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Regulation of Thyroid Cell Proliferation by TSH and Other Factors: A Critical Evaluation of *in Vitro* Models

TAKAO KIMURA, ALEXANDRA VAN KEYMEULEN, JACQUELINE GOLSTEIN, ALFREDO FUSCO, JACQUES E. DUMONT, AND PIERRE P. ROGER

Institute of Interdisciplinary Research (IRIBHN) (T.K., A.V.K., J.G., J.E.D., P.P.R.), School of Medicine, Université Libre de Bruxelles, Campus Erasme, B-1070 Brussels, Belgium; and Dipartimento di Biologia e Pathologia Cellulare e Moleculare (A.F.), Facoltà di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli, I-80131 Napoli, Italy

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TSH via cAMP, and various growth factors, in cooperation with insulin or IGF-I stimulate cell cycle progression and proliferation in various thyrocyte culture systems, including rat thyroid cell lines (FRTL-5, WRT, PC Cl3) and primary cultures of rat, dog, sheep and human thyroid. The available data on cell signaling cascades, cell cycle kinetics, and cell cycle-regulatory proteins are thoroughly and critically reviewed in these experimental systems. In most FRTL-5 cells, TSH (cAMP) merely acts as a priming/competence factor amplifying PI3K and MAPK pathway activation and DNA synthesis elicited by insulin/IGF-I. In WRT cells, TSH and insulin/IGF-I can independently activate Ras and PI3K pathways and DNA synthesis. In dog thyroid primary cultures, TSH (cAMP) does not activate Ras and PI3K, and cAMP must be continuously elevated by TSH to directly control the progression through G_1 phase. This effect is exerted, at least in part, via the cAMP-dependent activation of the required cyclin D3, itself synthesized in response to insulin/IGF-I. This and other discrepancies show that the mechanistic logics of cell cycle stimulation by cAMP profoundly diverge in these different *in vitro* models of the same cell. Therefore, although these different thyrocyte systems constitute interesting models of the wide diversity of possible mechanisms of cAMP-dependent proliferation in various cell types, extrapolation of *in vitro* mechanistic data to TSH-dependent goitrogenesis in man can only be accepted in the cases where independent validation is provided. (*Endocrine Reviews* 22: 631–656, 2001)

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I. Introduction

ABNORMAL THYROID CELL proliferation has a very important role in human disease. Its dysregulation causes goiter, thyroid adenomas, and carcinomas or primary hypothyroidism resulting from hypoplasia. To understand the pathogenic processes, endocrinologists are therefore trying to elucidate the mechanisms of the control of normal and pathological human thyroid cells *in vivo*. With regard to proliferation and differentiation, this is difficult or impossible for ethical and experimental reasons. We thus resort to the use of *in vitro* models or *in vivo* transgenic mice models. The aim of this review is to critically summarize our knowledge and the relevance *in vivo* of the various available *in vitro* models of thyroid cells.

Our analysis is mainly focused on the regulation of cell proliferation by TSH acting through cAMP and IGF-I, which are the main controls considered *in vivo*. Understanding the respective roles of TSH and IGF-I in their synergistic regulation of cell proliferation is indeed considered a question of major interest in the field of thyroid regulation. A central question addressed to the different models is whether TSH and IGF-I, through distinct signaling cascades, exert similar or complementary functions required for cell proliferation. In the latter hypothesis, a second question is whether one of these factors merely amplifies the effect of the other, which is thus qualified as the only genuine mitogen. Although specifically considered here in various thyroid cell systems, the complex problem of the identification of the integration of cAMP and IGF-I signaling cascades has a broader relevance to other endocrine cells targeted by both IGF-I and pituitary trophic hormones, including ACTH, LH, and FSH.

Unexpectedly, the mechanisms demonstrated in the various *in vitro* thyroid models are different.

II. The *in Vitro* Models

The *in vitro* models used for the study of thyroid cell proliferation and differentiation belong to two main classes, the cell lines and the primary cultures. Tissue slices from dog, calf, sheep, and human thyroid have been extensively used for functional studies. As they have no time to reprogram themselves, they are very good replicas of the tissue *in vivo*. However, they survive 24 h at most, and the cells do not enter DNA synthesis.

Cell lines are derived from normal and cancer tissues. Human cancer cell lines can obviously not be used to study the normal process of thyrocyte growth and division. Differentiated sheep thyroid cell lines (OVNIS) have been little used for proliferation investigations (1). The most studied models are immortal rat thyroid cell lines [FRTL-5 (2), WRT (3), and PC Cl3 cells (4)], which present a very appealing set of properties that resemble those ascribed to normal differentiated thyrocytes (5–8), such as TSH dependence for growth and differentiated functions, iodide uptake, and thyroglobulin and thyroperoxidase gene transcriptions. Because of their simplicity and accessibility, because they allow permanent transfections and genetic experiments, and also because of the increasing difficulty in obtaining animals for experimentation, these rat thyroid cell lines are now the preferred and often only used systems in the majority of *in vitro* studies of thyroid biology (>800 entries in MEDLINE concern FRTL-5 cells). Due in part to the availability of these untransformed rat cell lines, the proliferation of thyroid epithelium has been studied *in vitro* to a greater extent than that of any other endocrine gland.

The immortality of the cell lines is sufficient evidence that they have lost some of the basic mechanisms of cell cycle control, PC Cl3 cells to a lesser extent than the apparently similar FRTL-5 cells. The transformation of PC Cl3 cells requires the combination of two retroviral oncogenes, while only one is sufficient for the full transformation of FRTL-5 cells, which suggests their precancerous nature (4). In fact, in nude mice FRTL-5 cells present a thymidine labeling index 4-fold higher than endogenous thyrocytes (9) and develop TSH-dependent tumors (10). It is also important to keep in mind that most cell lines result from selections, fortuitous or not, and that they are often maintained only when they present desired characteristics, *i.e.*, when they correspond to *a priori* expectations. Depending on the culture medium (presence or not of serum), primary cultures of rat thyrocytes develop cell lines that stably display the phenotypes of FRTL cells (unpolarized but expressing thyroid differentiation) or FRT cells (morphologically polarized but lacking the expression of differentiation proteins) (2). FRTL-5 have been "adapted" to the presence of 5% serum and survive in the absence of TSH (2). However, they derive from FRTL cells for which TSH and insulin not only support proliferation, but also are necessary for survival (11). Some vital functions might thus have come to depend on these hormones, which were present during the establishment of the cell strain. Recently, a survival function of TSH has been unmasked in FRTL-5 cells (12). By contrast, the WRT cell line displays the interesting characteristics of slow proliferation in response to insulin alone, and survival in the absence of hormones while retaining the capacity to respond to TSH as a full mitogen (3). Nevertheless, the inventors of this cell line were correct to point out that the WRT clone was the only one (of 27 clones obtained by limited dilution plating) presenting these characteristics (3). Two other clones with characteristics more similar to FRTL-5 cells were discarded (3).

Although often left unsaid, it is widely acknowledged that cell lines may evolve and deviate from their parental counterparts. Perhaps due in part to their very broad dissemination, this is especially well documented in the case of FRTL-5 cells. This cell line was indeed reported to suffer from increasing instability (13, 14) and clonal variability (15–17), which explains the opposite results sometimes obtained in different laboratories. A monthly monitoring of thymidine incorporation characteristics has been recommended (5, 18). Several studies have now compared the characteristics of "young" and "aged" FRTL-5 cells (18–23). Repeatedly passaged FRTL-5 cells often display a larger size (16), lose the TSH responsiveness of growth, which then is only enhanced by insulin (24), lose thyroglobulin production (25) or the okadaic acid-induced apoptosis (24), or acquire the TSH-dependent capacity to grow in semisolid medium (26). In contrast to their initial characterization, FRTL-5 cells supplied by the ATCC (Manassas, VA) have been recently reported to be tetraploid (27). Aged WRT cells also lose the TSH responsiveness of growth (our unpublished observations). Until now, no such changes have been reported for the PC Cl3 cells.

Cells in short-term primary cultures are not selected by their propagation *in vitro* and are thus expected to be less remote from physiology. Although their environment and their organization may differ markedly from the *in situ* situation, they are probably not modified genetically or epigenetically. The contamination of primary cultures by nonepithelial cells should be evaluated, especially after long-term culture in the presence of serum. In the dog thyroid primary culture model, this problem has been solved by the initial enrichment of thyrocytes by the seeding of thyroid follicles rather than isolated cells, and plating and culture maintenance in serum-free conditions that do not support the attachment and proliferation of fibroblasts. The existence of rapidly proliferating phenotypic variants of thyrocytes reported in cultures of human thyroid might be more problematic (28).

Primary cultures can be distinguished by the species studied and by their architecture. Four species have been mainly used: man (29–32), dog (33, 34), pig (35–39), and sheep (40–42). Pig and calf (43) thyroid cultures, while useful for the investigation of function and gene expression, respond poorly to TSH as a growth stimulus, for still unknown reasons (44). In sheep thyroid primary cultures, TSH potentiates the increase of cell numbers induced by insulin and IGF-I (45). However, this effect might require very precise conditions, because it was not found in earlier reports by this group using the same system (41, 46), and it has not been further investigated. Although they were very useful for the

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demonstration of growth and differentiation effects of growth factors (47), pig, sheep, and calf thyroid cultures have generally been little used for the study of proliferative effects of TSH, and thus are no longer considered in the present review. Human cells are the obvious choice, but it is very difficult to obtain normal tissue in sufficient amounts; these cells, obtained mostly from rather elderly patients undergoing surgery for single nodules, grow poorly. Mostly dog thyroid cells have been studied by our group (34). As discussed in this review article, their properties are similar to those of human cells in several respects, but they grow much better and allow biochemical studies of the mechanisms. However, this material is also difficult to obtain in most centers.

Cells in primary cultures can be studied as monolayers (29, 34, 41), as reorganized follicles in suspension (36, 48, 49) or in collagen gels (32, 50), or as monolayers on filters set between two chambers (37, 51). The latter system is used mostly for transport studies. Reorganized follicles can produce high amounts of thyroid hormones and thus are used mostly for secretion and functional studies. Because they generally exhibit higher cell multiplication responses and are easily handled, monolayers are considered the material of choice for the investigation of cell proliferation mechanisms. However, as observed very early, thyroglobulin iodination and the synthesis and secretion of thyroid hormones are lost in monolayers in the absence of the spatial constraints of follicular architecture, even though the key enzymatic processes are preserved. Indeed, appropriately stimulated (TSH) dog thyrocyte monolayers have been demonstrated to perform all the thyroid-specific functions required for the synthesis and secretion of thyroid hormones, including thyroglobulin and thyroperoxidase gene expression, iodide uptake, H_2O_2 generation, iodide efflux, and macropinocytosis, and to remarkably retain the regulation of these functions (34). Nevertheless, the extent to which some of the proliferation characteristics of monolayer cell primary cultures might have been affected by their profoundly modified *in vitro* organization and environment has not been systematically investigated.

The confrontation of the different thyroid primary culture systems has pointed out the importance of possible species differences (52), but also the influence of culture conditions, and the fact that cells have a "memory," which means that their characteristics are not fully stabilized and may evolve depending on their previous *in vivo* and *in vitro* history. For instance, dog thyrocytes specifically lose their mitogenic response to TSH and cAMP (but not their response to growth factors or to the differentiation effects of TSH) after having proliferated in the presence of serum or growth factors (53). This might also apply to human thyrocytes, which have never been reported to maintain their responsiveness to TSH as a mitogen after exposure to high serum concentrations. The necessity to obtain fresh tissue for each culture constitutes a difficulty, especially when available quantities are low and scarce. Moreover, modern approaches based on gene transfection cannot be easily applied to primary cultures, although retroviral vectors allow efficient transfections of a fraction of human thyrocytes in primary cultures (54). The very limited proliferation capacity of thyrocytes in primary culture, of course, prevents permanent transfections. On the other hand, although there is some quantitative variability from one primary culture to another, such material exhibits a remarkable qualitative reproducibility over many years.

With all these caveats in mind, we shall analyze results obtained in the most studied models, the FRTL-5, WRT, and PC Cl3 cell lines, and the primary cultures of dog and human thyroid, and compare them with our real subject of interest, the elusive human thyroid cell *in vivo*.

III. Methods of Measuring Cell Proliferation

The best index of cell proliferation is the number of cells in a growing population. However, this measures the balance between cell proliferation and cell death, and, if used for evaluating cell proliferation, an important rate of cell death must be ruled out. Moreover, cells in primary cultures do not detach easily from the substratum or from each other. Substitutes of cell counting are therefore used, such as measurements of total DNA in a culture, or indexes of cell mass (protein content) or metabolic activities assayed using various colorimetric measurements. DNA measurements count multiploidy as cell division. Protein contents and metabolic activities measure cell volume independently of cell proliferation.

