Differential Utilization of Cyclin D1 and Cyclin D3 in the Distinct Mitogenic Stimulations by Growth Factors and TSH of Human Thyrocytes in Primary Culture

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Two distinct mitogenic modes coexist in thyroid epithelial cells. TSH via cAMP induces proliferation and differentiation expression, whereas growth factors including epidermal growth factor (EGF) induce proliferation and dedifferentiation. Divergent models of TSH/cAMP-dependent mitogenesis have emerged from different thyroid cell culture systems. In the FRTL-5 rat cell line, cAMP cross-signals with transduction pathways of growth factors to induce cyclin D1 and p21cip1 and downregulate p27kip1. By contrast, in canine primary cultures, mitogenic pathways of cAMP and growth factors are fully distinct. cAMP does not induce D-type cyclins and p21, it up-regulates p27, and it stimulates the formation and activity of cyclin D3-cyclin-dependent kinase (CDK) 4 complexes. In primary cultures of normal human thyrocytes, EGF/H11545 serum increased cyclin D1 and p21 accumulation, and it stimulated the assembly and activity of cyclin D1-CDK4-p21 complexes. By contrast, TSH repressed or did not induce cyclin D1 and p21, and it rather up-regulated p27. TSH did not increase cyclin D1-CDK4 activity, but it stimulated the activating phosphorylation of CDK4 and the pRb-kinase activity of preexisting cyclin D3-CDK4 complexes. As recently demonstrated in dog thyrocytes and other systems, cyclin D1 and cyclin D3 differently oriented the site specificity of CDK4 pRb-kinase activity, which might differently impact some pRb functions. Cyclin D1 or cyclin D3 are thus differentially used in the distinct mitogenic stimulations by growth factors or TSH, and potentially in hyperproliferative diseases generated by the overactivation of their respective signaling pathways. At variance with dog thyroid primary cultures, rat thyroid cell lines might not be valid models of TSH-dependent mitogenesis of human thyrocytes. (Molecular Endocrinology 20: 3279–3292, 2006)

A BNORMALLY ACTIVATED OR deregulated thyroid cell proliferation is associated with several pathological states including goitre and the various thyroid adenomas and carcinomas. Most if not all cell proliferation effects of TSH, TSH receptor-stimulating autoantibodies (TSAbs), activating mutations of TSH receptor, or Gs are mediated by elevation of cellular cAMP levels (1), according to a paradigm first demonstrated using primary cultures of canine thyroid cells (2) and confirmed in primary cultures of normal human thyrocytes (3). The clinical relevance includes TSH-dependent goitre, Graves’ disease (4), autonomous hyperfunctional adenomas (5), or congenital hyperthyroidism (6). Elevated TSH and TSAbs also favor the development of at least some differentiated thyroid carcinomas (7, 8). Whereas the cAMP-dependent stimulation is associated with enhanced thyroid function and differentiation expression (9), the cAMP-independent mitogenic stimulation by growth factors such as epidermal growth factor (EGF) is associated with reversible dedifferentiation of the cells (10, 11). The coexpression of some growth factors and their receptors might explain the relative TSH independence of multinodular goitre (12). Moreover, the subversion of tyrosine kinase pathways similar to those operated by local growth factors [i.e. the activation of Ret/PTC (13, 14), or TRK (15), the permanent activation of B-Raf by mutation (16) or chromosomal rearrangement (17), the overexpression of Met/hepatocyte growth factor receptor sometimes in association with hepatocyte growth factor (18), or ErbB1/EGF receptor in association with its ligand TGFα (19)] is causally associated with TSH-independent thyroid papillary carcinomas. To investigate cellular and molecular mechanisms of mitogenesis stimulated by TSH/cAMP, several cell systems have been used, including rat thyroid cell lines (FRTL-5, WRT, and PC Cl3 cells), and the primary cultures of dog and human thyrocytes that also allow...
inhibits the accumulation of D-type cyclins (43), but it egr-1 (41, 42). As a likely consequence, TSH rather different MAPKs (39). It down-regulates the expres-

sions by TSH or growth factors, respectively (51).

A crucial question is to discriminate which of these divergent models, if any, could apply to normal human thyrocytes. The induction of the different D-type cyclins and the expected resulting activation of CDK4 are the most generally considered endpoints of mitogenic signaling cascades (52). They thus reflect the activation state of the different mitogenic pathways. In the present study, we show the differential utilization of cyclin D1 or cyclin D3 in CDK4 activation in the mitogenic stimulations of primary cultures of human thyrocytes by EGF + serum or TSH. This observation indicates that the involved upstream signaling cascades must be largely distinct, as found in dog thyrocytes, but at variance with the situations reported from rat thyroid cell lines.

