2/M: 9   G2/M: 15   G2/M: 11

B

Release from Aph (h)   0   2   2   4   4   Resting B lymphocytes
Forskolin (h)   0   0   2   0   4

ppRb
pRb

C

Release from Aph (h)   0   2   2   4   4   Anti-Cdk2
Forskolin(h)   0   0   2   0   4

Histone H1

D

Release from Aph (min)   0   30   30   60   60   90   90   120 120
Forskolin (min)   0   0   30   0   60   0   90   0   120

Histone H1

ppRb
pRb

E

Release from Aph (h)   2   2
Roscovitine (h)   –   2
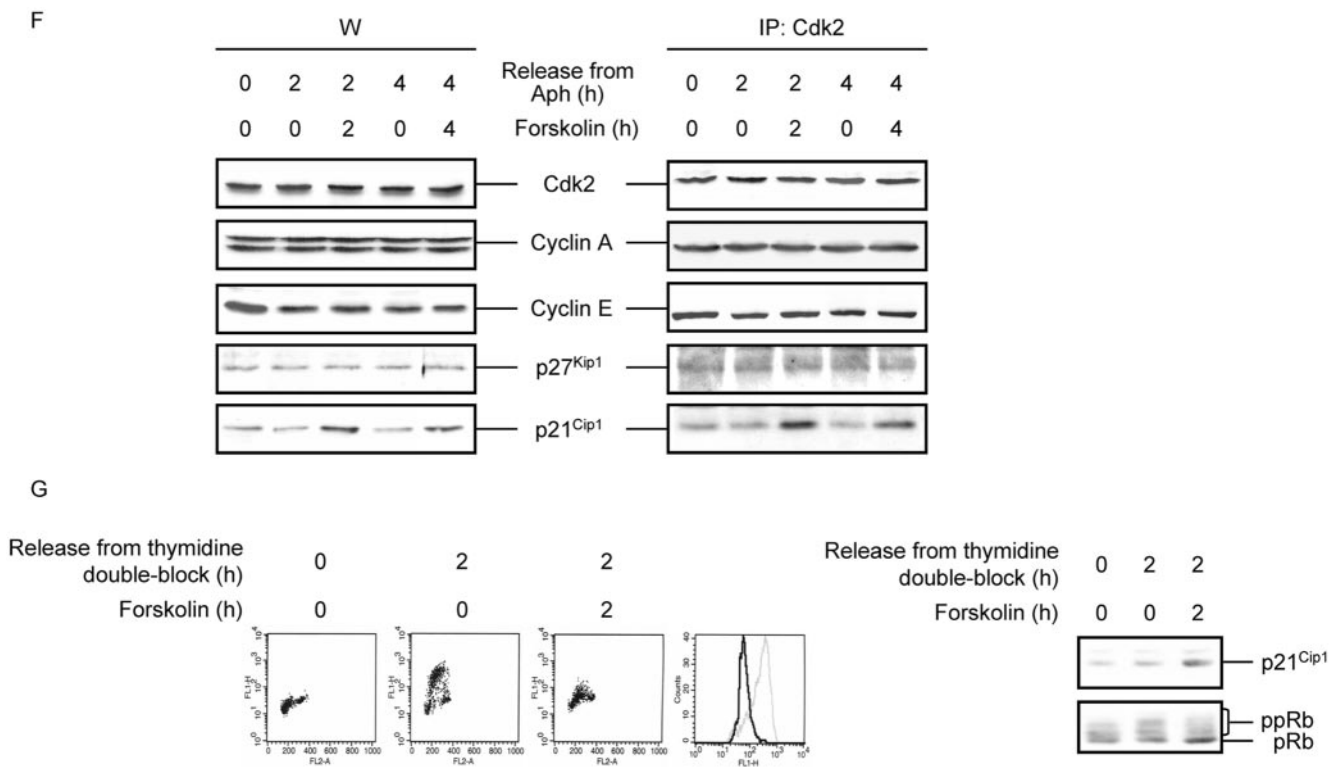
ppRb
pRb

Figure 2.

Figure 2 (continued from facing page). cAMP induces dephosphorylation of Rb and inhibits Cdk2 kinase activity in S phase cells. (A) Reh cells were synchronized in S phase by treatment with Aph. After synchronization, cells were treated with vehicle or forskolin for 2 or 4 h, and pulsed with BrdU for the final 30 min of treatment. Cells were then processed as in Figure 1A for detection of DNA synthesis and cell cycle phase determination. Shown are scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). The diagonal bar in each scatter plot separates BrdU-positive from BrdU-negative cells. (B) S phase–synchronized Reh cells were treated with vehicle or forskolin for the indicated times. Cells were harvested, lysates prepared, resolved by SDS-PAGE, and detected by immunoblotting with anti-Rb antibodies. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. Rb detected in the lysate of resting human B lymphocytes was used as control for pRB. (C) S phase–synchronized Reh cells were treated with vehicle or forskolin and harvested at the indicated times. Whole-cell extracts were prepared, immunoprecipitated with anti-Cdk2 antibodies, and assayed for H1 kinase activity. (D) S phase–synchronized Reh cells were treated with vehicle or forskolin and harvested at the indicated times. Each sample was split in two. Whole-cell extracts were prepared from one-half of each sample, immunoprecipitated with anti-Cdk2 antibodies, and assayed for H1 kinase activity (top panel). Lysates prepared from the other half of each sample were analyzed by immunoblotting with anti-Rb antibodies (bottom panel). pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. (E) S phase–synchronized Reh cells with treated with vehicle or 50 μM roscovitine for 2 h. Cell lysates were then prepared and processed for examination of Rb phosphorylation by Western blotting. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. (F) S phase–synchronized Reh cells were treated as in A. After treatment, each sample was split in two. Total cell lysates from one-half of each sample were resolved on SDS-PAGE and then subjected to immunoblotting with the indicated antibodies (left panel). Whole-cell extracts were prepared from the other half of each sample and immunoprecipitated with anti-Cdk2 antibodies. The recovered proteins were resolved on SDS-PAGE and then subjected to immunoblotting with the indicated antibodies (right panel). W, Western blotting; IP, immunoprecipitation. (G) Reh cells were synchronized in S phase by the thymidine double-block method as described in *Materials and Methods.* After synchronization, cells were treated with vehicle or forskolin for 2 h and pulsed with BrdU for the final 30 min of treatment. Each sample was split in two. Cells in one-half of each sample were processed as in Figure 1A for detection of DNA synthesis. Left: scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-Al). The relative rate of DNA synthesis was visualized by plotting FITC anti-BrdU staining in S phase cells as depicted in the histogram. Gray line, no treatment; black line, treatment with forskolin. Right: total cell lysates from the other half of each sample were resolved on SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

Northern Blot Analysis

Total cellular RNA was isolated using RNeasy (Qiagen, Chatsworth, CA) as outlined by the manufacturer, and 15 μg RNA per lane was fractionated on a 1.2% formaldehyde/agarose gel. After staining the gel to verify equal loading in each lane, RNA was transferred onto Hybond-N filter (Amersham Biosciences) in 20× SSC, and cross-linked by UV illumination. The filter was then hybridized with *p21^{Cip1}* cDNA probe following the Amersham Rapid Hybridization Protocol at 65°C in a buffer containing 10% dextran sulfate, 2% SDS, 5× SSPE, and 1× Denhardt's solution; washed to a final stringency of 1× SSC, 0.1% SDS; and autoradiographed. Human *p21^{Cip1}* cDNA probe was labeled with [α-^{32}P]dCTP (Amersham Megaprime Labeling System; Amersham Biosciences) according to the manufacturer's protocol.

Immunofluorescence Microscopy

Reh cells were cytospun onto poly-L-lysine–coated coverslips. 3T3 cells were seeded on poly-L-lysine–coated coverslips in six-well dishes. The coverslips were then washed once in cold PBS and extracted by incubation with buffer B (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl$_2$, 1 mM CaCl$_2$, 0.1% Triton X-100, 250 mM sucrose, 0.1 mM PMSF, 1 mM DTT) for 15 min at RT before fixing in 3% paraformaldehyde. Anti-PCNA and FITC-conjugated secondary antibodies (Sigma) were used at 1:50 and 1:100 dilutions, respectively. DNA was stained with 0.1 μg/ml Hoechst 33342. Observations were made on an Olympus AX70 epifluorescence microscope (Lake Success, NY) using a 100× objective, and photographs were taken with a Photonic Science CCD camera (Millham, UK) and OpenLab software (Improvision Inc., Lexington, MA).

