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Conditional Expression of RET/PTC Induces a Weak Oncogenic Drive in Thyroid PCCL3 Cells and Inhibits Thyrotropin Action at Multiple Levels

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Chromosomal rearrangements linking the promoter(s) and N-terminal domain of unrelated gene(s) to the C terminus of RET result in constitutively activated chimeric forms of the receptor in thyroid cells (RET/PTC). RET/PTC rearrangements are thought to be tumor-initiating events; however, the early biological consequences of RET/PTC activation are unknown. To explore this, we generated clonal lines derived from well-differentiated rat thyroid PCCL3 cells with doxycycline-inducible expression of either RET/PTC1 or RET/PTC3. As previously shown in other cell types, RET/PTC1 and RET/PTC3 oligomerized and displayed constitutive tyrosine kinase activity. Neither RET/PTC1 nor RET/PTC3 conferred cells with the ability to grow in the absence of TSH, likely because of concomitant stimulation of both DNA synthesis and apoptosis, resulting in no net growth in the cell population. Effects of RET/PTC on DNA synthesis and apoptosis did not require direct interaction of the oncoprotein with either Shc or phospholipase Cγ. Acute expression of the oncoprotein decreased TSH-mediated growth stimulation due to interference of TSH signaling by RET/PTC at multiple levels. Taken together, these data indicate that RET/PTC is a weak tumor-initiating event and that TSH action is disrupted by this oncoprotein at several points, and also predict that secondary genetic or epigenetic changes are required for clonal expansion. (*Molecular Endocrinology* 17: 1425–1436, 2003)

REARRANGEMENTS OF THE proto-oncogene RET resulting in its constitutive activation are believed to play a causative role in the pathogenesis of a significant proportion of papillary carcinomas of the thyroid (PTC) (1). The RET gene encodes a transmembrane tyrosine kinase (TK) receptor whose expression and function is normally restricted to a subset of cells derived from the neural crest. In thyroid follicular cells, RET activation occurs through chromosomal recombination resulting in illegitimate expression of a fusion protein consisting of the intracellular TK domain of RET coupled to the N-terminal fragment of a heterologous gene. Several forms have been identified that differ according to the 5′ partner gene involved in the rearrangement. RET/PTC1 is formed by a paracentric inversion of the long arm of chromosome 10, leading to fusion of RET with a gene named H4/D10S170 (2). RET/PTC2 is formed by a reciprocal translocation between chromosomes 10 and 17, resulting in the juxtaposition of the TK domain of c-RET with a portion of the regulatory subunit of RIαcAMP-dependent protein kinase A (3). RET/PTC3 is also a result of an intrachromosomal rearrangement and is formed by fusion with the RFG/ELE1 gene (4, 5). Recently, several additional variants of RET/PTC have been observed in papillary carcinomas arising in children exposed to radiation after Chernobyl (6, 7). The fusion proteins generated by these chimeric genes dimerize in a ligand-independent manner and constitutively activate the TK function of RET, thus exerting their transforming potential.

RET rearrangements are found in 5–40% of PTC in the adult population (1). By contrast, RET rearrangements are more common in pediatric PTC, and in cancers from children exposed to radiation after the Chernobyl nuclear accident (8–10). There are several lines of evidence pointing to RET/PTC as a key first step in thyroid cancer pathogenesis: 1) thyroid-specific overexpression of either RET/PTC1 (11, 12) or RET/PTC3 (13) in transgenic mice leads to development of tumors with histological features consistent with PTC. 2) There is a high prevalence of RET/PTC expression in occult or microscopic PTC (14, 15). 3) Exposure of cell lines (16) and fetal thyroid explants (17) to ionizing radiation results in induction of expression of RET/PTC within hours, supporting a direct role for radiation in the illegitimate recombination of RET. 4) The breakpoints in the RET and ELE1/RFG genes resulting in the RET/PTC3 rearrangements of radiation-

Abbreviations: BrdU, Bromodeoxyuridine; CREB, cAMP response element binding protein; DTT, dithiothreitol; IBMX, isobutylmethylxanthine; PKC, protein kinase C; PLC, phospholipase C; PT, pertussis toxin; PTC, papillary carcinomas of the thyroid; TSH-R, TSH receptor; TK, tyrosine kinase.

induced pediatric thyroid cancers from Chernobyl are consistent with direct double-strand DNA breaks resulting in illegitimate reciprocal recombination (18). Moreover, the H4 and RET genes, although lying at a considerable linear distance from each other within chromosome 10, are spatially juxtaposed during interphase in thyroid cells and presumably present a target for simultaneous double-strand breaks in each gene after ionizing radiation, thus giving rise to the RET/PTC1 rearrangement (19).

RET/PTC1 has been reported to confer PCCL3 cells with the ability to grow in a TSH-independent manner, and to decrease expression of the TSH receptor (TSH-R), thyroglobulin, and thyroid peroxidase (20). These effects of RET/PTC on thyroid cell growth and differentiation were observed in cells stably overexpressing the oncoprotein, and it is possible that some of the observed changes occurred during selection and adaptation to the genetic manipulation *in vitro*. To explore potential early changes associated with tumor initiation by RET/PTC, we developed rat thyroid cell lines with conditional expression of either RET/PTC1 or RET/PTC3. We report that RET/PTC is indeed associated with loss of thyroid-specific differentiation. Moreover, we show that RET/PTC interferes with TSH-R-mediated intracellular signaling at various levels, by blocking expression of the receptor, and by effects on adenylyl cyclase as well as distal to cAMP generation. Acute activation of RET/PTC is not, however, sufficient to allow cells to grow in the absence of TSH, as the oncoprotein stimulates both DNA synthesis and apoptosis in PCCL3 cells, suggesting that secondary events must be necessary to allow unregulated growth and clonal expansion.