Autoradiographs of cells incubated with labeled thymidine or immunocytochemistry of bromodeoxyuridine-labeled cells reveal the number of cells having entered into DNA synthesis. A 24- to 48-h incubation with the tracer labels all the cells having entered into DNA synthesis during this period, thus providing a cumulative measure that reflects the mathematical integration of the asynchronous cell cycle progression within the cell population. The continuous availability of the tracer during the incubation period must be checked. A half hour labeling provides a precise estimate of the number of cells synthesizing DNA at this time (this measurement thus represents a mathematical derivative rate of the overall cell proliferation process).

Measurement by fluorescence-activated cell sorter of DNA content allows a rough estimate of the number of cells in between the diploid and tetraploid state to be obtained at the time of measurement, *i.e.*, cells in DNA synthesis. This is also a derivative rate measure. Derivative measurements are especially useful for kinetic studies, but their use for an overall evaluation of the proliferation process requires many time points.

Incorporation of labeled thymidine into the whole DNA of the culture (or often into the trichloroacetic acid-precipitable material) is also often used as an index of proliferation. However, such measurements are sensitive to all the effects on cell pools of thymidine phosphates. For instance, they are often increased in the presence of hormones that enhance thymidine uptake. Several studies have shown qualitative dissociations of cell number increases and ^{3}H-thymidine uptake into acid-insoluble material (3, 18, 55–58). Unless specifically validated for each condition, the latter assay is unreliable.

A detailed analysis of these methods and their pitfalls has been presented previously (59). Because they are the most reliable, we shall take into account mainly studies based on

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DNA measurements (including by fluorescence-activated cell sorter), cell counting, and the frequency of labeled nuclei.

IV. Proliferation Characteristics of the Various Systems

Results obtained in different laboratories using the most reliable methodologies lead to the following conclusions:

A. FRTL-5 rat thyroid cell line

FRTL-5 cells (Table 1) proliferate rapidly (doubling time ≈ 36 h) in the presence of 5% FCS and the six hormone mixture (6H) containing TSH, high concentrations of insulin that activate IGF-I receptors (insulin/IGF-I), transferrin, somatostatin, gly-his-lys acetate, and hydrocortisone. According to the initial characterization, this proliferation was absolutely dependent on TSH, the cells remaining quiescent in the same medium as above without TSH (5H medium) (2, 5). Very soon, however, many reports of growth stimulation in the absence of TSH by insulin/IGF-I or serum, and additive effects of insulin and serum, have appeared, which correspond to the present characteristics of the cell line in most

TABLE 1. Variations of proliferation responses assayed by different methods in FRTL-5 rat thyroid cells in different laboratories

Mitogen	Methods		
	No. cells or DNA content	S-phase nuclei (FACS) ^{3}H-thymidine- or BrdU-labeled nuclei	^{3}H-thymidine incorporation into acid-insoluble material
TSH			
TSH alone	Increase (60, 61)	Induce S phase (62) (very weak), (24, 63)	Increase (64) (very weak), (65) (weak), (18, 56, 62, 63, 66–70)
	No effect (18, 57)	No effect (64)	No effect (57, 71)
TSH + insulin/IGF-I	Increase/additive (60)	Induce S phase/additive (24)	
	Increase/synergistic (57, 68, 72)	Induce S phase/synergistic (62–64, 73)	Increase/synergistic (56–58, 62–64, 66, 67, 71, 72, 74)
TSH + serum	Increase (56, 57, 60, 67, 70, 75–77)	Induce S phase/additive (73) (very weak)	Increase/additive (65, 76)
			Increase/synergistic (65, 76)
	No effect (78)		
TSH + serum + insulin/IGF-I	Increase/additive (56, 57, 60)		Increase/additive (57, 75)
	Increase synergistic (11, 18, 78–80)	Induce S phase/synergistic (69, 73)	
Insulin/IGF-I			
Insulin/IGF-I alone	Increase (57, 60)	Induce S phase (24, 63, 64, 73)	Increase (56, 57, 62–65, 67–72, 74, 81)
			No effect (82)
Insulin/IGF-I + serum	Increase/additive (56, 57, 60, 67, 68)	Induce S phase/additive (73)	Increase additive (57, 69)
	No effect (11, 18, 78–80)		Same as serum (65, 80)
Calf serum			
Serum alone	Increase (57) (weak)	Induce S phase (73)	Increase (57, 65)
EGF			
EGF alone		Induce S phase (73) (very weak)	
			No effect (63, 83)
EGF + insulin/IGF-I		Induce S phase/synergistic (73)	
			No effect (63)
EGF + insulin + serum	Increase (84) (weak)		Increase (58)
	No effect (58)		
EGF + TSH + serum	Increase (84)		
Phorbol esters (TPA)			
TPA alone		Induce S phase (73)	Increase (67) (weak) (69)
TPA + insulin/IGF-I		Induce S phase/additive (73)	Increase/additive (67, 82)
TPA + insulin/IGF-I + serum	Increase (67)	Induce S phase/additive (69, 73)	
bFGF			
bFGF alone			Increase (63)
bFGF + IGF-I			Increase/synergistic (63)
bFGF + insulin + serum			Increase (85, 86)
HGF			
HGF alone		Induce S phase (73) (very weak)	
HGF + insulin		Induce S phase/synergistic (73)	
			No effect (87)

FACS, Fluorescence-activated cell sorter; BrdU, bromodeoxyuridine.

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laboratories (56, 57, 63, 64, 73). As reported in 1990 by the laboratory of Kohn and associates (18), the basal incorporation of ^{3}H-thymidine into DNA supported by insulin and 5% serum strikingly increases during repeated passages of FRTL-5 cells, which is accompanied by a relative attenuation of the TSH response. The ability of insulin/IGF-I to increase ^{3}H-thymidine incorporation in "aged" cells even exceeded levels induced by TSH plus insulin/IGF-I in "young" cells. Although no such changes were observed by this group when growth was measured as cell number (implicitly casting grave doubts on the reliability of the thymidine incorporation assay) (18), many other investigators, including Tramontano and colleagues (60) and Takahashi *et al.* (63), report significant increases of cell number or DNA content by insulin/IGF-I in the absence of TSH.

Despite this controversy, the view that in FRTL-5 cells proliferation and DNA synthesis are synergistically activated by TSH and insulin/IGF-I is almost unanimously accepted. In some reports, TSH and insulin/IGF-I are only additive on cell number, while a marked synergy is shown by the incorporation of thymidine (56). Some confusion about the respective roles of TSH and insulin/IGF-I in this synergy also results from an additional controversy about the capacity of TSH to elicit a limited proliferation in the absence of insulin or IGF-I. While many investigators reported variable stimulations of thymidine incorporation by TSH alone (56, 61, 63, 66, 68), others did not find such an effect (57, 64, 71) or failed to reproduce it when measuring proliferation by cell number (18). According to Zakarija and McKenzie (57), TSH alone is devoid of stimulatory effects on thymidine incorporation and cell number, but as little as 0.2% FCS suffices to support these TSH effects in the absence of insulin. By contrast, Isozaki and Kohn (78) reported that 5% serum does not support TSH-dependent proliferation in the absence of insulin. The demonstration of an important mitogenic effect of TSH *per se* (but in the presence of 0.2% FCS) is facilitated by the removal of the cytostatic somatostatin from the usual 6H culture medium in the FRTL-5 cells used by Santisteban and collaborators (61). When observed, the effect of TSH alone on thymidine incorporation was found to depend, at least in part, on an autocrine IGF-II production (66). Collectively, results are therefore generally compatible with TSH merely amplifying the mitogenic response to insulin or IGF, either endogenous or exogenous.

Despite a first contradictory report (80), the mitogenic effects of TSH, either alone or in the presence of insulin/IGF-I, are reproduced totally (58, 62, 88), or only partially (76, 89), by various cAMP enhancers in FRTL-5 cells, as first demonstrated using dog thyroid primary cultures (90, 91).

Proliferation of FRTL-5 cells can be stimulated independently of TSH and cAMP. Phorbol ester and insulin effects are additive (67, 73). Basic fibroblast growth factor (bFGF) in synergy with insulin strongly induces DNA synthesis (63, 85, 86). We observed a similar stimulation using hepatocyte growth factor (HGF) (73), but others did not get this effect in FRTL-5 cells that, nevertheless, express functional HGF receptors (87). The recent findings of a slight to moderate growth stimulation by epidermal growth factor (EGF) in the presence of TSH or insulin (73, 84) contrast with earlier negative reports (63, 83), and might be a characteristic developed by "aged" FRTL-5 cells (92).

In conclusion, the marked regulatory differences between young and old cultures, as well as the discrepancies between effects of the same agents in different laboratories (Table 1), suggest that there are no "FRTL-5 cells," but different batches or subclones of such cells, each with its peculiar properties, and therefore that findings from one laboratory cannot necessarily be extrapolated to others. According to a majority of recent reports, FRTL-5 cells proliferate in response to insulin/IGF-I alone and the TSH/cAMP cascade amplifies this effect. By contrast, the group of Santisteban and colleagues (93) investigates the mechanisms involved in the stimulation of DNA synthesis in FRTL-5 cells that can proliferate in response to TSH alone (Fig. 1).

B. WRT rat thyroid cell line

WRT cells proliferate rapidly in the presence of TSH, insulin, and/or serum (doubling times 42 h with 5% serum and 80 h with 0.5% serum) (3). These cells proliferate more

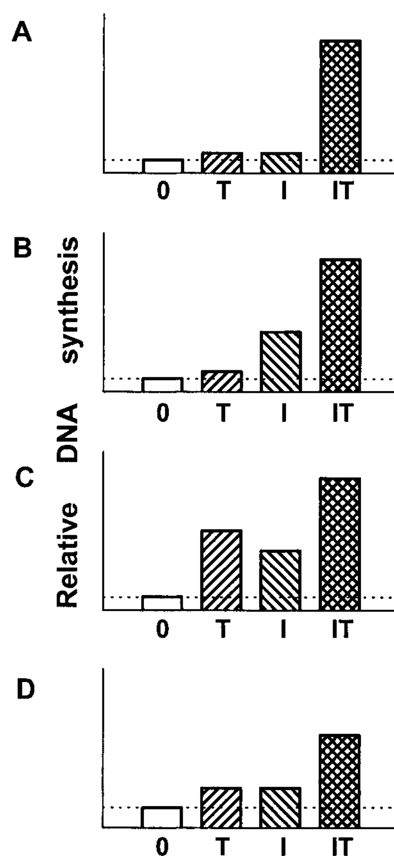

FIG. 1. Summary of relative DNA synthesis rates reported in the various *in vitro* thyroid culture systems, in response to TSH alone (T), IGF-I or high insulin concentrations alone (I), or the combination of both stimulations (IT) (0, no treatment). A, The situation corresponding to dog thyroid primary cultures and initial reports on FRTL-5 cells. B, The situation observed in rat thyroid follicles in suspension cultures, in PC Cl3 cells, and in FRTL-5 cells presently used in most laboratories. C, The situation studied in WRT cells, and in FRTL-5 cells used by Santisteban and collaborators. D, The situation generally observed by the present authors in primary cultures of normal human thyroid epithelium. See text for references.

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slowly, but can be propagated in the absence of TSH, using only insulin and a low serum concentration. TSH also can support a sustained proliferation in the presence of 0.5% serum but in the absence of insulin, and TSH alone can trigger DNA synthesis in WRT cells deprived of serum and insulin. Insulin/IGF-I and TSH synergistically increase thymidine incorporation but produce less than additive effects when proliferation curves are analyzed (3). At variance with the initial characterization of FRTL-5 cells, TSH may thus appear as a full mitogen in WRT cells (3, 94). Nevertheless, its mitogenic activity has been most frequently investigated in the presence of high insulin concentrations (95). Moreover, it is not totally clear whether the induction of DNA synthesis by TSH alone after insulin deprivation might depend on endogenous IGF production, as shown in FRTL-5 cells, or even on some persistence of a permissive effect of the high insulin concentration present in the 6H medium used for routine cell maintenance. cAMP enhancers fully mimic the mitogenic effects of TSH in WRT cells (94). In our hands, DNA synthesis in WRT cells is not stimulated by phorbol esters and HGF (our unpublished data). When maintained in low-serum conditions, they are also unresponsive to EGF. Nevertheless, two months after shifting to a 5% serum medium, WRT cells reversibly develop a moderate responsiveness to EGF proliferative effects (55).

In summary, in WRT cells, insulin/IGF-I and the TSH/cAMP cascade independently induce proliferation (Fig. 1).