Nuclear Fractionation and Chromatin Isolation

To isolate chromatin-bound proteins, cells were resuspended in buffer A (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl$_2$, 0.1 mM PMSF, 1 mM DTT). NP-40 (0.05%) was added, and the cells were incubated for 20 min on ice. Nuclei were collected by low speed centrifugation (200 × g) for 5 min at 4°C. To elute the weakly bound proteins, nuclei were resuspended in buffer B (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM MgCl$_2$, 1 mM CaCl$_2$, 250 mM sucrose, 0.1 mM PMSF, 1 mM DTT) plus 0.1% Triton X-100 for 15 min at RT. Nuclei were collected by low-speed centrifugation and washed once with buffer B. To distinguish between proteins that are attached to chromatin and those that are bound to some other insoluble structure in the nuclei, the nuclei pellet was resuspended in buffer B plus 5 U/ml MNase. After incubation at 37°C for 5 min, the nuclease reaction was stopped by addition of 5 mM EDTA. Nuclei were pelleted by centrifugation at 10,000 × g for 10 min at 4°C, and the MNase-solubilized fraction containing chromatin-bound proteins was analyzed by Western blotting with antibodies against PCNA and MCM2 proteins.

Measurement of Apoptosis

Typically cells exhibit a decrease in forward light scatter (FSC; caused by cytoplasmic shrinkage) and an increase in side light scatter (SSC; caused by increased granularity) when they undergo apoptosis. For measurement of apoptosis in Reh cells, we used an FSC and SSC gating strategy to gate apoptotic and viable cell populations (Darzynkiewicz *et al.*, 1992; Ferlini *et al.*, 1996). To validate the FSC/SSC quantitation method, duplicate samples were assessed using the PI exclusion method (Ormerod *et al.*, 1992; Darzynkiewicz *et al.*, 1994). Apoptosis in 3T3 cells was assessed by detection of subdiploid population. In brief, adherent and floating cells were harvested by centrifugation, washed once in cold PBS, resuspended in 100 μl PBS, and fixed in 4 ml of ice-cold 70% ethanol. After incubation overnight at −20°C, cells were washed once in cold PBS, twice in phosphate-citrate buffer (192 mM Na$_2$HPO$_4$, 4 mM citric acid [pH 7.8]), and then resuspended in PBS containing 50 μg/ml PI and 100 μg/ml RNase. After incubation for 30 min at RT, cells were analyzed on a FACS instrument for determination of cells with sub-G1 DNA content.

RESULTS

Forskolin Retards S phase Progression

We and others have previously reported that activation of the cAMP signaling pathway inhibits proliferation of lymphoid cells (Blomhoff *et al.*, 1988; Skalhegg *et al.*, 1992; Gutzkow *et al.*, 2002). To investigate in more detail cell cycle perturbations induced by cAMP, we treated the B-precursor cell line Reh with 100 μM forskolin, an activator of adenylyl cyclase, and pulse-labeled with BrdU for 30 min before collection of samples at regular intervals for a total of 36 h. Cells were then fixed, stained with PI and FITC-coupled anti-BrdU antibody, and analyzed by bivariant flow cytometry. After 1-h treatment with forskolin, the overall cell cycle distribution was not affected, whereas the rate of BrdU incorporation was significantly reduced compared with that of untreated cells (Figure 1A). By 2 h, forskolin led to a significant reduction in the rate of BrdU incorporation without having a pronounced effect on the cell cycle distribution of the cells. Within 4 h after forskolin treatment, cells had resumed to incorporate BrdU, albeit at a reduced rate relative to untreated cells. By 24 h, forskolin had led to a strong reduction in the number of S phase cells accompanied by accumulation of cells in G1. The cells remained arrested in G1 by the endpoint of the experiment at 36 h after treatment. Taken together, these results showed that treatment of cells with 100 μM forskolin led to a transient inhibition of BrdU incorporation into DNA. Similar results were obtained with human T and B lymphocytes, mouse embryo fibroblasts (MEFs), and the Jurkat cell line (unpublished data), suggesting that the inhibition of BrdU incorporation observed here represents a general action of forskolin.

We next wanted to determine the dose-response relationship for this inhibitory effect of forskolin on BrdU incorporation. To this end, Reh cells were treated with various concentrations of forskolin, pulse-labeled with BrdU for 30 min before collection of samples after 2 h, and evaluated for their ability to incorporate BrdU by flow cytometry. As shown in Figure 1B, inhibition of BrdU incorporation in Reh cells was dose-responsive to forskolin. Cells exhibited maximal inhibition of BrdU incorporation at 100 μM forskolin, and this concentration was therefore used in the following experiments.

BrdU is a thymidine analog that is incorporated in place of thymidine into synthesized DNA strands of actively proliferating cells. Incorporation of BrdU is therefore used as evidence of DNA replication. The ability of forskolin to reduce the incorporation of BrdU into DNA can thus be interpreted as the ability of forskolin to inhibit DNA synthesis. However, it can also be argued that forskolin may inhibit incorporation of BrdU into DNA but not DNA replication per se. To resolve this issue, we examined the effect of forskolin on cells that had already incorporated BrdU, thus circumventing the possible inhibitory effect of forskolin on incorporation of BrdU into DNA. We first synchronized Reh cells in S phase using an aphidicolin (Aph) synchronization protocol (*Materials and Methods*). The synchronized cells were washed, cultured in drug-free medium for 2 h, and then pulse-labeled with BrdU for 20 min. After incubation with BrdU, cells were washed and BrdU was chased by culturing the cells in the absence or presence of 100 μM forskolin for 2 h. Examination of cells by flow cytometry revealed that forskolin inhibited the progression of BrdU-positive cells through S phase, indicating that forskolin inhibited DNA synthesis (Figure 1C).

Overall, the results presented in Figure 1, A–C, suggest that forskolin leads to a transient cessation of DNA synthesis. Notably, the fraction of cells in G1, S, and G2/M phases of the cell cycle remained essentially unchanged during the transient inhibition of DNA synthesis induced by forskolin. This indicates that, in addition to S phase, forskolin also inhibits progression of cells through other phases of the cell cycle. Taken together, these results show that forskolin induces an immediate but transient multiphase cell cycle arrest in Reh cells.

Inhibition of DNA Synthesis by Forskolin Is Mediated via cAMP

In addition to its ability to increase intracellular levels of cAMP through direct activation of adenylyl cyclase, forskolin has also been shown to affect certain cellular processes through a mechanism that does not involve production of cAMP (Joost and Steinfelder, 1987; Hoshi *et al.*, 1988; Laurenza *et al.*, 1989; Beavo and Reifsnyder, 1990). To determine if the inhibitory effect of forskolin on DNA synthesis is mediated via cAMP, we treated Reh cells with 3-isobutyl-1-methylxanthine (IBMX), cholera toxin (CTX), and 8-CPT-cAMP. IBMX induces accumulation of cAMP by inhibiting phosphodiesterases, CTX ADP-ribosylates the α subunit of Gs protein, a GTP-binding protein that regulates the activity of adenylyl cyclase, thus causing intracellular increase of cAMP, and 8-CPT-cAMP is a membrane-permeable analog of cAMP (Beavo *et al.*, 1970; Seamon *et al.*, 1984; Beavo and Reifsnyder, 1990; Scott, 1991; Spangler, 1992; Negishi *et al.*, 1993; Coleman *et al.*, 1994). Cells were exposed to forskolin, IBMX, CTX, or 8-CPT-cAMP, pulse-labeled with BrdU for 30 min before collection of samples after 2 h, and examined for BrdU incorporation by flow cytometry. Exposure of cells to either 20 μM forskolin or 100 μM IBMX alone had marginal effect on DNA synthesis (Figure 1D). However, treatment of cells with a combination of forskolin and IBMX, which increases cAMP production by stimulating adenylyl cyclase and inhibiting phosphodiesterases, respectively, led to a substantial inhibition of DNA synthesis. Treatment of Reh

cells with CTX led to inhibition of DNA synthesis in a dose-dependent manner. When exposed to 0.5 μg/ml CTX, cells exhibited a slight reduction of DNA synthesis, whereas treatment of cells with 2 μg/ml CTX led to a significant inhibition of DNA synthesis. Similarly, exposure of cells to 8-CPT-cAMP led to a dose-dependent inhibition of DNA synthesis. These observations showed that 1) cAMP led to attenuation of DNA synthesis and 2) the forskolin-mediated inhibition of DNA synthesis was mediated via cAMP.

cAMP Induces Rb Dephosphorylation in S phase Cells

In addition to its role in regulation of G1/S transition, Rb has also been documented to inhibit S phase progression (Chew *et al.*, 1998; Knudsen *et al.*, 1998). To assess the involvement of Rb in cAMP-mediated inhibition of DNA synthesis, we first synchronized Reh cells in S phase using the Aph synchronization protocol. The synchronized cells were washed and cultured in the absence or presence of 100 μM forskolin for 2 or 4 h. Two hours after release of cells from Aph arrest, 85% of cells contained S phase DNA content and ~83% of cells incorporated BrdU (Figure 2A). In the presence of forskolin for 2 h, cells retained S phase DNA content, whereas only 12% of the cells incorporated BrdU. By 4 h after release of cells from Aph arrest, 65% of cells were in S phase and ~62% of cells incorporated BrdU. In the presence of forskolin for 4 h, 80% of cells were in S phase whereas only 25% of cells incorporated BrdU. We then examined the status of Rb phosphorylation in S phase–synchronized cells after 2 or 4 h treatment with forskolin. The Aph-arrested cells contained exclusively the hyperphosphorylated forms of Rb (Figure 2B). The phosphorylation of Rb remained unchanged in cultures that were released from Aph arrest for 2 or 4 h. Treatment of Aph-released cells with forskolin for 2 h led to accumulation of the hypophosphorylated form of Rb. The Aph-released cells that were treated with forskolin for 4 h maintained further the expression of hypophosphorylated Rb. These results demonstrate a correlation between the dephosphorylation of Rb and inhibition of DNA synthesis in cells that are treated with forskolin.

