Cyclic AMP Inhibits the Proliferation of Thyroid Carcinoma Cell Lines through Regulation of CDK4 Phosphorylation

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How cyclic AMP (cAMP) could positively or negatively regulate G1 phase progression in different cell types or in cancer cells versus normal differentiated counterparts has remained an intriguing question for decades. At variance with the cAMP-dependent mitogenesis of normal thyroid epithelial cells, we show here that cAMP and cAMP-dependent protein kinase activation inhibit S-phase entry in four thyroid carcinoma cell lines that harbor a permanent activation of the Raf/ERK pathway by different oncogenes. Only in Ret/PTC1-positive TPC-1 cells did cAMP markedly inhibit the Raf/ERK cascade, leading to mTOR pathway inhibition, repression of cyclin D1 and p21 and p27 accumulation. p27 knockdown did not prevent the DNA synthesis inhibition. In the other cells, cAMP little affected these signaling cascades and levels of cyclins D or CDK inhibitors. However, cAMP differentially inhibited the pRb-kinase activity and T172-phosphorylation of CDK4 complexed to cyclin D1 or cyclin D3, whereas CDK-activating kinase activity remained unaffected. At variance with current conceptions, our studies in thyroid carcinoma cell lines and previously in normal thyrocytes identify the activating phosphorylation of CDK4 as a common target of opposite cell cycle regulations by cAMP, irrespective of its impact on classical mitogenic signaling cascades and expression of CDK4 regulatory partners.

INTRODUCTION

Cyclic AMP (cAMP) is the first identified intracellular mediator (second messenger) of hormone action. In the seventies and early eighties, the observation that cAMP elevations may inhibit cell proliferation in various established cell lines, mostly of tumoral origin, has prompted an intense scientific activity, even becoming the main paradigm of cell cycle regulation (Pastan and Johnson, 1974; Pastan *et al.*, 1975; Friedman, 1976; Rebhun, 1977). More recent studies have ascribed the cell cycle inhibition by cAMP to the inhibition of several steps of the mitogenic cascades elicited by growth factors or oncogenic mutations (Roger *et al.*, 1995; Stork and Schmitt, 2002; Dumaz and Marais, 2005). Most generally considered mechanisms include inhibition of ERK1/2 pathway by inhibitory phosphorylations of c-Raf by cAMP-dependent protein kinases (PKA; Cook and McCormick, 1993; Graves *et al.*, 1993; Dumaz and Marais, 2003), leading to transcriptional repression of protooncogenic transcription factors (c-jun, c-myc, egr-1, . . .; Heldin *et al.*, 1989; Mechta *et al.*, 1989; Cowlen and Eling, 1992), repression of D-type cyclins (cyclin D1, cyclin D3; Cocks *et al.*, 1992; Sewing *et al.*,

1993; Ward *et al.*, 1996; L'Allemain *et al.*, 1997), and accumulation of the p27^{kip1} CDK inhibitor (Kato *et al.*, 1994; Ward *et al.*, 1996; van Oirschot *et al.*, 2001; Kuiperij *et al.*, 2005), which inhibits both CDK4 and CDK2 (Kato *et al.*, 1994; Kuiperij *et al.*, 2005) and thus inactivating phosphorylations of pRb. Inhibition of cyclin D1-CDK4 activity by cAMP-dependent accumulation of p27 has been ascribed to p27 impairing the activating T172-phosphorylation of CDK4 by the constitutively active CDK-activating kinase (CAK, cyclin H-CDK7-Mat1; Kato *et al.*, 1994). Though inhibition of ERK activity can generate all these distal outcomes, a few studies have indicated that ERK1/2 inhibition is not always a prerequisite for cAMP-induced growth arrest, even in those cells in which it occurs (Graves *et al.*, 1993; Dumaz *et al.*, 2002; Balmanno *et al.*, 2003).

In some other cell types, mostly differentiated cells of neuroendocrine origin, many epithelial cells and Swiss 3T3 cells, cAMP promotes cell cycle progression (Boynton and Whitfield, 1983; Dumont *et al.*, 1989; Roger *et al.*, 1995; Withers *et al.*, 1995), as also demonstrated by the multiple hyperproliferative disorders associated with the McCune and Albright syndrome (adenylyl cyclase activation by mutation of Gs α ; Weinstein *et al.*, 1991; Schwindinger *et al.*, 1992) and Carney complex (activation of PKA by inactivating mutation of R1 α ; Kirschner *et al.*, 2000). Thyroid epithelium is often considered the best example of a positive regulation of cell cycle by cAMP and PKA activation in response to stimulation by the physiological hormone thyroid-stimulation hormone (TSH; Roger *et al.*, 1983, 1988; Dumont *et al.*, 1989; Ledent *et al.*, 1992; Kimura *et al.*, 2001; Premier *et al.*, 2007). Deregulated adenylyl cyclase activity by various TSH recep-

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Abbreviations used: CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; FBS, fetal bovine serum; FSK, forskolin; PKA, cAMP-dependent protein kinase.

for mutations (Parma *et al.*, 1993; Duprez *et al.*, 1994), TSH receptor activating autoantibodies (Laurent *et al.*, 1991), or Gs α mutation (Lyons *et al.*, 1990) is associated with hyperfunctional adenomas, goiter in Graves' disease, and hereditary toxic thyroid hyperplasia, but it is infrequently observed in thyroid carcinomas (Mircescu *et al.*, 2000; Fuhrer *et al.*, 2003). Positive cross-signaling of cAMP pathway with ERK and PI3kinase cascades has been claimed to contribute to cAMP-dependent cell proliferation (Richards, 2001; Kimura *et al.*, 2001; Stork and Schmitt, 2002; Rivas and Santisteban, 2003). However, using serum-free primary cultures of canine and human thyrocytes stimulated by TSH, we have shown that cAMP does not activate Ras, ERKs, and PI3kinase/Akt pathways, represses early genes such as c-jun and egr-1 (reviewed in Kimura *et al.*, 2001), rather down-regulates D-type cyclins (Depoortere *et al.*, 1998; Van Keymeulen *et al.*, 1999; Paternot *et al.*, 2006b), and up-regulates p27 (Depoortere *et al.*, 1996; Paternot *et al.*, 2006b). Paradoxically, these various responses are similar to those associated with cAMP-induced cell proliferation arrest. Unlike growth factor-dependent mitogenesis, the differentiation-associated cAMP-dependent cell cycle progression of normal thyrocytes (Roger *et al.*, 1992, 1995) specifically requires cyclin D3 synthesized in response to insulin or IGF-I (Depoortere *et al.*, 1998; Van Keymeulen *et al.*, 1999). cAMP activates CDK4 by promoting the assembly of the cyclin D3-CDK4 complex (Depoortere *et al.*, 1998; Van Keymeulen *et al.*, 1999), its stabilization in nucleus through its binding to nuclear p27 (Depoortere *et al.*, 2000), and finally the activation of this complex through T172-phosphorylation of CDK4 by a CDK4-activating kinase that remains to be defined (Paternot *et al.*, 2003, 2006b; Bockstaele *et al.*, 2006a,b).

