Positive control of proliferation by the cyclic AMP cascade: An oncogenic mechnism of hyper-functional adenoma

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The activation of the cyclic AMP cascade in dog and human thyroid cells in primary culture induces the expression of differentiated gene expression (i.e. thyroglobulin, thyroperoxidase mRNA expression, iodide transport, etc), hyperfunction (i.e. H202 generation, protein iodination, iodotyrosine coupling and thyroid hormone secretion) and proliferation (as demonstrated by the increase in cell populations in culture) (1). These programs are developed simultaneously in EGF serum dedifferentiated cells: replacement of EGF by TSH in dog thyroid cell cultures treated with EGF serum both maintains the mitogenic rate and induces thyroglobulin mRNA accumulation with the same delay (24 hours), in the same cells (2). Such conditions are reproduced in human *in vivo* by TSAb, antibodies activating the TSH receptor, in Graves disease: these TSAb strongly activate the TSH receptor and the cyclic AMP cascade but even at double serum concentration do not stimulate the PIP2 cascade. Graves disease thyroids therefore represent good examples in humans *in vivo* of the effects of chronic stimulation of adenylate cyclase (3).

With those data in hand, we hypothesized that constitutive activation of any of the genes or proteins involved in the positive control of the cyclic AMP cascade could account for the pathogenesis of hyperfunctioning adenoma (4, 5). However extrapolating from *in vitro* experiments of a few hours or days to what happens over years *in vivo* is obviously very risky. In order to prove eperimentally the role of cyclic AMP we have used the receptor of adenosine A2 which we have cloned and characterized (6, 7). This receptor which activates adenylate cyclase is constitutively active in most cells because of the endogenous generation of adenosine.

Key-words Thyroid cell, oncogene, cyclic AMP adenosine receptor, hyperfunction adenoma, MAP kinase

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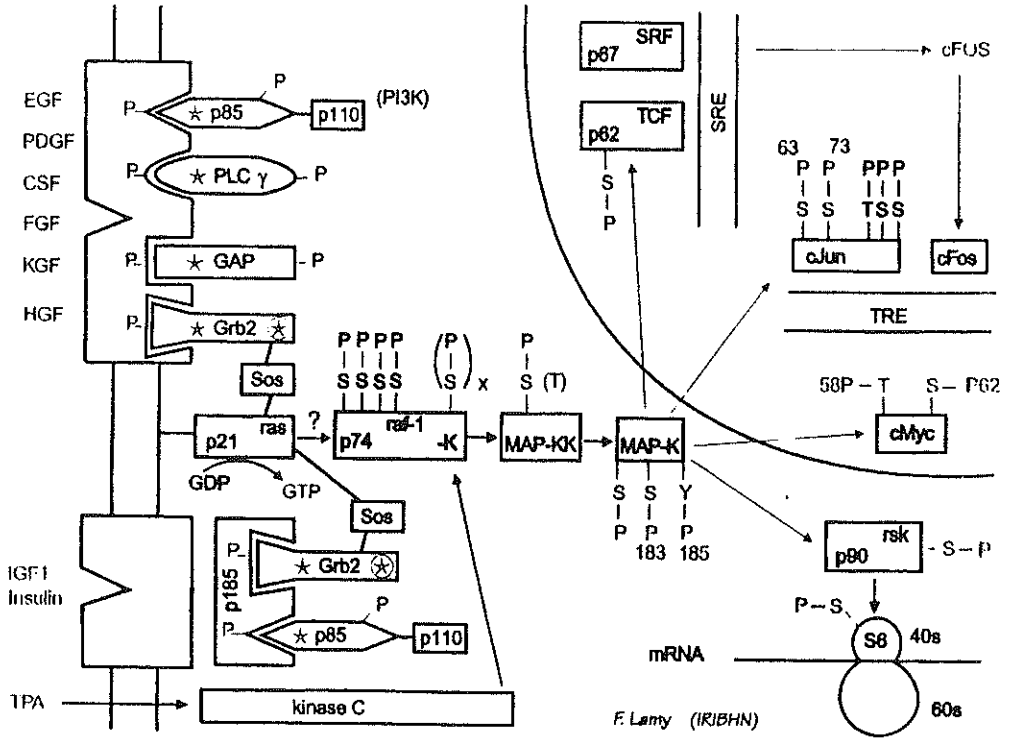
When its mRNA is microinjected in dog thyroid cells in primary culture, these cells enter into mitogenesis (7). We have generated transgenic mice by microinjection of the A2 adenosine receptor cDNA downstream and under the control of the thyroglobulin promoter. This promoter is only active in thyroid cells and regulated by cAMP (8). The adenosine A2 transgenic mice are hyperthyroid and develop a huge goiter. Their 3H thypidine labeling index is 8% (vs 0.1% in the control mice). Unless treated the mice die of hyperthyroidism. They represent good models of hyperfunctional autonomous adenoma of the thyroid, but involving the whole organ (9). This demonstrates *in vivo* the possible oncogenic role of the cyclic AMP cascade in cells in which this cascade stimulates proliferation. Beside these experimental arguments, if our predictions were valid, one should be able to demonstrate a constitutive activation of an element of the cyclic AMP cascade as the cause of a human hyperfunctioning adenoma. In parallel with our studies on proliferation, our group had cloned the cDNA of the dog and human TSH receptor (6, 10). To better understand the structure function relationship in this receptor we had to study mutants. Two strategies were possible. We could try a systemic rational mutagenesis and study of the function of such mutated receptors. This was the strategy pursued by Rapoport, Kohn et al. (11, 12). With a 700 aminoacid sequences and 19 possible replacements for each of these aminoacids, and an almost infinite number of possible deletions, additions or replacements for each of peptide parts, this strategy was not within reach of a rather small group like ours. We rather decided, that if our concepts were valid, in cells in which cAMP is mitogenic, any mutation conferring constitutive activity to the receptor should give a distinct advantage to the cell in which such a mutation would occur. This hyperfunctioning adenoma could be the result of a selection of such mutations. We therefore decided to search for mutations of the TSH receptor in the cDNA of thyroid hyperfunctioning adenomas. This was done by PCR amplification of segments of this