Induction of p21^{Cip1} and Inhibition of Cdk2-cyclin Kinase Activity by cAMP in S phase Cells

Cdk2 in complex with cyclin E or cyclin A is the kinase responsible for Rb phosphorylation in S phase (Mittnacht, 1998). To assess the mechanism responsible for cAMP-mediated Rb dephosphorylation in S phase cells, we measured the Cdk2 kinase activity in Aph-released Reh cells that were treated with forskolin for 2 or 4 h. The Aph-arrested cells contained substantial amount of Cdk2 kinase activity (Figure 2C). Cdk2 activity increased in cells that were released from Aph arrest for 2 h and remained at this level in cells 4 h after release from Aph arrest. Exposure of cells to forskolin for 2 h after Aph release led a reduction of Cdk2 kinase activity. Similarly, Aph-released cells that were treated with forskolin for 4 h expressed less Cdk2 kinase activity than did untreated cells. This result showed that forskolin inhibited Cdk2 kinase activity in S phase cells, which could account for forskolin-mediated Rb dephosphorylation in these cells. Next, we wanted to verify that forskolin-induced inhibition of Cdk2 activity was responsible for dephosphorylation of Rb in S phase. To do so, we first examined the time-course of Cdk2 kinase inhibition and Rb dephosphorylation after treatment of S phase–synchronized Reh cells with forskolin. Densitometric analysis of the result shown in the top panel of Figure 2D revealed that forskolin inhibited the activity of Cdk2 by ~20% between 60 and 90 min after treatment. Cdk2 kinase activity decreased further with longer exposure of

cells to forskolin, so that by 2 h after forskolin treatment, Cdk2 activity was inhibited by ~75%. As shown in the bottom panel of Figure 2D, by 90 min after forskolin treatment, cells expressed slightly higher levels of hypophosphorylated Rb than did control cells, and by 2 h after forskolin treatment, cells contained almost exclusively the hypophosphorylated form of Rb. This result establishes a close temporal correlation between inhibition of Cdk2 activity and dephosphorylation of Rb by forskolin in S phase Reh cells. To further demonstrate that inhibition of Cdk2 activity is responsible for dephosphorylation of Rb in S phase cells, Aph-synchronized Reh cells were treated with the specific Cdk2 inhibitor, roscovitine (Gray *et al.*, 1999), and examined for the phosphorylation of Rb. As shown in Figure 2E, treatment of S phase Reh cells with roscovitine for 2 h led to dephosphorylation of Rb. Thus, in agreement with previous reports, Cdk2 is the major enzyme responsible for phosphorylation of Rb in S phase cells (Mittnacht, 1998). Taken together, these results strongly suggest that in S phase cells, forskolin leads to dephosphorylation of Rb through inhibition of Cdk2 kinase activity.

The activity of Cdks is subject to regulation by a variety of mechanisms including the regulation of cyclin protein levels or complex formation with CKIs (Morgan, 1995; Sherr and Roberts, 1995). We therefore examined the protein levels of Cdk2, cyclin E, cyclin A, p27^{Kip1} and p21^{Cip1} proteins in S phase Reh cells after treatment with forskolin. Exposure of cells to forskolin for 2 or 4 h after release from Aph arrest had no effect on the levels of Cdk2, cyclin E, cyclin A or p27^{Kip1}, whereas it led to a moderate increase in the levels of p21^{Cip1} (Figure 2F, left panel). To ascertain the functional significance of the observed increase in p21^{Cip1} protein levels in forskolin-mediated inhibition of Cdk2 kinase activity, we immunoprecipitated Cdk2 from cells that were treated with forskolin for 2 or 4 h after Aph release and examined the relative amount of cyclin E, cyclin A, p27^{Kip1}, and p21^{Cip1} bound to Cdk2 by Western blot analysis. In agreement with the Western blot shown in the left panel of Figure 2F, the relative amount of cyclin E, cyclin A, and p27^{Kip1} in complex with Cdk2 remained unchanged both in control and forskolin-treated cells (Figure 2F, right panel). In contrast, the level of p21^{Cip1} coimmunoprecipitated with Cdk2 increased after treatment of Aph-released cells with forskolin. We also analyzed the relative content of phosphotyrosine in Cdk2 by immunoblotting with an antiphosphotyrosine antibody and found that forskolin did not affect the phosphorylation of Cdk2 on tyrosine (unpublished data). Thus, the increase in the level of Cdk2-cyclin-p21^{Cip1} complexes could account for the forskolin-mediated inhibition of Cdk2 kinase activity in S phase cells.

Finally, to verify that the effect of cAMP was not specific for Aph-synchronized cells, we used the thymidine double-

Figure 3. Induction of p21^{Cip1} mRNA in S phase cells by cAMP. S phase–synchronized Reh cells were treated with vehicle or forskolin and harvested at the indicated times. Total RNA was recovered, and 15 μg of the RNA from each sample was analyzed by Northern blotting using ^{32}P-labeled p21^{Cip1} probe as described in *Materials and Methods.* The ethidium bromide-stained 18S RNA was used as a loading control (bottom panel).
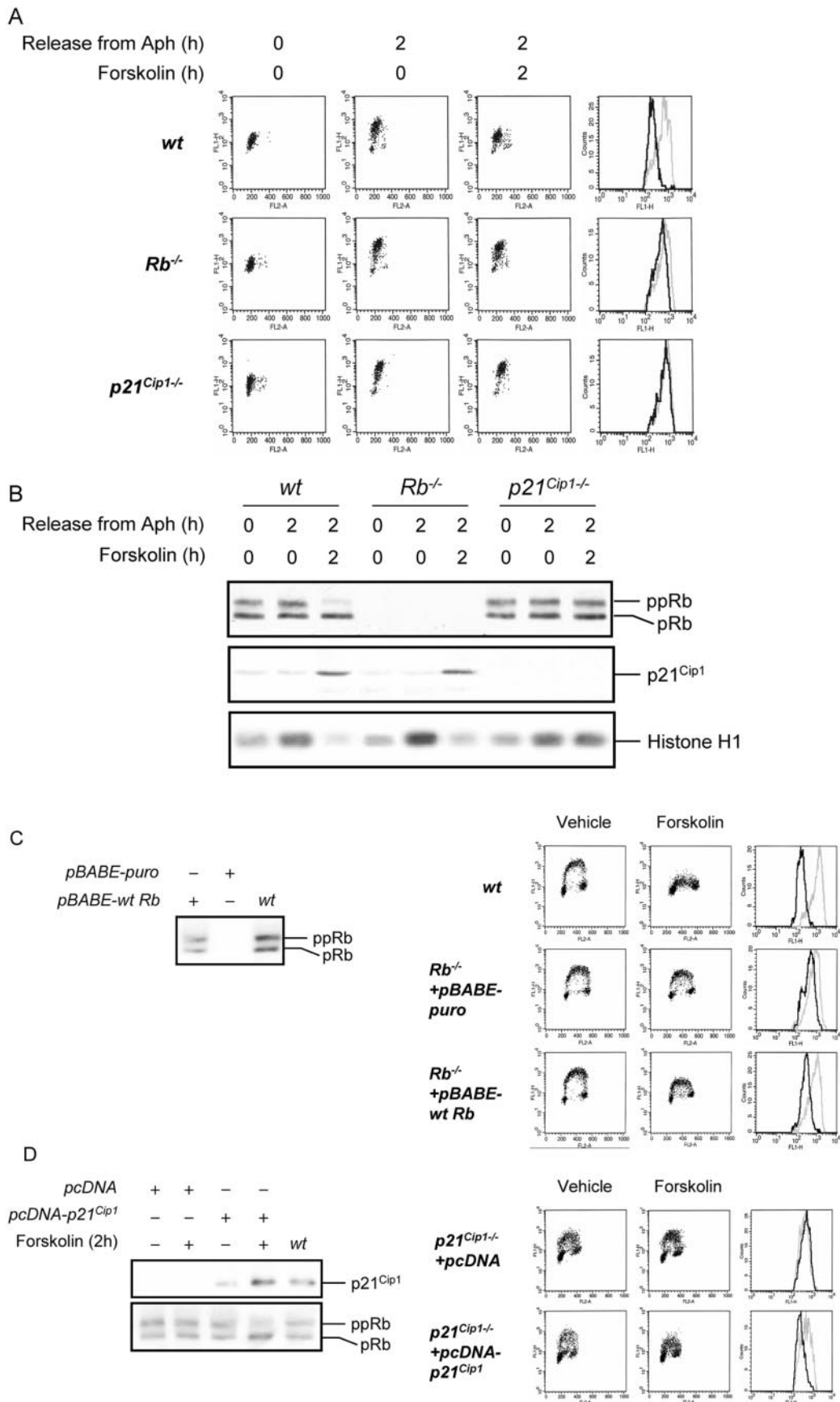
Figure 4.

