Distinct Specificities of pRb Phosphorylation by CDK4 Activated by Cyclin D1 or Cyclin D3

Differential Involvement in the Distinct Mitogenic Modes of Thyroid Epithelial Cells

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INTRODUCTION

The retinoblastoma tumor suppressor protein (pRb) plays a central role in the negative regulation of the cell cycle progression.¹-⁴ It also plays important roles in many stages of differentiation processes, including terminal cell cycle exit, protection from apoptosis and induction of tissue-specific gene expression through its interaction with a variety of transcription factors.⁵,⁶ In quiescent cells, pRb is underphosphorylated and inhibits transcription factor binding to the E2F sites (Ser807/811 and Thr826) required for its electrophoretic mobility shift. Therefore, different D-type cyclins could differently impact some pRb functions, which should be considered not only in the understanding of the relationships between cell cycle and differentiation expression in the distinct mitogenic modes of thyroid cells, but also in various development or differentiation models associated with dramatic switches in the expression of individual D-type cyclins.

ABSTRACT

Two distinct mitogenic modes coexist in the physiologically relevant model of primary cultures of dog thyroid epithelial cells. The differentiation-associated mitogenic stimulation by TSH and cAMP specifically requires the assembly and activation of cyclin D3-cyclin-dependent kinase (CDK)4 associated to p27kip1, while the dedifferentiating proliferation induced by growth factors is associated with induction of cyclin D1. Here, we suggest that the related CDK “inhibitors” p21cip1 and p27 are differentially utilized as positive CDK4 regulators in these mitogenic stimulations. p21 was induced by EGF + serum, but repressed by TSH, which, as previously shown, upregulates p27. In response to EGF + serum, p21 supported the nuclear localization, phosphorylation and pRb-kinase activity of CDK4. Unexpectedly, partly different site-specificities of pRb-kinase activity, leading to similar differences in the phosphorylation pattern of pRb in intact cells, were associated with cyclin D3-CDK4 bound to p27 in TSH-stimulated cells, or with CDK4 bound to p21 in growth factor-stimulated cells. These differences were ascribed to the predominant association of the latter complex to cyclin D1. Indeed, in different cell types and species, cyclin D1 varied from cyclin D3 by more efficiently driving the phosphorylation of pRb at sites (Ser807/811 and Thr826) required for its electrophoretic mobility shift. Therefore, different D-type cyclins could differently impact some pRb functions, which should be considered not only in the understanding of the relationships between cell cycle and differentiation expression in the distinct mitogenic modes of thyroid cells, but also in various development or differentiation models associated with dramatic switches in the expression of individual D-type cyclins.

KEY WORDS

CDK4, D-type cyclins, p21, p27, pRb, cell cycle, differentiation

ABBREVIATIONS

2D two-dimensional
BrdUrd bromodeoxyuridine
C AK CDK-activating kinase
CDK cyclin-dependent kinase
DTT dithiothreitol
EGF epidermal growth factor
FCS fetal calf serum
HDAC histone deacetylase
PAGE polyacrylamide gel electrophoresis
PCNA proliferating cell nuclear antigen
pRb retinoblastoma susceptibility protein
TSH thyroid stimulating hormone
TGFβ transforming growth factor β

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by all the cyclin-D-CDK complexes is the phosphorylation and inactivation of pRb. Nevertheless, functional roles of the different D-type cyclins might not be fully redundant. D-type cyclins have been suggested to play distinct roles in cell differentiation. Cyclin D3 is expressed in various nonproliferating cell types and during muscle differentiation cyclin D3 is strongly induced while cyclin D1 is repressed, which correlates with the exit of myoblasts from the cell cycle.

As initially considered, mitogens activate CDK4/6 not only by inducing at least one D-type cyclin, but also by downregulating or inactivating CDK inhibitors such as p27kip1. However, D-type cyclins and CDK4 poorly assemble in vitro or in the absence of a mitogenic stimulation. Moreover, they do not contain a nuclear localization sequence (NLS), whereas the activation of CDK4 requires its Thr172 phosphorylation likely by nuclear CDK-activating kinase (CAK, cyclin H-CDK7). Although there is still a consensus to consider p27kip1 as an inhibitor of CDK2, p27-related members of the CIP/KIP family, which possess a NLS and distinct binding domains for cyclins and CDKs, have been found to be associated with a pRb-kinase activity, and can stabilize cyclin D-CDK complexes in vitro or in cotransfected cells and/or target these complexes to the nucleus. D-type cyclins and cyclin D1-CDK4 complexes devoid of CIP/KIP proteins are active as pRb-kinases. Later in the progression, the sequestration of p27kip1 within D-type cyclin-CDK complexes contributes to activate cyclin E/A-CDK2.

