Cyclic AMP-dependent phosphorylation of cyclin D3-bound CDK4 determines the passage through the cell cycle restriction point in thyroid epithelial cells

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SUMMARY

According to current concepts, the cell cycle commitment after restriction (R) point passage requires the sustained stimulation by mitogens of the synthesis of labile D-type cyclins which associate with cyclin-dependent kinase (CDK) 4/6 to phosphorylate pRb family proteins and sequester the CDK inhibitor p27kip1. In primary cultures of dog thyroid epithelial cells, the cAMP-dependent cell cycle induced by a sustained stimulation by thyrotropin or forskolin differs from growth factor mitogenic pathways, as cAMP does not upregulate D-type cyclins but increases p27 levels. Instead, cAMP induces the assembly of required cyclin D3-CDK4 complexes, which associate with nuclear p27. In this study, the arrest of forskolin stimulation rapidly slowed down the entry of dog thyrocytes into S phase and the phosphorylation of pRb family proteins. The pRb-kinase activity, but not the formation, of the cyclin D3-CDK4-p27 complex was strongly reduced. Using two-dimensional gel electrophoresis, a phosphorylated form of CDK4 was separated. It appeared in response to forskolin and was bound to both cyclin D3 and p27, presumably reflecting the activating Thr172-phosphorylation of CDK4. Upon forskolin withdrawal or after cycloheximide addition, this CDK4 phosphoform unexpectedly persisted in p27 complexes devoid of cyclin D3, but it disappeared from the more labile cyclin D3 complexes. These data demonstrate that the assembly of the cyclin D3-CDK4-p27 holoenzyme and the subsequent phosphorylation and activation of CDK4 depend on distinct cAMP actions. This provides a first example of a crucial regulation of CDK4 phosphorylation by a mitogenic cascade and a novel mechanism of cell cycle control at the R point.
INTRODUCTION

The restriction (R) point has been originally defined as a critical no-return checkpoint in mid-to-late G1 phase at which cells are committed to initiate DNA replication and to complete one division cycle (1). While the progression through the pre-R portion of G1 strictly depends on the sustained cell stimulation by external growth factors and mitogens, the passage through the R point results in the triggering of an intrinsic machinery autonomously ordering cell cycle completion. This concept implies the peculiarly labile feature of a key event stimulated by mitogens in G1 phase to explain the necessity of their continuous presence until R point passage (2,3).

Accumulating evidence indicates that the phosphorylation and inactivation of the tumor/growth suppressors of the Rb family (pRb, p107 and p130) and activation of E2F-dependent gene transcription are major events underlying the transit through R point (4-6). Before R point the phosphorylations of pRb, p107 and p130 are initiated by CDK4 and 6 activated by D-type cyclins (D1, D2, D3) synthesized in response to growth factors (7-12). This triggers a positive feedback loop linking pRb to cyclin E. In this loop, cyclin E activates CDK2 and promotes its own expression by phosphorylating pRb thus relieving the inhibition of E2F-dependent transcription of cyclin E gene (4,13,14). From the R point the phosphorylations of pRb and p130 are maintained by cyclin E/A-CDK2 complexes and become independent of growth factors (12,15).

According to this model, the accumulation of labile D-type cyclins (rather than cyclin E (16)) over an inhibitory threshold imposed by INK4 CDK inhibitors constitutes the sensor element of the sustained action of mitogens that leads to the phosphorylation of pRb-related proteins and to R point passage (7,17-19). Nevertheless, the accumulation of a cyclin D does not suffice for CDK4/6 activation (20,21). Both the formation and nuclear import of cyclin D-CDK4/6 complexes have been demonstrated as additional regulated steps (22-26). Moreover,
the complete activation of nuclear D-type cyclin-CDK4 complexes requires the Thr172-phosphorylation of CDK4 by the nuclear CDK-activating kinase (CAK; cyclin H-CDK7) (20), which is generally considered to be constitutively active during cell cycle (27-29). The role of CDK « inhibitors » of the cip/kip family including p27kip1 in the passage through the R point is complex. Their downregulation by mitogenic factors (30) and/or their titration by cyclin D-CDK complexes participate to cyclin E-CDK2 activation (31,32). Their functions in the activation of cyclin D-CDK complexes are more controversial (19). Initially, p27kip1 was claimed to inhibit the activity of CDK4 by preventing its phosphorylation by CAK (33). More recently, p21cip1 and p27kip1 have been suggested as essential adaptors and/or nuclear anchors for cyclin D-CDK complexes (21,26,32,34-37). However, other groups still conclude that cip/kip proteins are not absolutely required for the assembly of cyclin D-CDK4 complexes (38,39) and that elevated p21 or p27 expression potently inhibits the activity of these complexes (40-42).