C. PC Cl3 rat thyroid cell line

Like FRTL-5 and WRT cells, PC Cl3 cells are routinely maintained in the 6H medium containing TSH, insulin, and 5% FCS (4). Insulin at high concentrations or IGF-I alone can stimulate DNA synthesis in the absence of serum (96). At variance with WRT cells, TSH or cAMP enhancers alone are almost inactive, but they markedly potentiate the effect of insulin/IGF-I (96), and they can stimulate DNA synthesis in the presence of a low, inactive *per se*, concentration of insulin (73). FGF stimulates the proliferation of PC Cl3 cells (97). In our hands, DNA synthesis is also induced by phorbol esters in the presence or not of insulin, but not by EGF or HGF (73). Thus, as in most FRTL-5 cells, insulin/IGF-I stimulates the proliferation of PC Cl3 cells, and this effect is revealed or amplified by the TSH/cAMP cascade (Fig. 1).

D. Rat thyroid follicles in primary culture

Rat thyroid follicles in suspension culture have been little used for cell multiplication studies, because of their poor capacity for proliferation and the insufficient amount of cell material they provide for biochemical studies. Nevertheless, this model has given us the first unambiguous *in vitro* demonstration of the mitogenic effect of TSH, observed in the presence of 0.5% serum (48). In serum-free medium, the stimulation of DNA synthesis by TSH absolutely requires the presence of insulin/IGF-I, which alone weakly increases the proportion of cells in S phase (98) (Fig. 1). The effect of TSH is mimicked by forskolin (99). EGF is devoid of mitogenic effect, either alone or in the presence of insulin (98).

E. Dog thyrocytes in primary culture

Dog thyrocytes in monolayer culture can proliferate rapidly (doubling time $\approx$ 36 h) in response to a combination of TSH, insulin, EGF, and serum (1–10%), and then abruptly stop growing after four to six generations (53, 100). After initial plating, cells remain quiescent and healthy in a serum-free medium supplemented or not with insulin. They have thus proliferated only slightly *in vitro* when stimulation is applied. Insulin, IGF-I, or IGF-II alone have in general only marginal effects on DNA synthesis, but they support the induction of DNA replication and cell cycle progression by TSH, EGF, bFGF, or phorbol esters (100–105). TSH and EGF triggering effects in the presence of insulin are additive (100). Insulin/IGF-I are generally required for the mitogenic stimulation by these various factors, but in one-third of the experiments a significant stimulation of DNA synthesis can be achieved in response to TSH alone (100, 105). When observed, this effect is inhibited in part by neutralizing antibodies blocking IGFs or IGF-I receptors (104). It thus depends, at least in part, on an autocrine IGF production, according to a paradigm first introduced by Eggo and collaborators (106) in the thyroid field. In general, the permissive effect of insulin is obtained at high concentrations and is mediated by IGF-I receptors, which are constitutively expressed in dog thyrocytes (104, 105). However, TSH in the absence of insulin induces the synthesis and accumulation of insulin receptors, which then allows low physiological insulin concentrations, instead of IGF-I, to act as a comitogenic permissive factor for the cell cycle progression induced by TSH (100). HGF is the only growth factor so far that can induce DNA synthesis and proliferation in dog thyrocytes cultured without insulin/IGF-I, thus acting as a full mitogenic factor (105, 107). In several experiments, its action is nevertheless potentiated by insulin (104). The mitogenic effects of TSH in dog thyrocytes are perfectly mimicked by forskolin, cholera toxin, and various cAMP analogs (90, 91, 102, 108), which has provided the first direct evidence that cAMP fully accounts for TSH-stimulated mitogenesis (109, 110).

Thus, in dog thyrocytes the cascade activated by IGF-I or insulin is necessary for the TSH/cAMP mitogenic effect, but, by itself, it is inactive on cell proliferation (Fig. 1).

F. Human thyrocytes in primary culture

We have restricted our analysis to studies performed using culture conditions that allow the *in vitro* demonstration of the mitogenic effect of TSH. Unlike growth factor effects, the stimulation of DNA synthesis and proliferation by TSH may be weak or absent if cells originate from goiter, from tissue from old people, and/or from previously frozen cells, subcultivated cells, or cells exposed to high serum concentrations (32, 50, 111–113). In serum-free primary cultures of normal human thyrocytes organized as cell monolayers or cell aggregates, the mitogenic effect of TSH is best demonstrated by the induction of DNA synthesis (29, 30, 114, 115). Nevertheless, marked stimulations of proliferation by TSH, as reflected by increases of cell numbers, were obtained using human fetal thyrocytes (114, 116). The mitogenic effect of

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TSH absolutely depends on the presence of IGF-I or insulin [including, at very low physiological concentrations, acting exclusively through insulin receptors (117)], which alone weakly stimulate DNA synthesis (29, 30, 105). In the absence of exogenous insulin or IGF-I, the weak stimulation by TSH of thymidine incorporation in human thyrocytes cultured with 1% serum was inhibited by an IGF-neutralizing monoclonal antibody, suggesting that it depended on autocrine IGF production (118, 119). In primary cultures of human thyroid follicular adenomas, this autocrine IGF production is exacerbated, leading to an escape from exogenous IGF dependence for proliferation, without reducing the requirement for TSH (30, 120). In monolayer cultures, the effect of TSH is mimicked in large part, but not totally, by cAMP enhancers [forskolin, cholera toxin, $(Bu)_2$ cAMP (29)]. cAMP-independent stimulations of proliferation and DNA synthesis can be achieved using serum, EGF in the presence of insulin and/or serum, or phorbol esters in the presence of insulin (29, 112, 121–123). HGF, even in the presence of insulin, fails to induce DNA synthesis, as observed in our group (S. Dremier, unpublished data). However, the Cardiff group has demonstrated a strong stimulation of DNA synthesis by HGF in the presence of 10% serum (124). Possibly, a serum factor might increase the abundance of HGF receptors (c-met), which are poorly expressed in normal human thyroid tissue, at variance with their high abundance in many papillary carcinomas (125).

Thus, as in dog thyroid cells, the signaling cascade of IGF-I or insulin is necessary for the TSH/cAMP-induced DNA synthesis, but it is weakly mitogenic by itself (Fig. 1).

G. Comparison of cell systems (Table 2)

In all these systems, at variance with thyroid cell cultures from porcine or bovine origins, TSH exerts a major stimulatory effect on cell proliferation, and cAMP is a sufficient mediator of at least a large part of this effect. In all the systems, the TSH/cAMP stimulatory effects on DNA synthesis and cell proliferation are best demonstrated in the presence of insulin or IGF-I, which reflects an important synergy between both kinds of factors. When studied, the effects of insulin/IGF-I have been related to an effect on the IGF-I receptor in rat thyroid cell lines and primary cultures (56, 98, 126). By contrast, in canine thyroid primary cultures and human thyrocytes, insulin receptors expressed in response to TSH can also mediate the insulin comitogenic effects (104, 117). Low concentrations of insulin were also reported to be mitogenic in sheep thyroid primary cultures (45).

Whether TSH or insulin/IGF-I alone can elicit a significant DNA synthesis response is particularly relevant to the question of the respective roles of these factors in their synergistic regulation of cell proliferation (Fig. 1). In the different rat thyroid cell lines and in rat thyroid primary cultures, insulin/IGF-I alone significantly stimulates proliferation. By contrast, it generally produces marginal effects on DNA synthesis in canine primary cultures. In PC Cl3 cells as in rat thyroid primary cultures, TSH alone is devoid of proliferation-inducing effect, but it merely potentiates the action of insulin/IGF-I. In FRTL-5 cells, the situation is more controversial, depending on the proliferation assay (^{3}H-thymidine incorporation *vs.* more reliable methods), the culture me-

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TABLE 2. Proliferation index of various mitogens in rat, dog, and human thyroid cells

	DNA synthesis or proportion of S-phase cells					
	FRTL-5	WRT	PC CI3	Rat	Dog	Human
TSH						
TSH alone	0/+	+	0	0	0/+	±
TSH + insulin	+++	++	+++	+++	+++	+++
Insulin/IGF-I						
Insulin/IGF-I alone	+/0	+	+	+	0/±	+
EGF						
EGF alone	0/±	0/+	0	0	0	nd
EGF + insulin	0/+++	nd	0 same as insulin alone	0	+++	+
Phorbol esters (TPA)						
TPA alone	+	0	+	nd	0	nd
TPA + insulin	++	0 same as insulin alone	++	nd	+++	+
HGF						
HGF alone	0/±	0	0	nd	+	nd
HGF + insulin	0/+	0 same as insulin alone	0 same as insulin alone	nd	++	0
HGF + serum	nd	nd	nd	nd	nd	+++
cAMP						
cAMP alone	0/+	+	0	0	0/+	0
cAMP + insulin	++(+)/+++	++	+++	+++	+++	++(+)
Calf serum						
Serum alone	+	+	+	nd	+	+

0, No effect; ±, very weak or marginal effect; +, increase; ++, additive increasing effects; +++, synergistic increasing effects; nd, not determined.

dium (presence of 0.2% serum, absence of somatostatin, possible incomplete removal of the very high concentration of insulin used for routine cell maintenance), and possibly the subclone used and the number of repeated passages. TSH alone may induce, or not, a significant mitogenic effect, which has been suggested to depend on an autocrine effect of endogenous IGF-II (66). By contrast, in WRT cells, TSH alone generates an important stimulation of proliferation, which is additive to the one exerted by high insulin concentrations. In the different rat thyroid cell systems, the respective roles of TSH and insulin/IGF-I might thus be different. In canine and normal human thyroid primary cultures, the triggering of DNA replication by TSH absolutely depends on insulin, IGF-I, or IGF-II. In some primary cultures of dog and human thyrocytes, as in FRTL-5 cells, endogenous IGF production may partially fulfill this requirement.

Thyroid cell proliferation is also stimulated independently of cAMP by various growth factors and phorbol esters. EGF in the presence of insulin/IGF-I stimulates DNA synthesis and proliferation in human and canine thyroid primary cultures, and in most other species (sheep, pig, calf), but not in rat thyrocytes in primary culture and in PC Cl3 cells. Moderate EGF responses in FRTL-5 cells might be restricted to "aged" cells, and in WRT cells *de novo* EGF responsiveness only appears after weeks of culture in the presence of high serum concentrations. The mitogenic responsiveness to bFGF in the presence of insulin first shown in dog thyrocytes is also observed in pig and calf (43) thyrocytes and in FRTL-5 and PC Cl3 cells, but it has not been reported so far in WRT cells and in human thyrocytes. The very potent mitogenic stimulation by HGF demonstrated in dog thyrocytes has been confirmed in human thyrocytes, but only in the presence of serum, and in porcine thyrocytes (87). Among the rat cell lines, we have found only FRTL-5 cells to be responsive to HGF. In our hands, phorbol esters stimulate DNA synthesis in all the cell systems including human thyrocytes, with the exception of WRT cells. There are thus again some important differences between the three rat cell lines, PC Cl3 cells being more similar to rat thyroid primary cultures. Dog thyrocytes are the only system responding to the full range of factors that stimulate DNA synthesis in human cells. This has prompted an investigation of the mitogenesis induced by TSH via cAMP by comparison with the more general mechanisms of growth factors and phorbol esters, which have been delineated in many other cell types.

V. Kinetics of TSH-Insulin/IGF-I Synergy and Cell Cycle Progression

In the following sections, we have only considered the stimulation of quiescent G_0 cells to enter the cell cycle and the DNA synthesis phase, not the control of the G_1 to S phase transit in continuously stimulated cycling cells, which has been little studied in thyroid culture models.

In the stimulation of quiescent BALB/c 3T3 fibroblasts, synergizing comitogens have been sorted in two categories. The first one increases the capacity (competence) of cells to respond to the second category, which supports cell cycle progression (127). For instance, platelet-derived growth factor stimulates proliferation, at least in part, by inducing IGF-I receptors, thus increasing cell competence to progress into the cell cycle in response to IGF-I (128). Similarly in FRTL-5 cells, a 12- to 24-h preincubation with TSH or a cAMP enhancer suffices to shorten G_1 phase and to strongly amplify the DNA synthesis response induced by insulin/IGF-I added afterward (62–64, 72, 129). The continuous presence of TSH is dispensable during cell cycle progression triggered and supported by insulin/IGF-I (63, 64, 72). TSH is thus identified as a competence factor that exerts a priming effect facilitating the action of the progression factor insulin/IGF-I. The mechanism is not fully understood. As reported by Takahashi and colleagues (130, 131), the TSH pretreatment does not increase the number and activity of IGF-I receptors, but it potentiates the IGF-I-dependent tyrosine phosphorylation of insulin receptor substrate (IRS)-2 and activation of PI3K, and the phosphorylation and up-regulation of Shc leading to increased binding of Grb2 to Shc and activation of p42/p44 MAPKs.