Although cAMP is a positive modulator for the multiplication of many normal differentiated cells, it often inhibits the proliferation of their derivative cancer cells (Roger *et al.*, 1995; Ohta *et al.*, 1997). The mechanisms of this inversion have not been defined. Most thyroid carcinomas are associated with mutations or rearrangements of genes that generate a permanent activation of ERK1/2 pathway. Nonoverlapping activating events involving the genes encoding the tyrosine kinase receptors Ret and NTRK1, B-Raf or one of the three Ras G-proteins are detected in 70% of cases (Soares *et al.*, 2003; Kondo *et al.*, 2006). In the present study, we show the inhibition of cell cycle progression by cAMP in four human thyroid carcinoma cell lines that express these different oncogenes, and we compare the effects of cAMP on signaling cascades, cell cycle regulatory proteins, and CDK4 regulation.

MATERIALS AND METHODS

Reagents and Antibodies

Forskolin (FSK) was obtained from Sigma (St. Louis, MO) and cAMP analogs specific for PKA (6-MB-cAMP) or EPAC (8-CPT-2'-O-Me-cAMP) were purchased from BIOLOG (Bremen, Germany). Wortmannin and rapamycin were from Calbiochem (San Diego, CA). PD184352 was kindly shared by Dr. Philip Cohen (University of Dundee, United Kingdom). The primary antibodies used were purchased from the following sources: anti-phospho and total p44/42 MAPK, anti-phospho (S473 and T308) and total-Akt, anti-phospho p70^{S6k1}, and anti-4EBP-1 were from Cell Signaling Technology (Beverly, MA); anti-p27 (C-15), anti-CDK4 (C-22), anti-p21 (C-19), anti-cyclin H (C-18), anti-CDK7 (C-4), and anti-actin were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibodies against cyclin D1 (DCS-6 and DCS-11), cyclin D3 (DCS-22 and DCS-28), CDK4 (DCS-31), cyclin A (E72), and α -tubulin were from NeoMarkers (Fremont, CA); the anti-phospho (T826) pRb antibody and anti-phospho (T246) PRAS40 were from Biosource (Camarillo, CA); the anti-total pRb (554136) was from BD-PharMingen (Erembodegen, Belgium). RAF kinase assay kit was purchased from Upstate Biotechnology (Charlottesville, VA) and used according to the manufacturer's instructions.

Cell Culture

Human thyroid carcinoma cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin, in a humidified atmosphere (5% CO₂) at 37°C. All culture reagents were from Invitrogen (Carlsbad, CA). All stimulations were performed according to the following procedure: cells were propagated in standard culture medium and then serum-starved for 12 h, after which the different agents were added in fresh serum-free medium. Cells were stimulated for different time points, and samples were collected according to the parameter to be analyzed. T98G cells were cultured and made quiescent by serum deprivation as described (Bockstaele *et al.*, 2006b).

DNA Synthesis

Cells in 3-cm Petri dishes were incubated with 10 μ M bromodeoxyuridine (BrdU) for 1 h before fixation. The incorporation of BrdU was detected by immunofluorescence, and BrdU-labeled nuclei were counted (1000/dish) as described (Roger *et al.*, 1992).

Western Blotting Detections of Proteins

Thirty micrograms of total proteins were separated by SDS-PAGE, and the proteins of interest were immunodetected after Western blotting as previously described (Coulonval *et al.*, 2003a). Secondary antibodies were either coupled to horseradish peroxidase (Amersham Biosciences, Uppsala, Sweden) for detection by enhanced chemiluminescence (Western Lightning, Perkin Elmer-Cetus, Boston, MA) or to DyLight 680 and 800 (Pierce Biotechnology, Rockland, IL) for infrared fluorescence detection using the Odyssey scanner (LI-COR, Lincoln, NE) according to manufacturer's protocol.

Coimmunoprecipitation and pRb-Kinase Assay

For analyses of protein complexes and their pRb kinase activity (Coulonval *et al.*, 2003a), subconfluent cultures in 9-cm Petri dishes that contain the same number of cells were lysed on ice in 1 ml NP-40 lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 5 mM NaF, 1 mM sodium orthovanadate, 10 mM DTT, protease inhibitors, and 10% glycerol. The homogenized (glass/glass) cellular lysates were sonicated twice, precleared with protein A Sepharose (Amersham Biosciences) and then incubated at 4°C for 3 h with protein A-Sepharose that had been preincubated overnight with 2 μ g of antibodies against cyclin D1 (DCS-11), cyclin D3 (DCS-28), p21 (C-19), or p27 (C-15). Washed complexes were resuspended in 40 μ l of kinase reaction buffer containing 2 mM ATP, 0.3 μ g of a 56-kDa fragment (aa 379-928) of pRb (QED Bioscience, San Diego, CA), 10 mM β -glycerophosphate, 0.1 mM orthovanadate, 1 mM NaF, 60 μ g/ml pefabloc and 1 μ g/ml leupeptin, and incubated for 30 min at 30°C with occasional gentle agitation. Reactions were stopped by adding 60 μ l of twice-concentrated Laemmli buffer and boiling for 5 min. Proteins were resolved by SDS-PAGE and transferred on PVDF membranes. The phosphorylation of the pRb fragment was detected using the phosphospecific (T826)-pRb antibody. Membranes were then re-probed for detection of CDK4 and D-type cyclins.

Two-Dimensional Gel Electrophoresis

Proteins were coimmunoprecipitated as above. Washed complexes were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated by isoelectric focusing as described (Coulonval *et al.*, 2003a,b) on immobilized linear pH gradient (pH 3-10) IEF strips (Amersham Biosciences). After loading onto SDS-polyacrylamide slab gels (12.5%) for separation according to molecular mass and transfer on PVDF membranes, CDK4 was detected with the C-22 polyclonal antibody (Santa Cruz).