receptor sequence in the DNA and sequencing of the amplied fragments (13). This was easy for the 7 transmembrane domains of the receptor, as it is coded by one exon. The 3rd intracellular loop of the receptor is in many cases involved in signalling to GTP binding proteins downstream. Moreover the group of Lefkowitz had found that direct mutagenesis of Ala 293 in the distal part of the 3rd intracellular loop of the alpha$_1$ adrenergic receptor leads to its constitutive activation. We therefore decided to look first for mutations in the distal part of the 3rd intracellular loop and 6th transmembrane segment of the TSH receptor. Mutations conferring a constitutive activity of the TSH receptor on adenylate cyclase have now been demontrated in 40% of human thyroid hyperfunctioning adenomas (13). This demonstrates both the clonal nature of the lesion and the pathogenetic role of the cAMP cascades in these human hyperfunctioning adenomas. This and other activating mutations have now been found in other thyroid tumors and in cases of congenital hyperthyroidism. We can safely predict that somatic mutations conferring constitutive activation of other elements of the cAMP mitogenic cascade (Gs, adenylate cyclase, protein kinases...) or inactivation of negative elements of this cascade (phosphodiesterases etc) will also lead to hyperfunctioning adenomas. This is proved by the GSP activating mutations in hyperfunctioning adenomas of the thyroid and pituitary (Suarez this symposium). Some of our old data showing enhanced cAMP response of such tissue to beta adrenergic suggests that heterotypic beta adrenergic receptors appearance could also explain some cases of hyperfunctioning adenoma (14).

During the last few years especially last year, our knowledge of the growth factors activated cascades has greatly expanded. In fact we know the almost complete pathway from the growth factor to early gene expression in these cascades (Fig. 1). In the dog thyroid the EGF and TPA growth pathways involve steps similar to those described in fibroblasts. The cAMP mitogenic pathway is strikingly different. At the level of protein phosphorylation there is not overlap in the patterns induced: e.g. MAP$_2$ kinase is not phosphorylated on tyrosine. At the level of protooncogene expression cmyc and JunB overexpressions are very short, shorter than cfos, and followed by an inhibition of expression; cjun expression is inhibited (15-17). cMax is slowly downregulated by TSH and cAMP while it is slowly upregulated by EGF or phorbol esters. The kinetics of the cell cycle is also very different with a longer S phase and a much delayed M (longer G2) inso the cAMP cascade. At the level of protein synthesis PCNA synthesis is stimulated in middle Gs

rather than in the S phase. Thus we now know very well that the cAMP mitogenic cascade is very distinct from the other cascades, both in its end points (division and differentiation expression) and in its intermediate steps. We know what this cascade is not. What we need to learn now is what it is, i.e. which are the proteins and genes involved. This is our next aim.

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