When TSH and insulin/IGF-I are administered simultaneously in FRTL-5 cells, the elevation of cellular cAMP levels is biphasic, as cAMP activates a type IV phosphodiesterase (132). While cAMP unambiguously mediates the mitogenic effect of TSH, preventing the further decline of cAMP levels by the administration of phosphodiesterase inhibitors or a phosphodiesterase-resistant cAMP analog impairs the initiation of DNA synthesis in both FRTL-5 (133, 134) and PC Cl3 cells (135). In our hands, when PC Cl3 cells are stimulated by the combination of insulin and the adenylyl cyclase activator forskolin, the washing out of forskolin 16 or 20 h afterward accelerates, rather than prevents, the entry of cells into DNA synthesis phase (S. Demartin and P. P. Roger, unpublished data). In FRTL-5 and PC Cl3 cells, cAMP effects on cell cycle are therefore biphasic. After its initial priming/competence effects, cAMP is no longer required for G_1 phase progression supported by insulin/IGFI, and it even inhibits it when maintained at too high a level. No such data are available from WRT cells.

The observation that the presence of TSH or cAMP enhancers is not continuously required for G_1 phase progression of FRTL-5 cells is compatible with the hypothesis that cAMP could also indirectly stimulate cell proliferation by inducing the production of autocrine growth factors (63). Part of the synergism between TSH and IGF-I is eliminated when the culture media are renewed every 4 h with fresh media (63). The conditioned medium from TSH-treated FRTL-5 cells potentiates the mitogenic effect of IGF-I on human fibroblasts (63). The priming action of TSH and its potentiation of IGF-I-dependent DNA synthesis were therefore suggested to be mediated, in part, by an autocrine amplification factor (63). bFGF was proposed as a likely candidate (63, 136). Not only does it strongly induce DNA synthesis in synergy with insulin/IGF-I, but also it is produced by FRTL-5 cells (136). During TSH-dependent thyroid hyperplasia in rats, mRNA expression of both bFGF and FGF receptor 1 are increased (137). Very recent findings confirm the induction by TSH and cAMP of the expression of both FGF and FGF receptor 1 in FRTL-5 cells (138). Immunoneutralization of bFGF slightly decreases the basal rate of DNA synthesis observed in the absence of TSH (136). Additional FGF immunoneutralization experiments are crucial to dem-

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onstrate to which extent this autocrine mechanism indeed contributes to the mitogenic action of TSH in this cell line. However, other possible explanations for the discontinuous requirement for TSH during the G_0-to-S phase progression have not been considered in FRTL-5 cells. For instance, one can imagine that TSH could be required for the assembly of a stable structure, such as a prereplication complex subsequently required for S phase initiation.

In canine thyroid primary cultures, quite at variance with FRTL-5 cells, the stimulation of DNA synthesis requires the *simultaneous* presence of TSH and insulin/IGF-I. When TSH is added 24 h after insulin/IGF-I or when insulin/IGF-I is administered 24 h after TSH, DNA synthesis follows with a similar 16- to 20-h lag phase the first time that TSH and insulin/IGF-I are present together, regardless of which factor is added first (100). Furthermore, in canine thyrocytes cultured with insulin, the induction of DNA synthesis by forskolin requires its continuous presence for at least 16 h until a very late G_1 phase restriction point situated approximately 2 h before DNA synthesis initiation. In response to forskolin, dog thyrocytes progress toward S phase, but if this adenylyl cyclase activator is withdrawn for as little as 2 h before cells reach the commitment point, they regress to an earlier part of G_1, from which they can be rescued by forskolin readdition (139). Moreover, elimination of forskolin at later time points arrest without detectable delay the entry of cells into DNA synthesis phase (140). cAMP thus directly supports G_1 phase progression in dog thyrocytes. This implies a very late rate-limiting event, which must be labile to explain the rapid consequence of forskolin deprivation on DNA synthesis initiation (139, 140). Similar observations were made in dog thyrocytes stimulated by the phosphodiesterase-resistant cAMP analog $(Bu)_2$cAMP in the presence of carbamylcholine, which can substitute for insulin as a supportive comitogenic factor (141). In this case, the immediate inhibition of carbamylcholine signaling by atropine, unlike the removal of $(Bu)_2$cAMP, still permitted the entry of G_1 cells into DNA synthesis phase for 6–8 h. This suggests that cAMP can exert alone the last crucial control that determines the cell commitment toward DNA replication (141).

Nevertheless, when TSH is administered to dog thyrocytes 24 h before insulin/IGF-I, a higher rate of DNA synthesis is often observed (100), and various responses to insulin, IGF-I, and IGF-II are enhanced, including autophosphorylation of insulin/IGF-I receptors, tyrosine phosphorylation of IRS-like proteins, MAPK activation, and c-Fos expression (104). In dog and human thyrocytes, TSH clearly increases insulin responsiveness by inducing the expression of insulin receptors, which allows low physiological insulin concentrations to exert a sufficient comitogenic activity (104). However, in dog thyrocytes as in FRTL-5 cells, the expression of IGF-I receptors is unaffected. The mechanism of the potentiation of IGF-I receptor activity by TSH remains to be defined in dog thyrocytes (104). It might be similar to the above detailed mechanism recently suggested in FRTL-5 cells by Takahashi and co-workers (131).

In dog thyrocytes, an additional synergy between TSH (cAMP) and EGF is observed in the presence of insulin, as evidenced by a shortening of G_1 phase and an increase of the fraction of cells that enter S phase (100, 142, 143).

In summary, in dog thyrocytes TSH through cAMP acts mainly as a progression factor. Secondarily, it also acts as a competence factor increasing responsiveness to insulin and IGF-I, which then cooperate with cAMP as progression factors (104). In most FRTL-5 cells, TSH through cAMP exerts a sufficient competence/priming action augmenting the responsiveness to IGF-I, but cAMP is not required for G_1 phase progression supported by insulin/IGF-I, which is inhibited even by the maintenance of high cAMP levels. Therefore, cAMP exerts opposite effects on G_1/S transition and DNA synthesis initiation in FRTL-5 cells and dog thyrocyte primary cultures (Fig. 2).

VI. Hypertrophy *vs.* Mitogenesis

For a cell to divide, or at least to sustain several divisions, it must double its mass as well as its DNA content before each division. Strikingly, while potently stimulating DNA synthesis in the presence of insulin/IGF-I, TSH does not increase the overall protein synthesis and protein accumulation in primary cultures of canine and human thyrocytes (105). Insulin and IGF-I, on the contrary, while not being sufficient mitogens, stimulate protein accumulation leading to the hypertrophy of these cells. This increase of cell mass is probably necessary, but clearly not sufficient, for the permissive action of these factors on the TSH/cAMP mitogenic response (105, 141).

The three rat thyroid cell lines are again different in this regard. Not only insulin/IGF-I, but also TSH, activate protein synthesis and induce cell growth (73). In our hands, these effects are less than additive (73). By contrast, Koide *et al.* (71) reported a marked synergy on FRTL-5 cells and a priming effect of IGF-I potentiating the stimulation of protein synthesis by TSH. Interestingly, the converse situation is observed for the induction of DNA synthesis (71).

Therefore, the regulations of protein synthesis and DNA synthesis are dissociated in all the systems. Nevertheless, as the final effects of hormones in the primary cultures and in

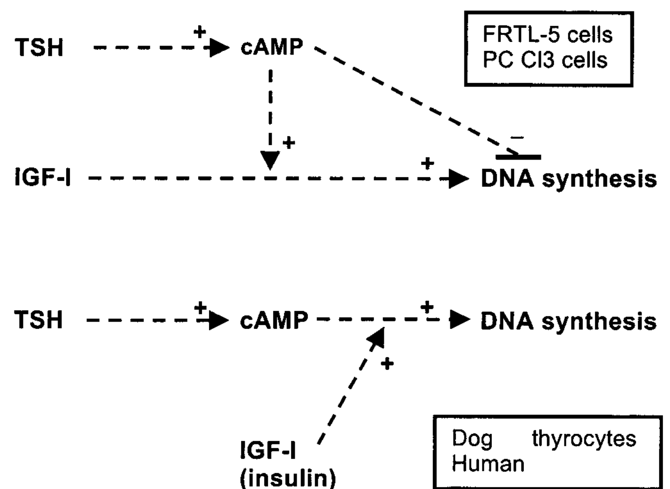

FIG. 2. Respective effects of TSH (cAMP) and IGF-I (insulin) on DNA synthesis in FRTL-5 and PC Cl3 cell lines *vs.* dog and human thyroid primary cultures. *Arrows pointing to other arrows* indicate permissive effects of the second factor, as recently proposed (144).

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the cell lines are different, their effects on the intracellular cascades are thus probably also different. Even in the different rat cell lines, different mechanisms might be involved. For instance, the stimulation of protein synthesis by insulin is blocked by the HMG-CoA reductase inhibitor lovastatin in FRTL-5 and WRT cells, but not in PC Cl3 cells (73).

Thus, in rat thyroid cell lines insulin/IGF-I and TSH independently stimulate protein synthesis, whereas only IGF-I or insulin do so in dog and human thyrocytes.

VII. Signaling Cascades

The problem of the delineation of the various signaling cascades involved in the mitogenic stimulation of thyrocytes is especially complex. It includes the identification of the respective intermediaries of the different comitogenic pathways (*i.e.*, TSH *vs.* insulin/IGF-I or growth factor *vs.* insulin/IGF-I) and of the possible cross-signalings by which one comitogenic factor influences the activity of another one. This may help to define the respective roles of such factors. Various pharmacological probes and genetic tools are available to "specifically" activate or inhibit most known signaling cascades. Nevertheless, it should be reemphasized that a very strong expression by transfection of a constitutively activated (mutated) intermediary of a cascade may produce effects that do not mimic a physiological activation. On the other hand, the simple fact that the action of a given stimulus is blocked by an inhibitor of a signaling cascade does not demonstrate that this stimulus transmits a signal through this cascade, as even its basal activity might exert a required permissive influence [*e.g.*, the simple observation of an inhibition of TSH-stimulated proliferation of FRTL-5 cells by wortmannin does not warrant the claim that TSH activates PI3K (93)]. Absence of oxygen blocks most cascades even though cell respiration is not part of these cascades. Moreover, the specificity of most inhibitors is only relative and should be checked for each model at the concentration used.

A. Expression of membrane receptors

The regulation of the expression of key membrane receptors markedly differs in the different systems. In FRTL-5 cells, insulin and IGF-I receptors are constitutively expressed as suggested by binding experiments (126, 145), and the expression of TSH receptor mRNA depends on insulin/IGF-I but is attenuated by TSH (146, 147). Such a down-regulation has not been observed in murine thyroid gland *in vivo* (148). Conversely, in dog and human thyrocytes the expression of insulin receptor protein is induced by TSH and inhibited by insulin (104, 117), while the constitutive expression of TSH receptor mRNA is independent of insulin but transiently and moderately enhanced by TSH (149, 150).

B. Coupling of TSH receptor

Studies using human and dog thyroid membrane preparations have shown that the TSH receptor can be coupled to G proteins of each of the four main classes, Gs, Gq, Gi, and G0 (151, 152). Nevertheless, in intact cells a more restricted selectivity of G protein coupling has been demonstrated (152). In all the thyroid cell systems, TSH activates the Gsα/adenylyl cyclase/cAMP cascade. In dog and human thyrocytes, TSH also activates Gi, as demonstrated by an inhibition of adenylyl cyclase, which partially opposes the stimulation through Gs and can be relieved by pertussis toxin (152). In human thyrocytes (153, 154), but not in dog thyrocytes (155, 156), TSH also stimulates the Gq/PLC/Ca^{++} cascade. This activation requires 10 times higher concentrations of TSH than adenylyl cyclase activation. A similar effect has been reported in FRTL-5 (Refs. 157 and 158 but see Ref. 159 for a contradictory report) and PC Cl3 cells (160), but with TSH concentrations 100–1000 times higher than those required for cAMP accumulation, which raises questions about the role of this effect in the cell lines and the possible effect of TSH contaminants. However, Sho *et al.* (161) have shown in FRTL-5 cells that adenosine through A1 receptors potentiates the stimulation by TSH of the PLC/Ca^{++} cascade and inhibits the activation of the adenylyl cyclase/cAMP cascade in a pertussis toxin-sensitive manner. A similar phenomenon has been shown in human thyrocytes (154). In FRTL-5 cells, the TSH receptors have also been claimed to be coupled to PLA_2 through a pertussis toxin-sensitive pathway leading to arachidonic acid release and PG synthesis (162), but no evidence of a direct coupling through G proteins was provided (163). On the contrary, in dog thyroid slices TSH and cAMP inhibit arachidonate release (164). Finally, the common $\beta\gamma$-subunits of the various G proteins are potentially coupled to different effector pathways including the Ras/Raf/MAPK pathway, raising the possibility of a Ras activation in response to TSH.