block as an alternative means to synchronize Reh cells in S phase. As shown in Figure 2G, exposure of cells to forskolin for 2 h after release from thymidine double-block led to induction of p21^{Cip1}, dephosphorylation of Rb, and inhibition of DNA synthesis.

cAMP Induces the Level of p21^{Cip1} mRNA in S phase Cells

To determine the mechanism by which cAMP induces the level of p21^{Cip1} protein, we examined the mRNA levels and the protein stability of p21^{Cip1} in S phase cells after treatment with forskolin. As shown in Figure 3, exposure of cells to forskolin for 2 h increased the p21^{Cip1} mRNA level. By 4 h after treatment of cells with forskolin, the level of p21^{Cip1} mRNA had declined slightly but remained above that of untreated cells. The changes in the expression of p21^{Cip1} mRNA correlated with the expression of p21^{Cip1} protein induced by forskolin (see Figure 2F). Examination of the stabilities of p21^{Cip1} transcript and protein revealed that forskolin did not affect the turnover of p21^{Cip1} transcript or protein in Reh cells (unpublished data).

Rb and p21^{Cip1} Are Required for cAMP-mediated Inhibition of DNA Synthesis

Our observation that forskolin-mediated inhibition of S phase progression was associated with the induction of

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Figure 4 (facing page). Rb and p21^{Cip1} are required for cAMP-induced inhibition of DNA synthesis. (A) *Wt*, *Rb*$^{-/-}$ or *p21*$^{Cip1-/-}$ 3T3 cells were synchronized in early S phase with Aph as described in *Materials and Methods*. Cells were released from Aph, cultured in drug-free medium in the absence or presence of 100 μM forskolin, and pulsed with BrdU for the final 30 min before harvesting at 2 h. Cells were then processed as in Figure 1A for detection of DNA synthesis and cell cycle phase determination. Shown are scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). The relative rate of DNA synthesis was visualized by plotting FITC anti-BrdU staining in S phase cells as depicted in the rightmost panel. Gray lines, treatment with vehicle; black lines, treatment with forskolin. (B) *Wt*, *Rb*$^{-/-}$, or *p21*$^{Cip1-/-}$ 3T3 cells were treated as in A. Whole-cell extracts were prepared and used for examination of the status of Rb phosphorylation, the level of p21^{Cip1} protein, and Cdk2-cyclin kinase activity as described in *Materials and Methods*. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. (C) *Rb*$^{-/-}$ cells harboring *pBABE-puro* or *pBABE-wt Rb* vectors were generated by retroviral infection of *Rb*$^{-/-}$ 3T3 cells. Cells were treated with vehicle or 100 μM forskolin and pulsed with BrdU for the final 30 min before harvesting at 2 h. Each sample was split in two. Left: whole-cell extracts from one-half of each sample were processed for examination of Rb phosphorylation by Western blotting. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. Right: cells in the other half of each sample were processed as in Figure 1A for detection of DNA synthesis and cell cycle phase determination. Shown are scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). The relative rate of DNA synthesis was visualized by plotting FITC anti-BrdU staining in S phase cells as depicted by the histograms. Gray lines, treatment with vehicle; black lines, treatment with forskolin. (D) *p21*$^{Cip1-/-}$ cells harboring *pcDNA* or *pcDNA-p21*^{Cip1} vectors were generated by transfection of *p21*$^{Cip1-/-}$ 3T3 cells. Cells were treated with vehicle or 100 μM forskolin and pulsed with BrdU for the final 30 min before harvesting at 2 h. Each sample was split in two. Left: whole-cell extracts from one-half of each sample were processed for examination of p21^{Cip1} and Rb phosphorylation by Western blotting. pRb, hypophosphorylated Rb; ppRb, hyperphosphorylated Rb. Right: cells in the other half of each sample were processed as in Figure 1A for detection of DNA synthesis and cell cycle phase determination. Shown are scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). The relative rate of DNA synthesis was visualized by plotting FITC anti-BrdU staining in S phase cells as depicted by the histograms. Gray lines, treatment with vehicle; black lines, treatment with forskolin.

p21^{Cip1} protein, accumulation of Cdk2-cyclin-p21^{Cip1} complexes, inhibition of Cdk2 kinase activity, and dephosphorylation of Rb suggested that p21^{Cip1} and Rb might be involved in inhibition of DNA synthesis by forskolin. To examine this possibility, we utilized 3T3 cells derived from *wt*, *Rb*$^{-/-}$, and *p21*$^{Cip1-/-}$ MEFs and assessed their response to forskolin-mediated inhibition of DNA synthesis.

Cells were first synchronized in S phase with Aph. These synchronized cells were washed and then treated with 100 μM forskolin, pulse-labeled with BrdU for 30 min before collection of samples after 2 h, and examined for BrdU incorporation by flow cytometry. As can be seen in Figure 4A, *wt* 3T3 cells exhibited reduced DNA synthesis, whereas *p21*$^{Cip1-/-}$ cells showed no inhibition of DNA synthesis after forskolin treatment. *Rb*$^{-/-}$ cells showed a partial inhibition of DNA synthesis in response to forskolin treatment compared with *wt* cells. This result shows that inhibition of DNA synthesis by forskolin is dependent on Rb and p21^{Cip1} proteins.

Next, we examined the status of Rb phosphorylation, the level of p21^{Cip1} protein, and the Cdk2 kinase activity in S phase–synchronized *wt*, *Rb*$^{-/-}$, and *p21*$^{Cip1-/-}$ 3T3 cells after treatment with forskolin. *Wt* and *p21*$^{Cip1-/-}$ cells that were released from Aph arrest for 2 h contained both hypophosphorylated and hyperphosphorylated Rb (Figure 4B). Treatment of Aph-released cells with forskolin for 2 h led to accumulation of the hypophosphorylated form of Rb in *wt* 3T3 cells but not in *p21*$^{Cip1-/-}$ cells. Exposure of cells to forskolin for 2 h after release from Aph arrest led to an increase in the levels of p21^{Cip1} in *wt* and *Rb*$^{-/-}$ cells. Furthermore, forskolin led to a reduction in Cdk2-cyclin kinase activity only in *wt* and *Rb*$^{-/-}$ cells. These results are consistent with a role of p21^{Cip1} protein in forskolin-mediated inhibition of Cdk2-cyclin kinase activity and Rb dephosphorylation in S phase 3T3 cells.

To further verify the requirement of Rb for forskolin-mediated inhibition of DNA synthesis, we examined whether expression of wt Rb in *Rb*$^{-/-}$ 3T3 cells would restore forskolin-mediated inhibition of S phase progression. *Rb*$^{-/-}$ 3T3 cells were infected with retrovirus expressing either *wt Rb* (*pBABE-wt Rb*) or empty vector (*pBABE-puro*) as control, and pools of cells with stably integrated virus were generated. Western blot analysis of *Rb*$^{-/-}$ 3T3 cells infected with *pBABE-wt Rb* revealed that the level of Rb expressed in these cells was ~50% of the level of endogenous Rb expressed in *wt* 3T3 cells (Figure 4C, left panel). When exposed to forskolin, *wt* 3T3 cells inhibited DNA synthesis, whereas *Rb*$^{-/-}$ 3T3 cells infected with *pBABE-puro* were impaired in their S phase response as expected (Figure 4C, right panel). Restoration of Rb expression in *Rb*$^{-/-}$ 3T3 cells by *pBABE-wt Rb* partially complemented this defect and caused the cells to partially inhibit DNA synthesis after exposure to forskolin. The lower level of wt Rb expressed in *Rb*$^{-/-}$ 3T3 infected with *pBABE-wt Rb*, compared with the level of endogenous Rb expressed in *wt* 3T3 cells, could account for the partial restoration of S phase response to forskolin in these cells. This result verifies the above observation that Rb is required for inhibition of DNA synthesis in response to cAMP.

