4 Discussion

The aim of this work was to examine the effects of the well-established thyroid growth factors TSH, insulin and EGF on the activity of the family of CRE-binding protein transcription factors, focussing in particular on possible differences in the behaviour of thyrocytes with pathological growth characteristics. For this, four thyroid carcinoma cell lines, one adenoma cell line, as well as human primary cells derived from paranodular and nodular goitre tissue were compared in their CREB/M- phosphorylation responses. Moreover, the impact of this phosphorylation response on gene transcription activity was evaluated by studying the expression of ICER and the induction of a CRE-reporter plasmid. Some of the findings made here confirm results reported previously, some are in excellent agreement with observations made by other experimental approaches, however, some are also unexpected, possibly demanding reconsideration of pre-existing hypotheses.

Control of thyroid function and growth under physiological conditions is exerted primarily by the pituitary hormone TSH. The main second messenger of TSH stimulation appears to be cAMP, as many of the TSH effects can be reproduced by stimulation with cAMP analogues or by activation of adenylate cyclase by forskolin. In addition, stimulation of proliferation by cAMP may be mediated by activation of cAMP-dependent protein kinases (Dumont et al., 1992). Accordingly, R. Armstrong *et al.* were able to demonstrate a strong CREB-phosphorylation response in TSH- treated FRTL-5 cells (Armstrong et al., 1995), a finding which was easily reproduced under the experimental settings employed here. Moreover, R. Armstrong *et al*. observed a subsequent refractory phase, which was found to be caused by a fivefold, selective downregulation of PKA catalytic subunit. This again underlines the important role PKA seems to play during TSH signalling. In agreement with the idea of PKA-mediated TSH effects are the results obtained with CRE-reporter plasmid transfected thyroid HTC-TSHr cells presented here: both TSH and forskolin, induced a rapid increase in reporter activity with a maximum at 8 hours, the main difference being the magnitude of the effect which was about fivefold lower in the case of TSH stimulation (25fold increase over basal *versus* 5fold, respectively). These findings are in good agreement with results reported previously for other cell systems: thus, P. Brindle *et al.* reported an approx. 10fold induction of a somatostatin CRE- CAT reporter in T- Jurkat cells after a 3 hrs stimulation with 10 µM forskolin (Brindle et al., 1995), while C. Q. Lee *et al.* examining JEG- 3 cells, a human choriocarcinoma cell line, observed an 11 times higher relative CAT activity after 12 hrs of 1

mM 8- br- cAMP treatment (Lee et al., 1990). It should be noted that the CAT- reporter used in this study possessed only a twice- repeated CRE as compared to the four copies in 4xSCE1/2T81.

When human primary thyrocytes isolated from paranodular goitre tissue were examined, TSH again was shown to be a strong inductor of CRE-binding protein phosphorylation. Also the different phases of induction, attenuation, and refraction described by R. Armstrong *et al*. were distinguishable in the response of human thyrocytes. However, the discovery of the biphasic nature of the TSH- induced phosphorylation reaction in human cells, which showed a first peak at 10 minutes, and a second peak at 30 minutes of TSH stimulation, was surprising. Further experiments employing specific protein kinase inhibitors then revealed the involvement of PKA and PKC. Moreover, it appears that both kinases are part of different branches in the TSH signal cascade, with the PKA- mediated response being responsible for the early phosphorylation peak, and the PKC involving pathway causing the second peak. While the TSH-receptor-Gsα-adenylate cyclase-PKA pathway corresponds to the „classic" CREB activation pathway, the transduction cascade leading to the TSH- triggered CREB/M phosphorylation *via* a PKC isoform is less obvious. On the basis of our current understanding of the thyroid signal transduction network, at least two alternative constellations are conceivable. First, in human thyrocytes TSH at high concentrations is known to stimulate the phosphoinositol pathway by activating phospholipase C_β via G_q (Allgeier et al., 1994). The release of DAG and IP_3 subsequently leads to the influx of Ca^{2+} from endoplasmatic reticulum stores, and DAG alone or in cooperation with Ca^{2+} then promotes the activation of novel and conventional PKC isoforms, respectively. In the thyroid, expression of PKC isoforms α, β, δ, ε, and ξ has been described previously, the exact isoform pattern depending on the species and stimulation status of the cell (Wang et al., 1995; Matowe et al., 1996; Feliers and Pavlovic-Hownac, 1994). Since the concentration of GFX used in this study was too low to permit inhibition of atypical PKC isoforms, PKC ξ may be excluded from the list of possible candidates promoting the observed effect. Of the remaining, only PKC α is expressed at significant levels in a species- and TSH- independent manner, which together with the reported redistribution of PKC α, ε, and ξ to the membrane fraction upon TSH or forskolin treatment (Feliers and Pavlovic-Hournac, 1994; Matowe et, al., 1996), makes it the most likely candidate for the TSH- triggered second phase of CREB/M- protein phosphorylation.

