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The cAMP in Thyroid

From the TSH Receptor to Mitogenesis and Tumorigenesis

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INTRODUCTION

The thyroid cell, as an object of study, is interesting in the following several respects:

1. It synthesizes and secretes the thyroid hormones, which control and are necessary for maintaining the metabolism and level of activity of most organs, and ensure brain and body development. The sorry state of the cretin (i.e., the child who has been hypothyroid before or since birth) proves the importance of the thyroid gland.
2. It is a model for specialized epithelial cells, differentiated, yet still able to proliferate (a non-stem-cell tissue).
3. Its very specialization and unique role in iodine metabolism allow the easy investigation of its function.
4. It responds to the major proliferation cascades.

In this short review, we intend to describe the regulation of the thyroid cell and to show how its study has led to results that may be of general and medical relevance.

Thyroid function *in vivo* is controlled mainly by thyrotropin (TSH) and iodide. Pi-

tuitary TSH activates the thyroid, and the secretion of TSH is inhibited by thyroid hormone in a classical negative feedback mechanism. Iodide, on the other hand, tonically inhibits the function of the gland, so that at low iodide (i.e., thyroid substrate) concentrations, the thyroid is stimulated. As is the case for most differentiated tissues, chronic or intense stimulation of thyroid function is also followed by hypertrophy (i.e., an increase in capacity). Thus, both chronic stimulation by TSH and chronic iodide deficiency induce thyroid hyperplasia and cell hypertrophy. This effect of TSH, as well as those of the thyroid-stimulating antibodies (TSAbs) of Graves disease, show that there is little *in vivo* desensitization and downregulation of the TSH receptor, as it has also been demonstrated *in vitro* (1).

Thyroid growth is also influenced by other factors. For instance, thyroid weight and cell population are increased in acromegaly and decreased in growth-hormone deficiency. Growth hormone presumably acts on the thyroid through locally induced insulin-like growth factor-1 (IGF-1) (2).

CASCADES REGULATED BY THYROID-STIMULATING HORMONE AND IODIDE

Very early after its discovery, cyclic adenosine monophosphate (cAMP) was shown by Sutherland and Butcher to be generated by thyroid adenylate cyclase in response to TSH (3). Gilman, at the time a doctoral candidate of Rall, demonstrated that TSH enhances cAMP accumulation in beef thyroid slices (4,5). Using dog thyrocytes as a model, our group performed the exhaustive task of testing the validity of Sutherland's famous criteria for the various effects of TSH. TSH was found to activate adenylate cyclase in acellular preparations and the accumulation of cAMP in whole cells. All of the functional effects of TSH that were studied turned out to be mimicked by cAMP, cAMP analogues, cholera toxin, and later, forskolin. These effects included the oxidation and binding to proteins of iodide, the secretion of thyroid hormones, the oxidation of glucose through the hexose monophosphate pathway, and others. Moreover, the effects of TSH were reproduced or enhanced by inhibition of phosphodiesterases (6–8). Contrary to our expectations, most effects of TSH were caused by cAMP in this system. Later, in other systems, Knopp et al. (9) showed that cAMP also caused the induction of iodide transport. We confirmed this in dog thyrocytes. Thus, all of the main steps of iodide metabolism in the dog thyroid were shown to be activated by TSH through cAMP (for review, see reference [6]). Later, similar results were obtained in mouse thyroid and in Fisher rat thyroid cell line (FRTL) cells. The effects of TSH were reproduced by the TSAbs of Graves disease patients (10). Effects of TSAbs on iodide uptake or cAMP accumulation in FRTL-5 cells or, more recently, in TSH receptor-expressing Chinese hamster ovary (CHO) cells, became the basis for the functional assay of these TSAbs. However, even in dog thyrocytes, there remains a few effects of TSH that are not accounted for by cAMP: calcium release from slices, phosphatidylinositol synthesis . . . (11).

We had predicted that calcium must also play a role in thyroid cell regulation (6).

Indeed, it was shown in human thyroid gland that TSH also stimulates the PIP_2–phospholipase C (PLC) cascade, releasing inositol trisphosphate (IP_3) and presumably diacylglycerol. This effect was obtained for higher concentrations of TSH than was the effect on cAMP accumulation (by a factor of 10). It was not reproduced by TSAb at concentrations even higher than those measured in the serum of very hyperthyroid patients. In human thyroid, stimulation of the PIP_2–PLC cascade accounts for the activation of protein iodination and thyroid hormone synthesis. These metabolic steps are controlled by H_2O_2 generation, which is stimulated by both Ca^{2+} and diacylglycerol. It is interesting that in the dog thyroid, in which TSH does not activate the PIP_2–PLC cascade, a positive control by cAMP exists, whereas it does not exist in the human cell, in which TSH activates both cascades (12).

