REVIEW

Thyrotropin-dependent proliferation of *in vitro* rat thyroid cell systems

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Abstract

This review is focused on the most recent knowledge on growth control of rat thyroid cell lines. We analyzed the effect of mitogenic as well as inhibitory agents, but mainly the proliferative effect elicited by thyrotropin (TSH). The classic cAMP-dependent protein kinase (PKA) signal transduction pathway involved in TSH-mediated cell growth is analyzed exhaustively. We have also reviewed new concepts about the participation of other effectors such as small GTPases and phosphatidyl inositol-3-kinase (PI3-K) and the new data about the existence of a cAMP-dependent but PKA-independent pathway. Finally, we give information about TSH induction of cell cycle-related genes, such as G1 cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors.

European Journal of Endocrinology **143** 161–178

Introduction

The different extracellular signals that activate proliferation in mammalian cells are coordinated with the cell cycle machinery by different transduction pathways. This activation process involves transcriptional and post-transcriptional events that finally overpass the first GTPase-activating protein (GAP) (G1 phase) and initiate DNA synthesis (S phase). Key regulators of G1 progression include the D- and E-type cyclins, which respectively assemble with cyclin-dependent kinases (CDK)-4/6 during G1 or with CDK-2 at the G1–S boundary. The activation of CDKs, by binding to G1 cyclins, promotes the phosphorylation of necessary substrates like retinoblastoma (Rb) protein. In the absence of phosphorylation, Rb will negatively regulate the activities of transcription factors implicated in cell cycle control like E2F–DP1 heterodimers, which are essential for the G1–S transition. Alternatively, cyclin–CDK complexes can be inhibited by two families of cyclin-dependent kinase inhibitors defined by their structural homology and CDK targets. The first family includes the inhibitor kinase (INK) proteins that specifically bind and inhibit CDK4 and CDK6 but not other CDK or cyclins. The INK family includes four proteins with multiple ankyrin repeat sequences: $p16^{INK4a}$ (1), $p15^{INK4b}$ (2), $p18^{INK4c}$ (3, 4), and $p19^{INK4d}$ (4, 5). This family contrasts with more broadly acting inhibitors, the Cip/Kip family, whose action affects the activities of E and A cyclin-dependent CDK2 and positively regulates D cyclin-dependent kinase (6). The Cip/Kip family includes $p21^{Cip1}$ (7–12), $p27^{Kip1}$ (13, 14), and $p57^{Kip2}$ (15, 16), all of which have characteristic motifs that enable them to bind to cyclin as well as to CDK subunits (17–22).

This review is focused on *in vitro* experimental models of rat thyroid cell lines including the Fisher rat (FRTL-5) (23), the Wistar rat (WRT) (24) and PC C13 (25) cell lines. Although the FRTL-5 cell line is the most frequently used system, all of them retain most of the features of differentiated follicular thyroid cells, such as thyrotropin (TSH)-dependence for growth and function, iodine uptake, and thyroglobulin (Tg) and thyroperoxidase (TPO) gene expression. The simplicity of these systems allows stable transfections and genetic experiments and also explains why they are the preferred models in most of the *in vitro* studies of thyroid biology.

The second part of this review is focused on the more recent advances in our understanding of the mitogenic effects of TSH, the signal transduction pathways involved in this response, including the role of the Ras superfamily, and its effects on the main cell cycle-related genes. Thus, classically, binding of TSH to the TSH receptor (TSH-R) stimulates both growth and function of thyroid cells. TSH-R coupled to adenylyl cyclase elevates cAMP levels promoting the activation of cAMP-dependent kinase (PKA) and the consecutive

phosphorylation of transcription factors such as cAMP response element binding (CREB) proteins. This phosphorylation is required to activate the expression of important genes implicated in normal growth and phenotype differentiation in thyroid cells (26). We have also reviewed the role of the small GTPases involved in TSH-mediated proliferation and found that the classic view summarized above now seems to be more complex. Thus, TSH-mediated proliferation requires Ras activation but not through typical Ras effectors such as Raf-1/ mitogen-activated protein kinase (MAPK). Some evidence indicates that other Ras effectors, such as RalGDS (Ral GDP dissociation stimulator), phosphatidylinositol-3-kinase (PI3-K) or members of the Rho family, could be involved in the TSH actions. These observations, together with the new characterization of a family of cAMP-binding proteins that directly activate Rap1, open a new field of study on the role of these proteins in the thyroid. In this way, recent results suggest the need to reformulate concepts about cAMP-mediated signalling. Finally, we go over the effect of TSH on the cell cycle machinery. Thus, TSH induces cell cycle-related gene expression such as G1 cyclins (D1, D3 and cyclin E) and CDK2, as well as down-regulates the universal CDK inhibitor $p27^{Kip1}$. The resulting increases are reflected by an elevation in the hyperphosphorylated form of Rb.

Rat thyroid cell systems

There is a broad range of *in vitro* models for studying thyroid growth and function. We can distinguish between primary cell cultures and established cell lines. Both have been widely used and have some advantages and handicaps, basically in the comparison of *in vitro* and *in vivo* situations, although the accumulated data proceeding from both experimental approaches should help to obtain a clearer view of thyroid physiology. The most frequently used rat cell line is FRTL-5 (23), which was obtained from a primary culture from 5- to 6-week-old rats of the NIH Fischer 344 inbred strain. Rat thyroid glands were excised, cut into small pieces and digested using standard methods for the isolation of primary cell lines (27, 28). Cells were cultured in a modified Ham's F-12 medium (29) supplemented with 0.1–0.5% donor calf serum and a hormone mixture, derived from the initial cocktail from Hayashi & Sato (30) including 10 ng/ml glycyl-L-histidyl-L-lysine, 5 µg/ml transferrin, 10 nM hydrocortisone, 10 ng/ml somatostatin, 10 µg/ml insulin, and 1 nM TSH. Primary culture was done in a low serum medium to avoid the selection of fibroblast-like cells, which are better adapted to growth in conventional culture conditions. The primary thyroid culture was completely dependent on TSH and insulin for growth and survival. Thus, the persistence of morphological and differentiated characteristics of the cells was

dependent on TSH dose starting with 0.5 mU/ml, and maximal growth stimulation was obtained between 5.0 and 10.0 mU/ml.