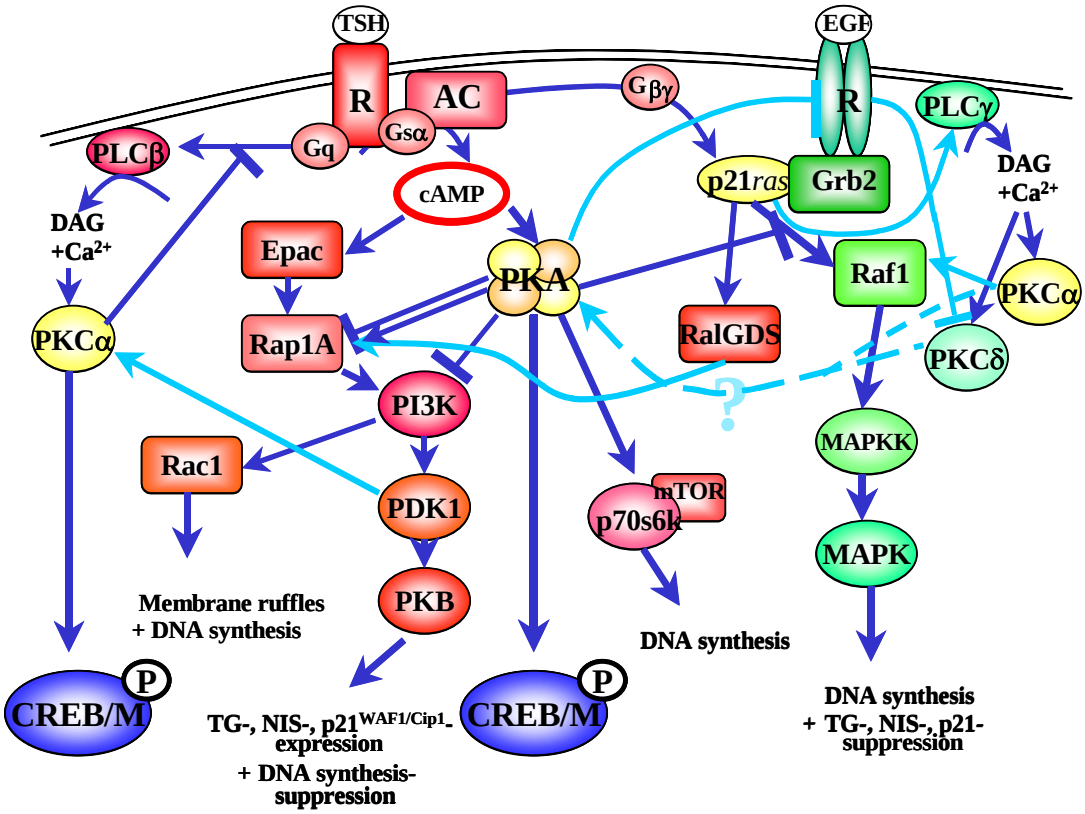

FIG.24: Schematic diagramm of the hypothetical signal transduction network of the thyroid. Parts of the TSH -induced cascade are shown in red shades, those of the EGF- pathway in green, and points of overlap in yellow. Dark blue arrows represent connections already demonstrated in the thyroid, light blue arrows show connections found in other organs, but likely to exist in the thyroid.

Alternatively, the second phase of CREB/M- phosphorylation may be cAMP- dependent, yet PKA- independent: as first shown by F.C. Mei *et al*. some of the cAMP- stimulated effects in thyrocyte are actually mediated by the so-called „exchange protein directly activated by cAMP“ (Epac) (Mei et al., 2002). Epac contains a cAMP-binding domain that is homologous to the R subunit of PKA and a guanine exchange factor (GEF) domain. Upon binding cAMP, Epac activates the downstream target Rap1, which in turn leads to activation of phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase (PDK1), and finally PKB (also known as Akt). Interestingly, PDK1 has been reported to phosphorylate PKC α and βII within the activation loop (Dutil et al., 1998). In favour of this second possibility is the occasional observation of a biphasic response upon forskolin stimulation made in FRTL-5 cells during this study.

Elucidation of the actual pathway employed, and final identification of the PKC isoform involved will certainly require further experiment, although the significant downregulation of PKCα during prolonged TSH- stimulation in HTC-TSHr cells clearly argues in favour of this isoform. In Fig. 24 an attempt was made to integrate the findings made in this work with the data so far published on the signalling network of the thyroid.
With the exception of a single „hot“ adenoma, which displayed raised basal CREB/M-phosphorylation levels, and which was actually inhibited by further TSH- stimulation, all other primary cultures tested showed good responses to TSH, independent of the morphological or scintigraphic classification. The basis of such constitutive activation observed in “hot” adenoma is not clear, but several activating mutations within the TSH-PKA cascade have previously been described (for review see, for instance, Derwahl et al., 1998). In good agreement with the observation made here, J. Bertherat *et al.* examining growth hormone secreting pituitary adenoma, previously reported abundant phospho-CREB staining in the nuclei of 70-80 % of functioning adenoma, but only low levels of phospho-CREB immunoreactivity were detected in non-functioning adenomas (Berherat et al., 1995).

