Forskolin-mediated G1 arrest in acute lymphoblastic leukaemia cells: phosphorylated pRB sequesters E2Fs

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Summary

Increased intracellular levels of cAMP, induced by forskolin, lead to permanent G1 arrest of Reh cells. As expected, we observed a rapid dephosphorylation of the retinoblastoma protein (pRB) within 2 hours of forskolin treatment concomitant with reduced activity of the pRB-specific kinases. Interestingly, however, the dephosphorylation of pRB, as well as the inhibition of the kinase activities, was only transient, despite the permanent arrest of cells in G1. Importantly, although the pRB-specific kinases were fully active after 48 hours, pRB became only partially rephosphorylated.

The transient dephosphorylation of pRB could be explained by the transient decrease in the activities of the pRB-specific kinases, but to understand why pRB became only partially rephosphorylated, despite fully activated kinases, we postulated that cAMP could activate a pRB-directed phosphatase. It was therefore interesting to find that the phosphatase inhibitor, tautomycin, was able to abolish the forskolin-mediated dephosphorylation of pRB, without increasing the activities of the pRB-specific kinases.

To understand how Reh cells expressing hyperphosphorylated forms of pRB can remain arrested in G1, we used three different methods to test for the ability of pRB to form functional complexes with the family of E2F transcription factors. As expected, we observed an increased complex formation between E2F-1, E2F-4 and pRB after 2 hours when pRB was in its most dephosphorylated state. Surprisingly, however, prolonged treatment with forskolin, which induced partial rephosphorylation of pRB, in fact further increased the complex formation between the E2Fs and pRB, and this also resulted in reduced E2F-promoter activity in vivo. These data imply that in Reh cells, partially phosphorylated forms of pRB retain the ability to inhibit E2F-promoter activity, and thereby prevent cells from entering into S-phase.

Key words: Forskolin, G1-arrest, pRB, E2F

Introduction

Normal proliferation of eukaryotic cells depends on a precisely controlled regulation of the cell-cycle machinery. The uncontrolled proliferation characteristic of cancer cells is reflected by frequent mutations in genes that regulate the cell cycle (for a review, see Bishop, 1987; Takahashi et al., 1991). The regulation of cells entering from the G1 phase of the cell cycle into S phase is particularly important (Sherr, 1996), as the cells normally must pass through a restriction point in late G1 to progress to the S phase (Pardee, 1974; Pardee, 1989). The central event in this process is the phosphorylation of the retinoblastoma gene product, pRB, leading to E2F-mediated transcription of S phase genes (Weinberg, 1995). The immediate upstream regulators of pRB phosphorylation are D-cyclins, cyclin-dependent kinases 4 and 6 (CDK4 and CDK6, respectively) and CDK inhibitors (CKI) of the INK4 family (Buchkovich et al., 1989; DeCaprio et al., 1992; Weinberg, 1995). It appears that this 'RB pathway' is mandatory for preventing neoplastic growth, as most if not all cancers harbour mutations in one of the genes involved in this pathway (Maelandsmo et al., 1996; Pokrovskaja et al., 1996; Sellers and Kaelin, Jr, 1997; Easton et al., 1998).

The retinoblastoma protein is a member of a family of pocket proteins, including p107 (Ewen et al., 1991) and p130 (Hannon et al., 1993; Mayol et al., 1993). Together with pRB itself, these proteins regulate the activity of the E2F family of transcription factors (Dyson, 1998). The RB protein binds directly to E2F and represses E2F-mediated transcription, a function that is prevented by the phosphorylation of pRB by CDKs (Hatakeyama et al., 1994; Lundberg and Weinberg, 1998). Transfection experiments have shown that overexpression of E2Fs or CDKs promote S-phase entry (Johnson et al., 1993), whereas overexpression of the pRB family of proteins leads to G1 arrest (Goodrich et al., 1991; Qin et al., 1992). The growth-inhibitory function of pRB, p107 and p130 maps to the domain known to involve E2F binding (Weintraub et al., 1992; Sellers et al., 1995), suggesting that the inhibitory effects of the pRB family proteins on proliferation are mediated by their suppressive effects on E2F-induced gene expression.

Numerous studies have shown that inhibition of cell-cycle progression of both normal and malignant cells frequently occurs in G1, linking this to an inhibition of pRB phosphorylation (Miyatake et al., 1995; Li et al., 1997; Kawamata et al., 1998). Using normal human lymphocytes we have previously shown that increased levels of cAMP lead to inhibition of pRB phosphorylation and arrest of cells in G1 (Naderi et al., 2000). By contrast, we observed that cAMP-

mediated G1 arrest of a cell line derived from an acute lymphoblastic leukaemia, Reh, was accompanied by a transient inhibition of pRB phosphorylation (Christoffersen et al., 1994; Naderi and Blomhoff, 1999). In these cells, cAMP, through activation of PKA, induced dephosphorylation of pRB within 2 hours of treatment, followed by partial rephosphorylation of pRB within the next 24 hours (Naderi and Blomhoff, 1999).

To investigate the mechanisms responsible for forskolin-mediated transient dephosphorylation of pRB, we examined the activity of pRB-specific kinases after forskolin treatment of Reh cells. Forskolin inhibited the activity of these kinases, the kinetics of which seemingly paralleled the dephosphorylation of pRB. This inhibition was transient, because within 72 hours of treatment the activity of these kinases was restored to the levels found in untreated cells. Interestingly, these cells, despite the presence of active CDKs, failed to phosphorylate pRB to the levels found in control cells and exhibited only a partially rephosphorylated pRB. These observations raised the following two questions that we have addressed in this study: how can pRB remain only partially rephosphorylated when the CDKs are fully active, and how can the Reh cells expressing phosphorylated forms of pRB remain permanently arrested in G1?

Materials and Methods
Reagents and antibodies
Forskolin and tautomycin were purchased from CALBIOCHEM, La Jolla, CA. FITC-conjugated anti-bromo-deoxyuridine (anti-BrdU) was purchased from Pharmingen, San Diego, CA. Propidium iodide (PI) and BrdU were purchased from Sigma. Anti-cyclin E (HE12 for western blot analysis and HE111 for immunoprecipitation), anti-cyclin A (C-19), anti-cyclin D2 (34B1-3), anti-cyclin D3 (C-16 for western blot analysis and D-7 for immunoprecipitation), anti-cdk2 (M2-G), anti-cdk4 (C-22), anti-cdk6 (C-21), anti-p21^{Cip1} (C-19), anti-p27^{Kip1} (C-19), p130 (C-20)X, p107 (C-18)X, E2F-1(C-20)X, E2F-4 (A-20 for western blot analysis and C-20X for EMSA) and DP-1 (K-20)X were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-pRB (G3-245 for western blot analysis and XZ133 for EMSA), anti-cyclin A (BF683) was obtained from Pharmingen. E2F-1 Gel Shift Oligonucleotide (sc-2507) was purchased from Santa Cruz Biotechnology. Phospho-Rb (Ser 780, Ser 795, Ser 807/811) antibodies were purchased from NEB (New England BioLabs) and phospho-Rb (Thr 821) was a kind gift from Steve Crouse, Biosource International (Camarillo, CA).