The cAMP-dependent cell cycle best characterized in primary cultures of dog thyroid epithelial cells (43-46) has raised the necessity to reexamine, at least in some systems, the nature of the critical labile events that couple at R point the mitogenic cascades with the autonomous part of the cell cycle (47). In this physiologically relevant system (48,49), the cAMP-dependent mitogenic stimulation by thyrotropin (TSH) or the general adenylyl cyclase activator forskolin depends on CDK4/6 activity (50) and involves the phosphorylations of pRb, p107 and p130 (51), but it strikingly differs from the most generally envisaged growth factor cascades as it stimulates the accumulation of p27kip1 (52) but not of D-type cyclins (23). In the presence of permissive comitogenic factors (insulin, carbachol) that support the accumulation of the required cyclin D3 (25,53), TSH and cAMP promote the assembly and nuclear import of cyclin D3-CDK4 complexes that associate with nuclear p27 (23,26). However, an additional cAMP-dependent labile event responsible for a last control of R point
passage remains to be uncovered. Even after the required induction of stable, or continuously formed, nuclear cyclin D3-CDK4-p27 complexes, dog thyrocytes still depend on sustained elevation of cAMP levels for entry into DNA synthesis phase (47,54) and pRb phosphorylation (47). Whereas the progression through a first part of G1 phase depends on the synergy of both cAMP and supportive comitogens integrated by the accumulation of cyclin D3-CDK4 complexes, the transit through the R point can be controled by cAMP alone (53). In the present study, we identify the phosphorylation of CDK4 within the cyclin D3-CDK4-p27 holoenzyme as the target of this crucial control. Moreover, we provide an explanation for the paradox that the disappearance of CDK4 phosphorylation upon arrest of cAMP stimulation could be perceived only in cyclin D3-bound CDK4 but not in total CDK4 or p27-bound CDK4.
EXPERIMENTAL PROCEDURES

Primary cultures – Follicular cells were obtained from dog thyroid tissue as described (55), cultured in monolayer in the «control» medium, i.e. DMEM+Ham’s F12+MCDB104 medium (2:1:1 by vol.) supplemented with bovine insulin (Sigma; 5 µg/ml), ascorbic acid (40 µg/ml), and antibiotics (55); and quiescent cells at day 4 or 5 were stimulated with the general adenylyl cyclase activator forskolin (Calbiochem; 2 × 10⁻⁶ M) as detailed in the figure legends. Previous experiments on dog thyrocytes have shown that forskolin quantitatively reproduces all the cAMP-dependent effects of TSH on cell cycle progression, function, and differentiation expression (reviewed in (49)); and that the activation of adenylyl cyclase by forskolin and cellular cAMP generation are very fast (maximum within 5 min in dog thyrocytes), do not desensitize for at least 48 h, and are completely reversible (within 10 min) after washing of the cells (54,56). Percentages of cells in the different phases of cell cycle were determined from the different patterns of proliferating cell nuclear antigen (PCNA) immunofluorescent staining exactly as described (57,58) by counting at least 500 cells per dish. Cell cycle progression was corroborated by the incorporation of 8-bromodeoxyuridine detected by immunofluorescence (59).

Western blotting analyses of cyclin D3, CDK4, p27, pRb, p107, p130 were performed as described (26). The Ser 807/811 phosphosite-specific pRb antibody was from Cell Signaling Technology (Beverly, MA).

Immunoprecipitation - For analyses of protein complexes and pRb-kinase activity, subconfluent cultures of thyrocytes in 10-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, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 10 mM DTT, protease inhibitors (pefablock, leupeptin), and 10% glycerol. The homogenized (glass/glass) cellular lysate was sonicated twice, precleared with protein A
sepharose (Amersham Biosciences, Uppsala, Sweden) and then incubated at 4°C for 3 h with protein A-sepharose which had been preincubated overnight with 2 µg of antibody (monoclonal antibody against cyclin D3 (DCS-28) (NeoMarkers, Fremont, CA), or a mixture of the K25020 anti-p27 monoclonal antibody from Transduction Laboratories and the C-15 p27 polyclonal antibody from Santa-Cruz. Alternatively, for analyses of the 2D-gel electrophoresis pattern of the whole population of cyclin D3, p27 or CDK4, cultures of thyrocytes in 10-cm Petri dishes were washed with PBS and scraped in 200 µl of denaturing lysis buffer (50 mM Tris-HCl (pH 7.5), 0.6% SDS, 10 mM DTT, 50 mM NaF, 100 µM vanadate and protease inhibitors), boiled for 5 min and frozen. Cell lysates containing 300-500 µg of protein were cleared by centrifugation and diluted by adding five volumes of RIPA buffer without SDS (10 mM Tris-HCl (pH 7.5), 1% Na deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM NaF, 100 µM vanadate and protease inhibitors). They were then subjected to immunoprecipitation as above, except that a mixture of DCS-28 and DCS-22 (NeoMarkers) was used for cyclin D3 and the C-22 polyclonal antibody for CDK4.