RESULTS

The present study was based on the analysis of 19 primary cultures obtained between 1998 and 2005 from histologically normal tissue. As previously described (3, 53, 54), the seeding of follicles in low-serum conditions and the subsequent culture in serum-free medium ensure that the primary cultures consisted of at least 99% of cytokeratin-positive thyrocytes that can express thyroid differentiation (thyroglobulin, thyroperoxidase, and iodide transport in response to TSH stimulation). In all these primary cultures, cells were stimulated at d 5 or 6 by TSH or EGF + serum in the presence of insulin. As previously shown, both treatments could induce DNA synthesis at levels that approach those observed in canine thyroid primary cul-
tures (2) and rat thyroid cell lines (see Fig. 4). However, as compared with our previous experience with similar primary cultures obtained from normal thyroid glands of donors deceased for non-thyroid-related causes (3, 53) (mostly traffic accident victims; this material was no longer available in the present study, as an indirect consequence of a modification of Belgian laws), DNA synthesis responses were more variable (Fig. 1). In several primary cultures, DNA synthesis was increased either by TSH (and the adenyl cyclase activator forskolin) or by EGF + serum (Fig. 1). Thus, no correlation could be observed between the cell responsiveness to cAMP-dependent or cAMP-independent mitogenic stimulations. In two experiments, both TSH and EGF + serum failed to appreciably induce DNA synthesis. When investigated, an increased proportion of DNA-replicating cells [bromodeoxyuridine (BrdU)-positive nuclei] was first detected about 26 h after stimulation both by TSH (or forskolin) and EGF + serum, and this proportion only slowly but steadily augmented afterwards (data not shown). Primary cul-
tures that are numbered in Fig. 1 are those for which the investigation of cell cycle regulatory proteins is further illustrated in this study.
Expression of Cell Cycle Regulatory Proteins in Human Thyrocytes Stimulated by TSH or EGF/Serum

In 13 of these primary cultures, the phosphorylation of pRb and the expression of proteins that regulate the activity of CDK4 (and CDK2 in a subset of these experiments) were investigated by Western blotting. The most comprehensive analysis was obtained 26 h after stimulation in culture no. 1 (see Fig. 1), which responded well to the mitogenic stimulation by TSH and more weakly to EGF + serum (Fig. 2). Both mitogenic treatments induced the hyperphosphorylation of pRb (as shown by the appearance of a slower migrating band), consistent with the increased entry of cells in S phase. Cyclin E presence was only slightly increased by both mitogenic stimulations (Fig. 2), probably due to the weak synchronicity of the cell cycle progression. CDK4 and CDK2 were slightly increased by TSH or EGF + serum, respectively, but these weak effects were not consistently observed in other experiments. Cyclin H and CDK7 that constitute the CDK activating kinase (CAK), were not up-regulated by mitogenic treatments (CDK7 level was even moderately reduced by EGF + serum) (Fig. 2), as observed in many cell systems. CDC25A, a phosphatase involved in CDK2 activation, was unexpectedly down-regulated by EGF + serum, as previously observed in dog thyrocytes (our unpublished data). On the other hand, cyclin D1 accumulation was stimulated by EGF + serum but not by TSH, whereas cyclin D3 expression was constitutive (Fig. 2). Cyclin D2 was undetectable by Western blotting in human thyrocytes (negative data not shown), whereas CDK6 was very weakly expressed compared with other human cells (not shown). In another primary culture (no. 2 in Fig. 1), cyclin D1, cyclin D3, and CDK4 were analyzed 8, 16, or 26 h after stimulation by fetal calf serum (FCS) (10%), forskolin, or TSH (Fig. 3). After 16 and 26 h of stimulation, cyclin D1 was induced by FCS, but markedly repressed by forskolin and TSH, despite a stronger stimulation of DNA synthesis by forskolin and TSH compared with FCS. Cyclin D3 and CDK4 levels did not show such a down-regulation (Fig. 3). Overall, cyclin D1 accumulation was stimulated by EGF + serum in all the primary cultures (13 cultures) but two (in which no DNA synthesis was induced). It was never increased by TSH and forskolin, and in eight of 13 experiments, it was even repressed by TSH. Conversely, cyclin D3 expression was never stimulated by EGF + serum, but

Fig. 1. DNA Synthesis Responses to TSH (T), Forskolin (F), EGF + Serum (ES), or None (Control; C) from 19 Independent Primary Cultures of Human Thyrocytes

Cells were stimulated at d 5 or 6 for 48 h, with BrdU for the last 24 h. The percentage of nuclei having incorporated BrdU was determined. When indicated, ranges are from duplicate Petri dishes. Numbered cultures are those for which the investigation of cell cycle regulatory proteins is further illustrated in this study.

Expression of Cell Cycle Regulatory Proteins in Human Thyrocytes Stimulated by TSH or EGF + Serum