Cell culture of Reh cells
The B-lymphoid precursor cell line Reh was originally derived from a patient with acute lymphoblastic leukaemia (Rosenfeld et al., 1977) and was kindly provided by M. F. Greaves (Imperial Cancer Research Fund Laboratories, London, UK). The cells were cultured at a density between 0.15×10^6 and 1.5×10^6 cells/ml in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM glutamine, penicillin (125 U/ml) and streptomycin (125 µg/ml) at 37°C in a humidified incubator with 5% CO_2.

Assessment of cell proliferation
Cell counting was measured through determination of DNA synthesis and was analysed by incorporation of [^{3}H]thymidine into DNA. Cells were cultured in microtiter plates at an initial density of 1×10^4 cells/0.2 ml, and were pulsed with 0.2 µCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) for the last 20 hours of a 72 hour incubation. The cells were then harvested and counted on a cell harvester and scintillation counter (Topcount; Packard).

Flow cytometric analysis of cell-cycle distribution
The cell-cycle distribution of forskolin-treated Reh cells was assessed by pulse labelling the cells with BrdU 1 hour before harvesting. The cells were fixed in 70% ethanol before staining the cells with a FITC-conjugated anti-BrdU antibody and PI (Ohtani, 1999). The cell-cycle distribution was analysed using a FACScan flowcytometer (Becton Dickinson, San Jose, CA) according to the manufacturer's procedure.

Immunoblot analysis
The expression of the different cell-cycle regulatory proteins was carried out by immunoblot analysis as described by Naderi and Blomhoff (Naderi and Blomhoff, 1999).

Detection of different phosphorylation forms of RB was performed by preparing whole cell extracts as described by Naderi and Blomhoff (Naderi and Blomhoff, 1999). Equal amounts of lysate (50 µg) were run on a 10% SDS-PAGE and the resolved proteins were transferred to a nitrocellulose membrane (Amersham) using a semidry transfer cell (Bio-Rad, Hercules, CA). Unspecific sites were blocked by incubating the membrane in blocking buffer (1×Tris buffered saline (TBS), 0.1% Tween with 5% nonfat dry milk) for 1 hour, followed by washing the blot three times in 1×TBS containing 0.1% Tween (TBST). The immunoblot was then incubated at 4°C overnight with primary antibody diluted 1:1000 in blocking buffer. After washing the immunoblot three times in TBST, the blot was incubated for 1 hour at room temperature with horse radish peroxidase (HRP)-conjugated secondary antibody diluted 1:7000 in blocking buffer. The immunoreactive proteins were visualised with the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Immunoprecipitation
For immunoprecipitation of E2F-1 and E2F-4, cell pellets were resuspended in Triton X-100 lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 10 µg/ml leupeptin, 9.5 µg/ml aprotinin, 35 µg/ml phenylmethylsulfonyl fluoride, 5 mM NaF, 0.1 mM orthovanadate, 10 mM β-glycerophosphate). The samples were placed on ice and vortexed at 5 minute intervals for 20 minutes. After removing the insoluble material by centrifugation, the lysates were precleared by incubation with 25 µl of a 1:1 slurry of protein G-sepharose (Pharmacia, Sweden) for 30 minutes at 4°C. The protein content was assessed by the Bradford method (Bio-Rad). Cell lysates (600 µg) were immunoprecipitated with the appropriate antibody (2 µg/sample) for 2 hours at 4°C. The immunocomplexes were absorbed to 30 µl of a 1:1 slurry of protein G-sepharose for 1 hour at 4°C, collected by centrifugation at 2,000 *g* for 5 minutes and washed twice with the appropriate lysis buffer. The beads were then resuspended in 1×SDS sample buffer and boiled, and the proteins were subjected to SDS-PAGE and immunoblot analysis as described by Naderi and Blomhoff (Naderi and Blomhoff, 1999).

Kinase assays
Measurements of cyclin E- and cyclin A-associated kinase activity were performed essentially as described by Naderi and Blomhoff (Naderi and Blomhoff, 1999), using histone H1 as substrate.

Cyclin D3-associated kinase activity was analysed using glutathione-S-transferase-tagged RB (amino acids 769-921), denoted GST-RB (Santa Cruz Biotechnology, Santa Cruz, CA), as substrate. Whole cell extracts were prepared by sonication in equal amounts of lysis buffer (50 mM HEPES pH 7.5, 50 mM NaCl, 10% glycerol,

0.1% Tween 20, 1 mM dithiothreitol (DTT), 20 mM Na-pyrophosphate, 50 mM NaF, 0.3 mM orthovanadate, 80 mM β-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml antipain, 10 µg/ml chymostain, 10 µg/ml pepstatin A, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 µg/ml Benzamidin). The supernatant after centrifugation was precleared by incubation with 25 µl of a 1:1 slurry of protein G-sepharose (Pharmacia, Sweden) for 30 minutes at 4°C and analysed for protein content by the Bradford method (Bio-Rad). Cell lysate (1 mg) was immunoprecipitated with the appropriate antibody (2 µg/sample) for 2 hours at 4°C. The immunoreactive complexes were absorbed to 30 µl of a 1:1 slurry of protein G-sepharose for 1 hour at 4°C, collected by centrifugation at 2000 g for 5 minutes. The immunoreactive complexes were washed four times with lysis buffer and once in 50 mM HEPES (pH 7.5) containing 1 mM DTT. The washed complexes were resuspended in 30 µl of kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl$_2$, 1 mM DTT, 2.5 mM EGTA, 1 mM NaF, 0.3 mM sodium orthovanadate, 10 mM β-glycerophosphate, 20 µM ATP) and the kinase reactions were initiated by adding 10 µCi of [γ-^{32}P]ATP and 2 µg of GST-RB (amino acids 769-921, Santa Cruz Biotechnology) to each reaction. After 30 minutes at 30°C, the reactions were stopped by adding 15 µl 3×SDS sample buffer. The samples were boiled for 5 minutes and subjected to SDS-PAGE. Following electrophoresis, gels were stained with Coomassie blue, dried and subjected to autoradiography.

Electrophoresis mobility shift assay (EMSA)

Whole-cell extracts were prepared essentially as described by Pagano et al. (Pagano et al., 1992); 20×10^6 Reh cells were collected, washed once in phosphate-buffered saline (PBS) and resuspended in 200 µl of lysis buffer A (20 mM HEPES pH 7.9, 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM DTT and 1 mM PMSF). Cells were incubated for 20 minutes on ice, frozen in liquid nitrogen and placed at –70°C. Cell extracts were thawed on ice and the suspensions were vigorously mixed, followed by centrifugation for 10 minutes at 13,000 g. The supernatant was collected and the protein content measured by the Bradford method (Bio-Rad). Total lysate, 20 µg, was incubated with approximately 0.5 ng (40,000 c.p.m.) of ^{32}P-labelled double-stranded oligonucleotide, containing a consensus E2F-binding site (Santa Cruz Biotechnology), in a final volume of 30 µl. Sonicated salmon sperm DNA (10 µg) was added as competitor DNA. Incubations were carried out at room temperature for 20 minutes in 20 mM HEPES, 20 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 1 mM DTT, 1 mM PMSF and 20 µg BSA (Lam and Watson, 1993). Supershift assays were performed by incubating the reaction mixture with the appropriate antibody, on ice for 1 hour, before adding the labelled oligonucleotide. The DNA-protein complexes were resolved on a 4% polyacrylamid gel in 0.25×TBE (Tris borate-EDTA) buffer at 4°C, and the protein/DNA complexes were visualised by autoradiography.