Two distinct modes of mitogen stimulation coexist in the physiologically relevant model of primary cultures of dog thyroid epithelial cells. Thyrotropin (TSH) via cAMP induces proliferation and differentiation expression, whereas growth factors including EGF induce proliferation and dedifferentiation. Both mitogenic stimuli require the activity of CDK4, but the positive control of DNA synthesis initiation and G1/S-replicative phase progression by cAMP is unique until now as it does not activate Ras-dependent signalling and upregulates p27kip1 instead of D-type cyclins. The expression of cyclin D3 supported by facilitative comitogenic factors (insulin and carbachol) is specifically required in the cAMP-dependent cell cycle, but not in the mitogenic stimulation by growth factors that induce cyclin D1. cAMP promotes the assembly of cyclin D3-CDK4 complexes, their nuclear import associated with their binding to p27, and then stimulates the Thr172 phosphorylation and activity of CDK4 within this complex.

In this study, two new essential features of these distinct mitogenic modes are reported, initially deriving from the same experiments. First, we suggest a differential positive involvement of p27kip1 and p21kip1 in the nuclear import and activation of CDK4 complexes required for the mitogenic stimulations of dog thyrocytes by TSH (cAMP) or by growth factors. Second, different patterns of pRb-kinase activity of CDK4 were associated with p27 in TSH-stimulated cells and with p21 in growth factor-stimulated cells. Unexpectedly, these different pRb-kinase activities turned to result from their predominant association with cyclin D3 or cyclin D1, respectively. Furthermore, we generalize to other cell systems the novel observation that different D-type cyclins support distinct pRb-kinase activities of CDK4.

**MATERIALS AND METHODS**

**Cell cultures and transfections.** Follicular cells were obtained from thyroid tissue of dogs or African green monkeys (obtained from a local pharmaceutical company) as described, cultured in monolayer in the control medium, i.e., DMEM + Ham’s F12 + MCD104 medium (2:1:1 by vol.) supplemented with bovine insulin (Sigma; St. Louis, MO; 5 μg/ml), ascorbic acid (40 μg/ml), and antibiotics: quiescent cells at day 4 or 5 were stimulated with bovine TSH (Sigma, 1mU/ml), the general adenyl cyclase activator forskolin (10 M, Calbiochem), or the combination of murine epidermal growth factor (EGF; Sigma, 25 ng/ml) and 10% fetal calf serum (FCS). To assay the induction of DNA synthesis, cells in 3-cm Petri dishes were incubated for 24 h before fixation in the presence of BrdUrd. The incorporation of BrdUrd was detected by immunofluorescence, and BrdUrd-labeled nuclei (1000/dish) were counted as described. Human diploid fibroblasts (IMR-90) (American Type Culture Collection, Manassas, VA) were cultured as described.

After reaching semiconfluency, they were synchronized by starvation in 0.2% FCS for three days. Quiescent cells were then growth stimulated by addition of FCS (20%). CHO (Chinese-hamster ovary) cells were transfected using fucgene 6 (Roche Diagnostics, Mannheim, Germany) followed by a biotinylated anti-mouse immunoglobulin antibody and fluorescein-conjugated streptavidin. CDK4 was simultaneously revealed using a selected batch of the C-22 polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA), followed by a Cy3-conjugated anti-rabbit immunoglobulin antibody. For double staining of proliferating cell nuclear antigen (PCNA) used as a cell cycle marker and p21 using two mouse monoclonal antibodies, fixed cells were incubated overnight at 4°C with the p21 monoclonal antibody from Transduction Laboratories (BD-Biosciences, Erembodegem, Belgium), and then for two hours at room temperature with biotinylated anti-mouse immunoglobulins and for 1 h with Texas Red streptavidin. Washed cells were then successively incubated for 30 min with normal mouse serum (1/100), for two hours with unconjugated anti-mouse immunoglobulin G Fab fragment (50 μg/ml; Jackson Immuno-Research Laboratories, West Grove, PA), for 1 h with PC10 (1/400; DAKOPATTS, Copenhagen, Denmark), and for 1 h with fluorescein-conjugated anti-mouse immunoglobulin (1/50; Amersham International). This “sandwich” procedure is designed to avoid binding of PC10 (the PCNA monoclonal antibody) to the first secondary antibody and binding of the second secondary antibody to the p21 antibody.