The requirement of cAMP-mediated induction of p21^{Cip1} for dephosphorylation of Rb and attenuation of DNA synthesis by cAMP was further demonstrated by examination of *p21*^{Cip1} 3T3 cells that were transfected with a vector carrying *p21*^{Cip1} cDNA driven by the human cytomegalovirus (CMV) major promoter/enhancer (*pcDNA-p21*^{Cip1}). An 8-base pair core palindrome of CMV promoter has been shown to act as a cAMP-responsive element (CRE; Ficken-

scher *et al.,* 1989). Elevation of intracellular concentrations of cAMP results in upregulation of CRE and enhancement of the transcriptional activity of the CMV promoter. As shown in the left panel of Figure 4D, the forskolin-mediated induction of *p21^{Cip1}* in cells that were transfected with *pcDNA-p21^{Cip1}* was comparable to the level of p21^{Cip1} in *wt* 3T3 cells after treatment with forskolin (see Figure 4B). Importantly, *p21^{Cip−/−}* 3T3 cells transfected with empty vector (*pcDNA*) failed to exhibit inhibition of DNA synthesis after forskolin treatment (Figure 4D, right panel). In contrast, after exposure to forskolin, *p21^{Cip−/−}* cells harboring *pcDNA-p21^{Cip1}* vector expressed dephosphorylated Rb and inhibited DNA synthesis. Thus, p21^{Cip1} is required for cAMP to induce the dephosphorylation of Rb and inhibit DNA replication.

cAMP Induces Dissociation of PCNA from Chromatin in S phase Cells

The aforementioned results indicate that cAMP, through Rb, negatively regulates the DNA replication machinery. Rb is implicated in transcriptional repression of E2F regulated genes (Kaelin, 1999). Furthermore, both PCNA and MCM2, two essential components of DNA replication machinery, are regulated by E2F (Helin, 1998; Leone *et al.,* 1998). Therefore, we hypothesized that cAMP might inhibit DNA replication through inhibition of PCNA or MCM2 expression. To examine this possibility, S phase–synchronized Reh cells were treated with forskolin for 2 h and then analyzed for the expression of PCNA and MCM2. Surprisingly, forskolin treatment of Reh cells did not affect the levels of PCNA and MCM2 proteins (Figure 5A). Because PCNA and MCM2 exert their effect only when tethered to DNA (Todorov *et al.,* 1995; Dimitrova and Gilbert, 2000), we proceeded to examine the influence of cAMP on association of PCNA and MCM2 with chromatin by two different approaches. First, nuclei were isolated from S phase–synchronized Reh cells that were treated with forskolin for 2 h and subjected to Triton X-100 extraction to remove soluble and weakly bound nuclear proteins. The nuclei were then treated with micrococcal nuclease (MNase) to solubilize the chromatin. The MNase-solubilized fraction containing chromatin-bound proteins was then analyzed by Western blotting with antibodies against PCNA and MCM2 proteins. The MNase-solubilized fraction from cells that were treated with forskolin contained less PCNA, indicating reduced association of PCNA to chromatin in these samples (Figure 5B). In contrast, forskolin treatment of cells had no effect on MCM2 binding to DNA. To further confirm this result, S phase–synchronized Reh cells that were treated with forskolin for 2 h were subjected to in situ extraction to remove unbound proteins. The cells were then examined by immunocytochemistry using antibodies against PCNA. As shown in Figure 5C, the chromatin association of PCNA was significantly reduced in cells that were treated with forskolin as evidenced by greatly diminished staining of PCNA.

As shown above, Rb- and p21^{Cip1}-deficient 3T3 cells were unable to inhibit DNA replication in response to cAMP. Because disruption of PCNA tethering to chromatin closely correlated with cAMP-mediated abrogation of DNA synthesis, we wanted to examine the association of PCNA to chromatin in *Rb^{−/−}* and *p21^{Cip1−/−}* cells after treatment with forskolin. To do so, S phase–synchronized *wt, Rb^{−/−},* and *p21^{Cip1−/−}* 3T3 fibroblasts were treated with forskolin for 2 h. Examination of the MNase-soluble fraction of these cells showed that forskolin treatment of *wt* 3T3 cells led to disruption of PCNA tethering to chromatin (Figure 5D). In contrast, forskolin did not disrupt binding of PCNA to chromatin in *p21^{Cip1−/−}* 3T3 cells. Treatment of *Rb^{−/−}* 3T3 cells

with forskolin led to a marginal inhibition of PCNA association with chromatin. The differential response of *wt, Rb^{−/−},* and *p21^{Cip1−/−}* 3T3 fibroblasts to cAMP-induced dissociation of PCNA from chromatin was also demonstrated by immunostaining of cells for PCNA. In agreement with the data presented in Figure 5D, forskolin inhibited association of PCNA with chromatin in *wt* 3T3 cells, whereas PCNA tethering was unaffected in *Rb^{−/−}* and *p21^{Cip1−/−}* 3T3 cells after treatment with forskolin (Figure 5E). Taken together, these results show that forskolin specifically inhibits association of PCNA to chromatin in an Rb- and p21^{Cip1}-dependent manner and establish a link between forskolin-mediated inhibition of DNA synthesis and the DNA replication machinery.

PGE₂-mediated Inhibition of DNA Synthesis

Under physiological conditions, generation of cAMP is controlled by the activity of certain receptors, such as prostaglandin E (PGE) receptors, that via regulation of GTP-binding proteins, activate adenylyl cyclase enzyme and thus lead to generation of cAMP (Negishi *et al.,* 1993; Coleman *et al.,* 1994; Regan, 2003). We therefore used PGE$_2$ to examine whether stimulation of such receptors would lead to attenuation of DNA replication. S phase–synchronized Reh cells were treated with PGE$_2$, pulse-labeled with BrdU for 30 min before collection of samples after 2 h, and examined for BrdU incorporation by flow cytometry. As shown in Figure 6A, treatment of cells with PGE$_2$ led to inhibition of DNA synthesis in a dose-dependent manner. When exposed to 30 μM PGE$_2$, cells exhibited a slight reduction of DNA synthesis, whereas treatment of cells with 60 μM PGE$_2$ led to a significant inhibition of DNA synthesis. This result indicates that generation of cAMP through stimulation of G protein–coupled receptors leads to inhibition of DNA synthesis. Next, we wanted to examine whether PGE$_2$-induced attenuation of DNA synthesis occurred via the same mechanism as that found with forskolin. To do so, we treated S phase–synchronized Reh cells with 60 μM PGE$_2$ for 2 h and examined them for the expression of p21^{Cip1}, phosphorylation of Rb, and association of PCNA with chromatin. We used 60 μM PGE$_2$ because, as shown above, this concentration of PGE$_2$ was found to substantially inhibit DNA synthesis in Reh cells. As shown in Figure 6B, PGE$_2$ treatment of Reh cells led to induction of p21^{Cip1}, dephosphorylation of Rb, and reduction of PCNA association with chromatin.

cAMP Protects Cells from Apoptosis Induced by S phase–specific Cytotxic Drugs in an Rb- and p21^{Cip1}-dependent Manner