In 13 of these primary cultures, the phosphorylation of pRb and the expression of proteins that regulate the activity of CDK4 (and CDK2 in a subset of these experiments) were investigated by Western blotting. The most comprehensive analysis was obtained 26 h after stimulation in culture no. 1 (see Fig. 1), which responded well to the mitogenic stimulation by TSH and more weakly to EGF + serum (Fig. 2). Both mitogenic treatments induced the hyperphosphorylation of pRb (as shown by the appearance of a slower migrating band), consistent with the increased entry of cells in S phase. Cyclin E presence was only slightly increased by both mitogenic stimulations (Fig. 2), probably due to the weak synchronicity of the cell cycle progression. CDK4 and CDK2 were slightly increased by TSH or EGF + serum, respectively, but these weak effects were not consistently observed in other experiments. Cyclin H and CDK7 that constitute the CDK activating kinase (CAK), were not up-regulated by mitogenic treatments (CDK7 level was even moderately reduced by EGF + serum) (Fig. 2), as observed in many cell systems. CDC25A, a phosphatase involved in CDK2 activation, was unexpectedly down-regulated by EGF + serum, as previously observed in dog thyrocytes (our unpublished data). On the other hand, cyclin D1 accumulation was stimulated by EGF + serum but not by TSH, whereas cyclin D3 expression was constitutive (Fig. 2). Cyclin D2 was undetectable by Western blotting in human thyrocytes (negative data not shown), whereas CDK6 was very weakly expressed compared with other human cells (not shown). In another primary culture (no. 2 in Fig. 1), cyclin D1, cyclin D3, and CDK4 were analyzed 8, 16, or 26 h after stimulation by fetal calf serum (FCS) (10%), forskolin, or TSH (Fig. 3). After 16 and 26 h of stimulation, cyclin D1 was induced by FCS, but markedly repressed by forskolin and TSH, despite a stronger stimulation of DNA synthesis by forskolin and TSH compared with FCS. Cyclin D3 and CDK4 levels did not show such a down-regulation (Fig. 3). Overall, cyclin D1 accumulation was stimulated by EGF + serum in all the primary cultures (13 cultures) but two (in which no DNA synthesis was induced). It was never increased by TSH and forskolin, and in eight of 13 experiments, it was even repressed by TSH. Conversely, cyclin D3 expression was never stimulated by EGF + serum, but
slightly enhanced by TSH (maximum 50%) in about half the cultures (seven of 13). The levels of the CDK inhibitors of the CIP/KIP family (p21, p27, and p57) were not decreased after stimulation. Indeed, p21 expression was even increased after EGF + serum (reproduced in five of five cultures), and p27 was most often (seven of 10 cultures) up-regulated by TSH (Fig. 2).

The observations described above are reminiscent of the situation found in dog thyroid primary cultures where D-type cyclins are not induced during the cAMP-dependent mitogenic stimulation, whereas cyclin D1 is induced by growth factors (43). Also in canine thyrocytes, p21 expression is increased by EGF + serum, whereas TSH up-regulates p27 (44, 51).

Comparison with the Regulation of Cell Cycle Regulatory Proteins in FRTL-5 Cells

The regulation by TSH of the expression of D-type cyclins and p21 and p27 is reported to be very different in rat FRTL-5 cells (21). We have thus analyzed the accumulation of these proteins and their association with CDK4 in response to insulin and TSH in FRTL-5 cells that have been made quiescent either by deprivation of serum, TSH and insulin (Fig. 4B), or deprivation of serum and TSH while maintaining insulin (Fig. 4C). The latter condition more closely corresponded to the treatment of human thyrocytes by TSH in the presence of insulin, as performed in the present study. In these FRTL-5 cells, the induction of DNA synthesis absolutely depended on the combined stimulation by both TSH and insulin (Fig. 4A), in accordance with the initial characterization (55). Similarly, CDK4 activity, as reflected by the CDK4-specific Thr826-phosphorylation of pRb (56), was strongly stimulated by TSH in the presence of insulin, but not in response to insulin or TSH used alone (Fig. 4, B and C). However, insulin alone moderately induced cyclin D1 and cyclin D3 and their complex with CDK4, and it decreased p27 levels (Fig. 4B). TSH alone was almost inactive on these proteins (but it up-regulated p21) (Fig. 4B). In the presence of insulin, TSH strongly increased the accumulation of cyclin D1, cyclin D3, and p21, which associated with CDK4, and it further reduced p27 levels (Fig. 4C). These results are consistent with previous reports (30–32) and sharply contrast with the opposite regulation of these proteins observed in human thyrocytes (Figs. 2 and 3).

Cyclin D3 Supports the TSH-Stimulated pRb-Kinase Activity of CDK4, Whereas Cyclin D1 Supports the Activity of CDK4 Stimulated by EGF + Serum in Human Thyrocytes

In human thyrocytes, none of the modulations of the levels of investigated cell cycle regulatory proteins could explain the stimulatory effects of TSH on pRb phosphorylation and DNA synthesis. We have thus compared, from human thyrocytes stimulated by TSH or EGF + serum, the composition and the pRb-kinase activity of CDK4 complexes coimmunoprecipitated using cyclin D1, cyclin D3, p21, and p27 antibodies. Sufficient amounts of cells for such an analysis were only obtained in four different occasions during the last 3 yr, and the detection of the pRb-kinase activity had required an improvement (51) of the classical method based on 32P labeling of pRb detected by
autoradiography (57). Cyclin D1/D3-CDK4 complexes were also investigated in two other primary cultures, with similar results (not shown), but their activity was not assessed. Because of the variations between individual primary cultures, these four different primary cultures have been compared in Fig. 5, A–D. In the cultures of Fig. 5, A (culture no. 1 in Fig. 1 also illustrated in Fig. 2) and B (culture no. 3 in Fig. 1), TSH was the most potent mitogen. TSH was a very weak DNA synthesis inducer in Fig. 5C (culture no. 5 in Fig. 1) where an appreciable mitogenic response to EGF + serum was observed, and in Fig. 5D (culture no. 4 in Fig. 1) DNA synthesis was more potently stimulated by EGF + serum in the presence of insulin (I). A, DNA synthesis was evaluated in cells that were stimulated for 48 h. BrdU was present for the last 24 h and the percentage of nuclei having incorporated BrdU was determined (mean + range of duplicate dishes). B and C, Cells were stimulated for 20 h, and the Thr826-phosphorylation of pRb (specifically ascribed to CDK4) (P-Rb826), CDK4, cyclin D1 (cyc D1), cyclin D3 (cyc D3), p21 and p27 were detected by Western blotting from whole cell extracts. CDK4 was also detected from cyclin D1 immunoprecipitations (IP) (cyc D1-bound CDK4), cyclin D3 IP (cyc D3-bound CDK4) and p21 IP (p21-bound CDK4).