**Immunoprecipitation and pRb-kinase assay.** Subconfluent cultures of thyrocytes, IMR-90 or CHO cells 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, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 10 mM DTT, protease inhibitors, 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 D1 (DCS-11) (Biosource International, Camarillo, CA), monoclonal antibody against cyclin D3 (DCS-28) (NeoMarkers, Fremont, CA), C-19 p21 polyclonal antibody (Santa Cruz), a mixture of the K25020 anti-p27 monoclonal antibody from Transduction Laboratories (BD-Biosciences, Erembodegem, Belgium) and the C-15 p27...
Figure 1. p27 or p21 support the pRb-kinase activity and phosphorylation of CDK4 respectively stimulated by TSH or EGF-serum. (A) CDK4, cyclin D1 (cyc D1), cyclin D3 (cyc D3), and p27 were detected from whole cell extracts [WCE] of dog thyrocytes stimulated or not [Cont] with TSH for 24 h or with EGF + serum for 16 h (when a maximum of cells were in late G1 stage in both stimulations). (B and C) Extracts of dog thyrocytes stimulated or not [Cont] with TSH for 24 h or with EGF+serum for 16 h were immunoprecipitated (IP) with anti-cyclin D3 (D3), anti-p21 or anti-p27 antibodies, assayed for pRb-kinase activity, separated by SDS-PAGE and immunoblotted. p21, p27, cyclin D1 (cyc D1), cyclin D3 (cyc D3), CDK4 and the pRb fragment phosphorylated in vitro at Ser780 (P-Rb780) were detected using specific antibodies. (D) Dog thyrocytes were stimulated or not (Cont) with TSH for 24 h or with EGF+serum for 16 h. Cell lysates precipitated (IP) with anti-cyclin D3 (D3), anti-p21 or anti-p27 antibodies, separated by 2D-gel electrophoresis, and CDK4 was immunodetected. Form 3 of CDK4 was associated with p27 (Fig. 1C), as previously shown.50 Instead, in TSH-treated cells (and to a lesser extent in EGF+serum-stimulated cells), the stimulated pRb-kinase activity of CDK4 was associated with p21 (Fig. 1B and C). In response to EGF + serum, abundant complexes containing p21, cyclin D1 (or to a lower extent cyclin D3) and CDK4 were formed, and a strong pRb-kinase activity was associated with p21 (Fig. 1B and C). By contrast, only very weak presence and activity of CDK4 were associated with p21 in TSH-stimulated cells. Instead, in TSH-treated cells (and to a lesser extent in EGF + serum-stimulated cells), the stimulated pRb-kinase activity of CDK4 was associated with p27 (Fig. 1C), as previously shown.50
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The nuclear import of CDK4 is associated with p21 in thyrocytes stimulated by EGF + serum, but not in cells stimulated by TSH. In dog thyrocytes stimulated by TSH, the extent of the nuclear translocation of CDK4 and cyclin D3 strictly correlates in individual cells with the presence of nuclear p27 synthesized in response to TSH.\textsuperscript{50} TGFβ does not prevent the TSH-dependent formation of cyclin D3-CDK4 complexes but reduces their association with p27 and their nuclear import.\textsuperscript{12,28} These previous observations support a positive role of p27 in the nuclear translocation of cyclin D3-CDK4 complexes in response to TSH. As cyclin D1 and p21 seemed particularly important for the activation of CDK4 by growth factors in dog thyrocytes (Fig. 1), we have examined the colocalization of these proteins with CDK4 (Fig. 2). Both forskolin (which perfectly mimics all the cAMP-dependent effects of TSH in dog thyrocytes) and EGF + serum stimulated the nuclear translocation of CDK4, but only EGF + serum enhanced the nuclear presence of cyclin D1 (not shown; consistent with the induction of cyclin D1 expression by growth factors but not by TSH or forskolin\textsuperscript{53} (Fig. 1A)). The double immunofluorescent labelling of cyclin D1 and CDK4 demonstrated a very good correlation in individual cells of the nuclear labelling of both proteins in cells treated by EGF + serum, but not in cells stimulated by forskolin (not shown). EGF + serum but not forskolin increased the nuclear presence of p21 (Fig. 2), consistent with the induction of this protein by EGF + serum but not by TSH (Fig. 1B and C). As shown by the double immunofluorescent labelling of CDK4 and p21, the nuclear translocation of CDK4 induced by EGF + serum closely correlated with the variable intensity of the nuclear labelling of p21 (Fig. 2). This supports the hypothesis that the nuclear localization of cyclin D1-CDK4 complexes formed in EGF + serum-stimulated cells could indeed depend on the nuclear content of p21 induced by this mitogenic treatment.