Enhanced chemiluminescence detections of Western blots were quantitated using a GS-800 densitometer and the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

CAK Activity Assay

Inactive cyclin D3 complexes containing CDK4 or CDK6 were immunoprecipitated from serum-starved T98G cells (Bockstaele *et al.*, 2006b) as above in NP-40 lysis buffer. These complexes were used as a substrate for activation by recombinant CAK (positive control) or CAK complexes immunoprecipitated from thyroid carcinoma cell lines. Their *in vitro* activation was then assessed by their pRb-kinase activity. Cyclin D3 complexes from T98G cells were thus washed three times with NP-40 lysis buffer and then three times with CAK buffer (80 mM β -glycerophosphate, pH 7.3, 15 mM MgCl₂, 20 mM EGTA, and 5 mM DTT; Matsuoka *et al.*, 1994). The beads were resuspended in 30 μ l of CAK buffer containing protease and phosphatase inhibitors with or without 1.4 μ g of recombinant CAK (CDK7-cyclin H-MAT1 complex; Upstate Biotechnology) or mixed with cyclin H-CDK7 complexes (coimmunoprecipitated as above using the C-18 cyclin H antibody) from thyroid carcinoma cells treated or not with forskolin. After addition of 1 mM ATP, the suspensions were incubated at 30°C for 30 min. After three washes in CAK buffer and three washes in pRb kinase buffer, the immunoprecipitated proteins were assayed for pRb kinase activity as above.

p27 Knockdown

Cell lines were plated at a density of 1.1×10^6 cells per well in a six-well plate. Cells were transiently transfected for 12 h in RPMI 1640 (without serum and antibiotics) with Lipofectamine 2000 (Invitrogen) and small interfering RNA (siRNA) according to the manufacturer's instructions. Briefly, 50 ng of each siRNA, scrambled sequence or p27 siRNAs (ON-TARGETplus, Dharmacon Research, Boulder, CO; or sc-29429, Santa Cruz Biotechnology) was added. The medium was then renewed with serum-free medium, and the cells were treated with vehicle, forskolin, or PD184352 for 24 h before analysis of BrdU incorporation and Western blotting detection of p27.

RESULTS

cAMP and PKA Activation Inhibit DNA Synthesis and pRb Phosphorylation in Thyroid Carcinoma Cells

The thyroid carcinoma cell lines used in the present study have been previously characterized: B-CPAP (from papillary carcinoma) and 8505C (from undifferentiated carcinoma) harbor the V600E B-Raf mutation, TPC-1 (from papillary carcinoma) the Ret/PTC-1 rearrangement and C643 (from undifferentiated carcinoma) the G13C H-RAS mutation (Meireles *et al.*, 2007). These cell lines maintain DNA synthesis and proliferation despite serum deprivation. Because they lack TSH receptor expression like most cell lines from thyroid carcinomas (Meireles *et al.*, 2007), we used the general activator of adenylyl cyclase FSK to specifically increase cellular cAMP levels, which perfectly mimics the effects of TSH on normal thyrocyte proliferation (Roger *et al.*, 1983; Dremier *et al.*, 2007).

In the absence of serum, FSK inhibited DNA synthesis (Figure 1) and cell proliferation (not shown) in the four thyroid carcinoma cell lines. In TPC-1 cells, DNA synthesis was inhibited from the first 4 h of FSK administration, and this inhibition increased until complete cell cycle arrest was achieved at 12 h (Figure 1). More partial and/or delayed inhibitions were observed in the other cell lines (Figure 1). These inhibitions were reversible; after 24 h of FSK removal in TPC-1 cells, BrdU incorporation levels rose from 0.5 to 85% in the presence of 10% fetal bovine serum (FBS) and 18% in the absence of serum. In the four cell lines, the kinetics and amplitude of DNA synthesis inhibition correlated with a similar inhibition of pRb phosphorylation by FSK (Figure 1). On the other hand, FSK did not increase apoptosis as evaluated using the TUNEL assay (not shown).

cAMP effects can be mediated by PKAs or by exchange proteins activated by cAMP (EPACs; Bos, 2006). We thus treated the cells with cAMP analogs that selectively activate PKAs (6-MB-cAMP) or EPACs (8-pCPT-2'-O-Me-cAMP; Christensen *et al.*, 2003; Dremier *et al.*, 2007). In the four cell lines, the inhibitions by FSK of DNA synthesis (Figure 2A) and pRb phosphorylation and cyclin A accumulation (Figure 2B) were perfectly mimicked by the PKA activator but not at all by the EPAC activator. Like the induction of DNA synthesis by TSH and FSK in normal thyrocytes (Dremier *et al.*, 2007), the inhibition by cAMP of DNA synthesis and pRb phosphorylation in thyroid carcinoma cells thus appeared to be mediated by PKA activation.

cAMP Inhibits the ERK and mTOR Pathways in TPC-1 Cells But Not in B-Raf-mutated Cells

The activations of ERK, PI3K/Akt and mTOR pathways were compared in the four thyroid carcinoma cell lines after serum deprivation (Figure 3A). ERK1/2 activating phosphorylations were easily detected. With the exception of a moderate stimulation in H-Ras-mutated C643 cells, it was not further increased by serum readdition, consistent with the permanent activation of the ERK pathway by the different oncogenes expressed in these different cells (Kondo *et al.*,

Figure 1. cAMP inhibits DNA synthesis and pRb phosphorylation in thyroid carcinoma cell lines. Cells were deprived of serum for 12 h and then treated with FSK (10 μ M) for the indicated times. BrdU was added 1 h before fixation, and the percentage of nuclei having incorporated BrdU was determined. In parallel, pRb was detected by Western blotting and its phosphorylation was shown by its upward migration shift (arrows). A complete cell cycle arrest was observed in FSK-treated TPC-1 cells (top), whereas inhibition by FSK was more partial and/or delayed in the other cell lines.