C. Involvement of cAMP and PKA (Fig. 3)

As stated above, cAMP enhancers (forskolin, cholera toxin, cAMP analogs . . .) mimic totally or in great part the effects of TSH on DNA synthesis and cell proliferation in the different experimental systems. In FRTL-5 cells, whether cAMP may totally account for the effects of TSH and thyroid-stimulating Igs remains a matter of controversy. The additional involvement of PLC and PLA_2 cascades has been suggested (163), but the high concentrations of TSH required make this very doubtful. The cyclooxygenase inhibitor indomethacin is claimed to partially inhibit the stimulation of thymidine incorporation by TSH, suggesting a role of PG synthesis (165). Pertussis toxin was also reported to inhibit DNA synthesis in FRTL-5, but this inhibition was related to the G_1 phase progression supported by IGF-I, not to the priming effect of TSH (72). In sharp contrast, in dog primary thyrocytes, the mitogenic effects of TSH are perfectly mimicked by the cAMP enhancers (34). TSH does not activate PLC (155), nor does it enhance PG production (166). Proliferation effects of TSH in the presence of insulin are insensitive to indomethacin (166) and pertussis toxin (152) in dog thyrocytes. As exemplified by this system and WRT cells, cAMP is thus a fully sufficient mediator of the comitogenic effects of TSH.

In WRT cells, dog thyrocytes, FRTL-5 cells, and human thyrocytes, the activity of PKA is required for the mitogenic stimulation by TSH (Refs. 93, 108, 167, and 168 and A. Van Keymeulen, S. Dremier, and P. P. Roger, unpublished data), possibly through downstream targets such as CREB/CREM

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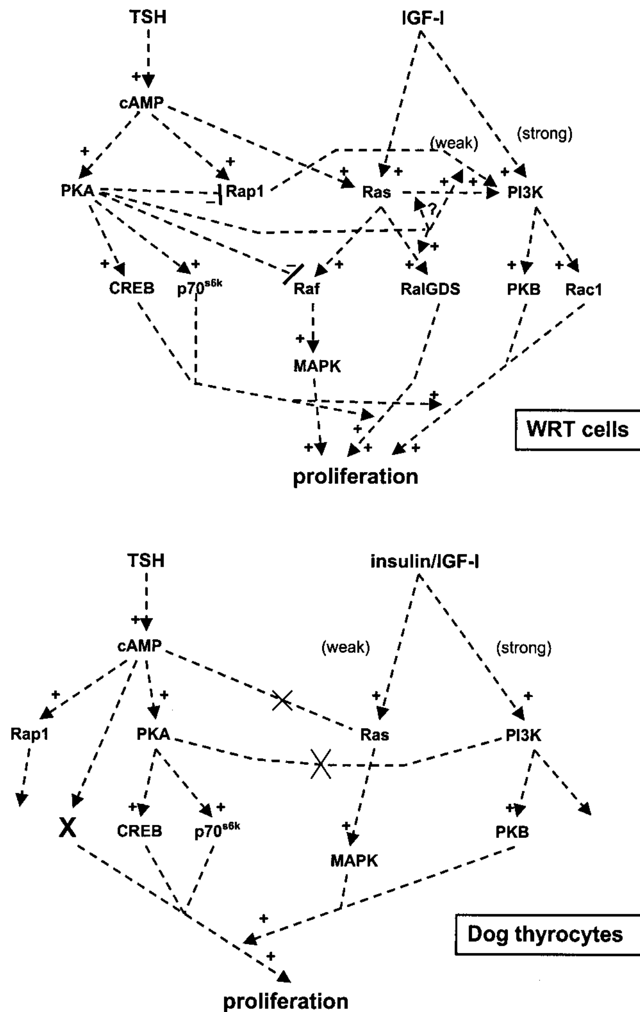

FIG. 3. Mitogenic signaling cascades of TSH (cAMP) and insulin/IGF-I suggested in WRT cells *vs.* dog thyrocytes in primary culture. The "**X**" event(s) in dog thyrocytes represents a unknown cAMP-dependent, but PKA-independent, effect required for cAMP-dependent mitogenesis. *Arrows pointing to other arrows* indicate potentiating permissive effects, and *crossed out lines* represent demonstrated absence of effects (144). In our tentative representation of the complex situation resulting from the intricate signaling cascades reported in WRT cells, we have been unable to depict the apparent paradox recently raised by the observations that the cAMP-dependent activation of Rap1 appears to be necessary and sufficient for the activation of the PI3K pathway by cAMP (174), but is neither sufficient nor even necessary for the PI3K-dependent mitogenesis elicited by cAMP (174). See text for references, details, and explanations.

(169, 170) or p70$^{S6\,k}$ kinase (95, 171). However, as first shown in dog thyrocytes, PKA activation is not sufficient for cAMP-dependent induction of DNA synthesis (167, 172), and the role of other cAMP-dependent but PKA-independent pathways remains to be defined.

In WRT cells, TSH and cAMP weakly stimulate the activating phosphorylation of protein kinase B (PKB)/Akt (173), as a likely consequence of PI3K activation. This effect is probably superfluous in the presence of insulin, which strongly activates the PI3K/PKB pathway. Nevertheless, it might help to explain the relative insulin independence, which is a peculiarity of the cAMP-elicited mitogenesis in WRT cells. Initially, the cAMP stimulation of PKB/Akt phosphorylation was reported to be independent of PKA because it was not inhibited by the PKA inhibitor H89 used at 25 μM (172, 173). However, in a more recent report by the same authors, 10 μM H89 abolished the cAMP-dependent phosphorylation of Akt in WRT cells, which is now interpreted as indicating a requirement for PKA activity in this process (174). This illustrates the weakness of evidence based solely on the use of such inhibitors. By contrast, in dog thyrocytes, TSH and cAMP do not activate PI3K and PKB/Akt (171). In this system, the inhibition of cAMP-dependent mitogenesis by PI3K inhibitors such as wortmannin thus bears on the permissive activity of PI3K strongly stimulated by insulin/IGF-I (171).

cAMP activates the small G protein Rap1 independently of PKA in dog thyrocytes (175) and WRT cells (172). However, Rap1 activation is a common step of various signaling cascades, which have different effects on dog thyrocytes, and it is thus neither characteristic nor sufficient for mitogenic stimulation (175). Moreover in WRT cells, expression of activated Rap1A (A63E) or a putative dominant negative Rap1A (A17N) did not affect the TSH stimulation of DNA synthesis in the presence of insulin (174). Because Rap1A (A17N) abolishes the PI3K-dependent phosphorylation of Akt by TSH (174), this also raises questions with regard to the involvement of the latter event in TSH-stimulated DNA synthesis, at least in the presence of insulin.

D. Involvement of Ras and its effector pathways

Ras has long been thought to play an important role in the regulation of proliferation and differentiation of thyrocytes. Activating mutations of Ras genes are a frequent early event in thyroid follicular adenomas and carcinomas (176). Activated (val12 mutation) H-Ras induces a sustained proliferation compatible with differentiation expression in primary cultures of normal human thyrocytes (177, 178), while it provokes both a TSH/insulin-independent growth and a suppression of differentiation expression in rat thyroid cell lines (4, 179–181). In WRT cells, each of various potential effectors of Ras has been reported by Meinkoth and collaborators (182) to be sufficient to elicit a TSH-independent proliferation, including Raf/MAPK kinase (MEK)/MAPK, PI3K (183) and Ral GDS (184). Nevertheless, this group did not consider the paradox between the potent induction of proliferation by Ras Val12 mutants defective for binding of Raf (183, 184) and the inhibition by strategies that block the Raf/MEK pathway of the DNA synthesis caused by overexpression of wild-type Ras (185). This might indicate that high concentrations of microinjected Ras mutant proteins could signal through effectors not normally activated by wild-type Ras. In FRTL-5 cells, constitutively activated MEK only weakly affects proliferation (186), and in human thyrocytes Raf/MEK is a necessary, but not sufficient, intermediary in the stimulation of proliferation by oncogenic Ras (187). Effects of oncogenic Ras are thus partly different in human thyrocytes *vs.* rat cell lines, and, even among the latter, downstream mechanisms may vary. In dog thyroid primary cultures, normal Ras activation by extracellular stimuli, including EGF and the very potent phorbol ester,

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12-*O*-tetradecanoylphorphol 13-acetate (TPA), is not sufficient to trigger mitogenesis (188).

As demonstrated in dog thyrocytes, unlike growth factors and TPA, TSH and cAMP do not stimulate the phosphorylation and activity of p42/p44 MAPKs (189), which was a first indication of a lack of Ras activation in this pathway. However, in WRT cells Ras is suggested by Meinkoth and collaborators (168) as an intermediary in the cAMP-dependent mitogenesis, because microinjected neutralizing antibodies and dominant interfering mutants of Ras partly inhibit TSH/cAMP-stimulated DNA synthesis. In this cell line, the lack of MAPK activation by cAMP is ascribed to PKA inhibition of c-Raf and redirection by cAMP of Ras signaling toward other effectors such as Ral GDS and PI3K (182). Very recently, Meinkoth and collaborators (172) have demonstrated the activation by TSH and cAMP of human H-Ras ectopically overexpressed in stably transfected WRT cells. As in the case of Rap1 activation, this effect is resistant to PKA inhibitors (172). However, the endogenous Ras proteins and thus their activation were undetectable (172), and a possible alteration of signaling pathways by cellular Ras overexpression has yet to be excluded. In sharp contrast in dog thyrocytes, while the activity of endogenous Ras, as reflected by its GTP-loading, is strongly stimulated by EGF, HGF, and TPA and weakly by insulin, TSH and cAMP reduce the basal levels of GTP-Ras (188). This lack of Ras activation explains and confirms the lack of MAPK activation in the cAMP-dependent pathway, which therefore does not result from the uncoupling by cAMP of c-Raf from Ras. Such a uncoupling is also made unlikely by the fact that MAPK phosphorylation and nuclear translocation induced by EGF are not affected by TSH and forskolin in dog thyrocytes (143). In these cells, we have not excluded a requirement for Ras activity in the TSH-stimulated DNA synthesis. We have indeed consistently observed a low basal level of Ras-GTP in dog thyrocytes (188). In these cells [but not in FRTL-5 cells (93)], PD098059, which specifically inhibits a MAPK kinase and thus p42/p44 MAPKs, inhibits DNA synthesis triggered by TSH in the presence of insulin, even though TSH does not activate MAPKs. This suggests a requirement for a basal activity of MAPKs, and thus perhaps of Ras, as one condition permitting the cAMP-dependent mitogenesis. However, the activation of Ras and MAPKs does not contribute as a *signal* in the still enigmatic mechanism by which cAMP can trigger mitogenesis in dog thyrocytes.

In FRTL-5 cells, active Ras was very recently shown to be required for cAMP-dependent mitogenesis (190), as in WRT cells, but Ras was mentioned not to be directly activated by cAMP (190). TSH and cAMP, through a PKA-dependent mechanism, rapidly stimulate the formation of Ras-PI3K complexes, indicating that cAMP can redirect Ras signaling toward PI3K, but PI3K activity was not investigated (190). Again, the significance of this observation is unclear for the cAMP-dependent proliferation in the presence of insulin/IGF-I, which strongly activates PI3K through its association with tyrosine-phosphorylated IRS proteins, an effect enhanced by cAMP (131) as detailed in *Section V*.

Commercial preparations of bovine pituitary or human recombinant TSH were recently found to activate p42/p44 MAPKs in FRTL-5 cells, but this cAMP-independent effect was also observed in cell lines that do not express TSH receptors, and thus was ascribed to contaminants of TSH preparations (191). A cAMP-independent activation of p42/p44 MAPKs by TSH was also reported in human thyrocytes (192). In our laboratory this effect is not inhibited by antibodies neutralizing TSH or blocking TSH receptors (F. Vandeput, unpublished data). It may thus also reflect the contamination of TSH with a growth factor.

E. Summary

In all the systems, the mitogenic effects of TSH are mainly or totally mediated by cAMP and require PKA activity. However, as shown in dog thyrocytes and WRT cells, the activation of PKA is not sufficient to reproduce the cAMP-dependent mitogenic activity, and cAMP activates Rap1 independently of PKA. In all the systems, the cAMP-dependent signaling cascade does not activate the p42/p44 MAPKs, unlike insulin/IGF-I, growth factors, and phorbol esters. In other respects the various models are very different. In WRT cells, overactivation of Ras and each of its potential effectors (Raf/MEK/MAPKs, PI3K, RalGDS) is reported to be sufficient to elicit mitogenesis. In dog thyrocytes, normal activation of Ras, MAPKs, or PI3K by extracellular stimuli is possibly necessary but not sufficient for mitogenesis. In WRT cells, cAMP and insulin/IGF-I are reported to independently activate Ras and PI3K (PKB), and the lack of MAPK activation by cAMP is ascribed to inhibition of c-Raf by PKA. In dog thyrocytes, cAMP does not activate Ras and PI3K. In FRTL-5 cells, the effects of cAMP alone on the various signaling pathways have been poorly explored, but cAMP exerts delayed potentiating effects on the PI3K and MAPK pathways activated by insulin/IGF-I. In the different systems, the different relative effects of cAMP and insulin/IGF-I on the intracellular signaling cascades are thus consistent with their relative effects on proliferation.