The negative effect of forskolin on DNA synthesis suggested that activation of cAMP signaling pathway would render cells resistant to antitumor agents whose function depends on DNA replication. To assess this assumption, we examined the effect of forskolin on apoptosis induced by the S phase–specific agents, camptothecin (CPT), 5-fluorouracil (5-FU), hydroxyurea (HU), and the microtubule-damaging agents paclitaxel (PAC) and vinblastine (VB) that exhibit G2/M phase–specific cytotoxicity (Yarbro, 1992; Jordan *et al.,* 1998; Liu *et al.,* 2000; Jordan, 2002; Sampath *et al.,* 2003). We exposed asynchronously proliferating Reh cells to a 2-h pulse treatment with the cytotoxic drugs alone or together with 100 μM forskolin and analyzed sensitivity in terms of apoptotic response at regular intervals after recovery. As shown in Figure 7A, both S phase– and G2/M phase–specific drugs induced apoptosis in Reh cells. Interestingly, cotreatment of cells with forskolin led to a significant reduction in the level of apoptosis in response to S phase–specific
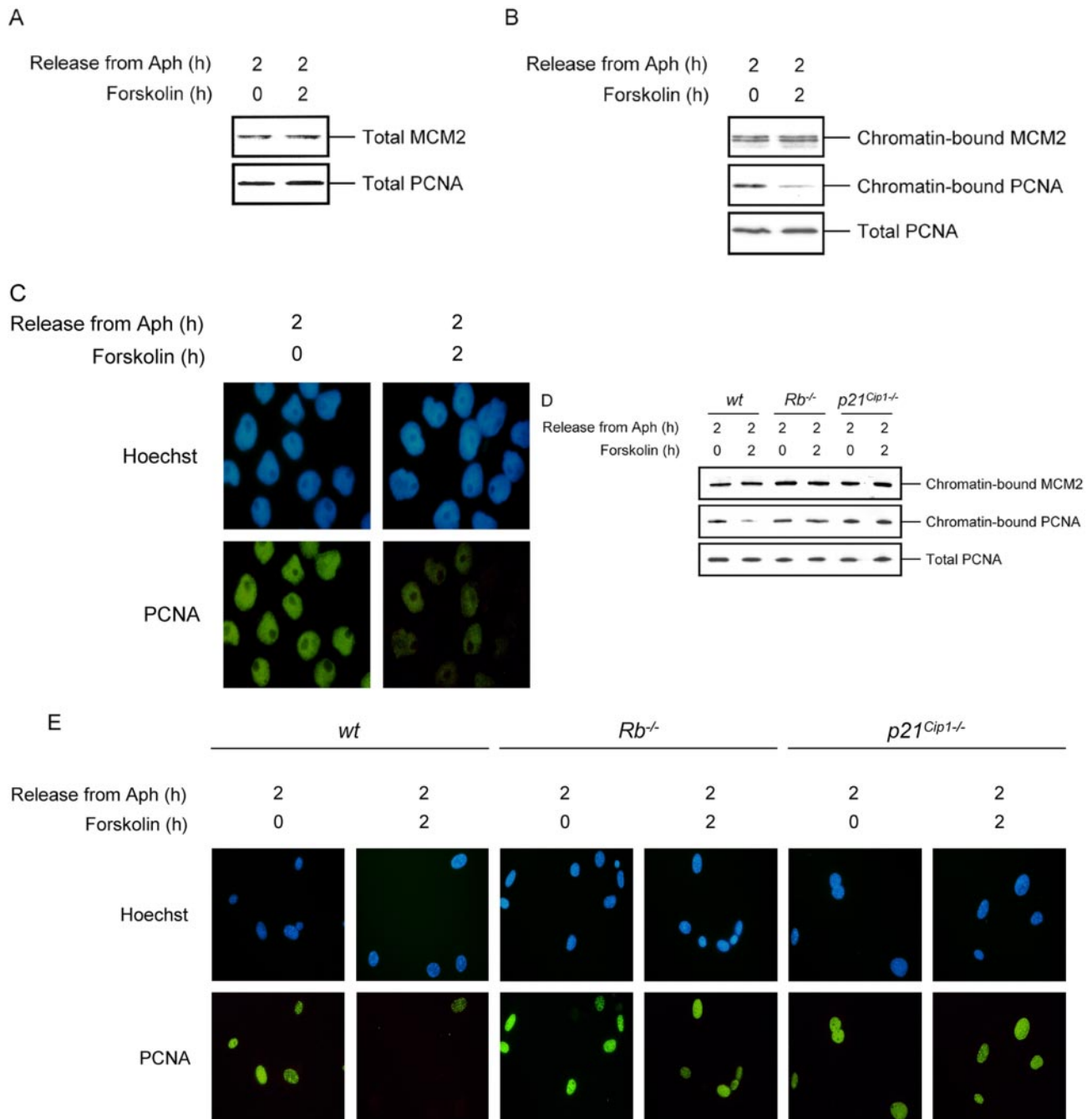
A

Release from Aph (h) 2 2
Forskolin (h) 0 2

— Total MCM2

— Total PCNA

B

Release from Aph (h) 2 2
Forskolin (h) 0 2

— Chromatin-bound MCM2

— Chromatin-bound PCNA

— Total PCNA

C

Release from Aph (h) 2 2
Forskolin (h) 0 2

Hoechst

PCNA

D

	wt		$Rb^{-/-}$		$p21^{Cip1-/-}$	
Release from Aph (h) 2 2 2 2 2 2
Forskolin (h) 0 2 0 2 0 2

— Chromatin-bound MCM2

— Chromatin-bound PCNA

— Total PCNA

E

wt		$Rb^{-/-}$		$p21^{Cip1-/-}$	

Release from Aph (h) 2 2 2 2 2 2
Forskolin (h) 0 2 0 2 0 2

Hoechst

PCNA

Figure 5. cAMP-mediated inhibition of DNA synthesis correlates with the disruption of PCNA tethering to DNA. (A) S phase–synchronized Reh cells were treated with vehicle or forskolin for 2 h. Cells were harvested, and lysates were prepared, resolved by SDS-PAGE, and detected by immunoblotting with PCNA and MCM2 antibodies. (B) Nuclei isolated from S phase–synchronized Reh cells that were exposed to vehicle or 100 μM forskolin for 2 h were treated with Triton X-100 to separate soluble proteins from the chromatin-bound pellet fraction (*Materials and Methods*). The extracted nuclei were further treated with MNase to solubilize the chromatin. The MNase-solubilized fraction was resolved on SDS-PAGE and immunoblotted with PCNA and MCM2 antibodies. (C) S phase–synchronized Reh cells were treated with vehicle or 100 μM forskolin and harvested after 2 h. Cells were cytospun onto coverslips, extracted, and then fixed in paraformaldehyde before staining with PCNA antibodies. (D) *Wt*, $Rb^{-/-}$, and $p21^{Cip1-/-}$ 3T3 cells were synchronized in S phase with Aph and then treated with vehicle or forskolin for 2 h. Nuclei were then isolated and processed as in B for detection of chromatin-bound PCNA by immunoblotting with anti-PCNA antibodies. (E) *Wt*, $Rb^{-/-}$, and $p21^{Cip1-/-}$ 3T3 cells were seeded on coverslips, synchronized in S phase with Aph, and then treated with vehicle or forskolin for 2 h. Coverslips were then processed as in C for immunofluorescence staining with PCNA antibodies.

agents, whereas it had no effect on apoptosis induced by G2/M phase–specific drugs. These results show that increase in intracellular levels of cAMP can protect cells from the cytotoxic effect of S phase–specific anticancer drugs.

Given the observation that cAMP-mediated inhibition of DNA synthesis was dependent on Rb and $p21^{Cip1}$, we next wanted to determine whether the inhibitory effect of cAMP on apoptosis induced by S phase–specific cytotoxic drugs
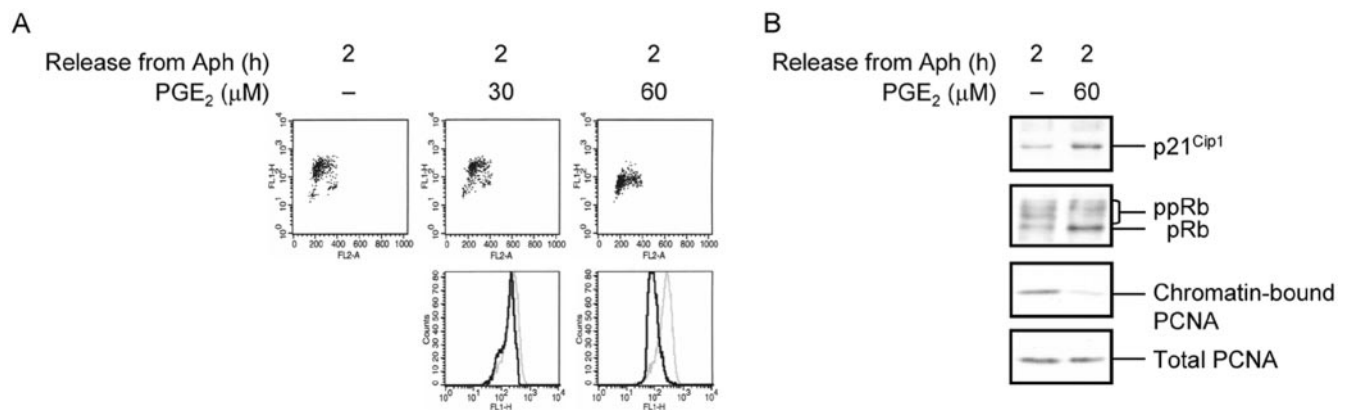
Figure 6. Inhibition of DNA synthesis by PGE$_2$. (A) S phase–synchronized Reh cells were treated with the indicated concentrations of PGE$_2$ for 2 h and pulsed with BrdU for the final 30 min of treatment. Cells were processed as in Figure 1A for detection of BrdU incorporation and cell cycle phase determination. Top: representative scatter plots with the log FITC anti-BrdU staining (FL1-H) versus PI staining (FL2-A). Bottom: the relative rate of BrdU incorporation was visualized by plotting FITC anti-BrdU staining in S phase cells. Gray lines, treatment with vehicle; black lines, treatment with forskolin. (B) S phase–synchronized Reh cells were treated with 60 μM PGE$_2$ for 2 h or left untreated. Each sample was then split in two. Total cell lysates from one-half of each sample were resolved on SDS-PAGE and then subjected to immunoblotting with the antibodies against p21^{Cip1}, Rb, and PCNA. Nuclei isolated from the other half of each sample were processed as in Figure 5B and then examined for chromatin-bound PCNA by immunoblotting with anti-PCNA antibodies.

required Rb and p21$^{Cip1.}$ To do so, *wt*, *Rb*$^{-/-}$, and *p21*$^{Cip1-/-}$ 3T3 cells were exposed to a 2-h pulse treatment with 5-FU alone or together with 100 μM forskolin. Samples were then collected at 72 h after recovery and analyzed for apoptosis. Cotreatment of *wt* cells with forskolin substantially inhibited apoptosis induced by 5-FU (Figure 7B). By contrast, forskolin had no effect on 5-FU–induced apoptosis in *Rb*$^{-/-}$ and *p21*$^{Cip1-/-}$ 3T3 cells. These results indicate that cAMP-mediated inhibition of apoptosis induced by S phase–specific cytotoxic agents is dependent on Rb and p21^{Cip1}.