Iodide inhibits both adenylate cyclase- and PIP_2–PLC-mediated pathways and their effects. In all cases these inhibitions are relieved by drugs that inhibit iodide uptake or iodide oxidation. This gave rise to the XI concept, according to which the effects of iodide are mediated by an unknown intracellular derivative of iodine, XI. The postulated XI could in fact be an oxidized form of iodide or iodinated protein targets (13). A search for a well-defined XI has led to two main candidates: an iodolactone and an iodohexadecanal (iHDA). The former reproduces several effects of iodine but is not synthesized in significant amounts in thyroid cells in the absence of exogenous arachidonate. The latter is formed in the absence of exogenous precursor (presumably from endogenous plasmalogens) and reproduces some effects of iodide (14).

The thyroid cell is also regulated by various neurotransmitters that modulate adenylate cyclase and/or PLC through their respective receptors. The nature of the existing receptors and therefore of the regulatory neurotransmitters varies greatly from one species to another. A physiologic role of any of these neurotransmitters remains to be demonstrated.

THE THYROID-STIMULATING HORMONE RECEPTOR

From the biologic data, it was quite conceivable that two different TSH receptors existed. In fact, when we finally first cloned the TSH receptor it turned out to be unique. It was able to activate both guanine nucleotide-binding proteins G_s and G_q and thus adenylate cyclase and PLC, respectively (15). This was demonstrated in CHO and African green monkey SV40-transformed kidney cells (COS cells) transfected with a plasmid encoding this receptor downstream from a simian virus 40 (SV40) promoter (16).

The TSH receptor is a classical seven-transmembrane-domain-type receptor. In fact, its cloning was based on homologies of its transmembrane domains II, III, IV, and VI with the corresponding domains of already known receptors. These homologies allowed cloning of the TSH receptor gene through the polymerase chain reaction (PCR) on multiple degenerate primers, which was later used by our group and others to clone the majority of the known seven-transmembrane-domain-type receptors (17). The TSH receptor itself is characterized by a very long extracellular N ter-

minal domain and a short third intracellular loop. It is very homologous to the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) receptors. The long N-terminal domain is sufficient to bind TSH (18). Expression of the receptor, although modulated positively and negatively to some extent by TSH and downregulated by dedifferentiating treatments, is very robust. This makes sense, since this protein is the main intermediate in the physiologic control of the thyroid cell (19).

It is rather striking that, when expressed in transfected cells, both the dog and the human TSH receptors activate adenylate cyclase and activate PLC only at higher TSH concentrations and for higher TSH receptor densities (20). TSH thus behaves as a full agonist for the TSH receptor effect on adenylate cyclase and as a partial agonist for PLC. The lack of effect of dog TSH receptor on PLC in dog thyroid cells is therefore not due to a defect of this receptor itself. Moreover, in isolated dog thyroid membranes, the TSH receptor activates Gs, Gq, Gi, and even other G proteins. There is no present explanation for this discrepancy, but we hypothesize that membrane microdomains may limit the accessibility of G proteins to the TSH receptor.

The TSAbs can activate the PIP_2–PLC cascade in CHO cells expressing TSH receptor, but this is true only for the most active TSAbs in cells that overexpress the receptor by a factor of 50 to 100. The coupling of TSAbs to the receptor is therefore much less efficient than the coupling of TSH for activating Gq and PLC. This *in vitro* effect therefore has little relevance to the situation *in vivo* (21).

CONTROL OF GENE EXPRESSION

The expression of genes encoding protein required for specialized thyroid functions, including those for the presumed but not cloned iodide transporter, the still unknown H_2O_2 generating system, thyroperoxidase, and thyroglobulin, is stimulated *in vivo* and *in vitro* by TSH. In some species, such as the rat, thyroglobulin gene expression is at its maximum at normal TSH levels and can be reduced only by thyroid hormone treatment and the consequent decrease in TSH serum levels. While stimulation of thyroperoxidase gene expression is rapid and does not require prior protein neosynthesis, the stimulation of thyroglobulin gene expression requires prior new protein synthesis and is slower in cells in culture. All of these positive modulations are reproduced *in vitro* by agents mimicking cAMP (cAMP analogues), or enhancing cAMP accumulation (cholera toxin, forskolin). These conclusions apply to all species studied so far. Thus, with regard to specific gene expression, the thyroid is highly controlled by the cAMP cascade (22).