In these four primary cultures (Fig. 5, A–D), complexes containing CDK4 and cyclin D3 or to a lesser extent cyclin D1, and p21 or p27 (IP p27 in Fig. 5, A and B) were already formed in control nonstimulated cells. A significant basal pRb-kinase activity was only detected in cyclin D3 coimmunoprecipitates (Fig. 5). In response to EGF + serum, the levels of cyclin D1 and p21 were increased at various levels (geometric mean of the stimulation ratios in the four experiments: cyclin D1, 1.9; p21, 1.4). The associations of CDK4 and p21 with cyclin D1, and cyclin D1 with p21, were more increased than the levels of these proteins in the four cultures (average stimulation ratios were: CDK4 in IP cyclin D1, 3.3; p21 in IP cyclin D1, 2.8; cyclin D1 in IP p21, 2.4), indicating that EGF + serum also stimulated the assembly of cyclin D1-CDK4-p21 complexes (Fig. 5, A–D). Both cyclin D1 and p21 supported the pRb-kinase activity stimulated by EGF + serum (Fig. 5, A–D). Interestingly, this activity was even more potently stimulated than the formation of the cyclin D1-CDK4-p21 complexes (average stimulation ratios were: 8.1 in IP cyclin D1 and 12.9 in IP p21). By contrast, EGF + serum did not increase the binding of CDK4 and p21 to cyclin D3, nor did it significantly stimulate the pRb-kinase activity associated with cyclin D3 (average stimulation ratio: 1.1) (Fig. 5, A–D).

On the other hand, TSH reduced or did not increase the levels of cyclin D1 and p21, and it did not at all stimulate the pRb-kinase activity associated with cyclin D1 (average stimulation ratio: 0.99) (Fig. 5, A–D), at variance with its effects in FRTL-5 cells (Fig. 4C). By contrast, TSH increased the pRb-kinase activity associated with cyclin D3 (Fig. 5, A–D). In the four primary cultures, this stimulated activity correlated with the amplitude of the DNA synthesis response to TSH (stimulatory factors ranged from 2.9 in the experiment of Fig. 5B in which DNA synthesis was the most potently induced by TSH, to 1.1 in the culture of Fig. 5C in which TSH only marginally increased DNA synthesis). Only in the cultures of Fig. 5, A and D, the presence of cyclin D3-CDK4 complexes was somewhat elevated by TSH, but this was accounted for by an increase of immunoprecipitable cyclin D3 in these two experiments, indicating that TSH did not stimulate the assembly of these complexes. TSH also increased the pRb-kinase activity associated with p27 (Fig. 5, A and B) or p21 (Fig. 5, A–C, but not in Fig. 5D, in which TSH more strongly reduced p21 levels). Nevertheless, these activities were weak compared with the strong pRb-kinase activity coimmunoprecipitated by the cyclin D3 antibody, or to the p21-associated pRb-kinase activity strongly stimulated by EGF + serum (Fig. 5, A–D).

In one of these primary cultures, we have also tested the effect of TGFβ1 in the presence of TSH on DNA synthesis and pRb-kinase activity (Fig. 5D). TGFβ inhibits cell proliferation in the different thyroid cell culture systems (48, 58). In normal human thyroid cells, TGFβ prevents both the DNA synthesis and the differentiation expression induced by TSH and cAMP (59). In the presence of TSH, TGFβ decreased DNA synthesis, as well as the pRb-kinase activity associated with cyclin D3, below their basal levels (Fig. 5D). TGFβ
also reverted the weak stimulatory effect of TSH on cyclin D3 accumulation and the reduction by TSH of the accumulation of cyclin D1 and p21 (Fig. 5D).

**Different pRb-Kinase Activities Are Associated with Cyclin D1 and Cyclin D3**

We have recently demonstrated that cyclin D1 and cyclin D3 drive partly different phosphorylations of pRb by CDK4 in dog and monkey thyrocytes and human fibroblasts (51). Whereas both cyclin D1 and cyclin D3 support the phosphorylation of pRb at Ser780, only cyclin D1-CDK4 efficiently phosphorylates pRb at Ser807/811. The phosphorylations on Ser807/811 and Thr826 but not on Ser780 or Ser795 contribute to generate an upward electrophoretic shift of the pRb fragment in pRb-kinase activity assays (51). Similarly, in the assays of pRb-kinase activity detected using the Ser780-phosphospecific pRb antibody, as shown in Fig. 5, A–D, the migration of the pRb fragment phosphorylated in vitro was different in immunoprecipitations of cyclin D1 (one upward-shifted band) or cyclin D3 (one more rapidly migrating doublet or large band). Thus, whereas the phosphorylation of pRb at Ser780 was stimulated by both TSH (supported by cyclin D3) and EGF + serum (supported by cyclin D1), additional phosphorylation(s) of pRb were induced only by EGF + serum through the activation of cyclin D1-CDK4 (Fig. 5). In immunoprecipitations of p21, which contain both cyclin D1 and cyclin D3, the signature of the pRb-kinase activity of cyclin D1-CDK4 activated by EGF + serum was prevalent, but a weaker contribution of the pRb-kinase activity of cyclin D3-CDK4 was also obvious (Fig. 5).