**pRb-kinase assay** - Immune complexes were washed three times with 0.5% NP40 lysis buffer supplemented with 1 mM DTT and three times with the kinase reaction buffer (50 mM HEPES, pH 7.5, 10 mM KCl, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM DTT). Washed complexes were resuspended in 40 µl of kinase reaction buffer containing 2 mM ATP, 0.5 µg 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 µg/ml pefabloc and 1 µg/ml leupeptine, and incubated for 30 min at 30°C with occasional gentle agitation. Reactions were stopped by adding 60 µl of twice-concentrated Laemmli buffer and boiling for 5 min. Proteins were resolved by SDS-PAGE, transferred on PVDF membranes, and the phosphorylation on Ser780 of the pRb fragment (specifically ascribed to CDK4 (60)) was detected using the phosphospecific-pRb (Ser780) antibody from Cell Signaling Technology. Membranes were
then reprobed using the C-22 polyclonal CDK4 antibody, the DCS-22 monoclonal cyclin D3 antibody and the C-15 polyclonal p27 antibody. Protein A (Pierce, Perbio Science, Erembodegem, Belgium) or an anti-mouse immunoglobulin antibody (Amersham Biosciences), both coupled to horseradish peroxidase, were used for detection by enhanced chemiluminescence (Western Lightning, Perkin-Elmer, Boston, MA). Various exposures of the bands corresponding to these proteins were collected on Hyperfilms (Amersham Biosciences) and quantitated by laser scanning densitometry (Ultroscan, Bio-Rad Laboratories, Hercules CA).

Two-dimensional gel electrophoresis - Immunoprecipitated proteins were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated by isoelectric focusing using the IPGphor apparatus from Amersham Biosciences and active in-gel rehydration as described (61) on immobilized linear pH gradient (pH 3 to 10) IEF strips (Amersham Biosciences). Isoelectric focusing was performed for a total of 28000 Vh, starting at 200 V and gradually raising the voltage to 8000 V. The IEF strip was then equilibrated for 15 min in 50 mM Tris (pH 8.5), 6 M urea, 30% glycerol, 1% SDS and 1% DTT, and for 15 min in 50 mM Tris, 6 M urea, 30% glycerol, 1% SDS and 5% iodoacetamide before loading onto SDS-polyacrylamide slab gels (12.5%) for separation according to molecular mass. After transfer on PVDF membranes, CDK4, cyclin D3 and p27 were detected as above.

Metabolic $^{32}$P-labeling of proteins - One hour before arrest, subconfluent thyrocytes in 10-cm Petri dishes were rinsed twice in phosphate-free DMEM and incubated in 3 ml phosphate-free DMEM supplemented with non-essential amino acids. $[^{32}\text{P}]$Phosphate (1.7 mCi/ml) was then added for the last 40 min. $^{32}$P-Labeled CDK4, cyclin D3 or p27 were immunoprecipitated, separated by 2D-gel electrophoresis and transferred to PVDF membranes exactly as above, and detected by film autoradiography (two-weeks exposure). The positions
of CDK4, cyclin D3 or p27 in these membranes were then defined by their immunodetection as above by enhanced chemiluminescence.
RESULTS AND DISCUSSION

Late G1 phase progression and phosphorylation of pRb family proteins depend on sustained cAMP stimulation – In response to TSH or forskolin, quiescent dog thyrocytes cultured in the presence of insulin progressively enter DNA synthesis phase after about 18 h (55,57)(Fig. 1A). Cells were stimulated with forskolin (F) for 16, 20 or 24h and then rapidly rinsed twice with prewarmed Hepes-buffered DMEM-F12 medium and subsequently incubated either in forskolin-free control medium (FC) or again with forskolin (FF). As shown in Fig. 1A, the fraction of cells having entered S phase gradually increased in cells replaced in the presence of forskolin whereas cells deprived of forskolin were largely prevented from entering S phase already 2 h after cell rinsing. This confirms that DNA synthesis stimulation by forskolin requires its continuous presence until a late G1 R point (54). This implies a rate-limiting cAMP-dependent event(s), which must be labile in order to explain the rapid consequence of forskolin deprivation on DNA synthesis initiation. As shown in Fig. 1B, this labile cAMP-dependent event(s) targeted the phosphorylations of pRb family proteins. Upon forskolin withdrawal, the slower migrating hyperphosphorylated forms of pRb, p107 and p130 stopped increasing and progressively disappeared in favor of a reaccumulation of the active hypophosphorylated forms of these proteins2. The phosphorylations of pRb at Ser807 and 811 also progressively declined (Fig. 1B)2. As these phosphorylations have been specifically ascribed to CDK4 but not CDK2 (8), this suggests that cAMP may positively control the R point transit by regulating the activity of CDK4.