Whereas the nuclear presence of p27 steadily increases in TSH-treated cells, including in cells progressing in G\textsubscript{1} and S phases,\textsuperscript{48} in EGF + serum-stimulated cells the increased nuclear presence of p21 was detected 8 h after stimulation, was maximum at 16 h, but the proportion of p21-positive cells declined thereafter (not shown). The double labelling of p21 and PCNA (previously demonstrated in dog thyrocytes as a cell cycle marker allowing to discriminate cells in late G\textsubscript{1}, S, G\textsubscript{2} and mitosis phases\textsuperscript{54,53}) showed that after EGF + serum-stimulation, p21 was found increased in late G\textsubscript{1}-phase cells (i.e., cells with an increased diffuse nuclear staining of PCNA), but it disappeared as soon as cells were detected in S-phase by the speckled appearance of the nuclear PCNA labelling (which reflects the association of PCNA at clusters of DNA replication sites\textsuperscript{53}) (Fig. 3). Thus, at variance with the situation observed for p27 in TSH-treated cells,\textsuperscript{48} the disappearance of p21 might well be required for the entry of thyrocytes into S-phase induced by growth factors.

Different pRb-kinase activities are associated with p21 in EGF + serum-stimulated dog thyrocytes and with p27 and cyclin D3 in TSH-stimulated cells. In the pRb-kinase assays illustrated in Figure 1B and C), we noticed that the migration of the pRb fragment phosphorylated in vitro on Ser780 was different in p27-immunoprecipitated complexes from dog thyrocytes stimulated by TSH (one main band) or in p21-immunoprecipitations from EGF + serum-stimulated cells (a doublet of two equivalent bands). By contrast, the pRb fragment phosphorylated on Ser780 by the immunoprecipitated cyclin D3 complexes was detected as only one main band from both TSH-stimulated cells and EGF + serum-stimulated cells (Fig. 1B). In parallel experiments performed using human fibroblasts stimulated by serum, the pRb fragment phosphorylated by immunoprecipitated cyclin D1 complexes also resolved as a doublet when detected using the Ser807/811 phospho-specific pRb antibody (see below), as from p21 complexes of dog thyrocytes stimulated by EGF + serum.

These puzzling observations suggested that CDK4 complexes, depending on their composition, could differentially phosphorylate pRb on distinct sites besides the Ser780. In dog thyrocytes stimulated by EGF or EGF + serum, we have thus reinvestigated the pRb-kinase activity of the different CDK4 complexes using phosphospecific antibodies that recognize other phosphorylations of pRb (Ser807/811 and Thr826) specifically ascribed to CDK4/6.\textsuperscript{55,56} The detection of the in vitro Thr826-phosphorylation of the pRb fragment by the p21 complex from EGF + serum-stimulated thyrocytes also displayed a strong additional band as compared to pRb-kinase assays performed with immunoprecipitations of p27 or cyclin D3 from TSH-stimulated cells (Fig. 4A). Moreover, p21 complexes from EGF + serum-stimulated cells more efficiently phosphorylated the pRb fragment on Ser807/811, compared to phosphorylation at Ser780, which was most actively performed by cyclin D3 and p27 complexes from TSH-treated cells (Fig. 4A).