Promoter reporter assay

Exponentially growing Reh cells were transiently transfected with the following luciferase constructs: pGL3TATAbasic-6xE2F (pGL3 containing a TATA box and six E2F binding sites, 5′-TTTCGCGCTTAA-3′, kindly provided by Ali Fattaey, Onyx Pharmaceuticals, Richmond, CA) and a pGL3TATAbasic (an E1b TATA box cloned into pGL3, Promega). An SV40-βGal construct (Promega Corp., E1081) was cotransfected and used as an internal control. Transfection was performed by electroporation at 250 V and 950 µF. The electroporated cells were transferred to fresh medium and incubated for 20 hours before the cells were treated with forskolin. Cells were harvested and resuspended in reporter lysis buffer, 20 µl/million cells, (Promega Corp., E3971). Luciferase activity was measured according to the manufacturer's protocol (Promega Corp., E1501) and by using an LKB Wallac 1251 luminometer (LKB,

Helsinki, Finland). The β-galactosidase activity was analysed according to the manufacturer's protocol (Promega Corp., E2000), where the absorbance at 405 nm was measured by spectrophotometry (Labsystems Multiscan, Bichromatic). Luciferase activity was normalized to β-galactosidase activity.

Results

Forskolin induces permanent accumulation of cells in G1

To show that forskolin (100 µM) inhibits proliferation of Reh cells, three different methods were used: [^{3}H]thymidine incorporation, direct counting of cells and flowcytometric analysis of BrdU-labelled and PI-stained cells. As shown in Fig. 1A, the incorporation of [^{3}H]thymidine was reduced by 75%-80% after 72 hours of forskolin treatment. Furthermore, treated and untreated cells were counted daily for 10 days (Fig. 1B). The number of cells treated with forskolin remained essentially at 0.2×10^6 cells/ml over a period of 10 days,

A

B
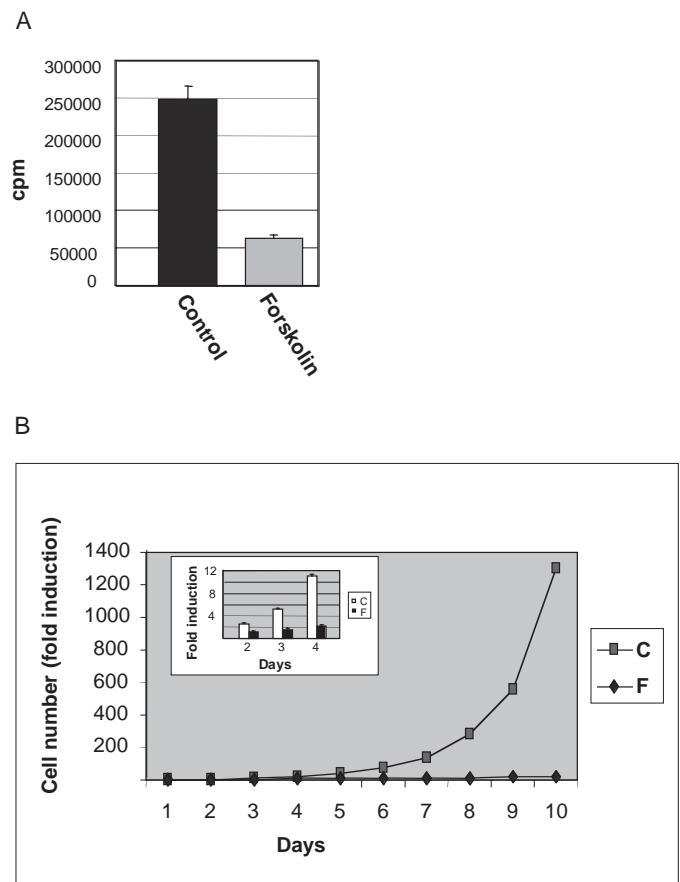
Fig. 1. Effect of forskolin on DNA synthesis. (A) Reh cells (0.1×10^5 cells/ml) were treated with forskolin (100 µM) for 3 days and DNA synthesis was measured as uptake of [^{3}H]thymidine, as described in Materials and Methods. Vertical bars indicate the s.e.m. of three experiments. (B) Reh cells (0.1×10^6 cells/ml), with or without forskolin (100 µM) treatment, were counted for 10 days and the cell counts are presented as fold induction of the starting culture (0.1×10^6 cells/ml). One representative experiment is shown. Inset shows the cell counts up to 4 days with or without forskolin (100 µM) treatment. The cell counts are presented as the mean from five separate experiments. The vertical bars represent standard error of the mean (s.e.m.). C, control; F, forskolin.

Table 1. Forskolin-mediated cell-cycle arrest measured by BrdU incorporation and PI staining

	A Forskolin (hours)					B Forskolin (days)	
	0	2	8	48	72	5	10
G1	53±0.88	55±0.19	60±0.19	63±1.34	69±1.16	77	77
S	39±0.84	33±0.33	27±0.19	28±0.96	21±0.83	5	6
G2	8±0.19	13±0.36	13±0.33	9±0.57	10±0.57	18	17

Cells were treated with or without forskolin (100 μM) for the indicated hours up to 72 hours (A) or up to 5 or 10 days (B). The cell-cycle distribution was assessed by flowcytometric analysis of cells stained with FITC-conjugated anti-BrdU and PI as described in Materials and Methods. The percentage of the cells in G1, S and G2/M is shown. In A, the mean±s.e.m. of five experiments is shown, whereas B shows one representative experiment of three.

whereas the untreated cells proliferated in an exponential manner and increased from $0.1×10^6$ to $1.15×10^6$ cells/ml in 4 days. The inability of forskolin-treated Reh cells to increase in number indicated that forskolin permanently blocked cell proliferation. To confirm this observation further, we treated Reh cells with forskolin and labelled the cells with BrdU to assess their cell-cycle distribution by flowcytometry. As shown in Table 1, after 72 hours of forskolin treatment the percentage of cells in G1 had increased from 53% to 69%. The percentage of cells in G1 increased even further after prolonged treatment with forskolin, as 77% of the cells were in G1 after 10 days of forskolin treatment, whereas less than 6% of the cells were in S-phase. The observed cell-cycle inhibition was not due to apoptosis because less than 10% of the cells were apoptotic after 72 hours of forskolin treatment as measured by TUNEL assays and scatter analysis (data not shown).