VIII. Immediate/Early Genes

Expression of immediate/early genes encoding potentially oncogenic transcription factors is generally considered to function as a third phase in the transduction of mitogenic stimuli. For instance, c-Myc is a strong inducer of proliferation, and its role in cell cycle control has been intensively investigated as it is believed to be critical for its oncogenic properties (193, 194). How c-Myc affects the cell cycle is still unclear. A plethora of mechanisms have been described, mostly derived from overexpression studies, whose physiological relevance is not always supported by genetic information. A sustained increase of c-myc expression is generally considered to be required for induction of important cell cycle-regulatory proteins (such as cyclin D2, E2F2, cdc25A, cyclin E . . .), G_1 phase progression, and DNA synthesis.

The expression of c-*myc* is stimulated by TSH and cAMP enhancers in FRTL-5 cells (77, 78, 195) and in dog (196, 197) and human thyrocytes (198). It is also induced by insulin/IGF-I and TPA in dog thyrocytes (196, 199, 200) and FRTL-5 cells (78), and by EGF and HGF in dog thyrocytes (199, 200). In FRTL-5 cells, TSH/cAMP effects on c-*myc* expression are sustained (77). By contrast, in dog thyrocytes, they are bi-

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phasic and the cAMP-dependent increase of c-*myc* expression is very transient, at variance with the sustained effects of growth factors and phorbol esters (197, 199). After a first phase of 1 h of higher levels of c-*myc* mRNA and protein, c-*myc* expression is even decreased below basal levels in TSH or forskolin-treated cells. At 1 h the effects of EGF and cAMP are additive, but at 3 h and thereafter cAMP markedly inhibits the stimulation of c-*myc* expression by EGF (197, 199). c-Myc expression is clearly not sufficient for mitogenesis in dog thyrocytes (155, 200), nor in PC Cl3 cells as shown by c-*myc* transfection (4). The involvement of c-*myc* in the cAMP-dependent mitogenic pathway of dog thyrocytes is unclear. c-Myc expression is too transient to explain the continuous requirement for high cAMP during the progression into G_1 phase (139). Unlike the relatively durable c-*myc* expression in TSH-stimulated FRTL-5 cells, in dog thyrocytes c-Myc is repressed by cAMP just when it is expected to transactivate genes encoding important cell cycle-regulatory proteins. Moreover, the activity of c-Myc as a transcription factor has been reported to require its stabilization by its phosphorylation by MAPKs (201), which are not activated by cAMP.

Dimers of proteins of Fos and Jun families compose the AP-1 transcription factors, which are also involved in the synthesis of cell cycle-regulatory proteins such as cyclin D1. c-Fos is induced by all studied (co)mitogenic stimuli, including TSH (cAMP), insulin/IGF-I, phorbol esters, serum and growth factors in FRTL-5 (77, 78, 202) and WRT cells (203), and in dog (143, 199, 200) and human thyrocytes (116, 204). c-Fos expression has been claimed to be required for TSH-dependent proliferation of FRTL-5 cells (205). However, in dog (143) and human thyrocytes (204) and in WRT cells (203), the effects of TSH and cAMP on c-*fos* expression are very weak compared with the effects of growth factors and phorbol esters, and to the effects of TSH in FRTL-5 cells. In FRTL-5 cells (78) and human fetal thyrocytes (116), TSH and insulin/IGF-I effects are additive, but in WRT cells cAMP represses the induction of c-*fos* mRNA by IGF-I (203). In dog thyrocytes, TSH and forskolin potentiate the induction of c-*fos* mRNA and protein by EGF (143, 199). By contrast, in human thyrocytes (in conditions which do not allow the demonstration of TSH-dependent mitogenesis), TSH inhibits the induction of c-*fos* by TPA and EGF (204). In contrast to c-*fos*, fos B is markedly induced by cAMP but weakly or not at all by other factors in dog thyrocytes. Interestingly, this effect is potentiated by insulin (200).

The expression of c-*jun* is stimulated by TPA, growth factors, and insulin/IGF-I in dog (200, 206) and human thyrocytes (204), and in WRT cells (203). By contrast, in these different systems basal and/or stimulated accumulations of c-*jun* mRNA are repressed by TSH and cAMP (200, 203, 204, 206). This difference between cAMP-dependent and -independent factors might be particularly significant, as c-*jun* is frequently considered the rate-limiting factor in the formation of AP-1 transcriptional complexes, downstream to the activation of MAPKs (207). Indeed, AP-1 transcriptional activity stimulated by serum or TPA is repressed by TSH in WRT cells (203). On the contrary, TSH induces c-*jun*-like c-*fos* in FRTL-5 cells (208, 209). Unlike c-*jun*, jun B expression is stimulated by cAMP-dependent and cAMP-independent factors in dog thyrocytes (210), WRT (203), and FRTL-5 cells (209). However, Jun B has been found as a repressor of AP1-activity by competition with c-Jun in Jun-Fos heterodimeric complexes, including on the cyclin D1 promoter (211, 212).

Egr-1 has also been reported as an important transactivator of the cyclin D1 promoter (213). Like c-*jun*, the expression of egr-1 is induced by insulin/IGF-I, growth factors, and TPA but repressed by TSH (cAMP) in WRT cells (203) and dog thyrocytes (200). By contrast, TSH has been found to stimulate egr-2 expression in FRTL-5 cells (208).

In no system, a single immediate/early gene expression could thus account for mitogenesis. Collectively, the investigations of the pattern of immediate/early gene expression in dog thyrocytes, WRT cells, and possibly human thyrocytes have shown some overlap, but also major differences, between the signaling pathways of TSH through cAMP, on the one hand, and of insulin/IGF-I, growth factors, and phorbol esters on the other hand. Remarkably, in these cells the expression pattern generated by cAMP is similar to the one observed in other cell types in which the proliferation is stimulated by growth factors but inhibited by cAMP (110, 214). At variance, in FRTL-5 cells available data indicate that TSH and cAMP induce an early gene expression pattern that resembles the one generally described in the response of fibroblasts to growth factors.

IX. Cell Cycle-Regulatory Proteins

The key events in the complex signaling cascades of TSH and insulin/IGF-I are still largely hypothetical. However, these cascades and those of growth factors are expected to finally modulate the level and activity of proteins that are the primary regulators of the cell cycle machinery. As generally considered, mitogenic signals regulate mammalian cell cycle by stimulating the accumulation of D-type cyclins and their assembly through a ill-defined mechanism with their partners the cyclin-dependent kinases (cdk) 4 and 6 (215). These complexes operate in mid-to-late G_1 phase to promote progression through the restriction point, and thus commit cells to replicate their genome. In the current model, this key decision depends on the initiation by cyclin D-cdk complexes of the phosphorylation of the growth/tumor suppressor protein pRb, which triggers the activation of transcription factors, including those of the E2F family, the synthesis of cyclin E and then cyclin A, and cdk2 activation by these cyclins. Activated cdk2, in turn, further phosphorylates pRb and other substrates and initiates and organizes the progression through the DNA synthesis phase (216, 217). The down-regulation of cdk inhibitors of the CIP/KIP family, including $p27^{kip1}$, by mitogenic factors and/or their sequestration by cyclin D-cdk complexes participate in cdk2 activation, but their proposed role of adaptor and/or nuclear anchor for cyclin D-cdk complexes suggests positive influences on cell cycle progression as well (218).

In dog thyrocytes, the different mitogenic stimulations (TSH, cAMP, growth factors) require the activity of cdk4 (219) and converge on the inactivating phosphorylation of pRb and related proteins p107 and p130 (220), on the phos-

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phorylation and nuclear translocation of cdk2 (221), and on the induction of cyclin A and cdc2 (221). These effects are dependent on insulin (220, 222). Cyclin D3 is the predominant D-type cyclin expressed in dog thyrocytes (223) and in mouse thyroid *in vivo* (224). TSH, unlike all the other known mitogenic factors, does not induce the accumulation of D-type cyclins (223), but it stimulates the expression of $p27^{kip1}$ (225). Nevertheless, cyclin D3 is required for the proliferation stimulated by TSH, but not in the proliferation of dog thyrocytes stimulated by EGF or HGF, which induce cyclins D1 and D2 in addition to increasing cyclin D3 levels (223). As depicted in Fig. 4, the formation and the nuclear translocation of essential cyclin D3-cdk4 complexes depend on the synergistic interaction of TSH and insulin in dog thyrocytes (222, 223). These complexes are absent from cells stimulated by TSH or insulin alone. Paradoxically, in the absence of insulin, TSH strongly inhibits the basal accumulation of cyclin D3 (222). In contrast, insulin alone stimulates the required cyclin D3 accumulation, and it overcomes in large part the inhibition by TSH (222), but it is unable to assemble cyclin D3-cdk4 complexes in the absence of TSH. In the presence of insulin, TSH (cAMP) unmasks some epitopes of cyclin D3 and induces the assembly of cyclin D3-cdk4 complexes and their import into nuclei (222, 223), where these complexes are presumably anchored by their association with $p27^{kip1}$ (Fig. 4), which is then sequestered away from cdk2 complexes (226), thus contributing to cdk2 activation. TGFβ selectively inhibits the cAMP-dependent proliferation of dog thyrocytes by preventing the association of cyclin D3 and cdk4 with nuclear $p27^{kip1}$, but it does not affect the assembly of cyclin D3-cdk4 complexes probably formed in the cytoplasm (226). Moreover, cAMP exerts an additional crucial function in very late G_1 phase, because pRb phosphorylation and DNA synthesis initiation still depend on sustained cAMP elevation, even after cAMP induction of stable nuclear cyclin D3-cdk4-p27 complexes (140). The investigation of cell cycle-regulatory proteins has thus clearly established that both cdk4 activation and pRb phosphorylation result from distinct but complementary actions of TSH and insulin, rather than from their interaction at an earlier step of the signaling cascades (141, 222) (Figs. 4 and 5).

In very sharp contrast, in FRTL-5 cells, TSH (cAMP) and insulin/IGF-I are both able to induce cyclin D1 and cyclin E (64, 93) (Fig. 4). In addition, TSH accelerates the IGF-I-stimulatory effects on cyclin D1 accumulation (64). These effects are probably related to the induction of *c-jun* and egr-2, which are known to transactivate the cyclin D1 promoter (212, 213). Cyclin D3 levels are also increased (227). In FRTL-5 cells, the activation of cdk2 is ascribed to the down-regulation of $p27^{kip1}$ induced by TSH alone (61, 93) and probably even more by the combination of TSH and IGF-I (228, 229). The delay of the onset of S phase provoked by the up-regulation of the PKA pathway by exogenous cAMP during G_1 phase was associated with an inhibition of p27 decay (134). In FRTL-5 cells, the synergy of TSH and insulin on cell cycle progression is thus mediated by an increase of cyclin D levels and a decrease of p27 levels likely resulting from an earlier integration of TSH and insulin cascades (Figs. 4 and 5). The mechanism of the TSH mitogenic action, therefore, much resembles the action of IGF-I in these cells and of growth factors in other cell types. The possible contribution of the autocrine loop involving FGF and FGF receptors should be examined. In PC Cl3 cells, as in FRTL-5 cells, TSH and insulin additively induce the expression of cyclin D1, cyclin D2, and cdk4 and enhance cyclin D3 levels (S. Demartin and P. P. Roger, unpublished data). However, in

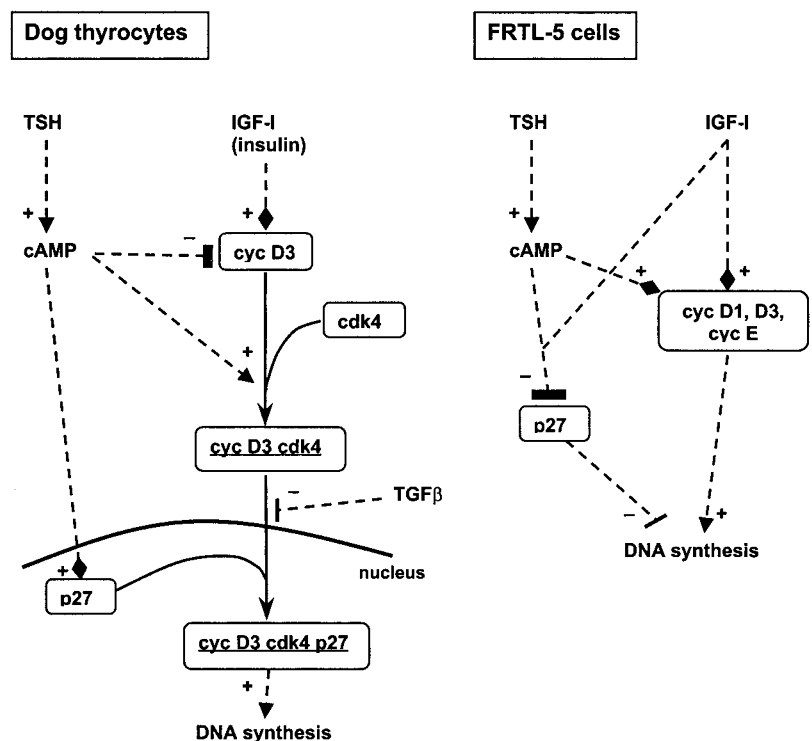

FIG. 4. Respective effects of TSH (cAMP) and insulin/IGF-I on cell cycle-regulatory proteins in dog thyrocytes in primary culture *vs.* FRTL-5 cells. *Straight lines* indicate transport or chemical associations or reactions. *Diamond/rectangle arrowheads* represent inductions/repressions; the other *dashed arrows* are activations (+) and inhibitions (−) (144). cAMP exerts opposite effects on the accumulation of $p27^{kip1}$ and D-type cyclins in dog thyrocytes *vs.* FRTL-5 cells.