CONTROL OF THYROID CELL GROWTH AND DIFFERENTIATION

It has long been known that chronic stimulation of the thyroid, either by repeated administration of TSH, by TSH-secreting pituitary adenomas, or by TSH secreted in response to a blockade of thyroid hormone synthesis, led to growth of the thyroid and the generation of a goiter. When our work on thyroid cell growth began in the 1980s, the prevalent dogma in the cell-proliferation field was that cAMP was a mediator of differentiation and growth inhibition. This concept certainly applies to fibroblasts and other types of cells of mesenchymal origin, but its generalization was certainly excessive. Moreover, the first attempts to stimulate thyroid cell proliferation with TSH failed in human cells. Thus, when we found that production of ornithine decarboxylase (ODC) was induced in dog thyroid slices by TSH- and cAMP-enhancing agents and was decreased by acetylcholine (Ach), which activates the PIP_2–PLC cascade, our findings were neglected and the use of ODC as a growth marker was disparaged (23). Later, using the methodology created by Kerkof et al. (24) and Fayet and Hovsépian (25), and the culture medium used by Ambesi-Impiombato et al. (26) to cultivate their FRTL-5 cells, we succeeded in establishing growing primary cultures of dog thyroid cells. The proliferation of these cells was stimulated by TSH, cAMP analogues, and cAMP enhancers. However, we had considerable difficulty in publishing our results. A referee at one journal thought that our work was "not original because everybody knows that TSH stimulates the growth of the thyroid," while another referee dismissed it because "it is well known that cAMP inhibits growth" (27). The difficulty was increased when many publications claimed that the TSH proliferative effect on FRTL-5 cells was not mediated by cAMP. It therefore took quite a few years to establish the concept that in some thyroid cells (dog, human, FRTL-5 cells), TSH acting through cAMP enhances both thyroid cell proliferation and the expression of differentiation (2).

We now know that the thyroid cell responds to three families of mitogenic cascades: the TSH–cAMP cascade, the growth-factor–protein tyrosine kinase (PTK) family of cascades, and the phorbol ester–protein kinase C (PKC) cascade (Fig. 1). Each of these cascades involves successive steps of intracellular signal generation, protein phosphorylation, gene induction, and protein synthesis, with a progressive overlap as one progresses downstream. The main distinction between these cascades is the end effect: proliferation and differentiation for the cAMP cascade, proliferation and loss of differentiation for the other two cascades. Differentiation is meant here as gene expression of the thyroid-specific proteins thyroglobulin (TG), thyroperoxidase (TPO), and iodide transporter, and also of the signal-transduction proteins including the TSH receptor, thyroid transcription factor-1 (TTF1), and cell–cell interaction proteins such as E-cadherin. The effects of each cascade on mitogenesis and differentiation can be dissociated in confluent cultures in which proliferative effects are repressed. The effects on differentiation are also fully reversible (2).

The first family of mitogenic cascades to consider in thyroid cell growth and differentiation is the growth-factor–PTK family, which is common to most cells. A first, simplistic schema of these families is now roughly defined, with a first cascade of protein tyrosine phosphorylations triggered by receptor dimerization, followed by the activation of Ras guanosine trisphosphate (GTP)-binding protein, a serine kinase cascade leading to the activation of the mitogen-activated protein (MAP) kinases, activation by phosphorylation of transcription factors, immediate early gene induction of the classical protooncogenes c-*jun*, c-*myc*, c-*fos*, and others, a cascade of induction of cyclins and cyclin-dependent kinases (CDKs), and the phosphorylation and inactivation of Rb and Rb-like proteins, releasing the E2F transcription factors, which then induce the repression of proteins necessary for deoxyribonucleic acid (DNA) synthesis.