The original FRTL strain grows with a doubling time of 5–7 days in the above-defined medium. The cells remain diploid with 42 chromosomes and the morphological characteristics of the primary FRTL culture remains unchanged. The individual cells are small with a small nucleus containing one or two nucleoli. The cells tend to grow in groups, forming follicle-like structures that grow on top after several weeks in culture, rather than expanding in a monolayer to fill the empty spaces on the dish. These cells maintain highly differentiated features typical of the thyroid phenotype such as Tg and TPO synthesis, iodine uptake and hormone dependence. Low serum conditions were critical during early passages, when the more differentiated cells are selected. Once isolated, a sub-strain of FRTL cells was grown in Ham's F-12, Coon's modified medium plus the six hormone mixture and 5% serum (complete medium). These cells were called FRTL-5 cells, had a doubling time of 30–40 h and remained diploid and differentiated (23, 31–34). The continuous presence of TSH is a necessary requirement for cellular growth. Thus, removing TSH from the culture medium arrests cellular replication and this arrest is reversed by the readdition of TSH. Cell growth then resumes with a 2 day 'lag phase' in the continued presence of TSH (32). Insulin alone is not a growth factor for FRTL-5 cells, but its removal from the complete medium significantly reduces the growth rate (23). Insulin appears to be a permissive factor in TSH stimulation of FRTL-5 cell growth (35). None of the other factors present in the complete medium is a mitogen by itself, or can positively influence TSH-dependent cellular growth.

Other rat thyroid cell lines isolated by similar methods are the WRT (Wistar rat thyroid) (24) and the PC C13 cell lines (25) (Table 1). The main difference among these systems is the selection by cellular cloning from fibroblasts in the case of WRT and PC C13, versus selection by a low concentration of calf serum in the case of the FRTL cells. In addition, PC C13 cells were obtained from 18-month-old rats whereas WRT cells were obtained from 3- to 4-week-old rats. Furthermore, insulin itself acts as a mitogen in WRT cells whereas in the FRTL-5 and PC C13 cells it is a permissive factor (35, 36). All are immortal cell lines from primary rat thyroid cultures which are considered 'normal': the cells are euploid, they do not grow in soft agar, do not show contact inhibition, and are not tumorigenic in euthyroid nude mice, although they can become tumorigenic when circulating TSH levels are artificially elevated after chemical destruction of the thyroid gland with $Na^{131}I$ (37) or after goitrogenic drug administration (38). The characteristics of the most useful rat thyroid cell lines appear in Table 1.

Table 1 Characteristics of rat thyroid cell lines. This table is a summary of data from references 23–25 and 38.

Cell line	Culture medium	Selected from fibroblast by	Age of donor rats	No. of chromosomes	Doubling time (h)	Tg secretion (a)	C/M ratio (b)	Tumorigenicity
FRTL-5	Ham's F12 + 5% calf serum and six hormones	0.5% calf serum	5–6 weeks	42	38	380	100	—
PC C13	Ham's F12 + 5% calf serum and six hormones	cellular cloning	18 months	42	24	780	100	—
WRT	Ham's F12 + 5% calf serum and six hormones	cellular cloning	3–4 weeks	42	42	670	100	—

(a) Expressed as ng Tg produced per ml by 10^6 cells, determined by RIA.
(b) C/M ratio is c.p.m. of ^{125}I per μl of medium/c.p.m. of ^{125}I per mg of cell pellet, as explained by Brandi *et al.* (24).

Extracellular signals involved in the control of thyroid cell proliferation

TSH, insulin and IGF-I The main regulators of thyroid growth and function (39) are TSH and insulin, which recognize both insulin- and IGF-I receptors. Both can produce a dose-dependent increase in DNA synthesis and cell proliferation. When added together, insulin and TSH cooperate synergistically inducing a level of proliferation that is much greater than the sum of the effects of each one alone (23, 24, 36). In PC C13 and FRTL-5 cells, insulin and TSH activate both growth in size and DNA replication (36). In FRTL-5, TSH alone promotes proliferation but this effect can be masked by the presence of the cytostatic agent somatostatin in the culture medium (40). Submitogenic concentrations of insulin (50 ng/ml) greatly potentiate the TSH effects in FRTL-5, PC C13, and WRT cells suggesting, once more, that insulin acts as a permissive factor for TSH action. Thus, it is possible to distinguish two major pathways stimulated at the level of the TSH-R or at the level of the insulin or IGF-I receptor.

TSH recognizes specific receptors on the thyrocyte surface and triggers the activation of adenylyl cyclase and other effectors via the heterotrimeric guanine nucleotide-binding protein (G-protein) (41). TSH-R belongs to a broad class of G-protein-coupled receptors. The basic structure of these receptors shows three domains: an extracellular segment at the N-terminus where the hormone binds, seven transmembrane helices, and three intracellular loops at the C-terminus (42, 43). Several experimental approaches demonstrated that the TSH-mediated response involves the activation of adenylyl cyclase and the increase of intracellular cAMP levels (39, 44). cAMP is the main second messenger in thyroid cells; thus agents which increase cAMP intracellular levels, such as forskolin or 8-bromo-cAMP, stimulate the expression of thyroid-specific genes, iodine uptake, the synthesis and secretion of thyroid hormones, and the growth of thyroid cells (34, 45, 46). In FRTL-5 cells, this second messenger stimulates both DNA synthesis and cell proliferation, and increases the levels of proto-oncogene mRNAs such as c-myc and c-fos (47, 48). In WRT cells, the micro-injection of an antibody for the stimulatory G-protein of adenylate cyclase G_s inhibits TSH-induced DNA synthesis (49). The importance of the TSH-R and cAMP-dependent pathway in the control of thyroid cell proliferation is also demonstrated by the observation that TSH-R gene mutations, which constitutively activate this receptor, have been associated with the development of thyroid tumors. Thus, mutations in the TSH-R gene have been observed in human adenomas and in congenital hyperthyroidism (50–56). These mutations in the TSH-R gene permanently activate adenylyl cyclase leading to TSH-independent proliferation and to constitutive cAMP accumulation, as well as phosphatidylinositide pathway activation in some cases

(57). Furthermore, the overexpression of TSH-R mutant proteins in FRTL-5 cells induce neoplastic transformation (58). Together, all these findings clearly suggest that the effects of TSH through its receptor are mainly due to increased intracellular cAMP levels.

The facts that insulin effects on thyroid cell growth are observed at pharmacological doses and mimicked by physiological doses of IGF-I suggest that insulin could act through the IGF-I receptors (59, 60). These receptors are glycoproteins that include two extracellular α-subunits which bind the ligand and two β-subunits with an intrinsic tyrosine kinase activity on their intracellular domain (61). After insulin stimulation tyrosine kinase activity promotes the autophosphorylation of the receptor and other proteins such as insulin receptor substrates (IRS), Vav proto-oncogen, and adapter molecules such as Shc (62). These events activate signal transduction pathways, such as Ras/MAPK, PI3-K, and PKC (63–65), that finally regulate insulin target genes. There are several indications of a role for PKC in insulin action in FRTL-5 and PC C13 cells. Both insulin and TSH induce the synthesis of 1,2-diacylglycerol, the endogenous activator of PKC (66), while PKC activators such as the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) mimic the effects of insulin in both cell lines. In fact TPA in PC C13 cells seems to be almost as potent as insulin (36, 67). In addition, co-stimulation with insulin and TPA in FRTL-5 or PC C13 cells does not synergize proliferation or protein synthesis, suggesting that both compounds activate the same pathway.