**Weak Association of CDK4 to p16 in Human Thyrocytes Compared with Human Fibroblasts**

High amounts of cyclin D3-CDK4 complexes were unexpectedly present in nonstimulated human thyrocytes (Fig. 5). p16, a CDK inhibitor of the CDK4 inhibitory (INK4) family (60), is an important regulator of CDK4 activity. It binds CDK4/6 and impairs their association with D-type cyclins (61). By Western blotting, we failed to detect the p16 protein in different primary cultures of human thyrocytes, although it was present in extracts of human normal diploid fibroblasts (IMR-90) put as a positive control on a same gel (not shown). We have thus compared the amount of CDK4 that could be coimmunoprecipitated by p16 and cyclin D antibodies from human thyrocytes and human fibroblasts (IMR-90) (Fig. 6). In human fibroblasts, CDK4 was mostly associated with p16 both in quiescent and in serum-stimulated cells. Even after stimulation, only a minor fraction of CDK4 was associated with cyclin D1 [and even less with cyclin D3 (51)]. By contrast, in human thyrocytes a larger amount of CDK4 was bound to cyclin D3 than to p16 even in nonstimulated cells (Fig. 6). This indicates that an insufficient concentration and CDK4-binding capacity of p16 in human thyrocytes could facilitate the constitutive formation of cyclin D3-CDK4 complexes.

**Activating Thr172-Phosphorylation of CDK4 Is Stimulated by TSH or EGF + Serum, and Inhibited by TGFβ**

The phosphorylation of CDK4 does not affect its electrophoretic migration in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Previously, using two-dimensional (2D) gel electrophoresis, we have separated different forms of CDK4. We have identified the form 3 of CDK4 as the Thr172-phosphorylated CDK4 using 

**DISCUSSION**

Primary cultures of normal human thyrocytes are a rather awkward experimental system, marred by...
scarce availability and generally insufficient amounts of the initial tissue left by the pathologist, the slow proliferation rates of the cells even after strong stimulations, and their very limited division capacity. Yet, the present primary culture system is so far the only in vitro model allowing the investigation, in normal (nontumoral) human cells, of the mitogenic action exerted by TSH and mediated by cAMP elevations (3, 21). The present study was also complicated by the variability of mitogenic responses observed in primary cultures of thyroid from different patients. Several cultures responded poorly to the mitogenic actions of TSH and forskolin (yet displayed the expected induction of differentiation genes) or EGF/H11001 serum. No correlation was observed between the responsiveness to either mitogenic treatments, arguing for their independent mechanisms. The reasons for this unexpected variability are unclear. The responsiveness of the cultured cell might depend on its in vivo history (e.g. age of patient; normal tissue surrounding a tumor that could produce growth factors; Graves’ disease tissue). For instance, the responsiveness of dog thyrocytes to cAMP as a mitogenic stimulus is specifically extinct after their proliferation stimulated by growth factors (63). Alternatively, transcriptome analyses of human thyrocytes stimulated by TSH or EGF/H11001 serum have suggested the induction of multiple growth limiting mechanisms, the impact of which could vary in different cultures (Ref. 64; and Hebrant, A., V. Detours, and C. Maenhaut, unpublished observations).

**Fig. 6.** Comparison of CDK4 Association to p16 in Human Fibroblasts (IMR-90) Stimulated or Not (C) for 16 h with 20% Serum (S), and Human Thyrocytes Stimulated or Not (C) for 26 h with TSH (T) or EGF + Serum (ES)

Cell extracts were immunoprecipitated (IP) with anti-p16, or anti-cyclin D1 (D1) or anti-cyclin D3 (D3) antibodies, separated by SDS-PAGE and immunoblotted. CDK4 was detected using a specific antibody.

At Variance with the Case of FRTL-5 Cells, TSH Does Not Induce Cyclin D1 Nor Repress p27 in Human Thyrocytes