Very much in contrast to this were the results obtained with insulin: insulin was able to elicit the phosphorylation of CRE-binding proteins in FRTL-5 as well as in human primary thyroid cells. The reaction was not seen with insulin dosages below 1 µg/mL, indicating the involvement of IGF-I receptors rather than the insulin receptor itself. However, while the extent and kinetics of the response were well- reproducible in FRTL-5 cells, human primary cells showed a very varied response, with maximal effects reached at either 10 or 30 minutes, and degrees of CREB/M- phosphorylation ranging from responses as low as observed for EGF to maxima coming close to those induced by TSH- stimulation. Moreover, experiments with protein kinase inhibitors again revealed the implication of PKA and PKC isoforms, however the relative contributions made by each kinase varied from isolate to isolate tested. One possible explanation of this finding may be the formation of chimera from insulin-, IGF-I, and IGF-II receptor monomers, the heterodimeric receptors differing in their properties from the homodimeric receptor forms, displaying slight differences in their relative affinities for insulin and the signal transduction pathways favoured (Belfiore et al., 1999).

Taken together, these findings support a previously proposed concept, suggesting that chronic stimulation of the TSH-cAMP- cascade primarily leads to diffuse thyroid hyperplasia, whereas subsequent nodular transformation is the result of local heterogeneity, with

individual thyrocytes differing widely in their intrinsic growth potential and proliferation rate (Derwahl and Studer, 1998; Studer and Derwahl, 1995). Interesting in this context, is the fact that the adult thyroid gland still harbours approximately 1% autonomous, i.e. TSH-independent, growing cells, where autonomous growth is actually the characteristic feature of embryonal thyrocytes. It is thus tempting to speculate that these cells have failed to switch from the fetal to the adult type of growth regulation (Derwahl and Studer,1998).
The clinical course of thyroid diseases that are accompanied by chronic stimulation of the TSH receptor-cAMP pathway, as in the case of iodine deficiency goitre, Graves´disease with TSH receptor- antibody secretion, autosomal-dominant hyperthyroidism due to TSH receptor gene mutations, TSH-secreting pituitary adenomas or generalized thyroid hormone resistance, supports this view: in all of these conditions, diffuse goitre development preceds nodular transformation by years or even decades (Derwahl et al., 1999; Derwahl et al., 1998). Furthermore, overexpression of IGF-I (Minuto et al., 1989; Williams et al., 1989), IGF-I receptor (Vannelli et al., 1990), basic FGF 1 and 2 (Gärtner et al., 1990; Thompson et al., 1998), basic FGF receptor (Thompson et al., 1998), EGF receptor (Di Carlo et al., 1990; Westermark et al., 1996), Gsα (Hamacher et al., 1995; Derwahl et al., 1996) and *ras* gene product p21 (Studer et al., 1992) has been detected in thyroid hyperplasia, nodules and adenomas. Enhanced proliferation will then favour other mutational events that finally lead to malignant transformation.

Since thyroid carcinomata are comparatively rare, the CREB/M-phosphorylation behaviour of four human thyroid carcinoma cell lines, two follicular and two anaplastic, were examined here. The XTC cell line was established from the breast metastasis of a Hürthle cell carcinoma. Hürthle cell cancers are a rare variant of the follicular thyroid carcinoma. They are typically well-differentiated, yet fail to take up radioiodine and metastasise early, and preferentially to the lymph nodes. The XTC cells were demonstrated to maintain a high degree of differentiation, producing and secreting thyroglobulin, and responding to TSH with an increase in intracellular cAMP, proliferation and invasiveness (Zielke et al., 1998). The FTC 133 cell line was derived from the primary tumour of a poorly-differentiated follicular carcinoma. These cells, too, have been described to be well-differentiated with maintenance of thyroglobulin synthesis and responsiveness to TSH by increases in intracellular cAMP and proliferation rate (Hoelting et al., 1994). However, it appears that when compared to primary cells, at least the FTC 133 clone used in this work, showed a clearly decreased cAMP

synthesis upon TSH treatment and expressed reduced numbers of TSH receptor (M. Broecker, unpublished data).
As the name suggests (Greek: *ana plassein* = to form backwards), anaplastic tumours are always of poorly differentiated phenotype. Of the two anaplastic thyroid cell lines examined here, the HTh 74 cells still displayed a very low expression rate of thyroglobulin and TSH receptor mRNA, in addition to aberrant features like the expression of functional PDGF receptors (Heldin et al., 1991). In contrast, the SW 1736 cells have lost any expression of thyroid differentiation markers. Their epithelial origin, however, was confirmed by positive immunofluorescence staining for cytokeratin (Heldin and Westermark, 1991).

Among these four carcinoma cell lines, the HTh 74 cells were found to show a special phenomenon: in these cells alone the exchange of the serum-reduced medium induced a strong round of autocrine CREB/M-phosphorylation that could not be increased significantly by additional administration of any of the growth factors tested here (data not shown). Moreover, when the conditioned and sterile-filtered medium from these cells was offered to FRTL-5 cells, these, too, reacted with an increase in CRE-binding protein phosphorylation. Hence, in HTh 74 cells autocrine loops exist that at least in part employ receptors found also in more differentiated thyroid cells.
In the remaining three cell lines, a concomitant loss of sensitivity to TSH and insulin was found to parallel an increasing degree of dedifferentiation. The basis of this most likely lies in the downregulation of TSH receptor levels: thus, XTC cells showed the best response to TSH, although the effect was clearly lower than in primary thyrocytes, while the TSH-induced CREB/M- phosphorylation in FTC 133 cells was only marginal, and in SW 1736 no reproducible effect at all was detected. The observed tendency of insulin sensitivity to follow the relative responsiveness to TSH probably reflects the strong positive regulation TSH exerts on the IGF/insulin pathway: so TSH was reported to enhance the expression of IGF-I (Hofbauer et al., 1995), upregulate insulin receptor mRNA (Burikhanov et al., 1996) and decrease protein levels of different IGF-I- binding proteins (Eggo et al., 1996), thereby probably raising the availability of free IGF-I (Clark, 1997).