Together with insulin and IGF-I, there are several other growth factors that may also activate proliferation via mechanisms that are triggered by the activation of the tyrosine kinases (68, 69). Some of these factors have an autocrine effect on proliferation (70, 71). Thus, although it has been suggested that FRTL-5 cells lose the epidermal growth factor (EGF) response (39), recently Kimura *et al.* (36) reported a slight increase in [³H]thymidine-labeled nuclei after EGF or hepatocyte growth factor (HGF) stimulation in FRTL-5 cells, and this increase was potentiated by insulin. Conversely, PC C13 cells do not respond to EGF or HGF (36).

Other growth factors and local signals Epithelial cell proliferation is controlled by extracellular signals that include a variety of regulator peptides. Some of these factors are synthesized and released by the epithelial cells themselves and they act as an essential part of an autocrine mechanism. It is thought that the regulatory function of these factors mediates or modifies the local action of classic hormones. Thus, the TSH-mediated proliferation of thyrocytes might partially be due to the release and autocrine effects of these growth-promoting factors (72). The following are some of the growth factors associated with TSH-mediated proliferation.

ATP. The purinergic agonist ATP is a potent activator of the phosphatidylinositol 4,5-biphosphate/inositol-1,4,5-triphosphate (PIP_2/IP_3) signal transduction pathway (73–76). In FRTL-5 cells, the activation of this pathway results in a substantial increase in intracellular free calcium levels. ATP acts as a potent co-mitogen in these cells, enhancing the incorporation of [³H]thymidine in the presence of insulin and TSH. This effect appears to be mediated by the activation of a MAPK pathway, although the mechanism of this activation remains unknown (77).

Sphingolipid metabolites. Sphingosine-1-phosphate (SPP) mobilizes sequestered calcium and stimulates proliferation in several different cell types. In FRTL-5 cells SPP rapidly mobilizes and sequesters calcium, which stimulates the entry of extracellular calcium and hereby increases the incorporation of [³H]thymidine in a dose-dependent manner (78).

Amyloid peptides. The proteolytically released ecto-domain of the β-amyloid precursor protein (APP) (72) is a single membrane-spanning glycoprotein known to serve as the macromolecular precursor for the amyloid peptide found in senile plaques and neurofibrillar tangles in the cerebral cortex of patients affected with Alzheimer's disease (79). APP is almost ubiquitously expressed and shows a high degree of evolutionary conservation (80, 81). Popp *et al.* (72) have demonstrated that APP promotes the growth of FRTL-5 cells and that the TSH-mediated proliferation might be related in part to APP activity.

Iodine. This element has an autoregulatory effect on various aspects of thyroid function and metabolism. Iodine deficiency may render a normal TSH concentration goitrogenic (39). Iodine inhibits thyroid growth and this is one of its major autoregulatory effects on this gland. This inhibition appears to be mediated by iodine interference with TSH-induced cAMP production although other inhibitory mechanisms may be involved (82). In addition, high iodine concentrations arrest the cell cycle at the G1 and G2–M phases, thus inhibiting FRTL-5 cell proliferation (83).

Other negative autocrine factors TGF-β. Transforming growth factor β (i.e. three closely related isoforms TGF-β1, -β2 and -β3) is a multifunctional cytokine with diverse biological actions that influence a wide range of cellular, physiological, immunological and developmental processes (84, 85). TGF-β is the most potent inhibitor of *in vivo* and *in vitro* growth for a wide variety of cell type. Expression of TGF-β precursor mRNA has been detected in most cell types, suggesting that it may mediate a negative autocrine loop that limits normal cell proliferation. TGF-β1 has been found to inhibit proliferation in all the normal thyroid cell systems and, in some of these systems (86), it also prevents thyroid specific differentiation. On the other hand, TGF-β1

precursor mRNA and/or immunoreactivity have been found in various pathological thyroid tissues, including follicular adenomas and malignant thyroid tumors (87). One example of the role of TGF-β1 in the control of proliferation appears in thyroid hyperplasia during the development of a goiter when thyroid follicular cells proliferate in response to a goitrogen (for review see 88). When the thyroid has reached a certain size, growth stops despite continuous administration of the goitrogen and this effect is accompanied by a wide-spread expression of TGF-β1 in the entire follicular epithelium; in normal thyroid the effect is restricted to parafollicular C cells (89). Withdrawal of the goitrogen results in a reduction in size of the thyroid gland that is partly mediated by cell death (39). This process correlates with high levels of TGF-β1 expression, suggesting that it plays a role in thyroid apoptosis. As in the *in vivo* situation, FRTL-5 growth is also inhibited by TGF-β1 in several physiological conditions. Thus, TGF-β1 induces apoptosis in quiescent cells and arrests growth in proliferating cells that are not stimulated by the presence of a stronger mitogenic stimulus such as TSH or TSH plus IGF-I (90–92). In all these situations TGF-β1 decreases p27^{Kip1} mRNA, cyclin D1, cyclin E–CDK2 activity and retinoblastoma phosphorylation. On the other hand, TGF-β1 increases p15 and c-myc mRNA expression (92). These findings suggest a physiological role for TGF-β1 in the control of thyroid growth through the modification of cell cycle regulatory proteins. In addition, Morris *et al.* (91) found that TSH stimulates the production of a TGF-β-like activity by FRTL-5 cells. This observation provides a good *in vitro* cell system to study the mechanism of TGF-β action in the control of thyroid proliferation.

Somatostatin. Somatostatin was initially identified as a potent growth hormone (GH) inhibitor in the hypothalamus. Soon after, it was identified in many tissues and cell lines exerting different biological actions, including inhibition of hormone secretion processes, control of neural cognitive and locomotor functions, and regulation of cell proliferation (93, 94). Somatostatin has also been found in the thyroid, more specifically in parafollicular C cells, leading to the speculation that this peptide could have local effects on thyroid hormone release, as well as a local action as a paracrine peptide (95). In FRTL-5 cells, somatostatin inhibits TSH- and IGF-I-mediated proliferation (96). More recently, the effect of somatostatin on cell proliferation has also been characterized in PC C13. In these cells, somatostatin inhibits insulin-dependent and insulin plus TSH-dependent cell growth, blocking the G1–S transition of the cell cycle (97). Somatostatin action is mediated by at least six different subtypes of receptors (called SSTR-1, -2A, -2B, -3, -4, and -5) (98). These receptors, belonging to the seven transmembrane domain superfamily, are coupled to different G-proteins that link SSTR to distinct cellular effector systems, such as adenylyl cyclase, protein tyrosine phosphatases, and the K^+ and Ca^{2+} channels. They are responsible for the broad spectrum of somatostatin actions. Overexpression of SSTR-2 in several cell lines inhibited growth and increased the expression of preprosomatostatin mRNA accompanied by somatostatin secretion (98). In addition, SSTR expression correlated with the constitutive activation of a tyrosine phosphatase (99). Furthermore, somatostatin increased SSTR mRNA in a pituitary cell line (100). More recently, we provided evidence that somatostatin is also expressed in FRTL-5 cells and its transcription is under the control of TSH. Experiments with conditioned medium from TSH-treated FRTL-5 cells demonstrate that this peptide has a cytostatic effect on cell growth that promotes cell cycle arrest at the G1 phase. Somatostatin action is mediated by the cyclin-dependent kinase inhibitor p27^{Kip1}, which is down-regulated by TSH to promote proliferative signals. These findings suggest that, within thyroid follicular cells, somatostatin expression is a local mechanism that regulates cell growth in an autocrine fashion (40).