We thus first hypothesized that the presence of p21 or p27 in CDK4 complexes could differently affect the specificity of their pRb-kinase activity. However, similar specificities of pRb-kinase activity were associated with cyclin D3 in thyrocytes stimulated by TSH or EGF + serum, i.e., in the presence of very different concentrations of p27 and p21 (Fig. 4). Furthermore,
the various differences in the phosphorylation pattern of the pRb fragment also correlated with the relative presences of cyclin D1 or cyclin D3 in CDK4 complexes precipitated using p27 or p21 antibodies from cells stimulated by TSH or EGF + serum (Fig. 4A). Indeed, due to its induction by growth factors but not by TSH in dog thyrocytes\(^{23}\) (Fig. 1A), cyclin D1 was markedly more represented in p21-CDK4 complexes from EGF + serum-stimulated cells. As quantitated by densitometry of the western blotting detections of the pRb-kinase assays, the normalized ratios of the pRb-kinase activities on Ser807/811 versus Ser780 were quite different in various p21- or p27-bound CDK4 complexes from diversely stimulated dog thyrocytes, and they closely correlated with the relative representation of cyclin D1 versus cyclin D3 in these complexes (Fig. 4B). This raises the possibility that the respective association of CDK4 with either cyclin D1 or cyclin D3, rather than its binding to p21 or p27, or any other factor related to the distinct signalling cascades activated by growth factors or TSH, might be the main factor orienting CDK4 activity towards a most efficient phosphorylation of pRb on Ser 807/811 or on Ser780.

Different pRb-kinase activities are supported by cyclin D1 and cyclin D3. No cyclin D1 antibodies are available to directly demonstrate the pRb-kinase activity associated with cyclin D1 in dog cells. We have thus used primary cultures of monkey thyrocytes stimulated by EGF + serum to directly compare the site specificity of the pRb-kinase activities supported by cyclin D1 or cyclin D3. Equivalent amounts of CDK4 were associated with cyclin D1 or cyclin D3 in response to EGF + serum in these cells (Fig. 5). Using the Ser780 phospho-specific antibody, the pRb phosphorylation pattern supported by cyclin D1 in monkey thyrocytes was found to be identical (one doublet) to the one associated with p21 from dog thyrocytes stimulated by EGF + serum, and the pRb phosphorylation patterns supported...
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Figure 5. Distinct patterns of pRb-kinase activity are associated with cyclin D1 or cyclin D3 in monkey thyrocytes. Quiescent monkey thyrocytes were stimulated or not [C] for 20 h with EGF + serum [EGF + S]. Cell extracts were immunoprecipitated [IP] with anti-cyclin D1 (D1) or anti-cyclin D3 (D3) antibodies, assayed for pRb-kinase activity, separated by SDS-PAGE and immunoblotted. Cyclin D1 (cyc D1), cyclin D3 (cyc D3), CDK4 and the pRb fragment phosphorylated in vitro at Ser780 (P-Rb780), Ser807-811 (P-Rb807-811) or Ser795 (P-Rb795) were detected using specific antibodies. For detections of pRb-kinase activities, the same membranes were first detected with a phospho Ser780 antibody [A], or using phospho Ser795 antibody and then phospho Ser780 antibody [A], or using phospho Ser795 antibody and then phospho Ser780 antibody [A]. Arrows indicate the position of the upper band in the doublet of phosphorylated pRb fragment.

These different observations were generalized in serum-stimulated human fibroblasts (IMR-90) (Fig. 6A and B). These cells more abundantly expressed cyclin D1 than cyclin D3, and 8 times as much cell extract had to be engaged in the immunoprecipitation of cyclin D3 than cyclin D1 to obtain similar amounts of CDK4 complexes (Fig. 6). Again, for a similar amount of coimmunoprecipitated CDK4, the detection with the phospho-pRb (Ser780) antibody revealed different patterns of pRb-kinase activities associated with cyclin D1 or cyclin D3 (Fig. 6). Cyclin D1 more efficiently supported the phosphorylation of the pRb fragment on Ser807/811, which was also reflected by an additional upper band in the detections of the phosphorylations on Ser780 and Ser795 (Fig. 6B). An intermediary situation was revealed by the Thr826 phosphospecific antibody. Migration of the Thr826-phosphorylated pRb fragment resembled the Ser807/811 phosphorylated fragment in the pRb-kinase assay of cyclin D1, while in the kinase assay of cyclin D3, it was more similar to the pRb fragment phosphorylated at Ser780 (Fig. 6B). CDK4 complexes immunoprecipitated using a p21 antibody were as active as the cyclin D1 complexes (Fig. 6A). Like in dog thyrocytes stimulated by EGF + serum (Fig. 1), p21 complexes also presented the pRb-kinase pattern characteristic of cyclin D1 complexes, in agreement with their predominant association with cyclin D1 (Fig. 6A).