Figure 2. cAMP inhibits DNA synthesis (A) and pRb phosphorylation and cyclin A accumulation (B) through PKA activation. Thyroid carcinoma cell lines were maintained with 10% FBS or were serum-deprived for 12 h and then treated for 24 h with vehicle (control, Ctr) or with FSK, a PKA-selective cAMP analog (6-MB-cAMP; 6 MB) or the EPAC-selective cAMP analog (8-CPT-2'-O-Me-cAMP; 8 Cpt) used at 100 μ M. BrdU was added 1 h before fixation, and the percentage of nuclei having incorporated BrdU was determined. In B, FSK was at 10 μ M, 6 MB at 1 mM, and 8 Cpt at 100 μ M. (A and B) The EPAC agonist had no effect, whereas the PKA agonist had a dose-dependent inhibitory effect comparable to that of FSK.

2006). As judged from the PDK1-dependent T308 phosphorylation of Akt and from the Akt-dependent T246 phosphorylation of the mTOR regulator PRAS 40 (Wang *et al.*, 2007; Sancak *et al.*, 2007; compared with the total levels of these proteins), Ret/PTC1-positive TPC-1 cells and H-Ras-mutated C643 cells, respectively, presented a weak and moderate constitutive activity of the PI3K/Akt pathway. By contrast the activity of this pathway seemed to be very low in nonstimulated B-Raf-mutated cells (B-CPAP and 8505C). It was markedly activated by serum in C643 and 8505C cells only. The activity of mTOR was reflected by the phosphorylations of p70^{S6K1} and 4EBP-1 (revealed by upward electrophoretic shifts), phosphorylation of ribosomal protein S6, as well as by the S473-phosphorylation of Akt by the mTOR-Rictor complex (Sarbasov *et al.*, 2005). It was highly active in nonstimulated (Ret/PTC1-positive) TPC-1 cells and (H-Ras-mutated) C643 cells, but not in B-Raf-mutated cells (B-CPAP and 8505C) where it remained inducible by serum. Overall, observations from the four cell lines were mostly consistent with the view that Ret/PTC1 and H-Ras mutation increase Ras activity and activate Ras effectors including Raf/ERK and PI3K/Akt cascades, which converge on mTOR pathway activation (Sabatini, 2006). By contrast, the B-Raf mutation only constitutively activates the ERK pathway.

In these serum-free conditions, a 1-h exposure of TPC-1 cells to FSK strongly inhibited the phosphorylations of ERK1/2, as well as the mTOR pathway (Figure 3A). As evaluated from S473-phosphorylation of Akt and T246-phosphorylation of PRAS 40, Akt activity might also be

slightly inhibited by FSK in these cells. Time-course experiments showed that the inhibition of ERK1/2 phosphorylation in FSK-treated TPC-1 cells remained stable for the first 4–8 h, after which ERK1/2 phosphorylation progressively returned to control values (Figure 3B). A much weaker inhibition by FSK of both ERK and mTOR pathways was also observed in H-Ras mutated C643 cells (Figure 3A). By contrast no inhibition of ERK, PI3K, or mTOR pathway by FSK was observed in B-Raf-mutated cells (B-CPAP and 8505C; Figure 3, A and B). We assayed the total Raf activity (i.e., the presence of an activity that phosphorylates MEK1 at S218/222 and MEK2 at S222/226) in total lysates of cells treated or not with FSK. In TPC-1 cells, FSK treatment resulted in total RAF kinase inhibition, whereas almost no inhibition was observed in B-CPAP cells (Figure 3C).

The Impact of cAMP and PKA Activation on CDK4 Regulatory Proteins Varies in Different Cell Lines

Although cyclin D1 and cyclin D3 are well established CDK4 activators, the CDK “inhibitors” p21 and p27 have both negative and positive impacts on the activity of CDK4 complexes (Sherr and Roberts, 1999), possibly depending on their binding stoichiometry (Zhang *et al.*, 1994; Blain *et al.*, 1997; Bockstaele *et al.*, 2006b). In primary cultures of normal thyrocytes, they support the nuclear localization and activity of cyclin D1/D3-CDK4 (Coulonval *et al.*, 2003a; Bockstaele *et al.*, 2006b; Paternot *et al.*, 2006a,b).

Because cAMP and PKA activation inhibited pRb phosphorylation (Figures 1 and 2), we next investigated the

Figure 3. Different impact of cAMP on cell signaling pathways in different thyroid carcinoma cell lines. (A) Serum-deprived cells were treated for 1 h with vehicle (C), 10 μ M FSK (F), or 10% FBS (S). The phosphorylation of various signaling proteins was detected from the same cell extracts using phosphospecific antibodies or was evaluated by their upward migration shifts (arrows indicating the different phosphorylation levels of p70^{S6K1} and 4EBP-1). When needed, Odyssey scans obtained using low (L) and high (H) sensitivity parameters are shown. A time course of ERK phosphorylation inhibition by FSK (10 μ M) is illustrated in B. In C, the total Raf kinase activity was evaluated from whole cell extracts 1 h after cell treatment with (+) or without (-) 10 μ M FSK. FSK markedly inhibited Raf activity, ERK phosphorylation, and readouts of mTOR activity (p70, S6, and 4EBP-1) in TPC-1 cells, but had weaker or no effect in the other cell lines (A–C).

accumulation of CDK4 regulators in response to FSK or the PKA activator 6-MB-cAMP in the four cell lines deprived of serum. In TPC-1 cells, FSK and 6-MB-cAMP (but not the EPAC activator) strongly repressed cyclin D1 and p21, while up-regulating p27 (Figure 4A). These effects were sustained and readily detected 8 h after FSK administration (Supplementary Figure S1). The repression of cyclin D3 by FSK and 6-MB-cAMP was more variable in different experiments (Figure 4A, Supplementary Figure S1). In H-Ras-mutated

C643 cells and B-Raf-mutated B-CPAP and 8505C cells, much weaker (if any) down-regulation of cyclin D1 and up-regulation of p27 were observed (Figure 4A, Supplementary Figure S1). These very weak effects correlated with the lack of appreciable cAMP effect on ERK, mTOR, and PI3K signaling cascades in these cells (Figure 3). However, p21 was reproducibly up-regulated 8–12 h after FSK addition in C643 cells (and in B-CPAP in some experiments; see Figure 5), at variance with its inhibited expression in TPC-1 cells (Supplementary Figure S1).