The activity, but not the assembly, of the cyclin D3-CDK4-p27 holoenzyme depends on sustained cAMP stimulation - To confirm that the arrest of stimulation by cAMP decreases the activity of CDK4, we measured the activity of CDK4 co-immunoprecipitated by the specific cyclin D3 monoclonal antibody DCS-28 using a 56 kDa fragment of pRb as a substrate. The phosphorylation of this fragment was detected with a phospho-pRb (Ser780)
antibody (60). As illustrated in Fig. 2, forskolin stimulated both the assembly of cyclin D3-CDK4-p27 complexes and the pRb-kinase activity associated with cyclin D3 (and also p27; unpublished data). The subsequent washing out of forskolin did not affect the presence or formation of these complexes for at least 7 h, but it progressively reduced the associated pRb-kinase activity of cyclin D3-bound CDK4 (Fig. 2B). We therefore conclude that cAMP separately controls the assembly, and then the activity, of the cyclin D3-CDK4 holoenzyme.

*cAMP stimulates the phosphorylation of CDK4* - *In vitro* experiments have demonstrated that the activation of CDK4 requires not only its association with a D-type cyclin but also its phosphorylation at Thr172 by CAK (20,29). In addition, an inhibitory phosphorylation of CDK4 at Tyr17 has been reported to be involved not only in DNA damage-induced G1 arrest, but also in regular cell’s arrest in quiescence (62), but this phosphorylation was not detected by other investigators (20,63). The phosphorylation of CDK4 does not affect its electrophoretic migration in SDS-polyacrylamide gels and thus it has been directly assessed on only very rare occasions in intact mammalian cells (33).

To investigate whether cAMP could modulate the phosphorylations of CDK4, we separated CDK4 using the high resolution power of 2D-gel electrophoresis. CDK4 from whole extracts of maximally stimulated dog thyrocytes presented 5 different isoforms that differ by their isoelectric point (Fig. 3A). Only the most negatively charged form 3 and minor form 4 were phosphorylated, as shown by 32P-orthophosphate metabolic labeling (Fig. 3A). The main phosphorylated form 3 of CDK4 was preferentially found in cyclin D3-CDK4 complexes (see Fig. 5, below). Moreover, the presence of the phosphoform 3 of CDK4 closely paralleled the modulation of the catalytic activity of CDK4 in different cells subjected to different treatments, including dog thyrocytes stimulated to proliferate by EGF+serum independently of cAMP, or arrested in G1 by TGFβ in the presence of TSH (K.C., L. Bockstaele, S.P., J.E.D., P.P.R., submitted), and serum-stimulated human fibroblasts (K.C.,
unpublished). It thus likely corresponds to the activatory phosphorylation of CDK4 by CAK at Thr172, which is the predominant phosphorylation site of cyclin D-bound CDK4 in infected insect cells (20) and murine macrophages (33), consistent with *in vitro* data showing that CAK phosphorylates and activates CDK4 complexed to D-type cyclins but not monomeric CDK4 (20).

Forskolin markedly increased the proportion of the CDK4 phosphoform 3 in whole extracts of dog thyrocytes but, contrary to our expectation, the presence of this form was only slightly affected by subsequent forskolin elimination (Fig. 3B). Although consistently observed in all the experiments, this weak reduction of CDK4 phosphorylation upon forskolin withdrawal appeared insufficient to explain the strong reduction of CDK4 activity shown in Fig. 2B.

We next investigated whether the control by cAMP of the activity of the cyclin D3-CDK4-p27 holoenzyme could involve changes in the posttranslational modifications of cyclin D3 or p27. 2D-gel electrophoresis revealed at least 8 forms of cyclin D3 (Fig. 4A) and 8 forms of p27 (Fig. 4B) with similar apparent molecular weight but different isoelectric points. Only the 5 more negatively charged forms of cyclin D3 and p27 were phosphorylated, as demonstrated by 32P-orthophosphate metabolic labeling (K.C., L. Bockstaele, S.P., J.E.D., P.P.R., submitted). No modifications of the relative abundances of these different forms of cyclin D3 or p27 were detected after stimulation of the thyrocytes by forskolin or upon forskolin withdrawal (Fig. 4A,B). All the most abundant (un)phosphorylated forms of cyclin D3 and p27 were found to be present, in unaltered relative proportions, in the co-immunoprecipitated cyclin D3-CDK4-p27 complexes from cells stimulated with, and then deprived of, forskolin (not shown).