Forskolin induces transient dephosphorylation of pRB

We have previously shown that forskolin treatment of Reh cells leads to a rapid, but transient dephosphorylation of pRB (Christoffersen et al., 1994; Naderi and Blomhoff, 1999). Consistent with our previous results, we observed a rapid dephosphorylation of pRB after 2 hours of forskolin treatment, followed by a rephosphorylation notable after 24 hours (Fig. 2A). However, the rephosphorylation was only partial, as the fully phosphorylated forms of pRB

were less apparent in forskolin-treated cells. Interestingly, pRB remained partially rephosphorylated 72 hours after addition of forskolin (Fig. 2A), at the time when the cells were growth-inhibited and accumulated in G1 (Fig. 1; Table 1).

To identify the various migrating forms of pRB that we observed in Fig. 2A, different controls were run together with lysates from Reh cells treated with or without forskolin for 2 hours (Fig. 2B). As a control for unphosphorylated pRB, we used lysates from unstimulated normal T-lymphocytes, known to be in G0. To rule out the possibility that the lower molecular weight form was not equivalent to the C-terminally cleaved form of pRB seen in apoptotic cells, we included lysates from Jurkat cells induced to undergo apoptosis by stimulation with the anti-Fas antibody, CH11, for 4 hours (Tan et al., 1997). As a source for fully phosphorylated form of pRB, we used lysates from normal T-lymphocytes activated into late parts of the cell cycle (S/G2/M) by stimulation with phytohemagglutinin-P (PHA)/ionomycin for 50 hours. From Fig. 2B it is evident that

Fig. 2. Forskolin mediates a transient dephosphorylation of pRB. Reh cells were treated with forskolin (100 μM) for the hours indicated. Total cell lysate (50 μg) from treated and untreated cells were subjected to immunoblot analysis as described in Materials and Methods (A), using monoclonal antibodies towards pRB. (B) Anti-RB immunoblotting of lysates from resting T-lymphocytes (lane 1), Reh-control lysate (lane 2), forskolin-treated Reh-cells for 2 hours (lane 3), PHA/ionomycin-activated T-lymphocytes for 50 hours (lane 4) and anti-Fas antibody (CH-11)-treated jurkat wt cells for 4 hours (lane 5). (C) An immunoblot with forskolin-treated Reh-lysates, as described above, was detected with the different anti-pRB-phosphorylated antibodies, as indicated in the figure. Total expression of pRB was detected on the same blot as a control for equal loading of protein in each lane. One representative experiment of five is shown.
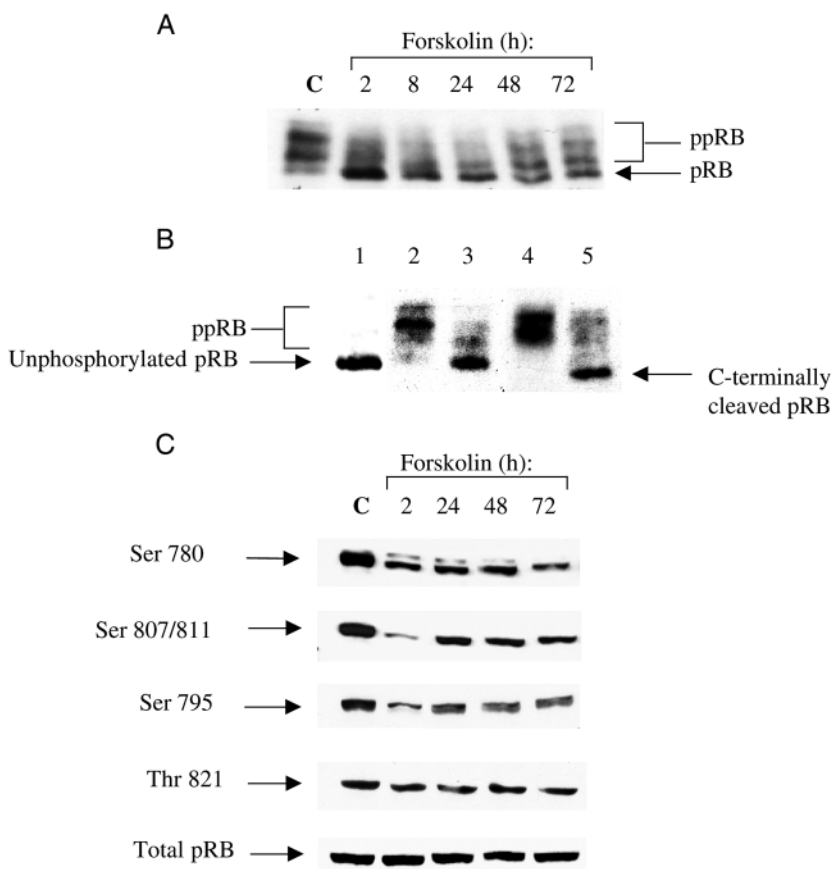
the lower molecular weight form of pRB, appearing rapidly after forskolin treatment, migrates as the unphosphorylated form of pRB found in resting T cells and not as the cleaved form of pRB.

To examine whether the partial rephosphorylation of pRB seen in Fig. 2A was due to some sites being permanently dephosphorylated, we performed immunoblot analysis of pRB using different antibodies directed against specific phosphorylated amino acids in the C-terminal region of pRB. It has been shown that several residues in this region are involved in the regulation of E2F-binding (Knudsen and Wang, 1997), and that several, if not all of these sites needs to be phosphorylated to disrupt the binding of E2F (Lundberg and Weinberg, 1998; Brown et al., 1999; Harbour et al., 1999). We therefore selected four commercially available antibodies directed against phosphorylated serine and threonine residues in this region (Ser 780, Ser 795, Ser 807/811, Thr 821). As shown in Fig. 2C, all four amino acids were phosphorylated in continuously growing cells. Ser 780, Ser 795 and Ser 807/811 were subjected to dephosphorylation at 2 hours of forskolin treatment, whereas only minor changes were observed in the case of Thr 821. Of note is that the dephosphorylation of Ser 780, Ser 795 and Ser 807/811 was transient, but the rephosphorylation of these sites was only partially restored after prolonged forskolin treatment. Apparently, none of these sites were permanently dephosphorylated.

Taken together, we believe that after 2 hours of forskolin treatment the pRB-population consists mainly of unphosphorylated and hypophosphorylated forms. After 48-72 hours of forskolin treatment we observe a reduction in the unphosphorylated and the most hypophosphorylated form of pRB, concomitant with an increase in the pRB-population that is rephosphorylated at several of the C-terminal residues. However, the amount of fully or hyperphosphorylated forms of pRB after 72 hours of forskolin treatment must be limited, as Fig. 2C shows that at least three of the C-terminal residues are not phosphorylated in a large portion of the pRB molecules.