In TPC-1 cells we compared the inhibitory effects of FSK on signaling cascades, CDK4 modulators, and DNA synthesis, to those produced by selective inhibition of ERK1/2 pathway (by the MEK inhibitor PD184352), of Raptor-mTOR complex (by rapamycin) and of both PI3K and mTOR (by wortmannin; Figure 4B). A low concentration of PD184352 (1 μ M) mimicked the effects of FSK on DNA synthesis, the repression of cyclin D1 and p21, the accumulation of p27, and the inhibition of pRb phosphorylation (including at T826, which is specifically performed by D-type cyclin-CDK4 complexes (Kitagawa *et al.*, 1996; Zarkowska and Mittnacht, 1997; Figure 4B). Interestingly, the MEK inhibitor such as FSK also strongly inhibited the mTOR pathway (Figure 4B). However selective inhibition of mTOR by rapamycin weakly affected the expression of cyclin D1 and p27, pRb-phosphorylation, and cell cycle progression (Figure 4B). Wortmannin also inhibited pRb phosphorylation and DNA synthesis in TPC-1 cells, but this was associated with a much weaker impact on cyclin D1 and p27 levels (Figure 4B). Therefore, in TPC-1 cells, the cell cycle arrest by cAMP and PKA might be essentially due to the inhibition of the Raf/MEK/ERK pathway, which leads to repression of cyclin D1 and p21 and accumulation of p27 as observed in this and other systems (Lavoie *et al.*, 1996; Woods *et al.*, 1997; Squires *et al.*, 2002; Vitagliano *et al.*, 2004).

Loss of p27 expression has been suggested to be crucial for deregulated proliferation of thyroid cancer cells associated with constitutively active ERK pathway (Vitagliano *et al.*, 2004; Motti *et al.*, 2007). In such cells including TPC-1 cells, antisense oligonucleotides to p27 were recently reported to suppress growth arrest by MEK inhibitors (Motti *et al.*, 2007). We thus wanted to evaluate whether the p27 up-regulation could also be critical for cell cycle arrest by cAMP. TPC-1 were transfected for 12 h with scrambled or p27 siRNAs and then treated or not with FSK or PD184352. As shown in Figure 4C, p27 siRNAs efficiently reduced p27 levels but did not prevent the inhibition of DNA synthesis by FSK or the MEK inhibitor. Similar results were observed using p27 siRNAs from Dharmacon (Figure 4C) or Santa Cruz (Supplementary Figure S2). The differences between our results and those of Motti *et al.* (2007) might be due to the fact that they performed their experiment in the presence of serum, or to a stronger knockdown of p27 expression in our experiments. In one experiment with a much weaker repression of p27, we indeed observed a partial reversal of the cell cycle arrest induced by PD184352, without any effect on the inhibition by FSK (not shown). In B-CPAP cells, p27 siRNA also did not affect the inhibition of DNA synthesis by FSK (Supplementary Figure S2). Therefore, p27 did not appear to be crucially involved in the cell cycle inhibition by cAMP in these cell lines.

cAMP Inhibits the pRb-Kinase Activity of D-Type Cyclin-CDK4 Complexes

In B-Raf-mutated B-CPAP and 8505C cells and in C643 cells, the weak or absent modulations of the levels of investigated cell cycle regulatory proteins could not explain the inhibi-

tory effects of FSK and PKA activation on pRb phosphorylation and DNA synthesis. We have thus compared, in the four thyroid carcinoma cell lines, the formation and pRb-kinase activity of CDK4 complexes coimmunoprecipitated using cyclin D1, cyclin D3, and p21 and p27 antibodies (the related CDK6 was very weakly detected in these cell lines; not shown). The pRb-activity was detected using an antibody directed against the CDK4-specific T826-phosphorylation of pRb. Because the inhibition by FSK of DNA synthesis and pRb phosphorylation was more rapidly observed in TPC-1 and B-CPAP cells (Figure 1), in the following experiments TPC-1 and B-CPAP cells, and C643 and 8505C cells, were treated for 8 and 16 h, respectively.

In the four cell lines deprived of serum, CDK4 was found to associate with cyclin D1, cyclin D3, p21, and p27. A high pRb-kinase activity was coimmunoprecipitated not only by cyclin D1 and cyclin D3 antibodies, but also by the p21 antibody in all the cell lines (Figure 5). An appreciable pRb-kinase activity was also coimmunoprecipitated by the

p27 antibody in TPC-1 and B-CPAP cells (Figure 5). A high pRb-kinase activity associated with p21 and p27 has also been observed in normal thyroid primary cultures (Coulonval *et al.*, 2003a; Paternot *et al.*, 2006a,b). As we have reported it in normal thyrocytes and other cell types (Paternot *et al.*, 2006a,b), the migration of the pRb fragment phosphorylated in vitro was different in immunoprecipitations of cyclin D1 (one upward-shifted band) or cyclin D3 (a doublet with a predominant lower band). This reflects the fact that cyclin D1-CDK4, more efficiently than cyclin D3-CDK4, drives the phosphorylation of pRb at S807/811, which is required for its upward electrophoretic shift (Paternot *et al.*, 2006a). Interestingly, as also observed in normal thyrocytes (Paternot *et al.*, 2006a,b), the profile of pRb-kinase activity coimmunoprecipitated by the p21 antibody much resembled the activity associated with cyclin D1, whereas the pRb-kinase activity coimmunoprecipitated by the p27 antibody resembled the activity associated with cyclin D3. This suggests that the pRb-kinase activity associated with p21 is mainly catalyzed

Figure 5. Impact of FSK (10 μ M) on the assembly and pRb-kinase activity of CDK4 complexes in thyroid carcinoma cell lines. Extracts from cells treated with FSK (+) or vehicle (–) for 8 h (TPC-1 and B-CPAP cells) or 16 h (C643 and 8505C cells) were immunoprecipitated (IP) with anti-cyclin D1, anti-cyclin D3, anti-p21, or anti-p27 antibodies, assayed for pRb-kinase activity, separated by SDS-PAGE and immunoblotted. Cyclin D1 (cyc D1), cyclin D3 (cyc D3), p21, p27, CDK4, and the *in vitro* phosphorylation at T826 of the pRb fragment (pRb-kinase) were detected using specific antibodies. In the top and middle panels, cyclin D1 and cyclin D3 were codetected using a mixture of their primary antibodies.

by cyclin D1-CDK4, whereas cyclin D3-CDK4 is mainly responsible for the pRb-activity associated with p27.