We next wanted to directly confirm that the different site-specificities in the pRb-kinase assays of CDK4 bound to cyclin D1 or cyclin D3 were independent of their association with p21 or p27. We also wanted to exclude the possibility of interference by some of the antibodies used to precipitate the different CDK4 complexes. CHO cells were transiently transfected with a vector encoding HA-tagged CDK4 alone or together with a vector expressing cyclin D1 or cyclin D3 fused to a flag tag. Cyclin D1 and cyclin D3 were precipitated using the same anti-flag antibody and the pRb-kinase activity of the cyclin D-CDK4 complexes was compared using the different phosphospecific pRb antibodies. CHO cells did not detectably express p21, and the expression of endogenous p27 was insufficient to significantly coimmunoprecipitate overexpressed cyclin D1/3-CDK4 (not shown). Again, the patterns of the pRb-kinase activity detected with the phospho-pRb Ser780, Ser795 or Thr826 antibodies were different in complexes of CDK4 bound to flag-cyclin D1 or flag-cyclin D3 (Fig. 7). Only flag-cyclin D1 was associated with a pRb-kinase activity targeting the Ser807/811 sites (Fig. 7). The different site-specificity of the pRb-kinase activity of CDK4 thus directly depended on its associated D-type cyclin isoform.
Figure 7. Distinct patterns of pRb-kinase activity of CDK4 are associated with cyclin D1 or cyclin D3 in transiently transfected CHO cells. CHO cells were transfected with a vector encoding HA-tagged CDK4 alone or together with a vector expressing a flag-tagged cyclin D1 or cyclin D3. Equivalent expression levels of CDK4 were demonstrated by immunodetection from whole cell extracts (WCE). Cyclin D1 and cyclin D3 complexes were precipitated using an anti-flag antibody, assayed for pRb-kinase activity, separated by SDS-PAGE and immunoblotted. Cyclin D1 (D1-flag) and cyclin D3 (D3-flag) were detected using an anti-flag antibody; CDK4 and the pRb fragment phosphorylated in vitro at Ser780 (P-Rb780), Ser795 (P-Rb795), Ser807-811 (P-Rb807-811) or Thr286 (P-Rb826) were detected using specific antibodies. Arrows indicate the position of the upper band in the doublet of phosphorylated pRb fragment.

Collectively, these results demonstrate that pRb-kinase activities supported by different D-type cyclins present a partly different site-specificity. The different representation of cyclins D1 or D3 in p21 or p27 complexes, as observed in dog thyrocytes stimulated by TSH or growth factors, thus also explains their qualitatively different associated pRb-kinase activities.

The distinct pRb-kinase activities of cyclin D1 or cyclin D3 lead to different phosphorylations of pRb in intact cells. To verify that the distinct pRb-kinase activities associated with cyclin D1 or cyclin D3, as observed in the in vitro assays, could indeed have a physiological relevance, we have compared the phosphorylation patterns of endogenous pRb in primary cultures of dog thyrocytes stimulated by TSH or EGF + serum. As previously shown and also illustrated in Figure 1, the mitogenic stimulation by TSH does not induce D-type cyclins but it specifically requires the assembly and activity of cyclin D3-CDK4. By contrast, the mitogenic stimulation by EGF + serum does not require cyclin D3 but it induces cyclin D1 in addition to increasing cyclin D3 levels (Fig. 8). As shown in Figure 8, TSH mostly stimulated the phosphorylation of endogenous pRb on Ser780. As in the pRb-kinase assays of cyclin D3-bound CDK4, this phosphorylation was mainly concentrated in the lowest band of pRb generally ascribed to hypophosphorylated forms. In response to EGF + serum, the Ser780 phosphorylation was distributed on several upper bands of endogenous pRb (Fig. 8), as observed in the pRb-kinase assays of cyclin D1-CDK4. On the other hand, the Thr286-phosphorylation, observed on upper "hyperphosphorylated" bands of pRb, was induced by EGF + serum but it was weaker and more delayed in response to TSH (Fig. 8). Identical results were obtained using a Ser807 phosphospecific pRb antibody (Fig. 8). In this experiment, phosphorylations of pRb on Thr826 and Ser807, but not on Ser780, correlated with the appearance of cyclin D1 (Fig. 8).