DISCUSSION

Elevation of intracellular cAMP leads to growth inhibition in a number of cell types including lymphocytes, and this growth inhibition has been linked to G1 arrest (Blomhoff *et al.*, 1987, 1988; Christoffersen *et al.*, 1994; Dugan *et al.*, 1999). We have previously shown that cAMP leads to a transient accumulation of Reh cells in G2, followed by permanent arrest of cells in G1 phase of the cell cycle (Blomhoff *et al.*, 1988). Here, we provide evidence for an inhibitory effect of cAMP on S phase progression. We have shown that elevation of cAMP inhibits DNA synthesis and arrests the cells transiently in S phase. This cAMP-induced S phase arrest requires Rb and p21^{Cip1} proteins and closely correlates with dissociation of PCNA from chromatin.

Our observation that cAMP-induced inhibition of DNA synthesis was associated with dephosphorylation of Rb in S phase cells, suggested the requirement of Rb for inhibition of DNA synthesis by cAMP. Indeed *Rb*$^{-/-}$ cells were partially defective in halting DNA synthesis after elevation of cAMP levels, and ectopic expression of wt Rb in these cells restored the ability of cAMP to inhibit DNA synthesis. In S phase, phosphorylation and inactivation of Rb is achieved through the action of Cdk2 in complex with cyclin E or cyclin A (Mittnacht, 1998). Thus, the observed cAMP-mediated inhibition of Cdk2-cyclin activity could be considered as the mechanism responsible for dephosphorylation of Rb in S phase cells. The cAMP-induced reduction in Cdk2-cyclin

activity correlated with induction of p21^{Cip1} expression and an increase in Cdk2-cyclin-p21^{Cip1} complexes, supporting a model wherein elevation of intracellular cAMP leads to induction of p21^{Cip1} protein expression. p21^{Cip1} then binds to Cdk2-cyclin complexes and attenuates the Cdk2-associated kinase activity. Reduction in Cdk2 kinase activity in turn leads to expression of dephosphorylated Rb and inhibition of DNA replication. The observation that *p21*$^{Cip1-/-}$ cells failed to 1) inhibit Cdk2-cyclin kinase activity, 2) dephosphorylate Rb, and 3) abrogate DNA synthesis after exposure to forskolin, together with the finding that cAMP-inducible expression of exogenous p21^{Cip1} in *p21*$^{Cip1-/-}$ 3T3 cells restored their ability to dephosphorylate Rb and inhibit DNA replication strongly support this model.

We have previously observed that treatment of Reh cells with forskolin induces the expression of p21^{Cip1} (within 2 h) and p27^{Kip1} (within 4–8 h) proteins and leads to dephosphorylation of Rb (Christoffersen *et al.*, 1994; Naderi and Blomhoff, 1999; Gutzkow *et al.*, 2002). In this study, we found that exposure of Reh cells to forskolin led to increase in expression of p21^{Cip1} and dephosphorylated Rb but had no effect on the levels of p27^{Kip1} protein. It is important to note that here we have used Aph-synchronized cultures of cells to specifically analyze the effect of forskolin on cells in the S phase of the cell cycle. In contrast, in our previous studies (Naderi and Blomhoff, 1999; Gutzkow *et al.*, 2002), we examined the effect of forskolin on asynchronous cultures of Reh cells, in which cells are present in all phases of the cell cycle. Taken together, our results indicate that cAMP-mediated induction of p27^{Kip1} is associated with G1 arrest and not with inhibition of DNA replication.

Both Rb (Chew *et al.*, 1998; Knudsen *et al.*, 1998; Saudan *et al.*, 2000) and p21^{Cip1} (Ogryzko *et al.*, 1997; Niculescu *et al.*, 1998) have previously been implicated in regulation of DNA replication. The mechanism by which Rb inhibits DNA synthesis is currently unknown. Activation of Rb has recently been shown to inhibit DNA replication by two kinetically and functionally different mechanisms; an acute pathway that targets PCNA loading onto chromatin and a more stable arrest program that involves a sustained attenuation of the
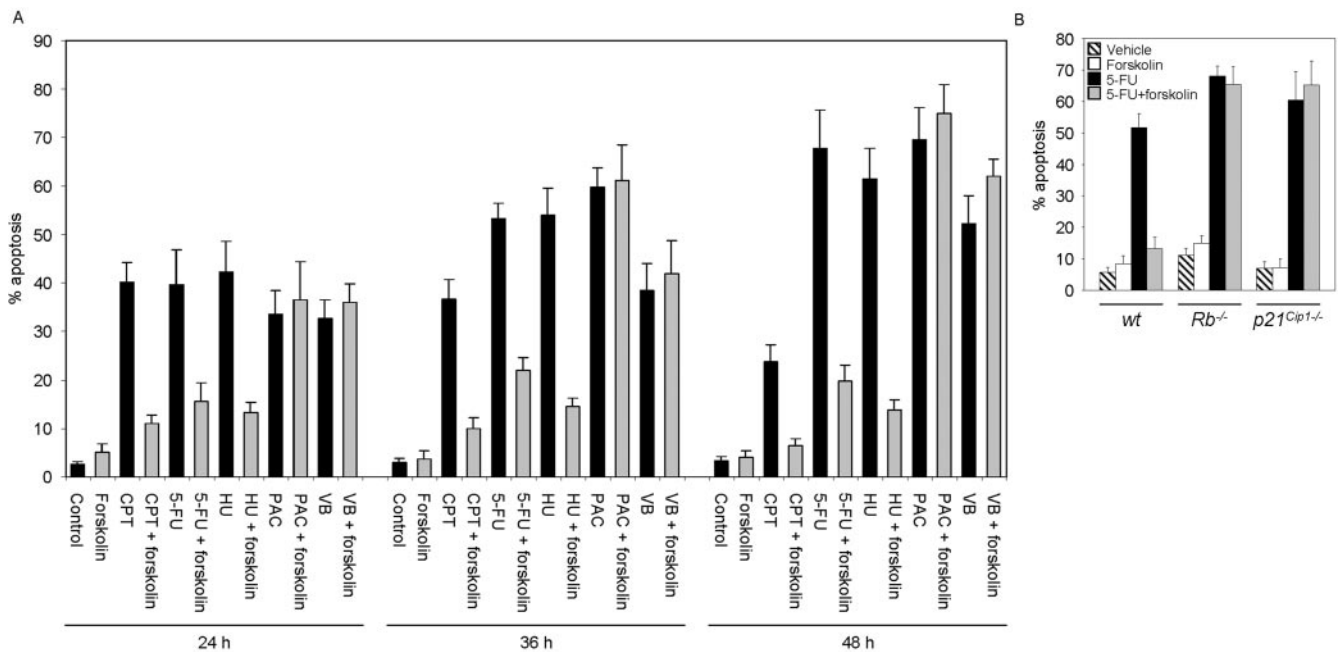
Figure 7. cAMP prevents cell death induced by S phase–specific cytotoxic agents in an Rb- and p21^Cip1-dependant manner. (A) Asynchronously proliferating Reh cells were treated with 100 nM CPT, 1 mM 5-FU, 110 mM HU, 1 μM PAC, or 25 nM VB alone or together with 100 μM forskolin for 2 h. Cells were then washed, cultured in drug-free medium, and harvested at the indicated recovery times for evaluation of apoptosis by flow cytometry. (B) Asynchronously proliferating *wt*, *Rb*$^{-/-}$, or *p21*$^{Cip1-/-}$ 3T3 cells were treated with 500 μM 5-FU alone or together with 100 μM forskolin for 2 h. Cells were then washed, cultured in drug-free medium, and harvested after 72 h for evaluation of apoptosis by flow cytometry. Data in A and B are from four independent experiments. Error bars, mean $\pm$ SE.