Signal transduction pathways

So far, we have described the main growth factors and inhibitory signals that control the complex regulation of thyrocyte proliferation. These factors exert their functions by activating several signal transduction pathways. Perhaps, as we have stated before, the TSH–cAMP–PKA pathway may be the most important signal in the control of thyroid growth and function but there are other pathways that have been implicated in this regulation, PKC, MAPK and, recently, the PI3-K pathway. In summary, control of thyroid growth and function involves PKA-dependent and -independent pathways.

PKA

The cAMP-dependent PKA is held to mediate cAMP action. There are at least two types of PKA enzymes, PKAI and PKAII, each has a different cellular location, species and tissue distribution, as well as cAMP affinities (101). PKAI is a soluble cytoplasmic enzyme (102), while PKAII, α and β, are often associated with the cytoskeleton and Golgi apparatus, centrosomes and the perinuclear area (103–105). The regulation and localization of the catalytic (C) subunit of PKA is typically mediated through interaction with an inhibitory regulatory (R) subunit, which sequesters the C subunit in an inactive state under physiological conditions (106–109). The inactive holoenzyme is a heterotetramer composed of two R and two C subunits. Activation is achieved when cytoplasmic cAMP binds to the R subunit, which then liberates the C subunit (110, 111). The RI subunit has a higher affinity for cAMP than RII α and β (112, 113) and a quicker turnover (114). These observations suggest that PKAI and PKAII

decode cAMP signals with different duration and targets.

Classically, PKA activation promotes the phosphorylation of specific substrates such as CREB (cAMP response element binding protein). CREB binds to specific DNA sequences, called CREs, present in the promoters of the genes that are required for the control of the growth and differentiated phenotype in thyroid follicular cells (26). Phosphorylation of CREB at Ser-133 (115) and activation of cAMP-responsive genes occur within 15–30 min of hormonal induction, coinciding with the time required for PKA to reach the nuclear compartment from the cytoplasm (116). In the continuous presence of hormones, transcription of cAMP-responsive genes is attenuated by protein phosphatase 1 (PP-1)-mediated, and, in some cases, PP-2A-mediated, desphosphorylation of CREB at Ser-133. Indeed, PP-1 inhibitors such as okadaic acid and the inhibitor-1 protein can reverse the attenuation phase, thereby maintaining high levels of phospho-CREB, and in consequence target gene transcription. After the attenuation phase, there is a refractory phase induced by progressive loss of PKA activity as a consequence of C subunit protein down-regulation (117). Proliferation and differentiation are the most important effects of PKA activation. Thus, CREB protein stimulates the expression of genes involved in cell division such as c-fos and c-myc, two genes induced by TSH and insulin in rat thyroid cell lines (47, 48). Data from *in vivo* and *in vitro* thyroid models suggest the preferential role of PKAI subtype in the control of cell proliferation (118). Microinjection of heat-stable PKA inhibitor (PKI) in WRT cells partially inhibited the TSH/cAMP-mediated DNA synthesis (119). Paradoxically, microinjection of the PKA catalytic subunit failed to stimulate proliferation (120), suggesting that there are other PKA-independent signals that can contribute to cAMP-stimulated cell cycle progression.

In parallel, PKA also has an important role in thyroid differentiation, thus several thyroid specific genes such as Tg, TPO, TSH-R, Na^+/I^- symporter (NIS) and thyroid-specific transcription factors such as TTF-1, TTF-2 and Pax-8 (121) are regulated by TSH/cAMP (122), via PKA, and may be regulated by CREB or members of the CREB family. Exclusion of the catalytic PKA from the nucleus, after Ras transformation or PKC activation, correlates with the loss of transcriptionally active forms of TTF-1 and CREB proteins (123). Conversely, overexpression of catalytic PKA or depletion of the PKC reverses inhibition of Tg promoter activity by Ras (124). These effects from PKA activity appear to be mediated by a Ras-dependent delocalization and ultimate loss of the PKAIIβ isoenzyme (125), again suggesting different functions for each PKA isoenzyme.

PKC

The PKC gene family is divided into three subgroups based on sequence homology and cofactor requirements:

conventional PKCs (α, βI, βII and γ) which are dependent on Ca^{2+} for activation; non-conventional PKCs (δ, ϵ, η and o) which are not dependent on Ca^{2+} for activation, and atypical PKCs (ζ and λ/ι) which are not stimulated by 1,2-diacylglycerol (DAG) or phorbol esters and are Ca^{2+} independent (126). Classically, PKC stimulation is dependent on inositol lipid hydrolysis. Thus, stimulation of phospholipase C catalyzes the hydrolysis of 4,5-bisphosphate (PIP_2) and generates inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (127). The resulting IP_3 facilitates an increase in intracellular Ca^{2+} (128), while DAG activates PKC (126). Several pieces of evidence suggest that TSH plus insulin/IGF-I can activate PKC by elevating DAG in FRTL-5 cells, but this increase is independent of phospholipase C (PLC) activation at normal TSH concentration (1−10 mU/ml) (66, 129). The observation that TSH and its agonists forskolin and dibutyryl-cAMP (dbcAMP) can activate another phospholipase, PLD (responsible for the production of DAG from phosphatidylcholine hydrolysis), suggests an alternative mechanism for TSH-dependent activation of PKC (130). The PLD activation depends on PKA and PKC as has been demonstrated in experiments with specific inhibitors of both kinases (131).

There is marked heterogeneity in PKC isoforms among the thyroids of various species (132). When FRTL-5 cells are chronically exposed to TSH, the α, δ, ϵ and ζ PKC isoforms are expressed and all, except the last isoform, are activated by phorbol esters (133). When TSH-depleted FRTL-5 cells are stimulated with this hormone there is no effect on the cytosolic fractions of any of the PKC isoforms identified, but the membrane-bound fractions of α, ϵ and ζPKC after short-term exposure, and of δPKC after long-term exposure do increase (134). PKC isozymes are involved in signal transduction pathways controlling growth, differentiation and apoptosis (135, 136). In thyroid cells, PKC has been implicated in differentiation, inhibiting TSH-mediated increase in iodine uptake and organification, cAMP production (137, 138) and Tg (139), and 5′ D-I expression (139). Moreover, the expression of PKC mimics the effects of Ras transformation, indicating a possible role as an effector downstream from Ras (122, 140, 141). In FRTL-5 cells, activation of PKC by phorbol esters have a mitogenic effect (67, 137). This effect is cAMP independent and also inhibits TSH-mediated cell growth, interfering with cAMP production (67). Although phorbol esters are pharmacological agents, their activation and effects may have a physiological relevance in view of evidence that other physiological agents, such as TSH, IGF-I or norepinephrine, may also activate PKC in the thyroid cell, (66, 129, 142, 143).