In TPC-1 cells, FSK treatment strongly inhibited the pRb-kinase activity associated with cyclin D1 and p21 (Figure 5, top). This could be largely due to the reduction of the amount of these complexes (Figure 5), which correlated with the down-regulation of cyclin D1 and p21 (Figure 4, A and B). By contrast, FSK did not inhibit the pRb-kinase activity and the presence of CDK4 in cyclin D3 and p27 complexes (Figure 5). Nevertheless, cyclin D3-CDK4 complexes were less abundant than cyclin D1-CDK4 complexes, as shown by the much lower amount of CDK4 coimmunoprecipitated by cyclin D3 antibody (Figure 5).

In the three other cell lines, FSK markedly inhibited the pRb-kinase activity associated with cyclin D1 and p21 (Figure 5), but this was not associated with a comparable reduction of the association of CDK4, indicating that FSK inhibited the activity but not the formation of cyclin D1-CDK4-p21 complexes. In C643 cells, but less in the B-Raf-mutated cell lines (B-CPAP and 8505C), the activity of cyclin D3-CDK4 complexes was also strongly inhibited by FSK (Figure 5).

To conclude, FSK inhibited the pRb-kinase activity of cyclin D1-CDK4-p21 complexes in the four cell lines. This was largely explained by a reduced presence of these complexes in TPC-1 cells, but not in the other cell lines. On the other hand, FSK markedly inhibited the activity, but not the formation, of cyclin D3-CDK4 complexes in C643 cells (and more weakly in 8505C cells), but not in the other cell lines.

cAMP Inhibits the Activating Phosphorylation of CDK4

In several systems including normal thyrocytes stimulated by TSH or FSK, we have recently identified the activating T172-phosphorylation of CDK4 as a crucial target for regulation of the activity of cyclin D3-CDK4, pRb phosphorylation, and cell cycle progression (Paternot *et al.*, 2003, 2006b; Bockstaele *et al.*, 2006b). The phosphorylation of CDK4 does not affect its electrophoretic migration in SDS-polyacrylamide gels. Previously, using two-dimensional (2D) gel electrophoresis, we have separated different forms of CDK4. We have identified a more negatively charged form as the T172-phosphorylated CDK4 using 32 P-phosphate incorporation, a new phosphospecific antibody, and 2D-gel analysis of T172A-mutated CDK4 (Coulonval *et al.*, 2003a; Bockstaele *et al.*, 2006b).

Because FSK inhibited the activity but not the formation of CDK4 complexes, we quantified the proportion of T172-phosphorylated CDK4 in coimmunoprecipitated cyclin D1-CDK4 and cyclin D3-CDK4 complexes in the four cell lines treated or not with FSK. As shown in Figure 6, FSK treatment strongly reduced the proportion of the T172-phosphorylated form of CDK4 bound to cyclin D1 in the four cell lines including TPC-1 cells. By contrast, in cyclin D3 complexes the proportion of phosphorylated CDK4 was reduced by FSK in C643 cells, more weakly in 8505C cells, but not in TPC-1 and B-CPAP cells (Figure 6). Therefore, the differential inhibitory effect of FSK on the activity of cyclin D1 or cyclin D3 complexes in the different cell lines perfectly correlated with a similar effect of FSK on the phosphorylation of CDK4 in these complexes. In TPC-1 cells, the abolition of cyclin D1-CDK4 activity appeared to result from both a reduction of cyclin D1 and thus cyclin D1-CDK4 complexes, as well as from an additional inhibition of T172-phosphorylation of CDK4 associated to residual cyclin D1. In the other cell lines, the inhibition of CDK4 phosphorylation appeared to essentially account for the inhibition of pRb-kinase activity by FSK in the absence of any prominent effect on the accumulation of CDK4 modulators and their association with CDK4.

cAMP Does Not Inhibit the Activity of the CDK-activating Kinase

The activating T172-phosphorylation of CDK4, like the analogous T-loop phosphorylations of the other CDKs, is considered to be performed only by CAK, the cyclin H-CDK7-Mat1 complex (Matsuoka *et al.*, 1994; Fisher, 2005). Nevertheless, the activity of CAK has generally been found to be constitutive and nonregulated during stimulations or inhibitions of proliferation (Kato *et al.*, 1994; Matsuoka *et al.*, 1994; Tassan *et al.*, 1994; Bockstaele *et al.*, 2006b).

We thus decided to directly assess the activity of cyclin H-CDK7 complexes immunoprecipitated from thyroid carcinoma cells treated or not with FSK. As a substrate in this immunoprecipitation/kinase assay, we used inactive CDK complexes coimmunoprecipitated by the cyclin D3 antibody from quiescent serum-starved T98G cells. Indeed, these complexes mainly contain the inactive nonphosphorylated forms of CDK4 or CDK6 but their pRb-kinase activity can be considerably increased by incubation with ATP and recom-

Figure 6. FSK inhibits the activating T172-phosphorylation of CDK4 in thyroid carcinoma cell lines. Cells were treated with (+) or without (-) FSK and CDK4 was coimmunoprecipitated (IP) using antibodies directed against cyclin D1 (cyc D1) or cyclin D3 (cyc D3) exactly as in Figure 5. The different forms of CDK4 were separated by 2D-gel electrophoresis. CDK4 was detected by Western blotting using a specific antibody. In the different panels, exposure times were different and adjusted to display roughly equivalent levels of total CDK4. Therefore, the detections allow the evaluation of the proportion of the different CDK4 forms, but not the relative amount of the different CDK4 complexes. Arrows indicate the T172-phosphorylated form of CDK4. Values indicate the proportion of this phosphorylated form relative to total CDK4 (% P-CDK4), as quantitated by scanning densitometry of CDK4 immunodetections. FSK inhibited the phosphorylation of CDK4 bound to cyclin D1 in the four cell lines, but more weakly or not the phosphorylation of cyclin D3-bound CDK4 in TPC-1, B-CPAP, and 8505C cells.

binant CAK (cyclin H-CDK7-Mat1 complexes; Bockstaele *et al.*, 2006b). In the present experiments, we thus incubated cyclin D3 complexes from serum-starved T98G cells with 1

mM ATP and either recombinant CAK (as a positive control) or cyclin H-CDK7 complexes that were coimmunoprecipitated from thyroid carcinoma cells treated or not with FSK. We then assayed the pRb-kinase activity of T98G cyclin D3 complexes by incubating the mixed immunoprecipitations with ATP and the pRb fragment. As shown in Figure 7, cyclin H-CDK7 complexes from TPC-1, B-CPAP, or C643 cells, like recombinant CAK, strongly increased the pRb-kinase activity of cyclin D3 complexes from quiescent T98G cells. This was not affected by treatment of the three thyroid carcinoma cell lines with FSK (Figure 7). As a control, we checked that cyclin H-CDK7 complexes had no intrinsic pRb-kinase activity when they were assayed on mock immunoprecipitation from T98G cells (Figure 7). We thus conclude that FSK did not inhibit the cyclin H-CDK7 activity in TPC-1, B-CPAP, and C643 cells.