Three kinds of (nonexclusive) mechanisms may mediate a positive action of cAMP on cell proliferation (65): 1) cAMP may act indirectly, for instance by up-regulating an autocrine growth factor or a growth factor receptor (see Ref. 12 for a review in thyroid); 2) as now most frequently considered, cAMP may also act by positively intervening at different steps of the signaling cascades of growth factors (66–68). This is the situation reported from FRTL-5 and WRT cells, although mechanisms are partly divergent in both rat cell lines and even in FRTL-5 cells used by different laboratories.
(21); 3) as demonstrated in canine thyroid primary cultures, cAMP could operate its own distinct mitogenic cascade and cell cycle regulation (21). The two first types of mechanisms are expected to converge with pathways generally operated by growth factors, and thus should culminate in the accumulation of cyclin D1, the transient induction of p21 [which stabilizes cyclin D1-CDK4 complexes in the nucleus (69, 70)], and the down-regulation of p27. These different effects are indeed observed in canine thyrocytes stimulated by growth factors (43, 51), but in response to TSH or forskolin in FRTL-5 cells (Fig. 4) (30–32). Activated (mutated) Ras has been previously shown in human thyrocytes to induce cyclin D1 and p21, but not cyclin D3, and to down-regulate p27 (71). In the present study, similar but weaker responses are generated by the stimulation of human thyrocytes by growth factors, but they are not at all observed in response to TSH. Indeed, TSH did not induce—or even markedly reduced in a majority of primary cultures—the accumulation of cyclin D1 and p21, and it rather up-regulated p27, in sharp contrast with the situation observed in FRTL-5 cells. This is consistent with the lack of MAPK activation in human thyroid primary cultures (39), and the repression by TSH and forskolin of c-Jun and c-Fos (72), which are important transactivators of the cyclin D1 gene. These major differences indicate that the poorly known signaling cascades of TSH-dependent mitogenesis must be quite distinct from growth factor pathways in human thyrocytes, as in canine thyroid primary cultures, but at variance with rat thyroid cell lines (21).

**Whereas Growth Factors Selectively Activate Cyclin D1-CDK4, TSH Selectively Activates Cyclin D3-CDK4 in Human Thyrocytes**

EGF + serum did not only increase levels of cyclin D1 and (to a lesser extent) p21; it also stimulated the formation of cyclin D1-CDK4-p21 complexes and even more potently their pRb-kinase activity. By contrast, TSH stimulated the activity of cyclin D3-CDK4, but little or not the presence of this abundant complex. Intriguingly, EGF + serum did not activate the abundant cyclin D3-CDK4 complexes, whereas TSH did not enhance the assembly and activity of cyclin D1-CDK4. The distinct mitogenic pathways of EGF + serum or TSH thus appear to be channeled to use only cyclin D1 or cyclin D3. Mechanisms responsible for the regulation of the assembly and activation of D-type cyclin-CDK complexes remain largely enigmatic (62). In dog thyrocytes, we have recently demonstrated that cAMP stimulates, whereas TGFβ inhibits, the phosphorylation and activity of CDK4 in cyclin D3-CDK4 complexes (49, 50, 62), at variance with the generally hold concept that the CDK activating kinase (CAK, consisting of cyclin H-CDK7-Mat1) is constitutively active during cell cycle progression and mitogenic stimulation (73, 74). In the present study, levels of CDK7 and cyclin H were also not increased by EGF + serum or TSH in human thyrocytes, but the stimulation of DNA synthesis by EGF + serum and TSH and its inhibition by TGFβ perfectly correlated with the activating phosphorylation of CDK4 (Fig. 7C). In human thyrocytes, as we recently observed it in other cells (62), the activating phosphorylation of CDK4, rather than the induction of D-type cyclins, could thus be the main regulated step that determines the activity of cyclin D1/D3-CDK4 complexes and the entry of cells into S phase. Very unexpectedly, our observations imply that the mechanisms of the assembly and activation of CDK4 complexes could be separate in the distinct mitogenic stimulations and specifically addressed to a particular D-type cyclin.

**Possible Implications of the Specific Involvement of Cyclin D3 in TSH-Dependent Mitogenesis**

The specific involvement of cyclin D3 in the mitogenic stimulation of human thyrocytes by TSH is congruent with our initial findings from dog thyroid primary cultures. In this system, cyclin D3 is specifically required for the mitogenic stimulation by TSH and cAMP, but the neutralization of cyclin D3 does not impair the response to various growth factors that induce cyclin D1 and more weakly cyclin D2 (43). A more partial requirement for cyclin D3 has been confirmed in the TSH-dependent proliferation of PC 113 cells, likely because TSH induces both cyclin D3 and cyclin D1 in this cell line (Ref. 75; and our own unpublished observations). D-type cyclins have been suggested to play distinct roles in cell differentiation (76). 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 (76–78). Cyclin D3 replaces cyclin D1 during myogenesis (79), adipogenesis (80), differentiation of the leukemia cell line HL-60 (76), or at the pre-TCR developmental stage during T lymphocyte maturation (81). It might thus be more specifically restricted to specialized cell cycles associated with maintenance or induction of differentiation. Using human thyrocytes, we confirm here our recent demonstration (51) that the differential utilization of cyclin D1 or D3 affects the site specificity of the pRb-kinase activity of CDK4. Besides Ser780, cyclin D1 but not cyclin D3 supported *in vitro* other phosphorylation(s) of pRb that affect its electrophoretic mobility. We did not identify these additional phosphorylations in the present study, but in dog and monkey thyrocytes, as in human fibroblasts and Chinese hamster ovary cells transfected with CDK4 and cyclin D1 or cyclin D3, cyclin D1-CDK4 more efficiently drives the phosphorylation of pRb at Ser807/811 and Thr826 (51). In general, the role of individual phosphorylations of pRb remains poorly defined or debated. Nevertheless, it is admitted that different functions of pRb could be regulated by phosphorylation on distinct residues (Ref. 82; and see a more complete discussion in Ref. 51). In addition to inhibiting E2F-dependent
gene transcription related to cell cycle progression, pRb plays positive roles in the induction of tissue-specific gene expression by directly interacting with a variety of transcription factors (83), including Pax 8 in thyroid cells (84). In the present primary culture conditions of human thyrocytes, TSH induces or maintains differentiation expression, whereas EGF strongly inhibits it (3, 53, 54, 85). Whether the selective utilization of cyclin D3 in the TSH cascade, associated with a more restricted pRb-kinase activity, could allow the preservation of some differentiation-related functions of pRb thus remains to be examined.