PI3-kinase

Phosphoinositide-3-kinases (PI3-Ks) are a subfamily of lipid kinases that catalyze the addition of a phosphate molecule specifically to the third position of the inositol

ring of phosphoinositide. To date, nine members of the PI3-K family have been isolated in mammalian cells and they are grouped into three classes according to the molecules that they preferentially use as substrates (144, 145). PI3-K was first described as a PI-K activity associated with the viral oncoproteins, v-Src, v-Ros, and middle T polyomavirus. Mutational studies of these oncoproteins indicated a critical role for their associated PI3-K in cell transformation (146).

Usually, mitogenic signals that exert their action through tyrosine kinase receptors stimulate PI3-K α/β while those that activate G-protein-coupled receptors stimulate PI3-Kγ (147–150). PI3-K is required for the mitogenic activity of many growth factors such as PDGF, EGF and insulin. Most of these factors, specifically those requiring PI3-K activity, stimulate lipid kinase activity. The inactivation of this pathway by pharmacological inhibitors or microinjection of PI3-K-specific inhibitory antibodies or proteins (151–153) impairs growth factor-stimulated mitogenesis. Although cAMP has only been reported to inhibit PI3-K activity, these studies were performed in cells where cAMP failed to stimulate proliferation (154, 155). The biological effects of PI3-K are mediated through downstream kinases such as PDK1 (156–158), Akt (159–161), and p70s6k (162, 163). The small GTPase Rac1 is also activated downstream from PI3-K, where it contributes to p70s6k activation and stimulates membrane ruffling (164–166). Recent reports using the WRT (167, 168) and FRTL-5 (169) cell systems suggest the possible implication of PI3-K in the TSH/cAMP-dependent growth of thyroid cells. Thus, TSH/cAMP stimulate membrane ruffling, Akt and p70s6k activity. Both the stimulation of membrane ruffling and Akt activity are PI3-K dependent, whereas the stimulation of p70s6k activity is PI3-K independent (although the possibility of some p70s6k activation by PI3-K cannot be discarded). In addition, treatment with the specific PI3-K inhibitor wortmannin or the expression of a dominant negative form of PI3-K causes a G1 arrest of FRTL-5 cells stimulated to proliferate with TSH (169). Moreover, TSH/cAMP increases p70s6k protein levels in a manner that appears to be cAMP dependent but PI3-K independent (167 and our unpublished observations). In all these cases, PI3-K activation by TSH is exclusively cAMP dependent and insulin independent because all these observations were performed in cells that had been arrested by the absence of insulin and TSH and that had been restimulated with TSH alone (168, 169).

Ras signaling and thyroid cells

General considerations Ras proteins are a group of 20–25 kDa guanine nucleotide-binding proteins that function as molecular switches in many cellular signaling pathways. Thus, GTP-binding protein cycles between the GDP-bound inactive state and the GTP-bound active state. Activation is dependent on external signaling and occurs when a guanine–nucleotide exchange factor, GEF, accelerates nucleotide release from the protein. Once activated, Ras proteins can interact with the effector molecules generating a signal pathway that involves a broad range of effectors. Return to the inactive state is due to the GTP-binding protein capacity to hydrolyze GTP to GDP, a GTPase reaction that is accelerated by GTPase-activating proteins (GAPs) which are either regulatory proteins or can themselves be effectors (170–172).

In mammals, this superfamily of proteins is comprised of over 60 members, which, based on sequence and functional homology, can be divided into several subfamilies: Ras, Rho, Rab, Arf, Ran and Rad/Gem. The genes coding for these proteins are not only the most frequently activated oncogenes in cancer, but their corresponding proto-oncogenes are expressed in most mammalian tissues. They codify proteins that interact with a wide spectrum of regulators and downstream effectors producing different normal cellular responses such as proliferation, differentiation or apoptosis (173, 174). The expression of transforming Ras oncogenes interacts with the establishment and the maintenance of cellular differentiation in different tissues (175–178), including the thyroid. In this particular tissue, Ras inhibits the expression of thyroid-specific genes, and confers a proliferative advantage over normal thyroid cells (123–125, 140, 141).

Inhibition of thyroid differentiation by Ras The growth of FRTL-5 cells transformed with a variety of activated oncogenes such as v-ras, v-src, v-raf and v-mos become TSH/cAMP independent. Furthermore, the thyroid differentiation markers are suppressed and so the transformed cells do not express Tg or TPO, do not respond to TSH and do not take up iodine (25). In parallel, the transcription factors controlling their expression are either not present or are inactive (140, 179, 180). In K-Ras transformed cells, both Pax-8 and TTF-1 mRNA are undetectable whereas in H-Ras, TTF-1 is present at normal levels and maintains its binding capacity although the cells lack the ability to express Tg and TPO (141). The mechanism inhibiting differentiation after Ras activation appears to down-regulate the catalytic PKA activity (123, 125). Inactivation of PKA decreases the phosphorylation of transcription factors such as TTF-1 and CREB, and inhibits the transcription of specific genes such as Tg, TPO or TSH-R that contain specific sites for these transcription factors (121, 124). The accumulated results suggest that by activating PKC, Ras finally inhibits the cAMP/PKA pathway The overexpression of PKC mimics the effects on thyroid cell differentiation exerted by the Ras transformation (124, 140, 141).