**DISCUSSION**

Differential positive involvement of p21 and p27 in the activation of CDK4 by growth factors and TSH. The role of the CDK "inhibitors" of the Cip/Kip family in the control of cell cycle remains a controversial issue. p21 and p27 were initially thought to inhibit the activity of cyclin D-, E- and A-dependent kinases, at least in part by preventing their activating phosphorylation by CAK. In a physiologically relevant model of thyroid epithelial cells in primary culture, our results demonstrate that, at least at cellular concentrations induced by mitogenic factors, endogenous p21 and p27 do not inhibit the activating phosphorylation of CDK4 and that they support the activity of CDK4 complexes. Whereas TSH and cAMP enhance p27 accumulation, growth factors (EGF + serum) increase the level of p21 in dog thyrocytes, as reported in fibroblasts and vascular cells. The nuclear translocation of CDK4 closely correlated with enhanced p21 levels in individual cells in EGF + serum-treated thyrocytes. The ability of p21 to target cyclin D1-CDK4 to the nucleus clearly depends on its NLS. It also may act by preventing the binding of Thr286-phosphorylated cyclin D1 to the nuclear exportin CRM1. By these mechanisms and possibly by enhancing the assembly of cyclin D1/3-CDK4, the increased p21 expression could play a positive role in the mitogenic stimulation by growth factors in thyrocytes, like this has been shown by the...
proliferation inhibition of vascular cells in response to inactivation of p21 by antisense oligonucleotides or RNA interference.34,57

In sharp contrast, p21 expression was not induced during the cell cycle progression stimulated by TSH, which demonstrates a new major qualitative difference between the distinct, cAMP-dependent or independent, mitogenic pathways of thyrocytes. In different cell systems, p53-independent transcriptional activation of p21 by growth factors depends on the activation of Ras/ mitogen-activated protein kinase signalling pathways59,60,33 and on the egr1 transcription factor.65 The lack of p21 induction in response to TSH is thus consistent with the absence of activation of Ras/ MAP kinases45,47 and the repression of egr162 by TSH and cAMP in dog thyrocytes. The reduced expression of p21 during the cAMP-dependent G0/S phase progression elicited by TSH reinforces our view that a similar positive role in the activation of CDK4 complexes can be fulfilled by p27, which accumulates in response to TSH but not growth factors48 and supports the nuclear import, phosphorylation and activity of cyclin D3-bound CDK4.28,50

Whereas the nuclear accumulation of p27 in TSH-stimulated dog thyrocytes is durable and compatible with the progression of cells in S phase,68 the induction of p21 by EGF + serum was transient and the nuclear p21 labelling abruptly disappeared at the G1/S transition. This might suggest that p21 must be degraded to allow DNA replication and possibly CDK2 activation. Interestingly, p21 but not p27 is able to bind and to inhibit PCNA, the processivity factor of DNA polymerase δ.63 In addition, since the sequestration of p27 within D-type cyclin-CDK complexes has been suggested to allow cyclin E-CDK2 activation,27 the opposite outcome of differentially expressed p27 and p21 implies that mechanisms underlying the coupling of CDK2 activity to the formation of CDK4 complexes could significantly differ in the distinct mitogenic modes of dog thyrocytes.

The subunit composition of CDK4 complexes markedly affects the site-specificity of their pRb-kinase activity. Distinct pRb-kinase activities were unexpectedly observed in p21-CDK4 complexes (mostly containing cyclin D1) of EGF + serum-treated thyrocytes and in p27-cyclin D3-CDK4 complexes of TSH-stimulated cells. We initially considered that p21 could alter the site-specificity of the pRb-kinase activity of CDK4 complexes, inasmuch as p21 but not p27 has been reported to directly interact with pRb.64 However, the different substrate-specificity of the pRb-kinase activity in dog thyrocytes stimulated by growth factors or TSH turned to be mostly explained by the preponderant association of CDK4 with cyclin D1 or cyclin D3, respectively, which are differently expressed in these mitogenic stimulations.25 Indeed, in different cell contexts, independently of the presence of p21 or p27 (see Results), cyclin D1 and cyclin D3 differently oriented the substrate specificity of CDK4. This novel observation is robust and was reproduced for CDK4 bound to overexpressed tagged D-type cyclins in transiently transfected CHO cells (which eliminates the possibility of interference with p21 or p27, as well as with the antibodies used to precipitate the complexes), and also for endogenously expressed D-type cyclin-CDK4 complexes in different cell types and species. Whereas the in vitro phosphorylation of pRb on Ser780 and Ser795 was supported by both cyclin D1 and cyclin D3, cyclin D1 complexes more efficiently phosphorylated the pRb fragment on Ser807/811 and Thr826 (to a lesser extent). This explains the differences of the migration pattern of the pRb fragment phosphorylated on Ser780 and Ser795 by cyclin D1 or cyclin D3 complexes, as the phosphorylations on both Ser807/811 and Thr826, but not other sites, are required for the electrophoretic mobility shift of hyperphosphorylated pRb.10,65 These different site-specificities observed in vitro pRb-kinase assays explain very similar differences in the phosphorylation patterns of pRb in thyrocytes stimulated by TSH or EGF + serum, which demonstrates their potential physiological relevance.