Preactivation of Primary Cultured Human Thyrocytes

As compared with canine thyrocytes in similar primary culture conditions, human thyrocytes displayed higher basal rates of DNA synthesis and appreciable levels of cyclin D1 and cyclin D3 in the present control conditions (with insulin), and even in the absence of insulin (our unpublished data). In nonstimulated human thyrocytes, CDK4 was mostly nuclear (immunofluorescence microscopy observations, not shown), and cyclin D3-CDK4 complexes were already assembled, which contrasts with the stimulation by TSH of both the nuclear import of CDK4 and its association with cyclin D3 in dog thyrocytes (43). In the present primary culture conditions, control human thyrocytes thus seemed to be primed for cell cycle progression. Several nonexclusive hypotheses could explain this preactivation: 1) contrary to canine thyrocytes which rapidly spread out when seeded in serum-free conditions, and thus likely secreted their own extracellular matrix proteins, human thyroid follicles require their seeding in the presence of 1% serum to allow the spreading of cells as a monolayer, and cells are then cultured without serum (3). Serum contains various extracellular matrix proteins, including fibronectin; moreover, human thyrocytes produce fibronectin only when stimulated by serum (86). In normal human thyroid cells, spreading of the cells on fibronectin and the resulting activation of integrins can stimulate MAPK and PI-3kinase pathways and suffice to support a weak proliferation (87); cultured human thyrocytes are reported to express a variety of growth factors, proteases that can activate growth factor precursors, and receptors for some of these growth factors, indicating that they could be subjected to several autocrine stimulations (88); and as also observed by Jones et al. (71), the INK4 protein p16 was weakly expressed in normal human thyrocytes and its levels were insufficient to bind most of CDK4. Although we could not investigate the other related INK4 proteins (p15, p18, p19), this is expected to facilitate the formation of cyclin D3-CDK4 complexes as we also observe it for the constitutive assembly of these complexes in p16-defective human T98G glioma cells (62).

Possible Relevance to Hyperproliferative Thyroid Diseases

The selective activation of CDK4 by cyclin D1 or cyclin D3, leading to partly different pRb-kinase activities in the distinct mitogenic stimulations by growth factors or TSH, could be relevant to human hyperproliferative thyroid diseases. Cyclin D1 is overexpressed in papillary carcinomas (89) caused by overactivation of growth factor signaling cascades by B-Raf mutation or Ret/PTC activation. It was proposed to predict the metastatic behavior of papillary microcarcinomas (90). On the other hand, cyclin D3 is prominent in hyperfunctional goitre caused in transgenic mice by thyroid-targeted overactivation of the cAMP pathway (91). It is also elevated during TSH-dependent goitrogenesis in rats, and overexpressed in human thyroid follicular adenomas (75). Further studies should investigate whether cyclin D3 could be specifically involved in thyroid proliferative diseases that maintain tissue follicular architecture and thyroid differentiated functions, by contrast with papillary carcinomas associated with overactivation of ERK/MAPK pathway and cyclin D1 accumulation.

MATERIALS AND METHODS

Cell Cultures

For primary cultures of human thyrocytes, thyroid tissue samples were obtained from 14 adult patients (aged 19–53 yr) undergoing surgery for a hypofunctioning nodule, following a protocol approved by the Ethics Committee of the Medical School of the Free University of Brussels. Only histologically normal perinodular tissue was used. In five other cases, thyroid tissue was obtained from Graves’ disease patients. The thyrocytes were cultured as described from follicles released by collagenase/dispase digestion of minced tissue (3). Follicles were seeded (at a density corresponding to about 2 × 10^5 cells/cm^2) in the control medium, i.e. DMEM + Ham’s F12 + MCDB104 medium (2:1:1 by vol) supplemented with bovine insulin (5 μg/ml; Sigma, St. Louis, MO) (82), human transferrin (1.25 μg/ml; Sigma), ascorbic acid (40 μg/ml), and antibiotics. One percent FCS was also added to ensure the spreading of the cells as a monolayer (3). After 1 d, medium was renewed and the culture was pursued in completely serum-free conditions with medium renewal every 2 d. In all the experiments, cells were stimulated at 5 or 6 with either bovine TSH (0.3 μI/ml; Sigma), the general adenyl cyclase activator forskolin (10^-5 M; Calbiochem, La Jolla, CA), 10% FCS or the combination of murine EGF (25 ng/ml) and bovine insulin (5 μg/ml) (83), human transferrin (1.25 μg/ml; Sigma), ascorbic acid (40 μg/ml), and antibiotics. One percent FCS was also added to ensure the spreading of the cells as a monolayer (3). After 1 d, medium was renewed and the culture was pursued in completely serum-free conditions with medium renewal every 2 d. In all the experiments, cells were stimulated at 5 or 6 with either bovine TSH (0.3 μI/ml; Sigma), the general adenyl cyclase activator forskolin (10^-5 M; Calbiochem, La Jolla, CA), 10% FCS or the combination of murine EGF (25 ng/ml; Sigma) and 10% FCS. In one experiment, cells were treated in the presence of BSA (500 μg/ml; crystallized; ICN Biomedicals, Irvine, CA) with TSH in the absence or presence of recombinant human TGFβ1 (2 ng/ml; R&D Systems, Minneapolis, MN).