Quite the opposite development was observed with EGF-stimulated CREB/M-phosphorylation, i.e. the EGF-induced effect in human primary cells generally was very low, with CRE-binding protein phosphorylation levels being maximal at 30 minutes and a concentration of 1 to 10 ng/mL. This is in good agreement not only with the reported K_D of

0.84 +/- 0.26 nM (Saller et al., 1993), but also with observations made *in vivo*, where in healthy thyroid tissue EGF and the EGF receptor are not detected or are detected in only a very few epithelial cells using immunohistochemistry techniques (Gärtner et al., 1990). A completely different situation was encountered in the three carcinoma cell lines: these generally showed a strong CREB/M- phosphorylation response upon EGF treatment, and the effect increased with the loss of differentiated function. So was the extent of CRE- binding protein phosphorylation observed in EGF- treated SW 1736 cells absolutely comparable to that achieved in primary thyrocytes by TSH treatment. This is of particular interest, as at least for FTC 133 cells, secretion of TGFα, a physiological ligand of the EGF receptor, has been demonstrated before (Broecker et al., 1998). Thus, similar autocrine loops as observed in HTh 74 cells may also exist in FTC 133 and SW 1736 cells. Since the signal transduction pathways induced by TSH or forskolin on the one hand, and those triggered by EGF or TPA on the other, have been shown to exert mutual antagonistic interactions (Kraiem et al., 1995), it is tempting to speculate that the observed loss in TSH/insulin- sensitivity may actually be connected to the concomitant gain in EGF effects.

One important point of overlap between the two pathways is the small GTP-exchange protein p21*ras*. Elucidation of the diverse functions of p21*ras* in the signal network of the thyroid came in particular from the group around J. L. Meinkoth: thus, it was demonstrated that p21*ras* influences cell cycle progression at various points (Cheng et al., 2003), that it has proapoptotic as well as mitogenic effects (Cheng and Meinkoth, 2001), and that it is part of the TSH as well as of the EGF signal pathways (Miller et al., 1998; Kikuchi and Williams, 1996). Taken together, it seems that PKA exerts its adverse effect on EGF-signalling by blocking the association of p21*ras* with Raf-1, while at the same time activation of $G_{\beta\gamma}$ by TSH promotes p21*ras* binding to RalGDS (see also Fig. 24).
Interesting in this context is the high incidence of activating point mutations of the *ras* protooncogene detected in thyroid adenomas and carcinomas, reaching in some studies 80% of the tumours examined (Lemoine et al., 1989; Lemoine, Mayall et al., 1988). There is also an intriguing preponderance of *ras* mutations at codon 61, which has not been reported in tumours of other tissues (Lemoine et al., 1989; Shi et al., 1991). Since the exact nature of the activating *ras* mutation actually seems to specify the signal pathway affected (Cheng and Meinkoth, 2001), this finding could be of particular importance. Moreover, the high frequency of *ras* mutations reported for thyroid adenoma, and even for nodules of multinodular goitre (Namba et al., 1990), indicates its involvement already during initiating

events in thyroid tumorigenesis. Certainly, the lack of lesions in the EGF receptor itself, i.e. the exclusion of *erbB* gene amplification or increases in the receptor affinity for EGF made in FTC 133 cells (Broecker et al., 1998), indicates the presence of an alteration in the downstream signal cascade as the cause of the observed EGF hyperresponsiveness.

Ample support of these findings comes from earlier investigations of clinical specimens from thyroid tumour patients: thus, thyroid cancer tissue was shown to have increased capacities for binding radiolabelled EGF when compared with adenomas or normal gland tissue (Kanamori et al.,1989; Mäkinen et al., 1988), TGFα and EGF receptor mRNAs were coexpressed at significantly higher levels in papillary carcinoma and their metastases than in normal thyroid tissue (Aasland et al., 1990), and, finally, N.R. Lemoine *et al*. confirmed these data on protein level, by detection of EGF receptor and TGFα immunoreactivity in the majority of a large series of thyroid carcinomas, with concomitant negative staining for all normal thyroid tissue samples (Lemoine et al., 1991). Moreover, the last group also noticed a trend to higher expression in more malignant neoplasms. In agreement with this observation, L.A. Akslen *et al*. identified cytoplasmic EGF receptor staining as a negative prognostic indicator in papillary carcinoma patients: thus, increased cytoplasmic, but not membrane, EGF receptor expression was significantly associated with extrathyroidal tumour growth and recurrences during the follow-up period (Akslen et al., 1995). Subsequently, G.O. Ness *et al*. were able to correlate this enhanced cytoplasmic EGF receptor detection in thyroid carcinoma with the presence of a 150 kDa degradation product, indicating an increased receptor turnover due to autocrine stimulation (Ness et al., 1996).