Forskolin induces a transient reduction in cyclin-associated kinase activity

To test whether the rapid dephosphorylation of pRB followed by incomplete rephosphorylation could be explained by the regulation of the activity of pRB-specific kinases, we analysed the effect of forskolin on various CDKs. As shown in Fig. 3, forskolin transiently inhibited the activity of the different cyclin-associated kinases. The activity of cyclin D3- and cyclin E-associated kinases were reduced threefold after 8 hours of forskolin treatment. However, the activity of these two kinases was fully restored after 72 hours. Cyclin A-associated kinase (Fig. 3) and CDK1 (data not shown) exhibited an alternating pattern of activity. At 2 and 24 hours post treatment these kinases showed a twofold reduction in activity, whereas at 8 and 72 hours post treatment, their activity was restored to control levels. Taken together, these results suggest that the immediate dephosphorylation of pRB by forskolin involves the reduction in the CDK kinase activity. However, as the dephosphorylation of pRB seen in Fig. 2A seems to be faster than the inhibition of the overall kinase activity, this may support our findings, presented below, that forskolin also

activates a pRB-specific phosphatase. This would also explain why we observe that pRB is only partially rephosphorylated after 48-72 hours of forskolin treatment, despite fully active CDKs.

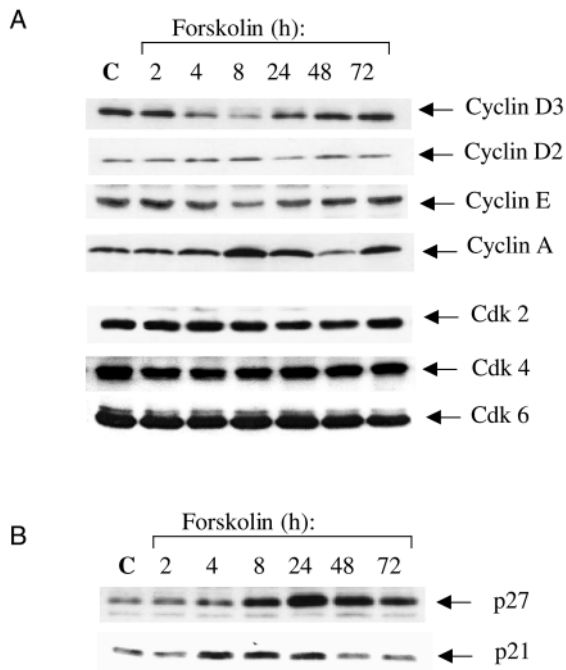### Effect of forskolin on protein expression of G1 cyclins, CDKs and CKIs

To understand how forskolin induces the transient inhibition of the CDK activities, we analysed the expression of the various cyclins, CDKs and CKIs. As shown in Fig. 4A, the expressions of cyclin D3 and cyclin E followed the activity level of their respective kinases. Within 2-8 hours of forskolin treatment, the level of these cyclins was reduced compared with untreated cells, whereas after 72 hours the expression levels were restored to the levels seen in untreated cells. Cyclin A expression generally showed the same cycling appearance as the activity of its associated kinase (Fig. 3). The protein levels of CDK 2, 4 and 6 were not altered during forskolin treatment (Fig. 4A), whereas the expression of both p21^{Cip1} and p27^{Kip1} were transiently increased with maximum levels at 8 and 24 hours, respectively (Fig. 4B).

The phosphatase 1 inhibitor, tautomycin, abolishes the effect of forskolin on RB-phosphorylation

Despite full restoration of the activity of pRB-specific kinases, Reh cells treated with forskolin for more than 24 hours contained only partially rephosphorylated pRB (Fig. 2). This observation suggested that forskolin-induced dephosphorylation of pRB also could involve activation of a protein phosphatase. To address this possibility, we examined the effect of several known inhibitors of PP1 and PP2A (okadaic acid, caliculin and tautomycin) on forskolin-mediated dephosphorylation of pRB. All the inhibitors tested completely abolished the effect of forskolin (okadaic acid and caliculin, data not shown). In Fig. 5A, pretreatment of Reh cells with the most specific of the PP1 inhibitors, tautomycin, prevented the forskolin-mediated dephosphorylation of pRB observed after
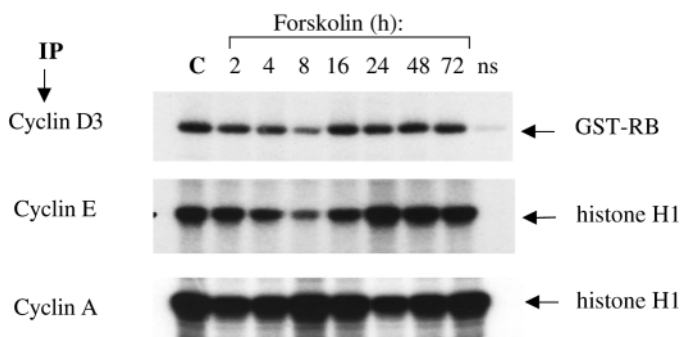
Fig. 3. Forskolin induces a transient inhibition of the cyclin-associated kinase activities. Reh cells were treated with forskolin (100 μM) for the times indicated. Total cell lysates (500 μg) were subjected to immunoprecipitation (IP) with the appropriate antibodies, and kinase assays were performed as described in Materials and Methods, using histone 1 or GST-RB as substrate. One representative experiment of three is shown. ns, nonspecific antibody used as a negative control.

Fig. 4. Effect of forskolin on the expression of G1 cyclin, CDK and CKI proteins. Reh cells were treated with forskolin (100 μM) for the hours indicated. Total cell lysates (50 μg) from treated or untreated cells were subjected to immunoblot analysis, as described in Materials and Methods, using antibodies specific for (A) cyclins and CDKs and (B) CKI proteins. One representative immunoblot of three is shown.

4 hours. Furthermore, tautomycin alone also induced hyperphosphorylation of pRB.

To exclude the possibility that the inhibitory effect of tautomycin on forskolin-mediated dephosphorylation of pRB was due to activation of the pRB-specific kinases, we measured the effect of tautomycin on the CDK activity. As shown in Fig. 5B, the cyclin-associated kinase activities were, in fact, reduced rather than activated by tautomycin, as were the expression of the cyclins also (Fig. 5C). Taken together, our results therefore suggest a role for protein phosphatase 1 or 2A, or both, in cAMP-mediated dephosphorylation of pRB.

## Transient dephosphorylation of p130 and p107 by forskolin

The pocket proteins p107 and p130 also showed a transient dephosphorylation after forskolin treatment, as did pRB. As shown in Fig. 6, a rapid dephosphorylation was evident after 2 hours of forskolin treatment and a rephosphorylation appeared after 24 hours of treatment. The rephosphorylation of p107 and p130 was only partial after 72 hours of forskolin treatment.