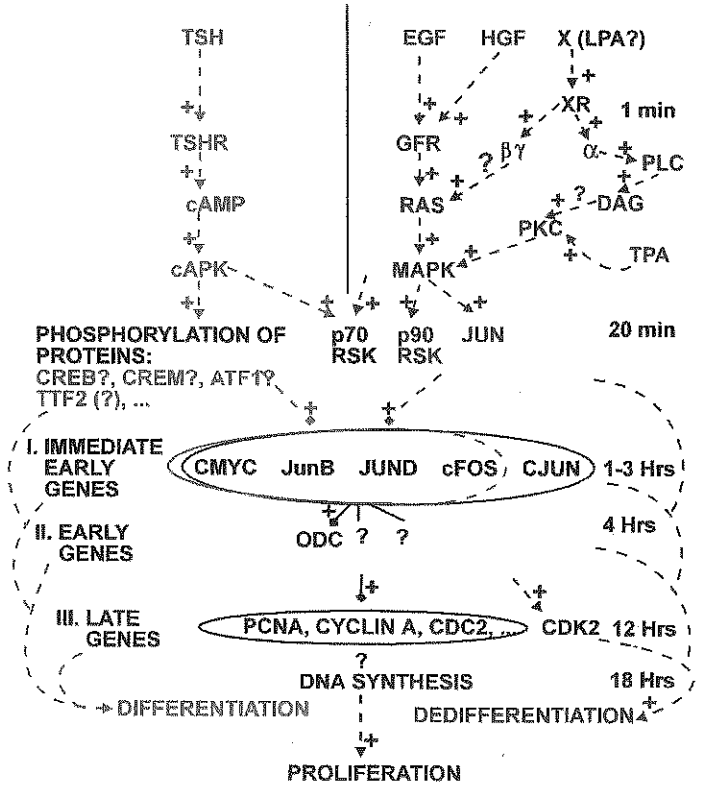

FIG. 1. Mitogenic pathways in the thyroid cell.

Constitutive activation of any of the positive factors in the cascade will lead to uncontrolled cell proliferation. Examples of such factors in thyroid tumors include Ret, which is a protein tyrosine kinase not normally present in thyroid cells, and activated Ras. Inactivation of a negative factor in the cascade (i.e., of an antioncogene) will give the same result. Known examples in thyroid tumors are Rb and p53, which induces CIP21, an inhibitor of CDKs.

Members of the G protein-coupled receptor family connect themselves on the growth factor cascade. Receptors activating Gi release βγ subunits, which through an unknown mechanism stimulate Ras. Additionally, receptors activating PLC release diacylglycerol (DAG); this activates PKC, which then stimulates Raf immediately downstream of Ras.

The TSH–cAMP cascade, which is the cascade that has been most extensively studied by our group, involves largely distinct steps until cyclin and CDK induction and activation. It involves the TSH receptor, Gs, adenylate cyclase, cAMP, cAMP-dependent protein kinases (PKA), and still unknown phosphorylated targets (perhaps transcription factors like cAMP-reponse element binding proteins [CREM], but not TTF- or the paired-domain transcription factor Pax8). This leads to some proto-oncogene expression and, through still unknown steps, to the common cyclin–CDK complexes (28).

Clearly, this outline is oversimplistic. For instance, several growth factors activate similar but not identical growth-factor cascades leading to very different results: growth and complete loss of differentiation for epidermal growth factor (EGF), growth and partial loss of differentiation for hepatocyte growth factor (HGF), and some differentiation but no mitogenesis for IGF-1 (2).

In our work, the mitogenic action of the cascades was demonstrated by cell-growth curves (DNA content) or by the autoradiographic counting of cells that incorporated tritiated thymidine. The mitogenic effect was confirmed in each biochemical experiment. In general, in dog thyroid, HGF is the strongest mitogen, followed by TSH and forskolin, and then by EGF and phorbol esters. Combinations of EGF, serum, and TSH are strongly synergistic, bringing almost all of the cells into DNA synthesis within 30 hr.

Expression of differentiation is evaluated by Northern blotting, *in situ* hybridization of thyroglobulin or thyroperoxidase messenger ribonucleic acid (mRNA), or iodide transport. In cultured cells, specific gene expression is greatly enhanced by TSH and greatly depressed by EGF and tetradecanoyl phorbol acetate (TPA), thus demonstrating the effects of these respective cascades on differentiation expression. It should be emphasized that the mitogenic and differentiating effects of TSH occur in the same cells. After pretreatment with EGF, the same cells that enter into DNA synthesis also begin to express thyroglobulin mRNA in the presence of TSH.

All of the effects of TSH on dog thyroid cells are reproduced by cAMP analogues, forskolin, and cholera toxin, thus demonstrating that they result from activation of the cAMP cascade (2).

Since the TSH receptor couples to Gi in cell membranes and, to some extent, in intact cells, stimulation of growth by TSH could be due to activation of Gi, release of βγ subunits, and subsequent activation of Ras and its cascade. However, pretreatment of dog thyroid cells with pertussis toxin, which completely inhibits the pure Gi effect of norepinephrine and further increases TSH-stimulated cAMP accumulation, fails to inhibit the TSH-induced proliferative effect. Moreover TSH does not activate MAP kinase in these cells (29).