Further proof of the existence of a functional autocrine loop comes from immunohistochemical investigations demonstrating an enhanced expression level of *c-myc* accompanying TGFα and EGF receptor coexpression in papillary carcinoma (Haugen et al., 1993). Taken together, it therefore can be stated that autocrine stimulation by overexpression of EGF receptor and TGFα proteins is a frequent finding in all forms of thyroid carcinoma and that high expression rates probably indicate poor prognosis, as they are associated with a more undifferentiated phenotype and high risk of extrathyroidal growth. Seemingly in contrast to this are the results reported by B. Saller *et al*.: examining EGF-binding to isolated membrane fractions from benign and malignant thyroid nodules, they not only found increased EGF-binding in thyroid carcinomas (18.4 +/- 16.7 fmol/mg protein as compared to 10.5 +/- 5.2 fmol/mg in normal paranodular tissue), with the highest binding capacities again in undifferentiated carcinomas, but also in autonomously functioning adenomas (14 +/- 8.2

fmol/mg protein as compared to 8.9 +/- 4.8 fmol/mg in corresponding normal tissue) (Saller et al., 1993).

However, autonomous function in toxic adenomas has been shown to be caused by activating mutations or overexpression of constituents of the TSH receptor signalling cascade (Derwahl et al., 1996; Parma et al., 1993). This may actually render these cells „immune“ to the dedifferentiating action of EGF. Therefore, the finding of B. Saller *et al*. do not exclude the possibility that disturbances within the EGF receptor signalling system are a key element in the pathogenesis of undifferentiated growth as previously suggested (Frauman and Moses, 1990). Rather, they support the view that autonomous growth and autonomous function are the result of different pathogenic mechanisms (Derwahl et al, 1998).

It could be shown by Southern blot analysis that neither gene amplification nor gross re-arrangements are responsible for the increased EGF receptor expression in thyroid tumours (Lemoine et al., 1990), suggesting that the defect is at the level of gene transcription and/or post-transcriptional processing. In this context it is interesting to note that *in vitro* transformation of a variety of cell types, including thyroid epithelial cells (Coletta et al.,1991), with *ras* oncogenes leads to up-regulation of TGFα expression.

Specific protein kinase inhibitors were again used to investigate the EGF-induced signal cascade leading to CRE-binding protein phosphorylation in some more detail.

As would have been expected, the phosphorylation response was blocked completely by preincubation of human primary thyrocytes with the PKC- inhibitor GFX. This is in good agreement with early experiments that demonstrate the reproducibility of EGF- induced effects on cell proliferation and differentiation by phorbol ester treatment (Roger et al., 1986). Moreover, M. Broecker *et al*. previously reported increased basal inositol phosphate generation due to autocrine EGF receptor- stimulation in HTC-TSHr cells, a subclone of the FTC 133 cell line (Broecker et al., 1997; Broecker et al., 1998). The affected cells were resistant to further stimulation by TSH, an effect that was reversible by pretreatment of HTC-TSHr cells with PKC inhibitors. Hence, these results also strongly suggest involvement of PKC in EGF-induced signalling in thyroid carcinoma cell lines. Surprising, however, was the discovery of complete inhibition of EGF- stimulated CREB/M-phosphorylation by blockage of PKA with H89. In addition, when TPA-induced CREB/M-phosphorylation was analysed, this, too, was completely inhibited by either GFX- or H89-pretreatment of cells. In conclusion, it appears that PKC and PKA act consecutively within one common pathway. Moreover, since neither EGF nor TPA are able to raise intracellular cAMP- levels

significantly, the activation of PKA has to be achieved by some other mechanism, as for instance phosphorylation. A second surprise was encountered when the effects of EGF and TPA on CRE-reporter plasmid transfected HTC-TSHr cells were studied. Since CRE-regulated gene activity was previously shown to be required throughout G1 up to the G1-S boundary (Desdouets et al., 1995), a correspondingly long observation period of 24 hours was chosen here. Indeed, very similar CRE-induction profiles were found in EGF- and TPA-treated cells. However, despite of the fast CRE-binding protein phosphorylation response elicited by EGF- and TPA, induction of the CRE- reporter plasmid was not observed before 20 hours of stimulation. The most obvious explanation for such a delay would be the requirement for the downregulation of some classic or novel PKC-isoform by prolonged stimulation. This, however, was excluded, since PKC δ levels were found to be unaffected by either EGF or TPA, while PKC α was downregulated much more rapidly by TPA as compared to EGF. Apart from these two, no other PKC isoform was detected in substantial amounts in HTC-TSHr cells.

The explanation of these unexpected results may lie in antagonistic functions fulfilled by PKA subforms (see Ciardiello and Tortora, 1998 for review and references therein): thus, the tetrameric holoenzyme PKA exists in two subforms, which are identical in their catalytic subunits (C), but differ within the regulatory subunits (termed RI in PKAI and RII in PKAII, respectively). Of these, predominant expression of PKAII is found in normal, non-proliferating tissues and in growth-arrested cells. In contrast, enhanced levels of PKAI are detected steadily in tumour cells and transiently in normal cells exposed to mitogenic stimuli. Moreover, the two subforms differ in their intracellular localization: thus, PKAII was found in association with the plasma membrane, the cytoskeleton, secretory granules, and the nucleus. In contrast, PKAI is broadly distributed in the cytoplasm, but may translocate to the cell membrane upon certain stimuli. This intracellular distribution of PKA appears to be determined by its association with other cellular components, which again is mediated by specific sequences within the amino- terminus of the respective R subunit. For the RIα subunit, a stretch of uncharged amino acids and an amino-terminal proline-rich sequence were proposed as potential SH3 domain binding sites, mediating the interaction of PKAI with the adaptor protein Grb-2. Grb-2 links autophosphorylated protein kinase receptors, which it recognizes via an SH2 domain, to intracellular signalling proteins harbouring proline-rich sequences like PI3K, PLCγ or Sos, that bind to one of two SH3 domains in Grb-2. Studying the role of PKAI during EGF-receptor signalling in MCF-10A cells, a well-differentiated,