Ras effectors and mitogenesis Rat thyroid cell proliferation is dependent on both PKA as well as Ras

activation. Overexpression of a constitutive inactive Ras form (Ras N17) significantly reduces TSH-mediated proliferation (120 and our unpublished observations). Concomitant overexpression of Ras N17 and a constitutive inactive PKA, fully suppress TSH-mediated proliferation, suggesting that both PKA and Ras are required for the full mitogenic action of TSH. Moreover, these results suggest that Ras function may be crucial in many G-protein-coupled signaling pathways in addition to its established role in tyrosine kinase-mediated pathways (120). Since TSH mitogenesis requires both signals, these must interact in some way. In several cell types in which intracellular cAMP levels inhibit growth, this second messenger exerts a negative effect on Ras-mediated signaling pathways. Several findings suggest that PKA inhibits the proper binding of Ras protein to Raf, blocking the activation of the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) and MAP kinases. In WRT cells, an increase in cAMP levels inhibits coupling of Ras to the Raf-dependent cytoplasmic kinase cascade (181–183). In primary dog thyrocytes, MAP kinase phosphorylation is not implicated in TSH-mediated proliferation (184). In FRTL-5 thyroid cells, the specific inhibition of MEK by the inhibitor PD85059 does not affect TSH-mediated proliferation (169). All these findings suggest that the TSH pathway is Ras dependent but Raf-1 and MEK independent and indicate that Ras signaling in TSH-treated cells might occur through an effector other than Raf. Some clues come from studies with mutant Ras effectors which differ in their ability to bind Raf (185). Thus, WRT cells overexpressing Ras (12V) or Ras (35S), which lead Ras to bind Raf, grow slowly in the presence of TSH, whereas another stable transfectant overexpressing Ras (37G) which only binds RalGDS, grows faster in the presence of TSH. These results suggest a role for a nucleotide exchange factor for Ral (RalGDS) in TSH-mediated proliferation. Several groups have shown that RalGDS is a Ras effector (186, 187). Dominant negative Ral mutants can suppress the oncogenic transformation induced by Ras in 50–75% (188). This indicates that Ral activation by Ras–RalGDS mediates some of the downstream effects of Ras, although the functions of Ral have long remained elusive (189). In WRT cells, microinjection of the dominant negative RalA (RalA N28) reduces TSH- and Ras-stimulated DNA synthesis (190). In addition, RalGDS can bind Rap1, a small Ras-like GTPase, identified as a protein that could suppress the oncogenic transformation of cells by Ras (188). Rap1 is activated by several extracellular stimuli and is involved in proliferation and differentiation. cAMP activates Rap1 in many cell types (191), including thyroid cells (168, 192), and cAMP-responsive Swiss 3T3 cells, where Rap1 acts as a mitogen (193) and as an oncogen (191). As we discussed before, recent reports have shown that one component of cAMP action is the inhibition of MAP kinase activation (194–196). Interestingly, the effects of

cAMP are very similar to those obtained after stable expression of a positive dominant form of Rap1 called Rap Val12. This mutant inhibits MAP kinase activation (197). This information tempts us to speculate that Rap1 plays a role in the cAMP-dependent inhibition of the MAP kinase pathway. The mechanism of this inhibition is unknown but one of the possible alternatives is that, upon activation, Rap may activate specific phosphatases that affect the Ras pathway at the Raf-1–MEK–MAP kinase activation step (191). Although the microinjection of wild-type Rap1 fails to induce proliferation or Tg expression in dog thyrocytes (192), this point remains to be studied in more detail using mutant forms of Rap1. All these recent findings open interesting questions about the effects of Rap1 overexpression on thyroid-specific markers under normal or Ras-transformed situations.

Recently, two different groups have reported (198, 199) that activation of Rap1 by forskolin and cAMP occurs independently of PKA. They cloned a gene encoding a guanine-nucleotide-exchange factor (GEF), called Epac (198), or cAMP-GEF (199), which contains a cAMP-binding site and a domain that is homologous to the GEF domain for Ras and Rap1. Thus, Epac seems to be a GEF for Rap1 that is regulated directly by cAMP. Epac is also expressed in the thyroid gland although its role is unclear and remains to be clarified. Thus, in this context, Epac may have an important function in the activation of proliferative or differentiative programs that are mediated by cAMP but are independent of PKA.

Rho family and thyroid The mammalian Rho family of GTPases, including RhoA, Rac1, and Cdc42, play pivotal roles in controlling many cellular functions, such as cell polarity, motility, proliferation, apoptosis, and cytokinesis (200). RhoA controls the assembly of actin stress fiber and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia (201, 202). All these proteins also stimulate progression through phases G1 to S of the cell cycle and their inhibitor forms can block serum-induced DNA synthesis (203). Rac1 and Cdc42 stimulate the JNK–SAPK cascade and lead to c-Jun transcriptional activity (204). RhoA is also involved in serum response factor (SRF) activation and acts synergistically with Rac1 and Cdc42 through a JNK-independent pathway (205).

All three proteins, Rho, Rac and Cdc42, cooperate with Raf in cell transformation and the dominant negative forms of RhoA, RhoB and Rac1 can inhibit Ras-induced transformation, indicating an essential role for these proteins in this process (206–210). Moreover, Rac1 and RhoA have also been implicated in the morphogenic and mitogenic responses to transformation by oncogenic Ras (165, 211). In the thyroid context, Cass *et al.* (168) described that activation of

Rac1 by a PKA-independent pathway, via PI3 kinase after TSH/cAMP stimulation, promotes the formation of ruffles on WRT thyroid cells.

In several cell types, such as neural cells and lymphocytes, RhoA can be specifically phosphorylated at Ser-188 by PKA. This effect decreases the binding of RhoA to the downstream effector ROKα, suggesting that RhoA and cAMP have antagonistic roles in regulating cellular processes in these cells (212, 213). However, the role of cAMP is completely different in the thyroidal context. This second messenger activates a proliferative response in most of the thyroid cells systems studied. Noguchi *et al.* (214) suggest that RhoA activation after growth stimulation plays an essential role in the degradation of p27^{Kip1} with the resulting progression from phase G1 to S in FRTL-5 cells. RhoA is geranylgeranylated and translocated to the membrane during this progression. Moreover, its activation is dependent on 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA-Red), the rate-limiting enzyme for the synthesis of isoprenoids such as the geranylgeranyl metabolites (215). This enzyme is transcriptionally regulated by TSH in FRTL-5 cells (216, 217). Results from our laboratory confirm the important role of RhoA in the control of TSH-mediated proliferation. Thus, overexpression of either the dominant negative form RhoA N19 or the specific inhibitor of RhoA activity, exoenzyme C3 (TC3), inhibits the FRTL-5 cells proliferation causing a G1 arrest. Moreover, the TSH-mediated activation of RhoA protein is PKA/HMG-CoA-Red dependent (169). The upstream regulators of RhoA are unknown but some authors, studying other cell systems, have proposed that Ras could activate RhoA-like proteins through its downstream effector RalGDS (218). Figure 1 presents a summary of our view on signaling in thyroid cells after the integration of the most recent observations.