DISCUSSION

How cAMP elevations could positively or negatively affect G1 phase cell cycle progression in different cell types or in cancer cells versus their normal differentiated counterparts has remained an intriguing question for decades. One recently proposed mechanistic explanation relies on opposite interferences of cAMP and its effectors PKA and EPAC on the Raf/MEK/ERK pathway activated by growth factors or various oncogenic mutations (Stork and Schmitt, 2002; Dumaz and Marais, 2005). At variance with the well-characterized induction of cell cycle progression by TSH and cAMP observed in normal thyroid epithelial cells, we show here that cAMP inhibits S-phase entry in four thyroid carcinoma cell lines. Our observation is consistent with previous reports from other thyroid carcinoma cells (Ohta *et al.*, 1997; Calebiro *et al.*, 2006), indicating that it could reflect a general characteristic of these cells. As shown by the same effector-selective cAMP analogs used at the same concentrations, both the mitogenic effect of cAMP in normal thyroid cells (Dremier *et al.*, 2007) and its antimitogenic effect in thyroid carcinoma cells (the present study) appear to be mediated solely by PKA activation.

In the four thyroid carcinoma cell lines, the cell cycle inhibition by cAMP and PKA activation perfectly correlates with the inhibition of pRb phosphorylation and pRb-kinase activity of D-type cyclin-CDK4 complexes. However, cAMP inhibits the Raf/ERK and mTOR pathways in RET/PTC1-positive TPC-1 cells (strongly) and H-Ras-mutated C643 cells (weakly) but not in B-Raf-mutated cell lines (B-CPAP and 8505C cells). Therefore, the cAMP-induced inhibition of

Figure 7. FSK does not inhibit CAK activity in thyroid carcinoma cell lines. The activity of coimmunoprecipitated cyclin H-CDK7 complexes (IP cyc H) was evaluated from thyroid carcinoma cells treated with (+) or without (-) FSK for 8 h (TPC-1 and B-CPAP cells) or 16 h (C643 cells) as in Figures 5 and 6. In this assay, these cyclin H-CDK7 complexes or a recombinant cyclin H-CDK7-Mat 1 complex (rec. CAK) were mixed and incubated with ATP and inactive cyclin D3-CDK4/6 complexes immunoprecipitated from quiescent T98G cells [IP cyc D3 (T98G); or similar mock immunoprecipitations [IP IgG (T98G)]] used as a substrate. The resulting activation of the cyclin D3-CDK4/6 complexes was then assayed by their pRb-kinase activity (second incubation in the presence of the pRb fragment and ATP). The mixtures were separated by SDS-PAGE and immunoblotted. Using specific antibodies, we then detected the presence of CDK7 coimmunoprecipitated by the cyclin H antibody (cyc H-bound CDK7) from thyroid carcinoma cells or recombinant CAK complex, the presence of the substrate, i.e., cyclin D3 complexes from T98G cells [cyc D3 (T98G)], and the in vitro activation of these cyclin D3 complexes reflected by the phosphorylation at T26 of the pRb fragment (pRb-kinase).

DNA synthesis (S-phase entry) in the latter cell lines does not result from inhibition of Raf and ERK1/2, in agreement with a few previous reports from other systems (Graves *et al.*, 1993; Dumaz *et al.*, 2002; Balmanno *et al.*, 2003).

TPC-1 cells appear to obey the paradigm of cAMP-induced cell cycle arrest through inhibition of Raf signaling by PKA (Stork and Schmitt, 2002; Dumaz and Marais, 2005). Nevertheless, the almost complete inhibition of total Raf activity by cAMP in these cells is rather unexpected. Raf knockdown experiments in rat thyroid PC Cl3 cells transfected with Ret/PTC oncogenes have suggested that Ret/PTC1 signals mainly through B-Raf (Mitsutake *et al.*, 2006), which unlike c-Raf is not inhibited by PKA (Stork and Schmitt, 2002; Dumaz and Marais, 2005). Thus, either c-Raf is the preponderant Raf isoform in human TPC-1 cells, or cAMP might inhibit undefined upstream stages of Ret/PTC signaling. The observed inhibition of mTOR pathway by cAMP might also contribute to arrest the cell cycle. Because it was mimicked by MEK inhibition by PD184352, it likely results from the inhibition of ERKs, which activate mTOR through phosphorylations of TSC2 (Roux *et al.*, 2004; Ma *et al.*, 2007). Nevertheless, inhibition of Raptor-mTOR complex activity by rapamycin was not sufficient to inhibit G1-phase progression in TPC-1 cells. As observed in other systems, the inhibition of MEK/ERK cascade by cAMP and PD184352 induced the accumulation of p27 and repression of both cyclin D1 and p21. Though the p27 accumulation would be expected to inhibit CDK2 activity (Sherr and Roberts, 1999)—even if it did not preclude cyclin D3-CDK4 activity in TPC-1 cells (Figure 5) as in normal thyrocytes (Coulonval *et al.*, 2003a), p27 knockdown indicates that it is not crucial for cell cycle arrest in the present experiments. Repression of both cyclin D1 and p21 is likely to be important for cAMP-induced cell cycle arrest. Beside cyclin D1, p21 indeed clearly supported the pRb-kinase activity of CDK4 (Figure 5) and its nuclear localization in TPC-1 cells (double immunofluorescence experiments not shown, performed as in Paternot *et al.*, 2006a), as observed in other cell systems (LaBaer *et al.*, 1997; Alt *et al.*, 2002) including normal dog and human thyrocytes (Paternot *et al.*, 2006a,b). Therefore, the more complete cell cycle arrest induced by cAMP and PKA in TPC-1 cells is likely explained at least in part by the inhibition of the Raf/MEK/ERK pathway, which results in repression of both cyclin D1 and p21, thus reducing the concentration of CDK4 complexes, and up-regulation of p27 which might inhibit CDK2.