Thus, *in vitro*, the TSH–cAMP cascade stimulates both thyroid cell function and proliferation, which led us to propose in 1989 that "it is quite possible that overactivity of the cAMP system will be found responsible for hyperfunctioning benign tumors or adenomas as opposed to dedifferentiated malignant tumors where the classical oncogenes are involved" (30). This was a daring extrapolation: from results with cells in culture to effects in cells *in vivo*; and from results with experiments done on a time scale of a few days to the behavior of cells over a span of at least 34 divisions and 20 yr . . .

To test our hypothesis *in vivo*, we relied on the adenosine A2a receptor, which we had cloned (17). Because cells release adenosine, this receptor is chronically stimulated in most systems. Preliminary experiments showed that the microinjection of adenosine A2a receptor mRNA into dog thyrocytes induced DNA synthesis (31). Ex-

pression of the receptor in the thyroid *in vivo* could therefore tell us about the results of a constitutive activation of the cAMP cascade *in vivo*. We therefore expressed the A2a receptor specifically in the thyroid glands of transgenic mice. As expected, mice expressing the adenosine A2a receptor specifically in the thyroid are hyperthyroid: thyroxine (T4) and triiodothyronine (T3) serum concentrations and iodide concentration in the thyroid are much higher than in control mice (31). Transgenic mice develop a goiter: the thyrocytes have a high labeling index, whereas it is negligible in control mice. Qualitatively similar results have been obtained in mice expressing constitutively activated Gαs specifically in the thyroid (32). Such mutations downstream from the receptor in the cascade further narrow the specificity in the cAMP pathway. Thus, constitutive activation of the cAMP cascade leads *in vivo* to a phenotype of hyperfunctioning adenoma involving the whole thyroid. There is considerable evidence that the cAMP pathway may have similar roles in other cell types (33).

THE cAMP PATHWAY IN THYROID TUMORS AND DISEASE

What about human hyperfunctioning thyroid adenomas? When a seven-transmembrane-domain-type receptor binds its agonist, it opens up, allowing activation of the G protein downstream. Lefkowitz et al. (34) had shown by directed mutagenesis that specific mutations in the adrenergic α1b or β2 receptors have the same result as agonist binding. Could a similar phenomenon occur in human hyperfunctioning adenomas? Investigating the same region (the third intracellular loop that Lefkowitz et al. had pinpointed) in the TSH receptor in thyroid adenomas, we found a mutation at the corresponding position (35).

Sequencing gel studies demonstrate such a mutation in toxic adenoma. The two nucleotide bands at the same position are similar in intensity. This suggests that: (i) only one allele is mutated, and that the mutation must therefore be dominant; and (ii) the two alleles are equally present, which means that all of the cells of the adenoma express the mutation (i.e., that all of these cells derive from a single cell in which this mutation took place, or that the tumor has a monoclonal origin).

Can the mutation explain the tumor? First it confers on the receptor a constitutive activation: cAMP accumulation is much greater in COS-7 cells expressing the mutated TSH receptor than in those that do not express it. Most of the mutations do not affect the PIP_2–PLC cascade (36). Moreover, when plasmids expressing the mutated receptor are microinjected into dog thyroid cells, many of these cells begin incorporating tritiated thymidine in their nuclei.

Thirteen such gain-of-function mutants have now been described, and such mutations explain nine cases in our series of 11 hyperfunctioning adenomas. This illustrates the interest in identifying mutations conferring a selective advantage on affected cells in pathologic tissues rather than in systematic mutagenesis. Thus, TSH-receptor-activating mutations account for the majority of hyperfunctioning thyroid adenomas. Some other cases of such adenomas are probably caused by mutations conferring constitutive activation to Gαs, as demonstrated by other workers

(37). Such mutations also explain congenital hyperthyroidism (Leclere's disease) (38,39). In the hereditary form of this disease, the defect is inherited as an autosomal dominant character. All patients presenting the mutation sooner or later exhibit the phenotype of goiter and hyperthyroidism. Thus, as predicted from the *in vitro* data, constitutive activation of one element of the cAMP cascade leads to thyroid cell growth and hyperfunction. It will now be interesting to seek mutations of adenylate cyclase in otherwise unexplained hyperfunctioning adenomas. The pathologic role of the TSH–cAMP cascade is also demonstrated in nongenetic diseases. TSH-secreting pituitary adenomas cause hyperthyroidism and thyroid enlargement. Moreover, Graves disease, also characterized by hyperthyroidism and goiter, is caused by activation of the TSH receptor by TSAbs (10).