G1 phase progression in thyroid cells

Key regulators of G1 progression in mammalian cells include three D-type cyclins (D1, D2 and D3), which
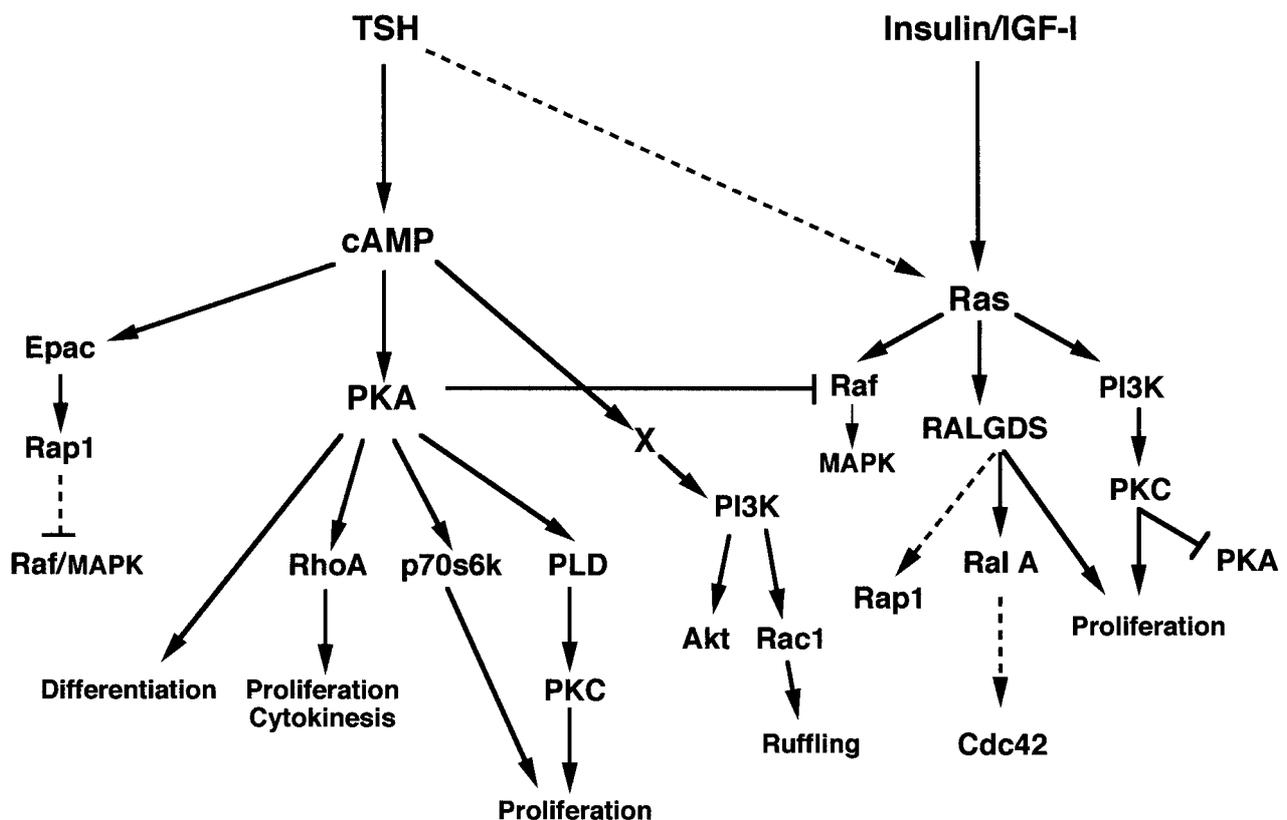

Figure 1 Hypothetical model of the signal transduction pathways induced by TSH and Insulin/IGF-I. TSH increases cAMP intracellular levels and eventually could activate Ras pathways. cAMP exerts its action through PKA-dependent and -independent pathways such as the cAMP–GEF (Epac) and PI3-K. All these pathways activate different downstream effectors that might interact with others pathways. In parallel, insulin activates Ras pathways and its downstream effectors (Raf, RalGDS and PI3-K). Finally all these pathways regulate several biological processes such as proliferation, differentiation and cytoskeletal remodeling. Symbols: arrows, activation; blocked bars, inhibition; dashed arrows, eventual pathways.
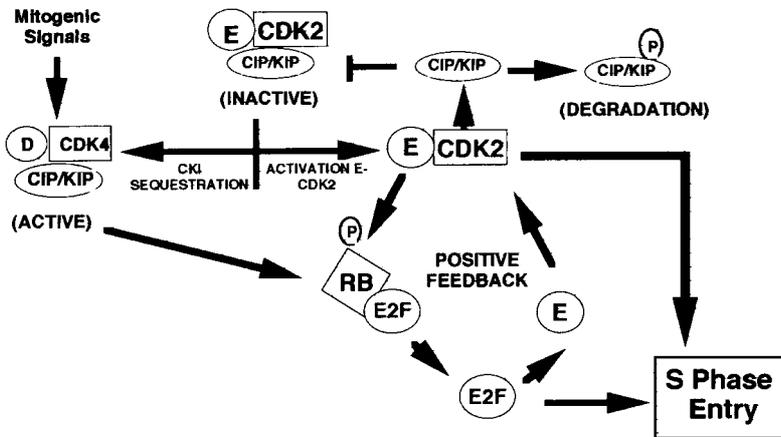
Figure 2 Regulation of the G1–S transition. Mitogenic signals promote the assembly of active cyclin D-dependent kinases containing either CDK4 or a Cip/Kip protein. Sequestration of Cip/Kip proteins lowers the inhibitory threshold and facilitates activation of the cyclin E–CDK2 complexes. Both cyclin D and E contribute sequentially to Rb phosphorylation, canceling its ability to repress E2F family members and activating genes required for entry into S phase. The latter includes cyclins E and A, as well as a set of important genes that regulate DNA synthesis (for review see Nevins 1992, 236). Moreover, cyclin E–CDK2 complexes can phosphorylate p27Kip1 triggering its proteolysis. The degradation of Cip/Kip proteins and the induction of cyclins by E2F (positive feedback) contribute to mitogen independence and to the irreversibility of the transition (modified from Sherr & Roberts 1999, 6). Symbols: arrows, activation; blocked bars, inhibition.

assemble into holoenzymes with either CDK4 or CDK6, and cyclin E, which combines with CDK2 and is expressed weakly at the G1–S boundary (219). The timing and periodicity of cyclin E expression in mammalian cells suggest that this cyclin may control the ability of the cells to enter into the S phase. Overexpression of D- or E-type cyclins can reduce the G1 phase, decrease cell size, and reduce the dependency of the cell on mitogens (220–222). Induction of D-type cyclin synthesis is strongly dependent on growth factors and D-cyclin levels decline precipitously when growth factors are withdrawn (223–225). Mitogen-induced Ras signaling promotes transcription and assembly of the cyclin D1 with CDK4 via Raf/MAPK (226–229). The degradation of D-type cyclins depends on a separate Ras signaling pathway involving PI3-K and Akt (230). These data suggest that D-type cyclins act primarily as growth factor sensors.