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our hands, the high levels of $p27^{kip1}$ are relatively unaffected by TSH and insulin alone or in combination. Cell cycle-regulatory proteins have not been analyzed in WRT cells.

In summary, the expression of G_1 phase regulatory proteins, which constitute the end points at which mitogenic signaling pathways are expected to be integrated, still markedly differ in FRTL-5 and PC-Cl3 cells *vs.* dog thyroid primary cultures. In FRTL-5 cells, D-type cyclins are induced, and p27 down-regulated, in response to both TSH and insulin/IGF-I, as generally observed in fibroblasts stimulated by growth factors. In dog thyrocytes, TSH does not induce D-type cyclins, but it increases p27 expression and activates cyclin D3 synthesized in response to insulin, which results in the assembly of required nuclear cyclin D3-cdk4-p27 complexes (Fig. 4).

According to our preliminary results in primary cultures of human thyrocytes, cdk4 is constitutively expressed, and cyclin D1 accumulation is stimulated by EGF and serum, but not affected or even markedly repressed by TSH and forskolin, as in dog cells. Cyclin D2 is undetectable, and the high basal accumulation of cyclin D3 is slightly enhanced by TSH or insulin in different experiments. p27 Levels are not down-regulated by TSH in the presence of insulin. Thus, these important cell cycle-regulatory proteins are not subjected to significant modulations of expression during TSH-stimulated G_1 phase progression of human thyrocytes. This is in sharp contrast to rat cell lines and also to the increased expression of cyclin D1 and down-regulation of p27 associated with the sustained proliferation induced by oncogenic Ras in human thyrocytes (178). The molecular target(s) of the mitogenic action of TSH through cAMP, therefore, remains to be identified in human thyrocytes.

X. *In Vivo* Models

The thyroid cell that obviously most interests us is the human thyrocyte *in vivo*. Clinical investigation has allowed us to validate in this context several concepts developed on the basis of *in vitro* models, especially human cells in primary culture.

To develop to adult stage, the human thyroid requires the number of divisions necessary to generate about 2.10^9 cells of the adult from 1 or a few cells in the embryo: about 30 divisions if there are no cell deaths. At the adult stage, the human thyrocytes divide once every 8 yr, *i.e.*, about six times (230). This by itself does not imply a limit of the lifespan. However in rats, chronic stimulation by TSH causes thyroid growth up to a plateau (231), and in dog thyrocyte primary cultures stimulated proliferation abruptly stops after four to six divisions (53), which might suggest such a limit. Autocrine mechanisms, such as the TSH-stimulated production by thyrocytes of growth inhibitors including TGFβ, could also be involved in the specific desensitization of the proliferative response to TSH, as first suggested from the FRTL-5 model (232), and validated in rats *in vivo* (233, 234).

The mitogenic role of the TSH receptor and of the cAMP cascade that it activates is supported, in man, by the growth of the thyroid in patients with TSH-secreting pituitary adenomas and in patients with Graves' disease (235). The serum TSAb found in the latter disease, *i.e.*, antibodies against the TSH receptor, stimulate predominantly the cAMP cascade (236). TSH and TSAb massively stimulate DNA synthesis in nonneoplastic human thyroid tissues xenotransplanted in nude mice (237). In contrast, TSH deficiency or TSH receptor-inactivating mutations are accompanied by thyroid hypotrophy (238, 239). The predicted role of the TSH receptor and its cAMP cascade has also been validated by the discovery of somatic and germline mutations of the TSH receptor (240, 241) and Gsα (242–245), causing constitutive activation of these proteins and their subsequent cascades in autonomous adenomas and congenital hyperthyroidism.

On the other hand, goiter is a frequent clinical finding in acromegalic patients, an effect mediated by chronically elevated IGF-I levels (246, 247). Nevertheless, the presence of basal TSH levels might be a prerequisite for the growth-promoting action of IGF-I, because a GH replacement therapy did not increase thyroid size in patients deficient for both GH and TSH (248). The anomalously low endemic goiter prevalence among pygmies living in iodine-deficient areas (249), who are genetically resistant to IGF-I, is also compatible with an *in vivo* permissive effect of IGF-I and IGF-I receptor on TSH mitogenic action. The *in vitro* demonstrated role of PI3K in the supportive proliferation effects of IGF-I and insulin is also consonant with the high incidence of thyroid tumors in patients with Cowden disease (250). These patients are congenitally deficient in PTEN (250), the 3′-phosphatase that catabolizes intracellular PIP3 and PIP2 signals generated by PI3K. Somatic hemizygous deletions of PTEN are also frequently found in follicular adenomas and a few thyroid carcinomas (251).

Transgenic and natural mutant mouse models have also validated *in vivo* many conclusions of *in vitro* studies. The role of the TSH receptor in thyroid growth is demonstrated by the hypotrophy of the thyroid in mice with natural TSH receptor-inactivating mutations (hyt/hyt) (252). The *in vivo* mitogenic role of the cAMP cascade is supported by the phenotype of TgA2R mice in which the constitutive activation of adenylyl cyclase by the adenosine A2 receptor expressed in thyroid leads to goiter and hyperthyroidism (253). Similar, albeit weaker, phenotypes are obtained in mice expressing constitutive Gs (the G protein activating adenylyl cyclase) (254), cholera toxin (255), or constitutive adrenergic α2 receptor (256) (which activates Gs). By contrast, transgenic mice overexpressing both human IGF-I and IGF-I receptor in their thyroid (TgIGF-I-TgIGF-IR) develop only a mild thyroid hyperplasia and respond to a goitrogenic effect of antithyroid drugs while maintaining a comparatively low serum TSH level. This indicates some autonomy of these thyroids, as in acromegalic patients, and a much greater sensitivity to endogenous TSH (S. Clément, S. Refetoff, B. Robaye, J. E. Dumont, and S. Schurmans, unpublished results). To distinguish the relative importance of the two mechanisms will require the cross of TgIGF-I-TgIGF-IR and hyt/hyt mice. Thus, the phenotype of TgIGF-I-TgIGF-IR mice supports the concept of the permissive role of the IGF-I system on the cAMP mitogenic cascade, and also of some independent effects of the overactivation of this system on growth and function. The functional and goitrogenic effects of endogenous TSH elevations by antithyroid drugs are im-

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paired in rats and mice made diabetic by streptozotocin. These defects are corrected by insulin (257, 258), but whether they primarily involve the thyroid gland, pituitary, or peripheral tissues remains unclear (259).

In rats, mice, and humans *in vivo,* chronic stimulation by TSH secondary to treatment with antithyroid drugs induces thyroid hyperplasia but also a marked hypertrophy of follicular cells. This apparent discrepancy with the lack of *in vitro* effect of TSH and cAMP on cell size in dog and human thyroid primary cultures (105) might be explained by the stimulation by TSH of IGF-I secretion by thyrocytes, which could activate the cells *in vivo* through an autocrine mechanism, but be diluted out *in vitro.* Stimulating effects of TSH on the accumulation by thyrocytes of IGF-I mRNA and peptide have indeed been demonstrated in mice (260).

Growth factor-signaling cascades demonstrated *in vitro* can exert similar effects *in vivo*. In nude mice, the injection of EGF promotes DNA synthesis in thyroid and inhibits iodide uptake in xenotransplanted rat (261) and human thyroid tissues (262). By contrast, the injection of FGF induces a colloid goiter in mice with no inhibition of iodide metabolism or thyroglobulin and thyroperoxidase mRNA accumulation (263). These effects are the exact replica of initial observations from the dog thyroid primary culture system (101, 102) and other thyroid primary culture systems (36, 41, 122, 123). EGF and FGF have since been found to be locally synthesized in the thyroid gland, as a possible response to T_4 (264) and TSH (137), respectively. Their exact role as autocrine and/or paracrine agents in the development, function, and pathology of the thyroid gland of different species has yet to be clarified (47, 265). The overexpression of both FGF and FGF receptor 1 in thyrocytes from human multinodular goiter might explain its relative TSH independence (266). On the other hand, the subversion of tyrosine kinase pathways similar to those normally operated by local growth factors [*i.e.*, the activation of Ret/PTC (267) and TRK (268), the overexpression of Met/HGF receptor sometimes in association with HGF (269), or erbB/EGF receptor in association with its ligand TGFα (270)] can be causally associated with TSH-independent thyroid papillary carcinomas, as demonstrated in transgenic mice in the case of different forms of Ret (271, 272).

The role of some *in vitro* studied downstream elements of the thyroid mitogenic cascades is also supported by studies of transgenic mice. The expression in thyroid of a dominant negative CREB provokes a marked thyroid hypotrophy, suggesting the crucial role of CREB and its activating phosphorylation by PKA (273). TgE7 mice, which express the HPV16E7 gene in their thyroid, develop an euthyroid goiter (148). The E7 protein sequestrates the Rb protein, thus releasing its negative control of E2F transcription factors. Rb protein inactivation by phosphorylation has been shown *in vitro* to be a common control point of all the thyroid mitogenic cascades. The massive cAMP-dependent thyroid hyperplasia of TgA2R mice is not associated with a down-regulation of nuclear $p27^{kip1}$ (224), as in TSH-stimulated dog thyroid primary cultures (225), but at variance with the stimulation of FRTL-5 cells by TSH (61, 228).

Thus, experimental evidence on mice *in vivo* and clinical evidence in human disease, when they exist, validate the mitogenic regulatory scheme derived from *in vitro* studies on dog and human thyroid cells in primary culture.

XI. Discussion

In this review article, we have examined the hypothesis that the hyperplasia of thyroid gland, as generated by the activation of TSH receptors, can be validly investigated by means of the available *in vitro* experimental models. Rat thyroid cell lines and primary cultures of canine and human origins maintain *in vitro* an excellent expression of TSH-dependent thyroid-differentiated functions. In all the systems, TSH, in large part or totally through cAMP, and insulin or IGF-I synergize and induce cell proliferation. Both in FRTL-5 cells (274) and in dog thyroid primary cultures (53), the TSH/cAMP-dependent mitogenesis has been described as a differentiated trait, which is adjunctive to the more general mechanisms of growth control by growth factors. Its unique characteristics might explain how it can be compatible with differentiation expression (275, 276), which is repressed by growth factors (277), phorbol esters (67, 103), or Ras activation (278). Quite unexpectedly, however, beyond this framework of common features, the confrontation of the experimental systems strikingly illustrates that similar phenotypes may rely on quite divergent mechanisms. In fact, it is now clear that the respective roles of TSH and insulin/IGF-I are different in the different systems.

The main divergences include 1) the insulin/IGF-I dependence of TSH receptor expression in FRTL-5 cells but not in dog and human thyrocytes and, conversely, the TSH dependence of insulin receptor expression and of insulin receptor-mediated comitogenesis in dog and human thyrocytes but not in FRTL-5 cells; 2) the increase of cell mass caused by both TSH and insulin/IGF-I in rat cell lines, but only by insulin/IGF-I in dog and human thyrocytes; 3) the independent activation of PI3K and Ras by both insulin/IGF-I and TSH (cAMP) in WRT cells, but not by TSH and cAMP in dog thyrocytes; 4) the profoundly different effects of TSH and insulin/IGF-I on cell cycle kinetics and cell cycle-regulatory proteins in FRTL-5 and PC Cl3 cells *vs.* dog and possibly human thyrocytes.