By contrast, in B-CPAP and 8505C cells that harbor the V600E B-Raf mutation, cAMP and PKA activation did not inhibit Raf activity and ERK phosphorylation, consistent with previous findings from uveal melanoma cells harboring the same mutation (Calipel *et al.*, 2006) and with the well-established observation that B-Raf is not inhibited by PKA (Stork and Schmitt, 2002; Dumaz and Marais, 2005). PI3 kinase/Akt and mTOR pathways also remained essentially unaffected by cAMP in B-CPAP and 8505C cells, as were the cellular concentrations of cyclin D1, cyclin D3, p21, and p27. In these cells (and also in C643 cells), the inhibition by cAMP of pRb phosphorylation and pRb-kinase activity of cyclin D1-CDK4 complexes thus could not be explained by appreciable modifications of the formation of these complexes or their association to p21 or p27. Instead, it did correlate with an inhibition of the activating T172-phosphorylation of CDK4 associated with cyclin D1. This phenomenon was observed in all the cell lines, including in the residual cyclin D1 complexes of FSK-treated TPC-1 cells. Moreover, the phosphorylation and activity of CDK4 associated with cyclin D3 was also inhibited by cAMP in C643 cells.

This inhibition of CDK4 phosphorylation by cAMP cannot be explained by current models. In animal cells, the only identified CDK4-activating kinase is the cyclin H-CDK7-Mat1 complex (CAK), which is also responsible for the activating phosphorylations of the other cell cycle CDKs (Matsuoka *et al.*, 1994; Kaldis, 1999; Fisher, 2005; Larochelle *et al.*, 2007). Nevertheless, CDK4 appears a relatively poor in vitro substrate for CAK, compared with other CDKs including CDK6 (Kaldis *et al.*, 1998; Bockstaele *et al.*, 2006b). In the present experiments, the activity of immunoprecipitated cyclin H-CDK7 was not affected by FSK, in agreement with the general observation that CAK activity is not modulated during cell cycle progression or in response to (anti)mitogenic treatments (Kato *et al.*, 1994; Tassan *et al.*, 1994; Matsuoka *et al.*, 1994; Nagahara *et al.*, 1999; Bockstaele *et al.*, 2006b). As in all these previous studies, it could not be formally excluded that a (undefined) regulatory protein might be lost during cyclin H-CDK7 immunoprecipitation. However, our unexpected observation that cAMP selectively inhibited the phosphorylation and activity of cyclin D1-bound CDK4 but less or not cyclin D3-bound CDK4 in three cell lines, would hardly suggest an implication of CAK. Inhibition of the activity and T172-phosphorylation of cyclin D1-bound CDK4 has been previously found during cAMP-induced G1-phase arrest in mouse macrophages (Kato *et al.*, 1994). However, this has been ascribed to the cAMP-dependent elevation of p27, which by binding to cyclin D1-bound CDK4 impairs its phosphorylation by constitutively active CAK (Kato *et al.*, 1994). This mechanism appears quite unlikely in thyroid carcinoma cell lines: 1) p27 levels were very weakly increased by cAMP in B-Raf-mutated cell lines; 2) p27 did not inhibit CDK4 activity (Figure 5) and CDK4 phosphorylation (not shown) in B-CPAP and TPC-1 cells, as observed during cAMP-dependent mitogenesis of normal dog thyrocytes (Coulonval *et al.*, 2003a; Bockstaele *et al.*, 2006b); 3) inhibition of CDK4 activity by cAMP was also observed in the very active p21 complexes that do not contain p27; and 4) p27 knockdown was insufficient to preclude cAMP-induced cell cycle arrest in TPC-1 and B-CPAP cells.

Similar modulations of CDK regulators (down-regulation of cyclin D1, p21 and more weakly cyclin D3, up-regulation of p27) are conspicuously observed in the cAMP-induced cell cycle arrest of TPC-1 cells and in the cAMP-dependent triggering of cell cycle progression of normal dog and human thyrocytes (Depoortere *et al.*, 1996; Van Keymeulen *et al.*, 2001; Paternot *et al.*, 2006a,b). Moreover, in both opposite situations, cAMP differentially regulates the activity and phosphorylation of CDK4 bound to cyclin D1 or to cyclin D3. Cyclin D3 is the predominant D-type cyclin in normal thyrocytes (Depoortere *et al.*, 1998; Van Keymeulen *et al.*, 1999; Motti *et al.*, 2003; Paternot *et al.*, 2006b). Although growth factors mostly activate cyclin D1-CDK4-p21 complexes, cyclin D3-CDK4 is specifically required and activated in the mitogenic stimulation by TSH and cAMP (Depoortere *et al.*, 1998; Motti *et al.*, 2003; Paternot *et al.*, 2006a,b). This crucially involves the cAMP-dependent phosphorylation of cyclin D3-bound CDK4 (Paternot *et al.*, 2003, 2006b; Bockstaele *et al.*, 2006b). This positive regulation is lost in papillary carcinoma cells. In TPC-1 cells (and B-Raf-mutated cell lines), cyclin D3-bound CDK4 are poorly affected by cAMP, but the phosphorylation of cyclin D1-bound CDK4 is markedly inhibited. Plausibly due to constitutive induction of cyclin D1 by permanently activated ERKs, cyclin D1-CDK4 is more abundant than cyclin D3-CDK4 in TPC-1 and B-CPAP cells (Figure 5), explaining the dominant char-

acter of the inhibition by cAMP of cyclin D1-CDK4 formation and/or activity.

To conclude, our studies in thyroid carcinoma cell lines and previously in normal thyrocytes have identified the T172-phosphorylation of CDK4 as the common target of the opposite cell cycle regulations by cAMP, irrespective of the impact of cAMP on classical signaling cascades of growth factors and the expression of CDK4 regulatory partners. Whether CDK4 phosphorylation inhibition could represent the long sought general mechanism of the antimitogenic effects of cAMP in other normal and cancer cell types should be examined. We have recently suggested that regulated CDK4-activating kinase(s) might remain to be uncovered (Bockstaele *et al.*, 2006a; Bockstaele *et al.*, 2006b). Full understanding of the opposite effects of cAMP/PKA on G1-phase progression might thus require the elucidation of the signaling cascades and mechanisms responsible for the activating phosphorylation of CDK4, which should also explain how they could be differentially wired to cyclin D1 or cyclin D3 complexes.

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