The D-type cyclins (231, 232), target CDK4 to Rb (233) and, unlike most CDKs, cyclin D-CDK4/6 phosphorylates Rb preferentially to histone H1 (234, 235). Rb binds to E2F-DP1 heterodimers and negatively regulates the activities of these transcription factors, with important functions in the G1–S transition. Phosphorylation of Rb on canonical CDK sites during middle to late G1 phase reverses the growth-suppressive effects of Rb by releasing E2F from its inhibitory constraint, thereby enabling the liberated factor to activate the genes required for DNA replication (236). Meanwhile, cyclin E preferentially forms complexes with CDK2 that phosphorylate substrates such as (i) histone H1 (237, 238), (ii) Rb (on sites other than where it is phosphorylated by D-CDK4/6) (239–243), (iii) the CDK inhibitor p27^{Kip1} (244, 245), and (iv) possibly other substrates that help trigger the firing of replication origins (246–251) during late G1 and early S phase (see Fig. 2). Once cells enter the S phase, cyclin E is degraded and CDK2 forms complex with cyclin A. The cyclin E–CDK2 and cyclin D–CDK4/6 complexes undergo phosphorylation on a single threonine residue, a

modification required for their activation. This activation involves a multisubunit enzyme designated CDK-activating kinase (CAK) composed of a catalytic subunit (CDK7) and a novel cyclin (cyclin H) (252).

In FRTL-5 cells, TSH, IGF-I, or a combination of both, induce G1 cyclin (D1, D3, and E) expression and accelerate the G1 phase (40, 92, 253). In quiescent FRTL-5 cells, levels of cyclin D1 and E are barely detectable; however, 24 h after TSH stimulation, their expression increases several fold (254). Stimulating growth in quiescent FRTL-5 cells with TSH, IGF-I and 5% calf serum results in an increase of CDK4 and CDK2 expression, and correlates with an increase in the phosphorylated form of Rb, all of which lead to cell cycle progression (92, 254). Moreover, results from our laboratory (169) indicate that the stimulation of quiescent FRTL-5 cells with TSH alone also increases both the expression levels of G1 cyclins (D1 and E) and CDK2 as well as the phosphorylation status of Rb. These results suggest that TSH increases the assembly and activity of cyclin D–CDK4 and cyclin E–CDK2 complexes. This is demonstrated by the increase of both CDK2 kinase activity (measured *in vitro* using histone H1 as a substrate) and Rb phosphorylation (measured as indirect index of cyclin D–CDK4 activity).

Another level of CDK regulation comes from the binding of CDK inhibitors (CKIs) to cyclin–CDK complexes that then either inhibit their kinase activities or prevent their activation by CAK (6). The CKIs comprise two classes of proteins, the Ink4 family, which specifically inhibits cyclin D-dependent kinases, and the Cip/Kip family, which can interact with virtually any cyclin–CDK complex. The Ink4 family includes 15–20 kDa proteins composed of repeated, loosely conserved ankyrin motif (1). The expression of p15^{Ink4b} is induced almost 30-fold in human keratinocytes treated with TGF-β. This induction is correlated with an increase of p15^{Ink4b} binding to CDK4/6 resulting in a loss of activity (2). These findings suggest that p15^{Ink4b} has a role as dominant effector of TGF-β-mediated cell cycle arrest in

these cells. Thus, it has been proposed that TGF-β treatment induces the p15^{Ink4b} expression that shifts p27^{Kip1} from cyclin D–CDK4/6 to cyclin E–CDK2 complexes (255). In FRTL-5 cells this mechanism has not been implicated among the known TGF-β effects and seems to be more related with the loss of p27^{Kip1}-free cyclin D–CDK4/6 complexes that leads to the shift of free p27^{Kip1} to cyclin E–CDK2 and its consequent inactivation (92).

The Cip/Kip family comprises p21, p27 and p57 gene products. These CKIs can interact with many different cyclin–CDK complexes, raising the possibility that they might act as a cyclin–CDK assembly factor. While cyclin–CDK complexes containing a single molecule of this family are catalytically active, those containing multiple molecules are not. Thus, changes in the stoichiometry of CKIs are sufficient to modulate the complex activation (256). Although several CKIs have been identified as potent inhibitors of cyclin–CDK complexes, p27^{Kip1} is the only CKI whose protein expression decreases when mitogen-induced cells enter the cell cycle (257–260). p27^{Kip1} levels are elevated in quiescent cells and decline during the G0–S phase interval (260, 261). If the synthesis of CDK4 is inhibited by TGF-β treatment, the free p27^{Kip1} levels inhibit cyclin E–CDK2 kinase activity and induce G1 arrest. Hence titration of p27^{Kip1} by cyclin D–CDK4/6 may promote cyclin–CDK2 kinase activity and help to establish the order of activation (13, 232). The decrease of p27^{Kip1} expression occurs through protein degradation via the ubiquitin–proteasome pathway (262). The existence of inhibitory levels of p27^{Kip1} appears to be involved in the growth-inhibitory properties of TGF-β, rapamycin, and cAMP (13, 14, 261). In contrast, overexpression of p27^{Kip1} antisense cDNA results in mitogen-independent G1 progression, demonstrating the importance of p27^{Kip1} for maintaining cell quiescence (263, 264). Although the mitogenic signals responsible for p27^{Kip1} degradation have not been defined clearly, in Chinese hamster embryo fibroblast cells (IIC9 cells) PDGF stimulation causes the rapid activation of Ras and subsequent downstream activation of ERK (227, 265). In addition, Ras also stimulates the downstream activation of the RhoA that regulates p27^{Kip1} degradation to ensure the proper activation state of cyclin D1–CDK4 complexes following mitogenic stimulation (265). Interestingly, in thyroid cells where the cAMP is mitogenic, TSH stimulation down-regulates p27^{Kip1} and this effect correlates with an increases in cell proliferation (40). TSH down-regulation of p27^{Kip1} appears to be mediated by the activation of RhoA protein after growth stimulation in FRTL-5 (254). These data indicate the important differences of cAMP signaling and the existence of specific circuits in different cell types.

Concluding remarks

In this work we have given an overview of the complex regulation of thyroid growth based on data accumulated using rat thyroid cell systems as an experimental model. These useful cell lines let us probe deeply into the main signal transduction pathways and cell cycle-related genes implicated in TSH-mediated proliferation of thyroid cells. The data basically describe two main pathways involved in TSH/cAMP-mediated proliferation in rat thyroid cells, one that is PKA dependent and another that is PKA independent. The first pathway could implicate the small GTPase RhoA whereas the second one could involve Rac1 and Akt activation through PI3-K. On the other hand, TSH/cAMP can activate the p70s6k phosphorylation necessary for rat thyroid cell growth. The role of Ras in this context must be clarified, since this protein is necessary for TSH-mediated proliferation although no direct activation of Ras after TSH treatment has yet been demonstrated. Moreover, the hierarchy of activation of the small GTPases, their downstream effectors, and possible crosstalk between different pathways remain elusive. To complicate the puzzle, the new cAMP–GEFs, which are also expressed in thyroid tissue, might have a role in cAMP-mediated proliferation that bypasses PKA and directly targets cAMP signal to small GTPases such as Rap1. The activation of these pathways finally converges and activates target genes involved in thyroid differentiation and cell cycle-related genes required for the G1–S phase transition.

Acknowledgements

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