In FRTL-5 cells, TSH induces early genes such as c-*jun* and c-*myc*, stimulates the expression of D-type cyclins, and down-regulates p27. Possibly because this reinforces similar actions of insulin/IGF-I, and also because cAMP augments the signaling pathways of IGF-I leading to the activations of MAPKs and PI3K (131) (Fig. 5), TSH exerts a priming effect, making the cell more competent to progress into G_1 phase in response to insulin/IGF-I alone, which can thus be qualified as the only genuine mitogen. Further TSH presence is dispensable during G_1 phase progression, and maintenance of high cAMP levels even delays DNA synthesis initiation. In fact, in FRTL-5 cells, TSH and insulin/IGF-I induce a similar pattern of responses, and TSH mostly potentiates IGF-I action (Fig. 5). Whether part of these TSH/cAMP comitogenic signaling events are indirectly mediated by autocrine growth factors such as FGF has yet to be definitively demonstrated.

In dog thyrocytes, TSH has a similar delayed potentiating action on IGF-I transduction (104). However, its main actions

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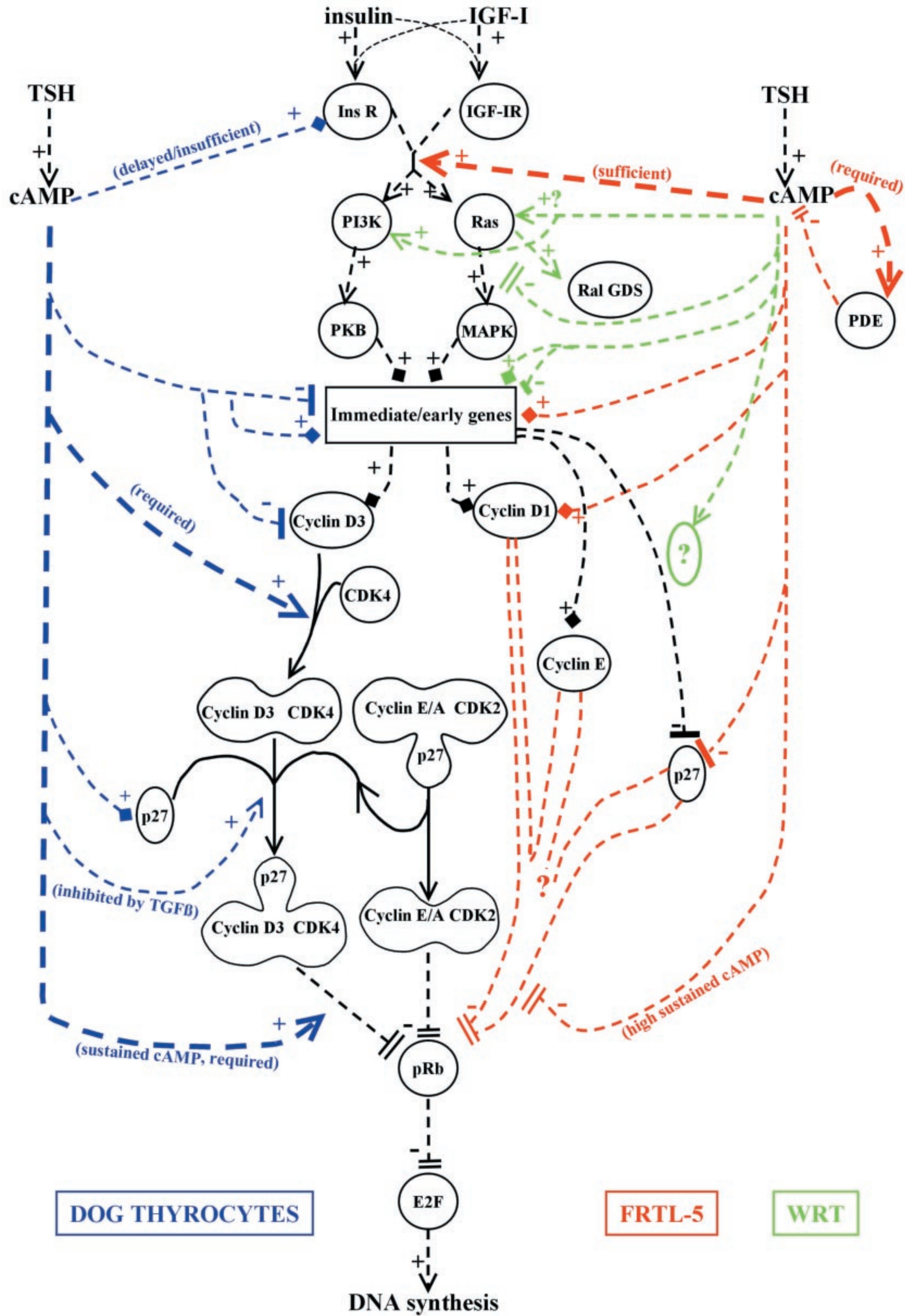

FIG. 5. Overall summary of synergistic interactions of comitogenic pathways of TSH and insulin/IGF-I mapped in dog thyroid primary cultures *vs.* FRTL-5 and WRT rat thyroid cell lines. Only the main regulations are included. TSH/cAMP actions demonstrated in dog thyrocytes, FRTL-5, and WRT cells are displayed in *blue, red*, and *green*, respectively. The symbolic representation of signal transduction controls is as proposed recently (144). *Straight lines* indicate transport or chemical associations or reactions. *Diamond/rectangle arrowheads* represent inductions/ repressions; the other *dashed arrows* are activations and inhibitions. See text for references, details, and explanations.

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are essentially different in these cells and involve more direct effects of cAMP on G_1 phase progression (Fig. 5). Unlike growth factors and/or insulin, TSH does not activate Ras, PI3K, PKB, or MAPKs. It down-regulates the expression of c-*myc* (after a short initial induction), c-*jun,* and egr-1. As a likely consequence, TSH rather inhibits the accumulation of D-type cyclins but stimulates the expression of $p27^{kip1}$. In dog thyrocytes, TSH must continuously elevate cAMP levels to directly control the passage through the restriction point. This requires, at least in part, critical actions on the assembly and nuclear translocation of cyclin D3-cdk4 complexes, which depend on the cAMP-dependent activation of the necessary cyclin D3, itself synthesized in response to insulin/IGF-I. Thus, at least in a first stage, the formation of cyclin D3-cdk4 complexes and the phosphorylation of pRb result from distinct but complementary actions of TSH and insulin/IGF-I, rather than their interaction at an earlier step of the signaling cascades. Together with the fact that the necessary increase of cell mass before division depends on insulin/IGF-I but not TSH, these observations provide a molecular basis for the well established physiological concept that in the regulation of normal thyroid cell proliferation, TSH is the "decisional" mitotic trigger, while locally produced IGF-I and/or circulating insulin are supporting "permissive" factors (52).

The reasons for these major differences between different experimental models of the same cell are unclear. They may obviously reflect species differences (52). Nevertheless, even among the apparently similar rat thyroid cell lines, major differences have been observed. The induction of c-*jun* by TSH/cAMP in FRTL-5 cells and its repression by cAMP in WRT cells, as in dog and human thyrocytes, likely reflect major differences in upstream signaling cascades and should result in divergent expression of downstream target genes, such as cyclin D1. Some signaling features, when they lead to selective proliferative advantages, might have been acquired during the establishment and continuous cultures of the cell lines and stabilized by subcloning. In the FRTL-5 cell line, this is facilitated by its instability exemplified by the spontaneous generation of variants with altered TSH dependence for growth or escaping from TGFβ inhibition (14, 17). Single mutations may completely change the complex interplay of signaling cascades. A model for this has been evidenced in WRT cells, where introduction of a mutated $Ras^{V12\,G37}$, but not other Ras^{V12} mutants, leads to a strong MAPK activation by cAMP (182), which is expected to profoundly influence the mechanism of cAMP-dependent mitogenesis. Among rat cell lines, PC Cl3 cells might more closely correspond to rat thyroid primary cultures. Unlike FRTL-5 cells, their transformation requires a two-hit mechanism, and their characteristics appear more stable until now. They are thus the most suitable for the investigation of mechanisms of multistep oncogenic transformation.

In the present analysis, we have considered the interest of model systems on the point of view of the closeness of their properties with those of the normal human cell *in vivo*. We were not concerned about the basic cell biology interest they have by themselves. Rat cell lines are valuable models for the investigation of mechanisms that underlie the thyroid-specific expression of differentiation genes (7, 279) [nevertheless oncogenic Ras was recently shown not to inhibit differentiation expression in human thyrocytes (177), at variance with rat cell lines]. Moreover, the different thyrocyte systems constitute interesting models of the wide diversity of possible mechanisms of the cAMP-dependent proliferation observed in other cell types including several endocrine epithelia (110, 280). However, clues gathered in the present review article are sufficient to suggest caution to the investigator contemplating the examination of human thyroid cell proliferation with rat thyroid cell lines as model systems. Although up-regulation of cyclin D1 or cyclin D3 and down-regulation or cytoplasmic retention of $p27^{kip1}$ may play an essential role in human thyroid tumorigenesis (281–283), they are not observed during the normal stimulation of human thyrocyte proliferation by TSH.

Dog thyrocyte primary cultures are the only system responding to the full range of (co)mitogens demonstrated in human thyrocytes. However, this system does not allow the analysis of the cAMP-independent component(s) of TSH action. It is marred by its restricted accessibility and its limited proliferation capacity. Most mechanisms demonstrated in this system so far apply to normal human thyrocytes, but much remains to be defined. This constitutes part of our current efforts, but will prove an especially difficult and frustrating task, because of the difficulty of obtaining normal tissue in sufficient amount, and the inherent variability between individuals (which nevertheless reflects rather than distorts reality). Moreover, comparison of data obtained for human thyrocytes in different centers is prevented by the absence of a consensus on culture protocols to be applied. However, the predictions based on the extensive *in vitro* investigation of dog thyroid cells in primary culture and the more scarce data on human cells have been supported by the available *in vivo* experimental data on transgenic mice and the clinical data in man. In both these systems, chronic activation of the TSH/cAMP cascade leads to hyperfunction and growth, and, conversely, in both systems IGF-I does not induce marked growth but sensitizes the thyroid to the action of TSH. One conclusion is also established: there is no such thing as "the thyroid cell" and many (but hopefully not all) extrapolations from results of *in vitro* model systems to the normal human thyroid cell are presently unwarranted, unless validated.

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Address all correspondence and requests for reprints to: Dr. J. E. Dumont or Dr. P. Roger at IRIBHN, Faculté de Médecine, Campus Erasme, 808 route de Lennik, B-1070 Bruxelles, Belgium, E-mail: proger@ulb.ac.be and jedumont@ulb.ac.be

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15th International Symposium of the *Journal of Steroid Biochemistry & Molecular Biology*

"RECENT ADVANCES IN STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY"

May 17–20, 2002—Munich (München), Germany

The 15th International Symposium of the *Journal of Steroid Biochemistry & Molecular Biology*. "*Recent Advances in Steroid Biochemistry & Molecular Biology*" will be held in Munich (München), Germany, on May 17–20, 2002. The following topics will be considered: 1. Steroid receptors, structure, gene expression, and mechanism of action; 2. Nongenomic effect of steroid hormones; 3. Steroid membrane receptors; 4. Steroids and cancer; 5. Steroids in the central and peripheral nervous systems; 6. Steroids and menopause; 7. Enzyme modulators; 8. Steroid hormones, phyto-, xeno-estrogens, and the environment; 9. Steroids and sport.

Lectures (approximately 30–35) will be by invitation of the Scientific Organizing Committee and, in addition, there will be poster sections. All posters presentations will be subject to selection by the Scientific Organizing Committee.

Abstracts (maximum 200 words) must be sent to Prof. J. R. Pasqualini by Monday, February 4, 2002 (postmark) (*original* + 12 copies).

For further information, please contact:

General Scientific Secretariat: Prof. J. R. Pasqualini, Steroid Hormone Research Unit, Institut de Puériculture, 26 Boulevard Brune, 75014 Paris, France. Tel.: (33-1)+45 39 91 09/45 42 41 21; Fax: (33-1)+45 42 61 21; E-mail: Jorge.Pasqualini@wanadoo.fr or the local organizing committee: Prof. H. Michna, Technical University of Munich, Olympiapark, Conollystr. 32, D-80809 München, Germany. Tel.: (49)+89/289-24601; Fax: (49)+89/289-24636; E-mail: michna@hrz.dshs-koeln.de

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Erratum

In the June 2001 issue of *Endocrine Reviews*, an error appears in Table 1 in the article by Palter *et al*. (Palter SF *et al*. 2001 Are estrogens of import to primate/human ovarian folliculogenesis? Endocr Rev 22:389–424). Under the heading "ERα status," the entry for Rosenfeld *et al*. (1999) should read N/A instead of TC.

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