protein levels of several E2F target genes involved in DNA replication (Angus *et al.,* 2004). Disruption of PCNA activity elicited by Rb is shown to depend on E2F-mediated transcriptional repression and the ability of Rb to inhibit the expression of cyclin A has been suggested to be such an event (Sever-Chroneos *et al.,* 2001; Angus *et al.,* 2004). As demonstrated here, the cAMP-mediated inhibition of DNA synthesis is dependent on Rb function and correlates with dissociation of PCNA from chromatin. It can therefore be suggested that cAMP utilizes Rb in its capacity to acutely disrupt the PCNA function and leads to cessation of DNA replication. This assumption predicts that Rb-mediated transcriptional repression contributes to cAMP-induced dissociation of PCNA from chromatin and inhibition of DNA replication. However, we cannot formally exclude the possibility that cAMP inhibits DNA replication independent of the transcriptional repression function of Rb. This notion is based on two lines of evidence: First, Rb dephosphorylation and inhibition of DNA synthesis occur almost simultaneously in forskolin-treated cells. Second, cAMP does not inhibit the expressions of cyclin E and A in S phase cells. Because cyclin E and A are known targets of Rb, this observation suggests that the ability of Rb to regulate gene transcription is distinct from the cAMP-mediated inhibition of DNA replication. It is conceivable that Rb might directly interfere with PCNA function to inhibit DNA replication at the elongation phase. Our data that cAMP-mediated dephosphorylation of Rb and inhibition of DNA synthesis coincided with dissociation of PCNA from chromatin is consistent with this notion. Further support for this idea is provided by the finding that cAMP failed to induce dissociation of PCNA from chromatin and inhibit DNA replication in *Rb*$^{-/-}$ cells. Indeed, the phenomenon of disruption of PCNA function and inhibition of DNA replication at the elongation phase has been reported previously. Activation of the S phase checkpoint by DNA damage has been shown to inhibit replication elongation and decrease the association of PCNA with DNA replication foci, an event that appears to depend on Rb signaling (Watanabe, 1974; Knudsen *et al.,* 2000; Sever-Chroneos *et al.,* 2001; Wang *et al.,* 2004). The exact mechanism by which Rb interferes with PCNA function is unclear. However, a potential insight into how Rb exerts its effect on PCNA might be provided by the report showing interaction of Rb with replication factor C (RFC; Pennaneach *et al.,* 2001). This study indicated that Rb interacts with RFC to induce cell cycle arrest and promote cell survival after DNA damage. Considering the function of RFC as the loader of PCNA onto DNA, it is tempting to speculate that Rb might inhibit association of PCNA with chromatin through its interaction with RFC and thus lead to inhibition of DNA replication. Further studies will be required to test this model.

Results from a number of studies suggest a role of Rb in inhibition of replication at the level of initiation. For instance, Rb has been reported to bind MCM7, a component of the prereplication complex, and inhibit in vitro DNA replication in an MCM7-dependent manner (Sterner *et al.,* 1998). In *Drosophila*, dDP–dE2F1–Rbf in complex with DmORC at the chorion origin of replication has been shown to limit replication initiation at the chorion locus, independent of transcription (Bosco *et al.,* 2001). Furthermore, after irradiation of S phase–synchronized cells, hypophosphorylated Rb has been reported to be directed to certain chromosomal replication initiation sites, at or after the time they had begun to fire, as part of the process whereby Rb suppresses abnormal rereplication activity (Avni *et al.,* 2003). Although the interaction of Rb with proteins at the replication origins or its presence at the replication foci has been disputed

(Dimitrova and Berezney, 2002; Angus *et al.*, 2004), we cannot exclude the possibility that after dephosphorylation, Rb might be recruited to replication initiation sites at or after the time they have begun to fire. This might, in turn, control any series of steps in initiation such as origin recognition, DNA unwinding, or it can block the switching from pol α–dependent primer formation to processive replication using pol δ and PCNA, and thus lead to inhibition of DNA replication.

When exposed to forskolin, $Rb^{-/-}$ cells exhibited an incomplete reduction in S phase arrest compared with *wt* cells. In contrast, $p21^{Cip1-/-}$ cells were irresponsive to the S phase inhibitory effect of forskolin. These observations suggest that inhibition of DNA replication by cAMP is partially dependent on Rb and indicate an Rb-independent role of $p21^{Cip1}$ in cAMP-mediated inhibition of DNA replication. In addition to binding to Cdk-cyclin complexes, $p21^{Cip1}$ also associates with PCNA (Flores-Rozas *et al.*, 1994). In vitro, interaction of $p21^{Cip1}$ with PCNA has been shown inhibit the RFC-mediated loading of PCNA onto DNA and also to prevent binding of pol δ to the PCNA clamp assembled on DNA (Waga *et al.*, 1994; Podust *et al.*, 1995). Similarly, a recent study has shown that, in vivo, direct binding of $p21^{Cip1}$ to chromatin-bound PCNA results in the loss of interaction with the catalytic subunit of pol δ (Cazzalini *et al.*, 2003). Based on these results, it can be postulated that $p21^{Cip1}$ contributes to the cAMP-mediated inhibition of DNA replication through its ability to directly interact with PCNA and thus abolish the replication complex formation involving PCNA, pol δ, and RFC. The possible role of $p21^{Cip1}$, as an inhibitor of Cdk-cyclin complexes, in attenuation of S phase progression should also be addressed. Several reports have suggested that the negative effect of $p21^{Cip1}$ on DNA synthesis relies primarily on its ability to inhibit Cdk-cyclin–dependent pathways (Luo *et al.*, 1995; Nakanishi *et al.*, 1995a, 1995b; Ogryzko *et al.*, 1997). Moreover, a number of studies have shown that, in addition to phosphorylation of Rb, Cdk2-cyclin complexes also regulate DNA replication in a manner that does not appear to require Rb function (Mimura and Takisawa, 1998; Zou and Stillman, 1998; Furstenthal *et al.*, 2001). One such report has shown that inhibition of Cdk2-cyclin A kinase has the potential to negatively regulate DNA replication through displacement of PCNA from chromatin (Sever-Chroneos *et al.*, 2001). On the basis of these observations and our finding that $p21^{Cip1}$-deficient cells were unable to disrupt PCNA association with chromatin in response to cAMP, we propose that the Rb-independent function of $p21^{Cip1}$ in cAMP-mediated inhibition of DNA synthesis might stem from its ability to attenuate Cdk2-cyclin activity and thus lead to disruption of PCNA tethering to chromatin.

In summary, we propose that cAMP inhibits DNA replication through its ability to induce the expression of $p21^{Cip1}$ protein and inhibit the activity of Cdk2-cyclin kinases (Figure 8). Abrogation of Cdk2-cyclin kinase activity in turn leads to expression of hypophosphorylated, active form of Rb, which leads to disruption of PCNA association with chromatin and thus inhibition of DNA replication. Furthermore, the inability of $Rb^{-/-}$ cells, as opposed to $p21^{Cip1-/-}$ cells, to completely alleviate the inhibitory effect of cAMP on association of PCNA with chromatin and DNA replication, argues for an additional, Rb-independent pathway through which $p21^{Cip1}$ can regulate PCNA activity and DNA replication.

A number of current cancer therapy regimes rely on the use of drugs whose cytotoxic activity depends on DNA replication. Our observation that elevation of intracellular cAMP inhibits DNA replication and blocks apoptosis in-
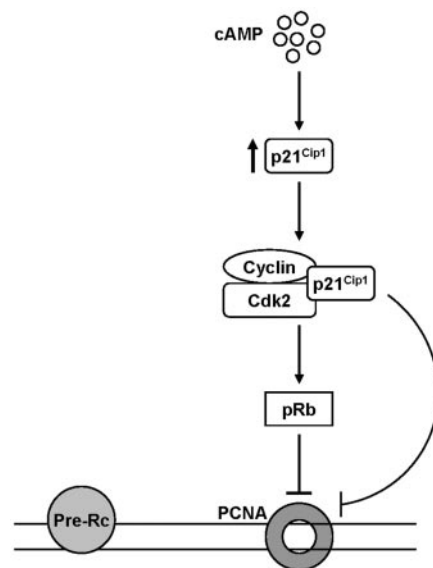

Figure 8. Suggested model for the cAMP-mediated inhibition of DNA replication. In S phase cells, elevation of intracellular levels of cAMP induces the expression of $p21^{Cip1}$ protein. This leads to inhibition of Cdk2-cyclin kinase activity. Abrogation of Cdk2-cyclin kinase activity in turn leads to expression of hypophosphorylated, active form of Rb (pRb), which leads to disruption of PCNA association with chromatin, and thus inhibition of DNA synthesis. Furthermore, data presented here argue for an additional, Rb-independent pathway through which $p21^{Cip1}$, possibly via inhibition of Cdk2-cyclin kinase, can regulate PCNA activity and DNA replication. Pre-Rc, prereplication complex.

duced by such S phase–specific anticancer agents may have potential practical implications for cancer therapy. These observations predict that conditions that lead to increase in intracellular cAMP would reduce the efficacy of such drugs against tumor cells. For instance, it is known that lymphocytes and macrophages are attracted to sites of neoplasia (Witz, 2001). At these sites, tumors stimulate macrophages to secrete a number of regulatory molecules including PGE_2 (Alleva *et al.*, 1993; Brum-Fernandes *et al.*, 1996; Elgert *et al.*, 1998). Most effects of PGE_2 are mediated by the PGE_2 receptors whose activation leads to elevation of intracellular cAMP (Brum-Fernandes *et al.*, 1996; Kiriyama *et al.*, 1997). Thus, it can be postulated that tumor-induced secretion of PGE_2 by macrophages might elevate cAMP levels inside tumor cells and render them resistant to S phase–specific antitumor drugs. Accordingly, a combinatorial approach, consisting of an S phase–specific antitumor agent and cAMP antagonists, can be considered as an effective regime to increase the sensitivity of tumor cells to such drugs.

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