*The phosphorylation of cyclin D3-bound CDK4 depends on sustained cAMP stimulation* - Because p27 binding to cyclin D-CDK4 has been shown to either prevent (33) or
support (64) the \textit{in vitro} activation of these complexes by CAK, perhaps depending on the stoichiometry of p27 relative to the cyclin D (41,64), we next compared the 2D-gel electrophoresis pattern of CDK4 co-immunoprecipitated with cyclin D3 or p27. As shown in Fig. 5\textit{A}, form 1 and even more the phosphoform 3 of CDK4 were preferentially associated not only with cyclin D3 but also with p27 in forskolin-stimulated dog thyrocytes. This clearly establishes that p27 can support rather than impair (33) the phosphorylation of CDK4 in a cAMP-stimulated cell. Upon forskolin elimination, the phosphorylated form 3, but not form 1, of CDK4 progressively disappeared from the cyclin D3 co-immunoprecipitates (Fig. 5\textit{A}). Nevertheless, CDK4 phosphorylation persisted in the p27 co-immunoprecipitates from the same cell extracts (Fig. 5\textit{A}), which was consistent with the only slightly reduced phosphorylation of total CDK4 after forskolin deprivation (Fig. 3\textit{B}). Seven hours after forskolin elimination, the most abundant CDK4 complexes thus consisted of unphosphorylated CDK4 form 1 bound to both cyclin D3 and p27, and of CDK4 phosphoform 3 bound to p27 but not to cyclin D3 (Fig. 5\textit{B}). Both CDK4 complexes are expected to be inactive, due either to lack of the activatory phosphorylation (20) or absence of the cyclin required for activation and binding to pRb proteins (65,66). Indeed, the disappearance of the cyclin D3-bound phosphoform 3 of CDK4 exactly paralleled the diminution of the catalytic activity of the cyclin D3 complex (Fig. 5\textit{C}). It thus appears as the target of the crucial cAMP-dependent event that controls the activity of CDK4, the phosphorylation of pRb family proteins and the passage through the R point.

\textit{Persistent association of phosphorylated CDK4 with p27 but not with cyclin D3} - The disappearance of phosphorylated CDK4 from the cyclin D3 complexes upon forskolin deprivation suggests that cAMP somehow controls the CAK activity. Nevertheless, this hypothesis, apparently, could not explain the paradoxical persistence of p27-bound phosphorylated CDK4. Moreover, to our knowledge, the existence of p27-CDK4 complexes
devoid of a cyclin D, as deduced from the comparison of cyclin D3 and p27 co-immunoprecipitates from forskolin-deprived cells (Fig. 5B), has never been reported (32). We have thus investigated the stability of phosphorylated CDK4 and CDK4 complexes in another situation. The inhibition of protein synthesis by cycloheximide mimics the effect of forskolin withdrawal on the kinetics of S-phase entry in dog thyrocytes (54). As D-type cyclins are expected to be more labile than CDK4 and p27 (7,18), we have examined whether p27-bound phosphorylated CDK4 could subsist after the disappearance of cyclin D3 provoked by an administration of cycloheximide to forskolin-stimulated dog thyrocytes. As shown in Fig. 6A, the addition of cycloheximide 20 h after forskolin reversed most of the phosphorylation of pRb within 3 h. This was associated with a strong reduction of cyclin D3 levels already 3 h after cycloheximide addition (Fig. 6A). By contrast, the presence of CDK4 was almost unaffected and only the steady accumulation of p27 that occurred during the continuous forskolin stimulation was prevented by cycloheximide (Fig. 6A). Seven hours after cycloheximide addition, the presence of cyclin D3 in p27 co-immunoprecipitates was more completely reduced than the presence of CDK4 in these complexes (Fig. 6A). As measured by laser-scanning densitometry, the cyclin D3 / CDK4 ratio in p27 co-immunoprecipitates was reduced approximatively 2.5 fold by the 7-h cycloheximide treatment. If p27 complexes normally contained equal amounts of cyclin D3 and CDK4 in the absence of cycloheximide, about 60% of p27-CDK4 complexes should thus be devoid of cyclin D3 7 h after cycloheximide addition. Moreover, contrasting with the almost complete reversal of pRb phosphorylation and the strongly diminished presence of cyclin D3 (Fig. 6A), the phosphorylation of CDK4 (i.e. the presence of the phosphoform 3) was only partially reduced by the cycloheximide treatment (Fig. 6B). Considering that the disappearance of cyclin D3 should also arrest the phosphorylation of CDK4 by CAK (as it occurs only within D-type cyclin complexes (20)), this suggests that the phosphorylation of CDK4 was relatively stable.
Indeed, as shown in Fig. 6C, in p27 co-immunoprecipitates from forskolin-stimulated cells, cycloheximide only slightly reduced the presence of CDK4 relative to p27 and did not reduce the proportion of phosphorylated form 3 compared to unphosphorylated form 1 of CDK4. Therefore, relatively stable complexes containing p27 and phosphorylated CDK4 can survive the disappearance of cyclin D3 and the arrest of CDK4 phosphorylation that it should provoke.