E2F-RB complex formation is increased by forskolin

Both pRB itself and the related pocket proteins, p130 and p107, exert their growth-inhibitory effects by binding to members of the E2F/DP1 family of transcription factors and leading to repression of transcription from E2F-regulated promoters (Chellappan et al., 1991; Weinberg, 1995; Harbour and Dean,
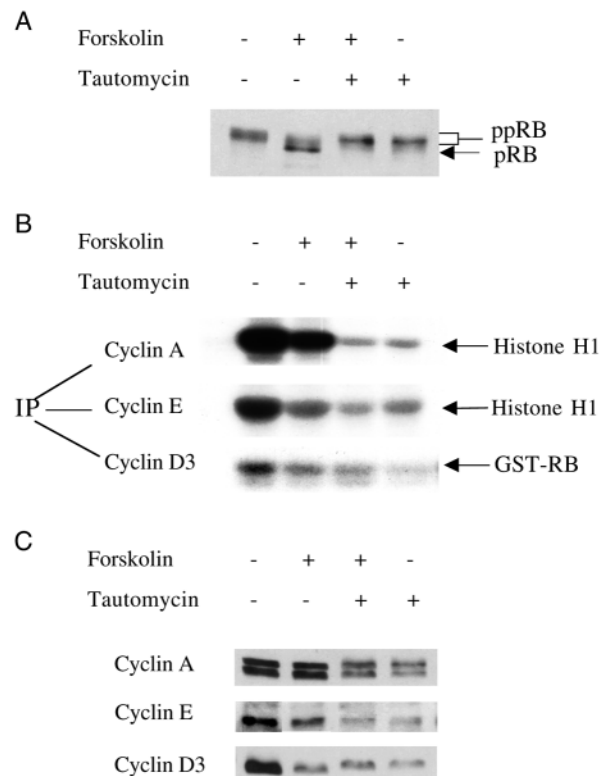

Fig. 5. Inhibitors of phosphatase 1 and 2A prevent forskolin-mediated dephosphorylation of pRB. Reh cells were treated for 4 hours with forskolin (100 μM), in the presence or absence of tautomycin (10 μM). Total cell lysate (50 μg) from treated and untreated cells were subjected to immunoblot analysis (as described in Materials and Methods), using antibodies specific for (A) pRB, or (C) cyclins as indicated. (B) For analysis of kinase activity, 500 μg of total cell lysate was subjected to immunoprecipitation (IP) with the appropriate antibodies, and kinase assays were performed, as described in Materials and Methods, using histone 1 or GST-RB as substrate. One representative experiment of three is shown.
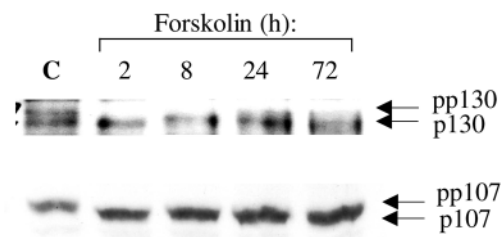
Fig. 6. Effect of forskolin on the expression of p130 and p107. Reh cells were treated with forskolin (100 μM) for the hours indicated. Total cell lysate (50 μg) from treated and untreated cells were subjected to immunoblot analysis (see Materials and Methods), using antibodies specific for p130 (upper) and p107 (lower). One representative immunoblot of three is shown.

2000). It is generally believed that pRB binds to E2Fs in its hypophosphorylated form, and that the binding is disrupted when pRB is hyperphosphorylated (Knudsen and Wang, 1997; Helin, 1998). As we showed that pRB (Fig. 2A), as well as p107 and p130 (Fig. 6), was partially rephosphorylated by prolonged treatment of forskolin, we wished to examine
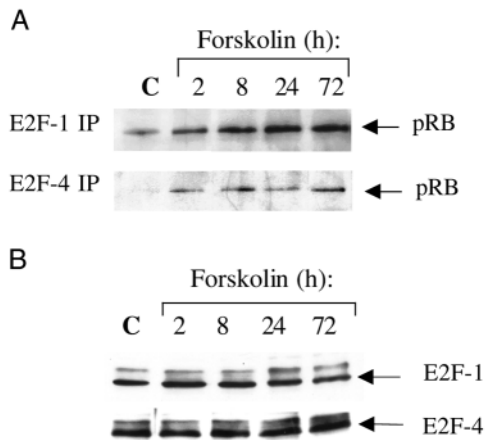
Fig. 7. Co-immunoprecipitation of pRB with E2F-1 and E2F-4 antibodies. Reh cells were treated with forskolin (100 µM) for the hours indicated. (A) Total cell lysate (500 µg) from treated or untreated cells were subjected to immunoprecipitation as described in Materials and Methods, using antibodies specific for E2F-1 or E2F-4 as indicated. The E2F-complexes were resolved on a 10% SDS-PAGE and the resulting immunoblots were detected with antibodies specific for pRB. (B) 30 µg of the same cell lysates were subjected to immunoblot analysis to detect the total amount of E2F-1 or E2F-4 as indicated. One representative experiment of three is shown.

whether these pocket proteins were still able to bind E2Fs. We used two different methods to test the complex formation between E2Fs and pRB, i.e. co-immunoprecipitation and gel retardation assay. Using the first method, E2F-1 and E2F-4 were immunoprecipitated and the resulting immunoblots were incubated with antibodies against pRB. As shown in Fig. 7A, complexes between pRB and E2F-1 or E2F-4 were noted already after 2 hours of forskolin treatment, but interestingly, the amount of pRB bound to both E2F-1 and E2F-4 increased with longer exposure of the cells to forskolin. As E2F-4 has also been shown to bind to other pocket proteins, such as p130 and p107 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Vairo et al., 1995), we also examined the complex formation between p130 or p107 and the E2F-4 proteins. The complex formation essentially followed that of pRB-E2F-4, but the amount of p130 or p107 associated with E2F-4 appeared to be much less than the amount of pRB bound to E2F-4 (data not shown). No complexes between p130 or p107 and E2F-1 were observed (data not shown). Notably, the expression levels of E2F-1 and E2F-4 remained unaffected by forskolin treatment (Fig. 7B).

In the second method, we used gel retardation assay to examine E2F-complexes following treatment of the cells with forskolin. Total extracts were incubated with radioactive labelled E2F-specific oligonucleotides and the resulting complexes were separated on a native polyacrylamid gel. We detected three specific complexes (I, II and III in Fig. 8A). All three complexes were differentially regulated by forskolin, as the formation of complex III decreased upon exposure to forskolin, concomitantly with increased formation of complexes I and II.

The composition of the different complexes were determined by gel retardation supershift assays, using a panel of specific antibodies directed against proteins known to form complexes with E2Fs and DPs. As shown in Fig. 8B, complex III was shifted by antibodies directed against E2F-1 and E2F-4, but not by antibodies specific for pRB. This complex was, to a certain extent, also shifted by antibodies specific for DP1 (Fig. 8C), but remained unaffected by antibodies directed against p107 or p130 (Fig. 8C). This suggests that complex III consists of E2F-1/E2F-4 and DP-1. Complex II was shifted by antibodies against pRB, E2F-1, E2F-4 (Fig. 8B) and DP-1 (Fig. 8C), but not by antibodies against p107 and p130 (Fig. 8C), and therefore represents the complex between pRB and E2F-1 or E2F-4, including DP-1. Complex I, however, was shifted by antibodies against p107, p130, E2F-4 and cyclin A (Fig. 8B,C).

Taken together, the gel retardation assay showed that the complex formation between pRB and E2F-1 or E2F-4 increases after 72 hours of forskolin treatment, at the time when pRB is partially rephosphorylated (Fig. 2A). Furthermore, complexes between p107 or p130 and E2F-4 are prominent already at 2 hours of forskolin treatment, and this complex formation is further increased upon longer exposure to forskolin (72 hours). Concomitant with the increased complex formation between pRB and E2Fs, the fraction of free E2Fs bound to the E2F-consensus site is reduced.