human mammary epithelial cell line, G.Tortora and co-workers were able to demonstrate that (a) RIα coprecipitates with Grb-2 independently from EGF-receptor activation, suggesting that RIα and Grb-2 form a complex already before ligand activation of the EGF-receptor, (b) both RIα and Cα, but not RIIβ, coprecipitate with the ligand activated EGF-receptor, thereby indicating the presence of the PKAI holoenzyme in the EGF-receptor macromolecular signalling complex, and (c) in immunofluorescence studies, RIα staining is superimposable to that of the EGF-receptor, and following ligand activation, translocates from the cell membrane to the cytoplasm by endocytosis. There is plenty of evidence also coming from other cellular systems in support of an important role of PKAI in EGF-receptor signalling: (1) in NRK rat fibroblasts TGFα- and p21*ras*- dependent transformation causes an early induction of RIα and PKAI expression and a parallel reduction of PKAII; (2) in NOG-8 mouse mammary epithelial cells, stable overexpression of TGFα is associated with a fall in RIIβ mRNA expression and a concomitant rise in RIα mRNA, while Cα expression remains unaltered; (3) in MCF-10A human mammary epithelial cells overexpression of TGFα or an activated p21*ras* gene causes neoplastic transformation, which is accompanied by an increase in PKAI expression and PKAII reduction; and, finally, (4) in MDA-MB468 human breast carcinoma cells, a cell line for which a functional TGFα-EGF-receptor autocrine loop was demonstrated, constitutive inhibition of EGF-receptor expression with a respective antisense vector is accompanied by a selective downregulation of PKAI. Furthermore, PKAI expression was demonstrated to be functionally involved in S-phase entry: when complete medium containing EGF was added to serum-starved, quiescent MCF-10A cells, RIα expression was induced 6 to 9 hours before cells entered S-phase, and entry was anticipated by PKAI translocation to the cell membrane. On the other hand, pretreatment with an anti- RIα antisense oligonucleotide blocked S-phase entry of MCF-10A cells following EGF addition, suggesting a role for PKAI in the EGF-receptor-triggered G1-to-S transition. Possibly, such cell cycle dependency may also be the basis of the delay observed here in CRE-reporter plasmid induction during EGF and TPA treatment, because control is certainly not restricted to the level of PKAI expression. Further experiments employing cell cycle phase inhibitors will be needed to address this question in more detail.

Apart from the differences in cellular localization, the contrary modes of expression, and the apparently opposing functions during growth and differentiation, PKAI and II subforms also differ in their regulation by cAMP: each regulatory subunit has two high-affinity binding sites for cAMP per monomer. In addition, the PKAII subform contains an autophosphorylation

site, which is phosphorylated rapidly in an intramolecular reaction, so that the PKAII holoenzyme will exist primarily in its phosphorylated state in the cell. Autophosphorylation of the RIIα, however, reduces the affinity for the C subunit by nearly 10-fold. Consequently, the phosphorylated PKAII holoenzyme dissociates at a lower concentration of cAMP than the dephosphorylated holoenzyme. In contrast, in the RI subunit the autophosphorylation site is replaced by a high-affinity MgATP-binding site. In addition to increasing the amount of cAMP necessary to induce its dissociation, MgATP also enhances the reassociation of the type I holoenzyme (reviewed in Taylor et al., 1990). cAMP was proposed to constitute an ancient hunger signal, regulating nutrition-dependent responses in organisms as diverse as bacteria (catabolite repression), slime moulds (slug formation), and mammals (glycogen breakdown). The dual regulation of PKAI by cAMP and MgATP may thus connect cell proliferation induced by tyrosine kinase receptors to the metabolic state of the cell, favouring cell cycle progression only in well- nourished cells.