**Mechanisms of cAMP-dependent activation of CDK4** - We propose that the unexpected stability of p27-phospho CDK4 complexes demonstrated above also explains the persistence of p27-bound phosphorylated CDK4 observed despite the arrest of CDK4 phosphorylation provoked by the interruption of forskolin stimulation (Fig. 5A,B). As schematized in Fig. 7, the decline of CDK4 phosphorylation could be more rapid within the cyclin D3-CDK4 complexes (Fig. 5A,C), due to their higher turnover rate driven by the intrinsic instability of cyclin D3 (Fig. 6A).

The present study and our previous reports have thus demonstrated that the cAMP-dependent activation of CDK4 in dog thyrocytes successively integrates the following steps, which are dissociated by their independent regulations: (i) the required synthesis of cyclin D3 supported by insulin/IGF-1 and other comitogenic factors but not by cAMP (25,53); (ii) the cAMP-dependent assembly of cyclin D3 with constitutively expressed CDK4 (23,53) and their nuclear import facilitated by their binding to nuclear p27 synthesized in response to cAMP (26,52)(K.C., L. Bockstaele, S.P., J.E.D., P.P.R., submitted). These steps were not reversed by the arrest of the cAMP stimulation (Fig. 2A)(47) despite the intrinsic instability of cyclin D3, which suggests that the unknown cAMP-dependent event responsible for the continuous formation of cyclin D3-CDK4-p27 complexes should be relatively stable (the X event in Fig. 7); and (iii) the crucial cAMP-dependent activating phosphorylation of CDK4 within cyclin D3-CDK4-p27 complexes. This last step, which is shown here to be separately
regulated by cAMP (Fig. 7), determines the catalytic activity of CDK4, the phosphorylation of Rb family proteins and thus the passage through the R point.

The mechanism by which cAMP regulates the phosphorylation of cyclin D3-bound CDK4 remains enigmatic. Modifications of phosphorylations of cyclin D3 or p27 did not appear to be involved (Fig. 4). In principle, cAMP might regulate the activity of CAK, its substrate specificity or its access to CDK4. In mammalian cells, the major CAK activity that phosphorylates CDK4/6, CDK2 and CDK1 is constituted of cyclin H-CDK7-MAT1, which are also subunits of transcription factor II H (67,68). However, as generally considered, mitogenic stimulations and progression through the interphase of cell cycle do not affect the expression and nuclear location of these different subunits, the activating Thr170 phosphorylation of CDK7 and the constitutive enzymatic activity of the CDK7 complex assayed in vitro (27-29,69-71). Nevertheless, a few recent studies have pointed out the Thr160 phosphorylation of CDK2 as a direct target of a TGFβ antimitogenic cascade (72) or treatments that arrest growth factor signal transduction (73-75). In some of these studies, the activation of CDK4 (72,73) and/or the in vitro assayed activity of CDK7 (72,75) remain unaffected, leading these authors to suggest the involvement of distinct CAK activities. A second mammalian CAK activity has been reported to be immunologically related to the monomeric budding yeast Cak1p (72,76), but its nature remains elusive and no metazoan Cak1p ortholog has been identified by analyses of genome sequences (77,78). In dog thyrocytes, CDK4 phosphorylation preferentially (only ?) occurs within cyclin D complexes. The involved CAK activity thus more likely corresponds to the heterotrimeric CDK7, which preferentially phosphorylates cyclin-bound CDKs, than to monomeric Cak1p-types CAK that exhibit a preference for cyclin-free CDKs (76,79). The identification of the cAMP target that mediates the induced phosphorylation of CDK4 may require some improvement of our understanding of the regulation of mammalian CAK(s).
To conclude, the present investigation of the cAMP-dependent cell cycle regulation of thyrocytes provides a first example where the phosphorylation and activity of CDK4 is regulated independently of changes in its association with cyclin and CDK «inhibitor», establishing a new mechanism of cell cycle control crucially implicated in the passage through the R point. The separation of phosphorylated and unphosphorylated forms of CDK4 using 2D-gel electrophoresis should prove a valuable tool to directly re-evaluate in different cell contexts the phosphorylation of CDK4 as an important target for mitogenic control.
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FOOTNOTES

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Abbreviations used are: R point, restriction point; pRb, retinoblastoma susceptibility protein; CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; TSH, thyroid stimulating hormone; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; 2D, two-dimensional; EGF, epidermal growth factor; TGFβ, transforming growth factor β.