Do TSH receptor mutations have a role in cancer? They may. We have crossed mice expressing the adenosine A2 receptor in their thyroid cells with mice expressing the E7 oncogene in this tissue. As mentioned earlier, the first mice exhibit a goiter and thyroid hyperfunction, but no carcinoma. The second mice, in which E7 relieves the inhibitory effect of Rb downstream in the different mitogenic cascades, develop a euthyroid colloid goiter and later a localized tumor (40,41). In crosses between the two types of mice, tumorigenesis and metastases are observed (Fig. 2).

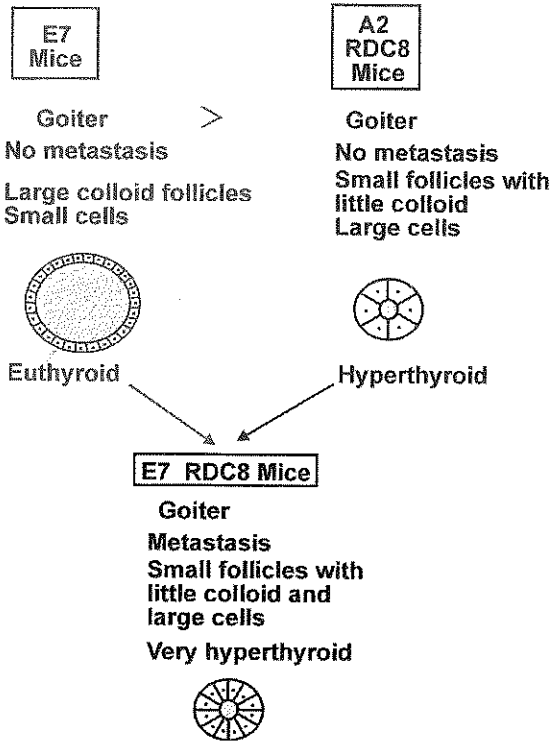

FIG. 2. Complementation of thyroid partial oncogenes (for the adenosine A2a receptor and HPV16–E7) on thyroid tumorigenesis.

Thus, two incomplete oncogenes complement each other and lead to invasive papillary carcinoma with lung metastases.

Do such mutations exist in human thyroid cancers? Studying exon 10 of the TSH receptor through the sequencing of tumor DNA of 15 thyroid cancers from Chernobyl, we have not so far found such a mutation. Complete sequencing is in progress. Exon 10 covers the entire seven-transmembrane domain of the receptor. Positive results have been obtained in thyroid cancers selected for increased adenylate cyclase activity in homogenates (Suarez, *unpublished results*).

Studies of mutated TSH receptors led to the investigation of the possible effects of normal receptors. Wild-type dog or human TSH receptors, when expressed transiently in COS-7 or permanently in CHO cells, increase cAMP accumulation. Thus, the normal receptor has a basal constitutive activity on Gs and adenylate cyclase (20). This is not true for all such receptors: the α-melanocyte-stimulating hormone (α-MSH) receptor has no such activity. Nevertheless, for receptors having such activity this raises interesting physiopathologic and therapeutic possibilities. Simple overexpression of the receptor could lead to the same disease as its constitutive activation. The thyroid could be inhibited by negative agonists of the TSH receptor.

THE THYROID-STIMULATING HORMONE–cAMP CASCADE IN HUMAN DISEASE

As seen from the findings described earlier, the cAMP cascade is implicated in normal mitogenesis and in tumorigenesis in human thyroid cells. Clearly, any activation at any step of the TSH–cAMP cascade, whether by a mutational event or through a physiologic or pathologic stimulation, will have the same consequences of thyroid functional stimulation and goiter (Fig. 3). Hypophyseal thyrotroph adenomas secreting TSH, as well as stimulating antibodies directed against the TSH receptor produced in Graves disease, are good examples. Activating mutations of the TSH receptor and Gαs have been described, and together should account for most hyperfunctioning adenomas (42). Similar mutations of adenylate cyclase or of cAMP-dependent kinases should be sought. Such mutations are dominant gain-of-function mutations. When hereditary they are autosomal dominant.

Inactivation of a negative controlling element of the cascade (e.g., the regulatory subunit of cAMP-dependent kinase, proteins of the iodide inhibitory pathway) would be a loss-of-function recessive mutation. To cause a hyperthyroid phenotype, double mutations would be necessary.