Forskolin inhibits E2F-promoter activity in vivo

To verify that the increased complex formation between the pocket proteins and E2Fs resulted in functional inhibition of E2F-mediated transcription, we examined the effect of forskolin on the E2F-promoter activity in vivo using a pGL3-Luciferase reporter construct containing a TATA-6xE2F-promoter fragment or a basic-pGL3TATA-Luciferase construct. As shown in Fig. 9A, the relative luciferase activity was reduced by 10% after 2 hours of forskolin treatment, whereas after 24 (Fig. 9B) and 72 hours (Fig. 9C) of treatment the E2F-promoter activity was reduced by 40% and 46%, respectively. Thus, we can conclude that the increased complex formation between the pocket proteins and E2F in the presence of forskolin resulted in functional inhibition of E2F-mediated transcription.

Discussion

In the present study we wished to address the mechanisms whereby elevated intracellular levels of cAMP leads to G1 arrest of Reh cells. The initial rapid dephosphorylation of pRB that we observed within the first 2-4 hours after addition of forskolin resembled what we had observed in normal lymphocytes (Naderi et al., 2000), and could be explained by the rapid inhibition of the activity of the relevant CDKs (cyclin D3-, cyclin E- and cyclin A-associated activity). In contrast to what we observed in the normal lymphocytes, prolonged treatment of Reh cells with forskolin induced rephosphorylation of pRB beginning after 24 hours. On the basis of both the phosphorylation pattern of total pRB after 72 hours of forskolin treatment, and by using antibodies directed against specific phosphorylation sites of pRB, we concluded that the rephosphorylation of pRB was only partial. The rephosphorylation of pRB was, however, preceded by complete reactivation of the relevant CDKs. To explain the discrepancy between fully active CDKs and partially rephosphorylated pRB, we postulated that cAMP could activate a pRB-directed

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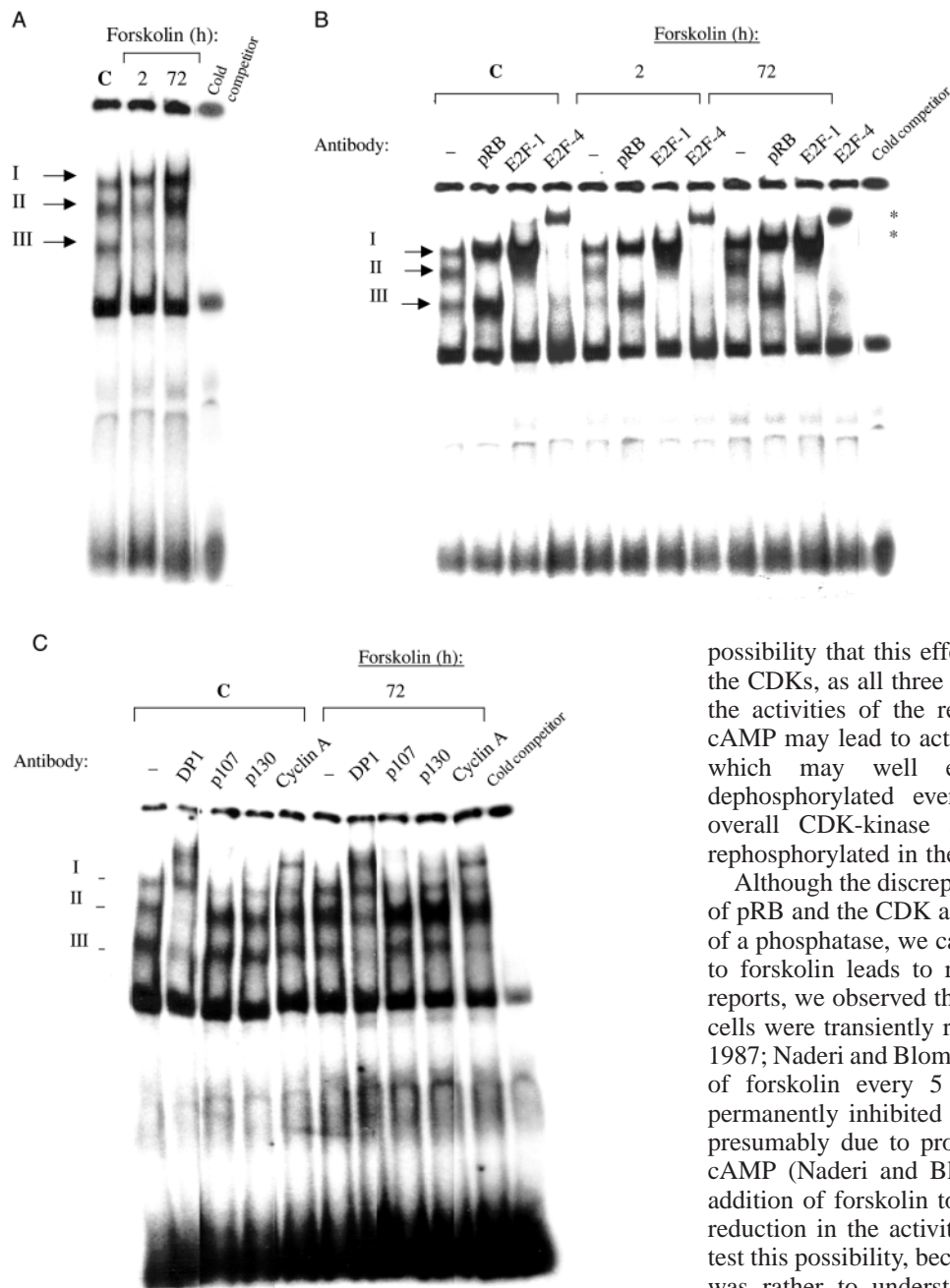
Forskolin (h):

Fig. 8. Gel retardation analysis of complex formation on the E2F-binding site. Reh cells were treated with forskolin (100 μM) for the hours indicated. (A) Total extracts were prepared as described in Materials and Methods and incubated with ^{32}P-labelled oligonucleotide probe containing the E2F-consensus binding site. (B,C) Supershift assays were performed by incubating total cell extract with the appropriate antibodies before adding the probe containing the E2F-binding site. In all three panels, the protein-DNA complexes were subjected to a native 4% polyacrylamid gel electrophoresis and visualised by autoradiography. One representative experiment of three is shown. I, II and III denote the different protein complexes bound to the E2F-probe. The asterisks indicate supershifted bands.

phosphatase. Continuous activation of this putative phosphatase upon prolonged treatment with forskolin would thereby prevent full phosphorylation of pRB even in the presence of active CDKs.