Due to a relative increase of total pRb content, 3 h after forskolin deprivation (FC3 in Fig. 1B), the still partial diminution of pRb phosphorylation was more apparent on the reaccumulation of the (active) hypophosphorylated form than on the reduction of the slower migrating hyperphosphorylated form also revealed using the Ser807/811 phosphosite-specific pRb antibody.

Two minor phosphorylated forms of p27 were detected by overexposure of the western blots but are not seen in Fig. 4B.
**FIGURE LEGENDS**

**Figure 1. Late G1 phase progression and phosphorylation of pRb family proteins depend on sustained forskolin stimulation.** *A*, initially quiescent dog thyrocytes were stimulated at 0 h with forskolin (*F*). At 16 h (●, ○), 20 h (●, ◊), or 24 h (▲, Δ) (arrows), cells were rapidly rinsed twice and immediately replaced in the culture medium with forskolin (*F-F*) (*filled symbols*), or in the control medium without forskolin (*F-C*) (*empty symbols*). At the indicated times, cells were fixed and the percentage of cells in S and G2 phases was determined from the pattern of PCNA immunofluorescent labeling (57). *B*, phosphorylation of proteins of the Rb family, as detected by their electrophoretic shifts evidenced by Western blotting (Santa-Cruz antibodies against total pRb, p107 or p130) or by the Ser807/811 phosphosite-specific pRb antibody (*PpRb 807-811*) (arrows). Dog thyrocytes remained in control medium (*C*), or were stimulated for 17 h with forskolin (*F*), then rinsed and replaced in forskolin medium (*FF*) or in control medium without forskolin (*FC*) for 3 h (*FF3, FC3*) or 7 h (*FF7, FC7*).

**Figure 2. Forskolin deprivation reduces the catalytic activity but not the assembly of cyclin D3-CDK4-p27 complexes.** Dog thyrocytes remained quiescent in control medium (*C*) or were stimulated with forskolin (*F*) for 17 h (*A*) or 24 h (*B*) and then rinsed and incubated in forskolin medium (*FF*) or in the control medium without forskolin (*FC*) for 3 h (*FF3, FC3*) or 7 h (*FF7, FC7*). *A*, the same cell lysates were subjected to immunoprecipitation (*IP*) with anti-cyclin D3 (*D3*) or anti-p27 antibodies. Western blotting analysis of the (co)-immunoprecipitated proteins was performed using cyclin D3, CDK4 and p27 antibodies. *B*, cell lysates were immunoprecipitated (*IP*) with anti-cyclin D3 antibody, assayed for pRb-kinase activity, separated by SDS-PAGE and immunoblotted. Cyclin D3, CDK4, p27 and the pRb fragment phosphorylated *in vitro* at Ser780 (*pRb kinase*) were detected using specific antibodies.
Figure 3. **Forskolin stimulates the phosphorylation of CDK4.** *A,* an extract of dog thyrocytes maximally stimulated for 20 h by TSH+EGF and metabolically labeled with $^{32}$P was immunoprecipitated with a CDK4 antibody, separated by 2D-gel electrophoresis and electroblotted. The membrane was exposed for autoradiographical detection of phosphorylated forms of CDK4 ($^{32}$P) and then total immunodetected CDK4 was revealed from the same membrane by enhanced chemiluminescence (*W.B.*). *B,* immunodetection of total CDK4 separated by 2D-gel electrophoresis (immunoprecipitated with anti-CDK4 antibody) from dog thyrocytes that remained quiescent in control medium (*C*) or were stimulated with forskolin (*F*) for 19 h and then rinsed and incubated in forskolin medium (*FF*) or in the control medium without forskolin (*FC*) for 3 h (*FF3, FC3*) or 7 h (*FF7, FC7*). Arrows indicate the phosphorylated form 3. Data are representative of 7 independent experiments.