FRTL-5 cells were initially received in 1994 from Dr. R. Di Lauro (Stazione Zoologica Anton Dohrn, Napoli, Italy) and kept frozen after a very few number of passages since this date. They were cultured as described (93) in Coon’s modified Ham F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% calf serum, transferrin (5 μg/ml), insulin (5 μg/ml), ascorbic acid (40 μg/ml) and bovine TSH (1 μI/ml) (H-medium). Cells were made quiescent by switching to medium containing transferrin, BSA (500 μg/ml), with or without insulin (5 μg/ml) for 3 d, and then stimulated for 20 h (analysis of cell cycle
regulatory proteins) or 48 h (evaluation of DNA synthesis) using insulin (5 μg/ml), TSH (1 μl/m) or both.

Human diploid fibroblasts (IMR-90) (American Type Culture Collection, Manassas, VA) were cultured as described (94). After reaching semiconfluence, they were synchronized by starvation in 0.2% FCS for 3 d. Quiescent cells were then growth stimulated by addition of FCS (20%).

**DNA Synthesis**

Cells in 3-cm Petri dishes were stimulated for 48 h and BrdU was added for the last 24 h. The incorporation of BrdU was detected by immunofluorescence, and BrdU-labeled nuclei (1000/dish) were counted as described (63).

**Western Blotting Detections of Proteins**

Ten to 20 μg of total proteins were separated by PAGE and the proteins of interest were immunodetected after Western blotting as previously described (49). The following antibodies were used: C-15 polyclonal anti-pRb (Santa Cruz Biotechnology, Santa Cruz, CA), DCS-6 monoclonal anticyclin D1 (from J. Bartek, Danish Cancer Society, Copenhagen, Denmark; or NeoMarkers, Fremont, CA), DCS-22 monoclonal anti-cyclin D3 (NeoMarkers), DCS-3 monoclonal anti-cyclin D2 (from J. Bartek), HE-12 monoclonal anti-cyclin E (from J. Bartek), C-18 polyclonal anti-cyclin H (Santa Cruz), DCS-156 monoclonal anti-CDK4 (Cell Signaling Technology, Beverly, MA) or C-22 monoclonal anti-CDK4 (Santa Cruz), M2 polyclonal anti-CDK2 (Santa Cruz), DCS-83 monoclonal anti-CDK6 (NeoMarkers), DCS-61 monoclonal anti-p21 (from J. Bartek), C-15 polyclonal anti-p27 (Santa Cruz), DCS-230 monoclonal anti-p57 (from J. Bartek) and DCS-120 monoclonal anti-CDC25A (NeoMarkers).

**Immunoprecipitation (49)**

For analyses of protein complexes and their pRb kinase activity, subconfluent cultures of thyrocytes or IMR-90 fibroblasts in 9-cm Petri dishes that contain the same number of cells were lysed on ice in 1 ml Nonident P-40 (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, 10 mM dithiothreitol, 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 that had been preincubated overnight with 2 μg of antibody [DCS-11 monoclonal anti-cyclin D1 antibody (Biosource International, Camarillo, CA), DCS-28 monoclonal anti-cyclin D3 antibody (NeoMarkers), C-19 polyclonal anti-p21 antibody (Santa Cruz), a mixture of the K25020 monoclonal anti-p27 antibody from Transduction Laboratories (BD Biosciences, Erembodegem, Belgium) and the C-15 monoclonal anti-p27 antibody from Santa Cruz, or DCS-50 monoclonal anti-p16 antibody (NeoMarkers)].

Alternatively, for analyses of the 2D gel electrophoresis pattern of CDK4, cultures of thyrocytes in 9-cm Petri dishes were washed with PBS, scraped in 200 μl of denaturing lysis buffer [50 mM Tris-HCl (pH 7.5), 0.6% SDS, 10 mM dithiothreitol, 50 mM NaF, 100 μM vanadate, and protease inhibitors], boiled for 5 min, and frozen. Cell lysates containing 150–300 μg of proteins were cleared by centrifugation and diluted by adding 5 vol 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 with the C-22 polyclonal anti-CDK4 antibody.

**pRb-Kinase Assay (51)**

Washed complexes were resuspended in 40 μl of kinase reaction buffer containing 2 mM ATP, 0.3 μg of a 56-kDa fragment (amino acids 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 and transferred on polyvinylidene difluoride membranes. The phosphorylation on Ser780 of the pRb fragment was detected using a phospho-specific-pRb antibody from Cell Signaling Technology. Membranes were then immediately reprobed using the DCS-156 CDK4 antibody, which allowed a direct internal comparison of the pRb-kinase activity with the amount of coimmunoprecipitated CDK4. D-type cyclins and p21 were detected on separate membranes from the same pRb-kinase immunoprecipitation sample, using the antibodies previously described for the Western blotting.

**2D Gel Electrophoresis**

Immunoprecipitated proteins were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated by isoelectric focusing as described (49, 94) 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 polyvinylidene difluoride 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).

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