The putative EGF-induced signal cascade is depicted in Fig. 25 *a*: EGF-triggered dimerisation and autophosphorylation of the EGF receptor leads to recruitment of the Grb-2/Sos/PKAI complex to the activated receptor. Localisation of the guanine nucleotide releasing protein Sos to the membrane-bound p21*ras* catalyzes formation of the active, guanine triphosphate (GTP)- bound form of p21*ras*. Activated p21*ras* in turn causes activation of PLCγ, and subsequently release of DAG and IP_3 from the plasma membrane promotes Ca^{2+}- influx and translocation of PKC α and δ to the membrane fraction. Both PKC isoforms have previously been linked to EGF receptor signalling: thus, PKC α has been shown to affect the MAPK-pathway by phosphorylating and stimulating the autokinase activity of Raf-1 (Kolch et al., 1993). On the other hand, tyrosine kinase receptor activation was found to result in tyrosine phosphorylation of PKC δ (Li et al., 1994). To further address this question, Y.Ueda *et al*. introduced activating point mutations into the pseudosubstrate regions of PKC α, δ and ε. Examining theses constructs in NIH3T3 and COS1 cells, they found that while PKC α and ε failed to activate MAPKK and MAPK, PKC δ was involved and sufficient for the activation of the MAPK- cascade (Ueda et al., 1996). Intriguingly, only PKC α and δ were detected here at high levels in HTC-TSHr cells by Western blot analysis, and expression of PKC δ was clearly aberrant, with high basal levels and no downregulation even upon 24 hours of TPA treatment. The macromolecular complex of Grb-2/PKAI/p21*ras*/PKC δ(α) then catalyzes the phosphorylation and activation of Raf-1, a process that was shown to require direct physical interaction between the amino-terminal end of Raf-1 and activated p21*ras* (Warne et al.,

1993; Van Aelst et al., 1993). Raf-1 in turn phosphorylates and activates MAPKK and this in turn MAPK (Kyriakis et al., 1992; Dent et al., 1992).

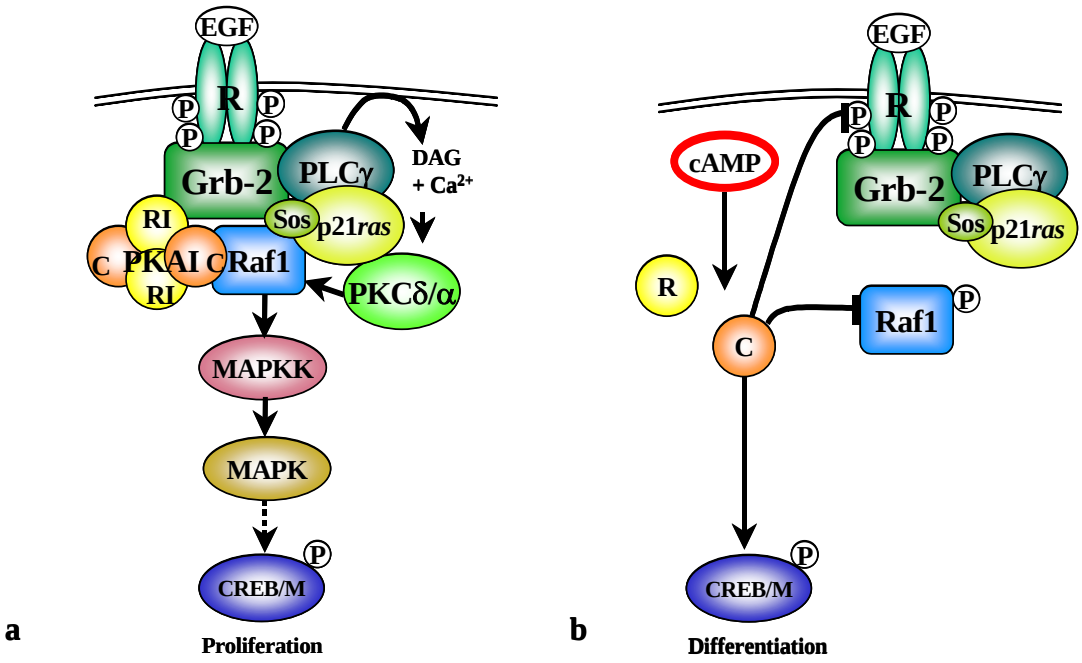

FIG.25 Hypothetical model of PKA involvement in EGF receptor signalling:
(a) Active participation of PKAI in EGF-triggered CREB/M-phosphorylation.
(b) Inhibition of EGF- induced cascade by free catalytic subunit upon cAMP rise.

It should be noticed that as PKAI is recruited to the macromolecular complex catalysing Raf-1 phosphorylation *via* the RI subunit, high cAMP levels may actually disrupt the functional complex by causing dissociation of PKAI subunits (Fig. 25 *b*). This would easily explain the seemingly paradox observation of an inhibitory effect exerted by cAMP analogues on the proliferation of some thyroid carcinoma cell lines (Derwahl et al., 1993; Endo et al., 1990).
In addition, the free catalytic PKA subunit has been shown to interfere with EGF-signalling by phosphorylation of the EGF receptor and of Raf-1. Such PKA- mediated phosphorylation of Raf-1 on serine 43 has previously been demonstrated to prevent its association with p21*ras* (Wu et al., 1993). In other cell types, CREB/M- phosphorylation after EGF- stimulation has been shown to be mediated by MAPK-activated p90S6 kinase (De Cesare et al., 1998; Xing et al., 1996). Whether this is also the case in thyrocytes will have to be examined in further experiments employing MAPK- pathway-specific inhibitors and studying phosphorylation and nuclear translocation of p90S6 kinase. However, the data presented here are at least not in disagreement with this possibility.