It has been suggested that protein phosphatase 1 (PP1) can interact with pRB in G1 (Liu et al., 1999), and it has been shown that PP1 is the phosphatase responsible for dephosphorylating pRB at the entry of cells into G1 from mitosis (Durfee et al., 1993; Nelson and Ludlow, 1997). To examine the possibility of phosphatases being involved in cAMP-mediated dephosphorylation of pRB, we treated the cells with inhibitors of PP1 and PP2A. The inhibitors, okadaic acid, calliculin and tautomycin, abolished the effect of forskolin on dephosphorylation of pRB. We excluded the possibility that this effect was due to the inhibitors activating the CDKs, as all three phosphatase inhibitors in fact inhibited the activities of the relevant CDKs. The results imply that cAMP may lead to activation of a pRB-directed phosphatase, which may well explain why pRB seems to be dephosphorylated even faster than the inhibition of the overall CDK-kinase activity, and why pRB is partially rephosphorylated in the presence of fully active CDKs.

Although the discrepancy between the phosphorylation state of pRB and the CDK activity could be explained by activation of a phosphatase, we can not explain why prolonged exposure to forskolin leads to reactivation of the CDKs. In previous reports, we observed that also levels of MYC and Mad in Reh cells were transiently regulated by forskolin (Blomhoff et al., 1987; Naderi and Blomhoff, 1999). We showed that re-addition of forskolin every 5 hours would keep the MYC levels permanently inhibited and the Mad levels permanently high, presumably due to prolonged elevated levels of intracellular cAMP (Naderi and Blomhoff, 1999). It is possible that re-addition of forskolin to Reh cells would lead to a permanent reduction in the activity of the CDKs. We did not, however, test this possibility, because the main issue in the present study was rather to understand how cells could be permanently inhibited in G1 when pRB is partially rephosphorylated.

To address this next question, we examined the ability of pRB to bind to the different members of the E2F/DP family of transcription factors, after prolonged treatment with forskolin. It is generally believed that pRB exerts its growth inhibitory effect by binding to the members of the E2F/DP family (Qian et al., 1992; Qin et al., 1992; Ohtani, 1999), and that pRB binds to E2F/DP in its hypophosphorylated state (Bagchi et al., 1991; Chellappan et al., 1991; Knudsen and Wang, 1997; Helin, 1998). Our finding that treatment of Reh cells with forskolin for 2 hours induced binding of pRB to E2Fs was therefore in line with data from other cell systems, having shown that growth inhibition is associated with hypophosphorylated forms of pRB binding to E2Fs (Hatakeyama et al., 1994; Beijersbergen and Bernards, 1996; Wu et al., 2000). The unexpected result was, however, that the partially
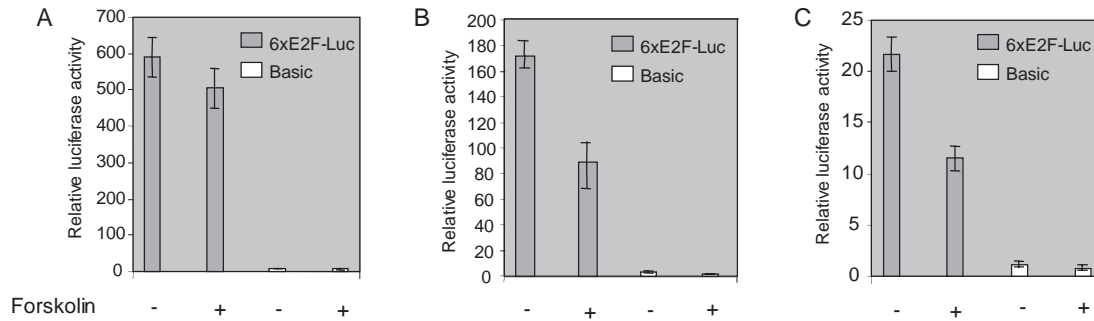
Fig. 9. Forskolin downregulates the E2F-promoter activity. Reh cells were transfected with the pGL3TATA-6xE2F-promoter luciferase construct or with the basic pGL3TATA vector. An SV40-βGalactosidase construct was cotransfected with the luciferase constructs as an internal control. 20 hours after transfection, the cells were treated with and without forskolin (100 µM) for (A) 2 hours, (B) 24 hours and (C) 72 hours. After forskolin treatment the cells were harvested and lysed in reporter lysis buffer. The luciferase activity was determined and normalized for transfection efficiency, with the activity of β-galactosidase being used as a reference. The values are presented as the mean from four experiments, and the vertical bars represent the s.e.m.

rephosphorylated forms of pRB present after 72 hours of forskolin treatment still and even more readily formed complexes with E2F-1 and E2F-4 than did the more dephosphorylated form of pRB present after 2 hours treatment with forskolin. We showed that the complex between pRB and E2F/DP-1 increased after 72 hours, concomitant with a reduction in free E2F/DP-1s. Also, the complexes between cyclin A, p130 or p107 and E2F-4 increased with longer exposure to forskolin. The functional implication of the increased complex formation between the pocket proteins and E2Fs was confirmed by the observed inhibition of E2F-promoter activity in vivo in the presence of high levels of cAMP. This may reflect a cAMP-mediated repression of one or several genes important for the G1-arrest in Reh cells. Interestingly, we can conclude that these genes are not cyclin A or cyclin E because these genes are highly expressed, and their associated kinase activities are fully restored at 72 hours of forskolin treatment.

We cannot at present fully explain the mechanisms behind the increased complex formation between partially rephosphorylated forms of the pocket proteins and E2Fs. The most obvious explanation would be that functionally important phosphorylation sites in the E2F-binding pocket of pRB were permanently dephosphorylated upon prolonged exposure to forskolin. From our results using four commercially available antibodies (Ser 780, Ser 795, Ser 807/811 and Thr 821) directed against different phosphorylation sites on pRB, known to be in a region involved in E2F-binding (Knudsen and Wang, 1997), we could conclude that none of the sites were permanently dephosphorylated in the total pRB-population. However, at least three of these sites seemed to be dephosphorylated in a large portion of the pRB molecules, even after 72 hours of forskolin treatment, and thus may account for the ability of pRB to still bind E2F at this time point. These results agree with the findings that phosphorylation on several residues in the large A/B-pocket of pRB is needed to disrupt the E2F-pRB binding (Knudsen and Wang, 1997). Recent results even indicate that complete phosphorylation of pRB is needed to relieve E2F and allow transcription from E2F-responsive promoters (Lundberg and Weinberg, 1998; Brown et al., 1999; Harbour et al., 1999). Our observation of increased complex formation between pRB and E2F at 72 hours, as compared with 2 hours of forskolin treatment, is supported by recent reports suggesting that E2Fs

more readily form complexes with hypophosphorylated forms of pRB than with unphosphorylated forms of pRB (Ezhevsky et al., 1997; Brugarolas et al., 1999; Ezhevsky et al., 2001). Thus, the fact that the most dephosphorylated forms of pRB became less apparent after 72 hours of forskolin treatment suggest that forskolin induced a shift in the fraction of unphosphorylated forms of pRB to more hypophosphorylated forms.

Taken together, we have shown that forskolin-treated Reh cells are permanently arrested in G1, despite pRB becoming partially rephosphorylated upon prolonged exposure to forskolin. The growth-inhibitory potential of partially rephosphorylated pRB could be explained by inhibited E2F-promoter activity, enforced by the strong ability of rephosphorylated pRB to form complexes with E2Fs.

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