On the other hand, any inactivation of a positive element of the cascade, whether genetic or otherwise, should lead to hypofunction and hypotrophy (Fig. 3), or at least, if compensated by pituitary thyroid hormone feedback, to high TSH levels. Two complementary hereditary inactivation defects of the TSH receptor have been described, both indeed leading to euthyroid high TSH levels (43). Blockade of the TSH receptor by autoimmune antibodies leads to hypothyroidism and atrophy. Thus, the majority of thyroid diseases are in fact signal-transduction diseases.

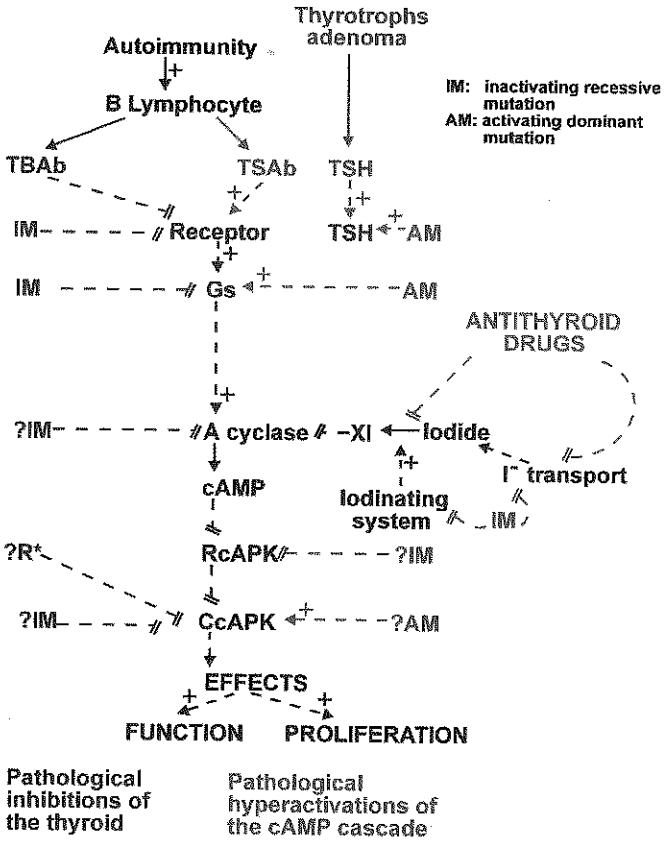

FIG. 3. The TSH–cAMP cascade and human thyroid disease. (——→) Positive control; (——|) negative control.

THE MITOGENIC cAMP PATHWAY: PROTEINS AND GENES INVOLVED

The TSH–cAMP mitogenic pathway involves the TSH receptor, Gs, adenylate cyclase, and cAMP. Stimulation of the cascade at the level of Gs by cholera toxin or mutations, at the level of adenylate cyclase by forskolin, or by cAMP analogues has the same stimulatory effect. Evidence for the involvement of cAMP-dependent kinases in the pathway is the correspondence in the specificity of pairs of cAMP analogues in specifically stimulating PKA I and (to a lesser extent) PKA II, and in the mitogenic actions of these analogues (44). Also, microinjection of Walsh protein-kinase-inhibitor peptide decreases the TSH-induced mitogenic stimulation. However, we do not know about the elements involved downstream from protein kinase A (PKA). Certainly, dominant negative CREB-expressing plasmids inhibit cAMP ac-

tion, but they also inhibit HGF-induced mitogenesis, and overexpression of CREB has the same effect (Dremier, *unpublished results*). Excess of transcription factors probably squelches the activity of other factors. Moreover, CREB-minus mice exhibit no obvious thyroid phenotype. If we do not know which protein phosphorylation may be involved in the TSH–cAMP mitogenic effect, we do know that this pathway does not induce phosphorylations identical to those resulting from activation of the growth-factor or tumor-promoter PKC cascades (45). In particular, the TSH–cAMP cascade does not lead either to p42 or p44 MAP kinases, or to p90 S6 kinase phosphorylation and activation (29). Similarly, immediate early gene inductions are different in the cAMP cascade from those in the EGF- and phorbol ester-activated cascades. If *jun* D and *jun* B are induced in both categories of cascades, c-*fos* is much less induced, c-*myc* induced only very transiently, and c-*jun* not at all in the cAMP cascade (2). Finally, the cAMP cascade is much more sensitive to the inhibitory effect of transforming growth factor-β (TGF-β) than are the EGF and phorbol ester pathways. The three pathways converge, at least qualitatively, at the cyclin/CDK induction and activation levels. They are therefore completely distinct at the level of receptor, signal generation, and protein phosphorylation, partially overlapping at the level of immediate early gene expression, and convergent at the level of the cyclin/CDK fundamental late G_1 mechanism. We have therefore defined, by exclusion, the steps in other cascades that are not involved in the cAMP mitogenic pathway. To identify the steps of this cascade, we are now looking at phosphorylated proteins and newly expressed or repressed genes (46).