Together with the previous report that cyclin D3 complexes more efficiently phosphorylate p130 than do cyclin D1 complexes,15 our results thus point to partly nonoverlapping functions of the different D-type cyclin-CDK complexes. As different functions of pRb could be regulated by phosphorylation on distinct residues,9 cyclin D1-CDK4 and cyclin D3-CDK4 could differently impact some pRb functions. D-type cyclin-directed phosphorylation in vitro and in vivo is restricted to a subset of sites on pRb.55,56,11,66 Phosphorylation of Ser795 by cyclin D1-CDK4 inactivates pRb-imposed growth suppression in a microinjection assay.57 Nevertheless, cumulative effects of different phosphorylations of pRb, including other phosphorylations specifically ascribed to CDK4 such as Ser780, appear to be required to abolish E2F-binding and cell cycle inhibition.55,65,68 By contrast, inhibition of pRb binding to LXCXE-containing proteins depends on phosphorylations of Thr821 and/or Thr826, while phosphorylation of Ser807 and/or Ser811 is required to abolish pRb binding to, and inhibition of, the nuclear tyrosine kinase c-Abl.10 Beside their overlapping effects on E2F-binding and cell cycle progression associated with phosphorylation of pRb at Ser780 and Ser795 and possibly other sites, cyclin D1 might thus differ from cyclin D3 by its higher capacity to modulate other pRb functions via Ser807/811 phosphorylations. pRb plays positive roles in the induction of tissue-specific gene expression by directly interacting with a variety of transcription factors, including myoD, C/EBPβ and NF-IL6 implicated in muscle, adipocytes and lymphocyte maturation.82 Whereas cyclin D3-CDK4/6 complexes differently oriented the substrate specificity of CDK4 such as Ser780, appear to be required to abolish E2F-binding and cell cycle inhibition.55,65,68 In such interactions, pRb is generally assumed to be hypophosphorylated as judged from its electrophoretic migration pattern, but this was not directly assessed using phosphospecific antibodies.71-74 In a background of six other phospho-mimetic mutations, the glutamic acid mutation of Ser807/811 suffices to completely prevent the differentiation-promoting function of pRb in Saos-2 cells.68 An intriguing possibility thus remains that the more restricted pRb-kinase activity of cyclin D3 could preserve some differentiation-related functions of pRb, and thus support specialized cell cycles involved in the proliferation of cells undergoing or maintaining differentiation.

Our present findings should be considered in the various development and differentiation models that are associated with dramatic D-type cyclins’ expression switches. Despite the restricted pattern of developmental defects in mice embryos lacking some or all D-type cyclins,75,76 the genes encoding the three D-type cyclins are diversely regulated during embryogenesis.77 In several adult mammalian tissues, cyclin D1 localizes to proliferative layers, whereas cyclin D3 is often highly expressed in the adjacent compartment where differentiation takes place.14,78-80 Cyclin D3 replaces cyclin D1 during myogenesis,20,19 adipogenesis,81 differentiation of the leukaemia cell line HL-60,14 or at the preTCR developmental stage during T lymphocyte maturation.82 Whereas cyclin D3-CDK4/6 complexes are inactive in terminally differentiated myotubes,20,83 they display a high pRb-kinase activity associated with the mitotic expansion phase of differentiating adipocytes,81 and they are required for cell cycle progression in maturing T cells.82

Similarly in dog thyrocytes, the differentiation-associated mitogenic stimulation by TSH and cAMP specifically requires cyclin D3-CDK4 activity associated to enhanced p27,23,50 while the dedifferentiating
pRb was reported to interact with Pax 8 and to potentiate its transcriptional activity on the thyroidperoxidase gene promoter.55 Further studies should investigate the involvement of pRb phosphorylations driven by cyclin D1 or cyclin D3, in the transcriptional regulation of thyroid differentiation genes.

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References

9. Adams PD. Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdk.


60. Woods D, Parry D, Cherwinski H, Bosh E, Lee E, McMahon M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21cip1. Mol Cell Biol 1997; 17:5598-611.