Figure 4. **Forskolin stimulation and deprivation do not affect the posttranslational modifications of cyclin D3 (*A*) and p27 (*B*).** Western blotting detection of total cyclin D3 (immunoprecipitated with anti-cyclin D3 antibody) (*A*) or total p27 (immunoprecipitated with anti-p27 antibody) (*B*) separated by 2D-gel electrophoresis from dog thyrocytes that remained quiescent in control medium (*C*) or were stimulated with forskolin (*F*) for 17 h and then rinsed and incubated in forskolin medium (*FF*) or in the control medium without forskolin (*FC*) for 7 h (*FF7, FC7*). Arrows indicate the phosphorylated forms of cyclin D3 and p27 (as identified by $^{32}$P metabolic labeling ; not illustrated). Data are representative of 3 independent experiments.
Figure 5. **Forskolin deprivation reduces the phosphorylation of cyclin D3-bound CDK4 but not of p27-bound CDK4.** Dog thyrocytes remained quiescent in control medium (C) or were stimulated with forskolin (F) for 17 h and then rinsed and incubated in forskolin medium (FF) or in the control medium without forskolin (FC) for 3 h (FF3, FC3) or 7 h (FF7, FC7). The same cell lysates were subjected to immunoprecipitation (IP) with anti-cyclin D3 (D3) or anti-p27 antibodies, separated by 2D-gel electrophoresis, and CDK4 was immunodetected. A, notice the enrichment of CDK4 phosphoform 3 (arrows) in both cyclin D3 IP and p27 IP of forskolin-stimulated cells (F, FF) and the progressive diminution of this form in response to forskolin deprivation (FC) in cyclin D3 IP but not in p27 IP. Data are representative of 6 independent experiments for cyclin D3 IP and of 4 independent experiments for p27 IP. B, schematic representation of the main CDK4 complexes present 7 h after forskolin readdition (FF7) or withdrawal (FC7), as deduced from the comparison of the detections shown in A of CDK4 co-immunoprecipitated with cyclin D3 or p27 antibodies. C, the data in A were subjected to densitometry analysis, illustrating the evolution of CDK4 phosphorylation (ratios of the CDK4 phosphoform 3 over total CDK4 (form 1 + form 3)) in cyclin D3 IP (IPD3) versus p27 IP (IPp27). The pRb kinase activity associated with cyclin D3 (normalized to cyclin D3-bound CDK4 detected on the same membrane), as quantitated by densitometry (data of Fig. 2B), is shown for comparison.

Figure 6. **Analysis of the stability of CDK4 complexes and CDK4 phosphorylation after protein synthesis inhibition.** Dog thyrocytes remained quiescent (C) or were stimulated for 20 h with forskolin (F) and then incubated for 3 or 7 h with (+) or without (-) cycloheximide (10 µg/ml) in the presence of forskolin. A, western blotting detection of pRb, cyclin D3 and CDK4 from whole cell extracts, and of p27, cyclin D3 and CDK4 from p27 co-immunoprecipitates (IPp27). B, immunodetection of total CDK4 separated by 2D-gel
electrophoresis (immunoprecipitated with anti-CDK4 antibody). C, CDK4 and p27 were (co)-immunoprecipitated with anti-p27 antibodies, separated by 2D-gel electrophoresis and simultaneously detected using a mixture of CDK4 and p27 antibodies. Notice the persistence in cycloheximide-treated cells of the CDK4 phosphoform 3 (arrows in B, C) and p27-CDK4 complexes (A, C), despite the strong reduction of cyclin D3 contents and the arrest of pRb phosphorylation (A).

Figure 7. **Suggested explanatory model of the different roles of cAMP in the activation of CDK4.** cAMP separately stimulates (i) the assembly and nuclear import of cyclin D3-CDK4-p27 complexes and (ii) the activating phosphorylation of CDK4 within these complexes. Upon forskolin withdrawal, the presence of the cyclin D3-CDK4-p27 complex is unaffected despite its unstability driven by the intrinsic lability of cyclin D3. This complex thus continues to be formed, which could imply the stability of an unknown cAMP-dependent intermediary (X). The arrest of CDK4 phosphorylation upon forskolin removal more rapidly concerns cyclin D3-CDK4 complexes due to their higher turnover rate, leading to the diminution of their catalytic activity. However, the stable phosphoform of CDK4 unexpectedly persists within a p27 complex devoid of cyclin D3.
Figure 2

A

| IP | D3   | CDK4 | p27  |
|----|------|------|------|
|    | D3   |      |      |
| IP | p27  |      |      |
|    | p27  |      |      |
|    | D3   |      |      |
|    | CDK4 |      |      |

C  F  FC3  FF3  FC7  FF7

B

| IP | D3   | CDK4 | pRb kinase |
|----|------|------|-------------|
|    | D3   |      |             |
| IP | D3   |      |             |
|    | CDK4 |      |             |
|    | pRb kinase | |             |

C  F  FC3  FF3  FC7  FF7
Figure 3

A

W.B.

32P

B

C

FC3

FC7

F

FF3

FF7
Figure 5

A

IP D3  
C  
F  
FC3  
FF3  
FC7  
FF7  

IP p27  

B

Continuous forskolin stimulation

FF7  

D3  
CDK4  
form 1  
P27  
CDK4  
form 3  
active  

FF  

C  

CDK4 phosphorylation

cyclin D3-bound pRb kinase activity
(normalized to CDK4)

Cyclin D3-bound pRb kinase activity
(normalized to CDK4)

cont  
Forsk  
F-F  
Pp27 F-F  
Pp27 F-C  

fold increase

hours
Figure 7

Continuous forskolin stimulation

Arrest of forskolin stimulation
Cyclic AMP-dependent phosphorylation of cyclin D3-bound CDK4 determines the passage through the cell cycle restriction point in thyroid epithelial cells

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