FUNCTIONAL ROLES OF THE THYROID-STIMULATING HORMONE–cAMP PATHWAY AND INSULIN–INSULIN-LIKE GROWTH FACTOR-1 CASCADE

To cultivate their FRTL-5 cells, Ambesi-Impiombato et al. (26) had shown that insulin was required for cell multiplication. We later showed (47), and Williams (48) and collaborators confirmed, that insulin at high concentration or IGF-I was necessary for the mitogenic effect of TSH or forskolin, EGF, or phorbol esters on dog and human thyroid cells. In some cells (e.g., the FRTL-5 rat thyroid cell line), but not in dog or human cells, IGF-I was sufficient by itself to induce mitogenesis. All of these effects are believed to be mediated by the IGF-I receptor, since they are induced by low concentrations of IGF-I or high concentrations of insulin. Studies by our group and others of the effects of TSH or other growth factors on the steps of the mitogenic cascade have always been conducted in cells incubated with insulin or IGF-I (2).

In order to ascertain the nature of the permissive IGF-I action in thyroid cell proliferation, we first investigated the effects of TSH or forskolin on the one hand and of high insulin concentrations on the other hand on the growth and proliferation of dog thyrocytes in primary culture. As expected, TSH or forskolin separately had no effect on protein or DNA accumulation. Also as expected, insulin had no effect on DNA accumulation. However, much to our surprise, insulin induced a marked pro-

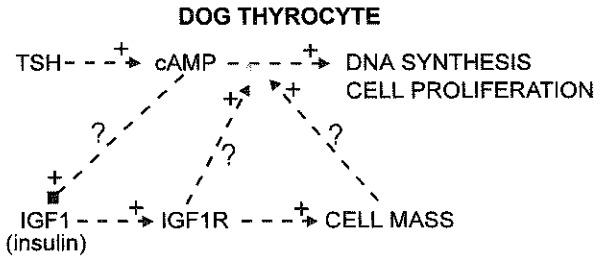

FIG. 4. Respective roles of TSH and the cAMP cascade on the one hand and insulin and/or IGF-1 on the other hand on the growth and division of the dog thyroid cell. (— — →) Positive control; (— — ♦) induction; (?) Inferred but not demonstrated relations.

tein accumulation, with the protein content of our cells increasing within a few days by a factor of 3- to 4-fold. The protein/DNA ratio in cells of similar size should remain constant whether these cells proliferate or not. Strikingly, this ratio increased markedly in insulin-treated cells. If such cells were also treated with TSH or forskolin, both DNA and protein accumulated, but the protein/DNA ratio did not increase. These data suggest that insulin and IGF-I are responsible for the growth in size of the thyrocytes. TSH and cAMP can only stimulate DNA synthesis and cell division in such cells. Thus, we have dissociated in our model the system responsible for cell growth and the cascade responsible for the induction of proliferation in such cells (Fig. 4). The latter cascade cannot operate without the action of the first (Taton, *unpublished results*). In yeast, mutations that allow cell division regardless of cell size lead to death. Most work on cell-proliferation cascades uses cell proliferation or DNA synthesis as an endpoint, and therefore does not distinguish between events leading to cell growth and events which in the same cells induce DNA synthesis and cell division. Our finding should therefore allow us to delineate both cascades.

CONCLUSION

We have demonstrated the mitogenic role of the TSH receptor–adenylate cyclase cascade *in vitro* in dog and human thyroid cells, *in vivo* in transgenic mice, and in human disease in autonomous adenomas and congenital hyperthyroidism. We have now outlined a new model of proliferative control involving the permissive action of IGF-1, which stimulates growth and the action of the cAMP cascade, with the latter then stimulating the division of the hypertrophic cells. However many questions remain. What is the relation between structure and function in the normal and mutated TSH receptors? Which are the steps downstream of cAMP-dependent protein kinases in the mitogenic and differentiating cascade? What limits the growth of autonomous adenomas? By which mechanism does iodide affect proliferation? These and other questions are currently being addressed.

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