In theory, two different situations are conceivable that would require thyroid growth *in vivo*: Firstly, insufficient endocrine performance would warrant an increase in thyroid mass in order to raise hormone production. Proliferation in this situation would need to maintain differentiated organ function. The primary growth trigger in this case is the chronic release of TSH by the pituitary gland with subsequent synthesis of local paracrine factors like IGF-I. In this study no significant variation in TSH-induced CREB/M- phosphorylation levels was observed when human primary cells were compared, and indeed thyroid hyperplasia in this situation is primarily diffuse. However, under prolonged stimulation local heterogeneity will finally lead to nodular transformation. An example of such heterogeneity was encountered here when the CRE-binding protein phosphorylation responses to insulin were compared. On the other hand, mechanical or inflammatory trauma accompanied by cell loss will necessitate fast cell proliferation irrespective of thyroid function in order to restore organ integrity. Apart from the relatively long half-life of thyroid hormones, differentiated function under these circumstances may be maintained by unaffected parts of the thyroid. In this repair process the TGFα-EGF receptor cascade appears to play a fundamental role: while there is no EGF receptor expression detectable in normal thyroid tissue, EGF receptor is always expressed in epithelial cells of follicles involved in inflammatory reactions as in Graves´disease or Hashimoto´s thyroiditis (Lemoine et al., 1991; Miyamoto et al., 1989). Similarly, chronic and acute pancreatitis has been found to be accompanied by an enhanced expression of EGF receptor and TGFα (Korc et al., 1994; Konturek et al., 1998). Moreover, EGF administration was shown to exhibit beneficial effects on the course of experimentally-induced acute pancreatitis in rats (Tomaszewska et al., 2002) and radiation- induced release of TGFα was demonstrated to activate the EGF receptor and MAPK pathway in mammary carcinoma cells, leading to increased proliferation and protection from radiation- induced cell death (Dent et al., 1999; Contessa et al., 1999; Reardon et al., 1999; Schmidt-Ullrich et al., 1999). Interestingly, there was also a post-irradiation increase in CREB phosphorylation noticed, which could be blocked by inhibitors of either tyrosine kinase receptor activity, p21*ras* or MAPKK. In addition, CREB phosphorylation was preceded by an increase in nuclear p90S6 kinase phosphorylation (Amorino et al., 2002). Here an enhanced responsiveness of human thyroid carcinoma cells to EGF-induced CREB/M-phosphorylation was observed. It thus appears that whereas differentiation-supporting growth factors play an essential role during benign growth processes in the thyroid, alterations in repair-induced proliferation mechanisms are of particular importance during malignant transformation (see also Fig. 26).

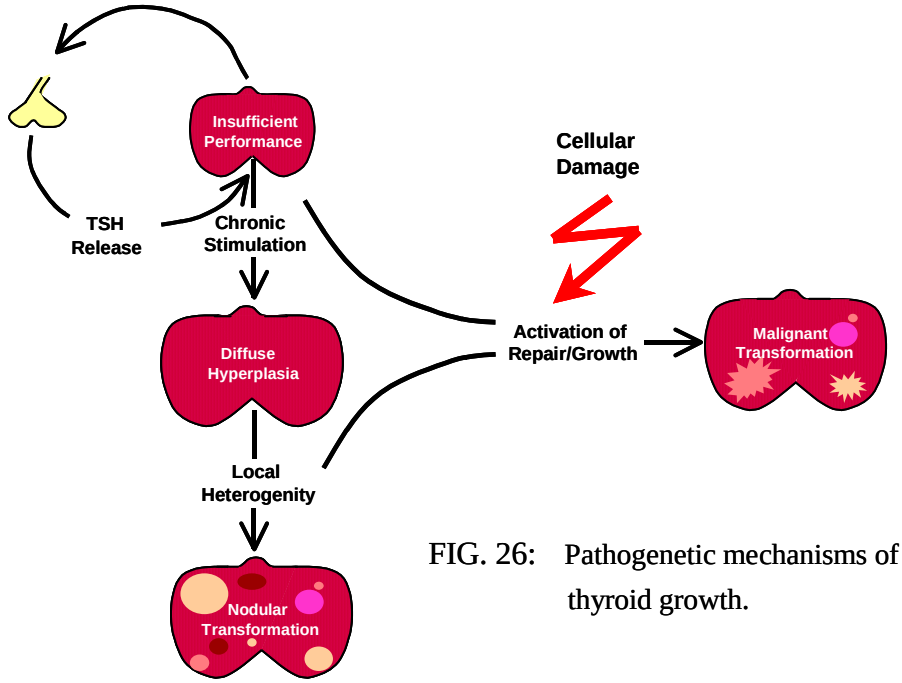

FIG. 26: Pathogenetic mechanisms of thyroid growth.

The involvement of both these conditions in thyroid growth transformation is also indicated by endemic observations made in Switzerland: as early as in 1923 iodine prophylaxis was started in some cantons, and retrospective examination of histological specimens in the 1970s then revealed not only the expected decrease in goitre incidence due to iodine deficiency, but also a shift in the relative frequencies of different forms of thyroid carcinoma. The authors of the study concluded that in areas of goitre, follicular carcinomata are the most frequent tumour form, while in areas with low goitre incidence, papillary carcinomata are the leading thyroid carcinoma form. Moreover, there was a decrease in anaplastic carcinoma of 13% (Bubenhofer and Hedinger, 1977).

Hence, it seems that a deeper understanding the molecular basis of thyroid carcinogenesis may be essential to the future development of more effective strategies of prophylaxis and treatment of malignant thyroid disease.