RESULTS

Inducible Expression of RET/PTC1 and RET/PTC3 in Rat PCCL3 Thyroid Cells

To investigate the early cellular responses and phenotypic changes evoked by RET/PTC activation, we developed cell lines by sequential stable transfection of PCCL3 cells with expression vectors for the tetracycline-dependent *trans*-activating protein rtTA, and then for either RET/PTC1 or RET/PTC3. Numerous clonal lines were obtained that expressed the respective oncoproteins in a doxycycline-dependent manner. Expression of the chimeric gene products was detectable as early as 2 h (not shown), sustained through an 8-d period in the continued presence of doxycycline (Fig. 1A), and dose dependent (Fig. 1B).

Constitutive activation of RET/PTC oncoproteins requires oligomerization and autophosphorylation (21). Incubation of protein extracts of doxycycline-treated RET/PTC1–31 and RET/PTC3–5 cells with the cross-linking agent glutaraldehyde for varying times demonstrated oligomerization of both RET/PTC1 and RET/PTC3 (Fig. 2A). The glutaraldehyde incubation time required for RET/PTC dimer formation was similar between both chimeric proteins, despite the fact that they differ in the structure of the N-terminal coiled-coil domains that mediate dimerization. Doxycycline-dependent RET autophosphorylation was confirmed in

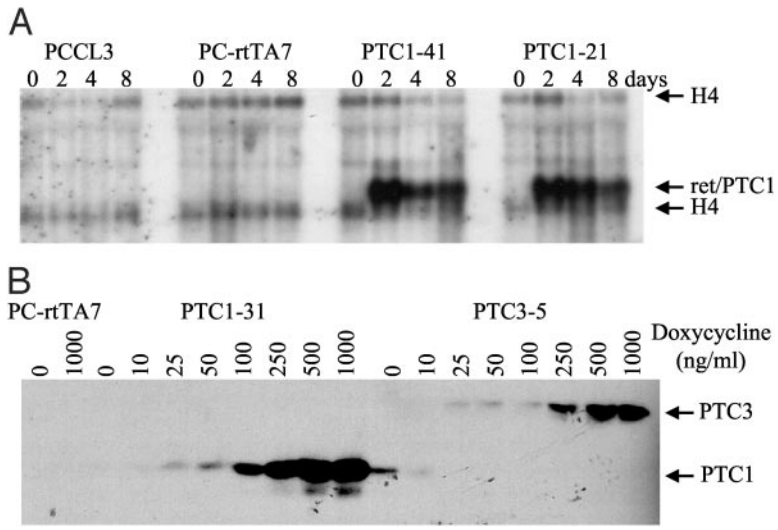

Fig. 1. Doxycycline-Inducible Expression of RET/PTC3 and RET/PTC1 in PCCL3 Thyroid Cells

A, Northern blots containing 10 μg of total RNA from the indicated cell line treated with doxycycline (1.0 μg/ml) for 2–8 d were hybridized with a ^{32}P-labeled RET/PTC1 cDNA. The positions of the endogenous H4 mRNA and the RET/PTC1 transgene are indicated by *arrows*. B, Forty-five micrograms of protein from total cell extracts prepared from the indicated cell lines incubated with the specified concentration of doxycycline for 24 h were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. RET/PTC1 and RET/PTC3 were detected using a rabbit polyclonal anti-RET and a horseradish peroxidase-conjugated goat antirabbit IgG.

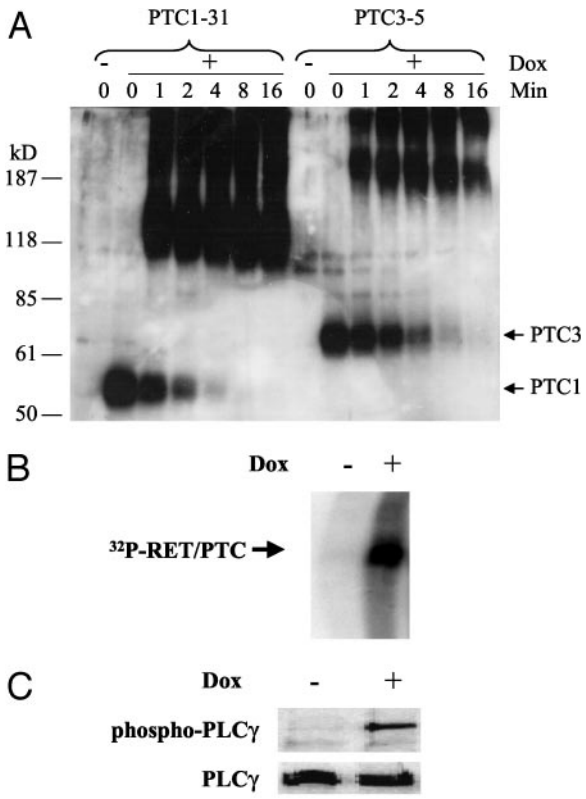

Fig. 2. Expressed RET/PTC3 and RET/PTC1 Dimerize and Retain Kinase Activity

A, Total cell extracts prepared from cells incubated with or without doxycycline were incubated for the indicated time with glutaraldehyde, a cross-linking agent. As a control for specificity (t = 0), cell extracts were denatured before a 5-min incubation with glutaraldehyde. The treated extracts were then subjected to SDS-PAGE and transferred to a nitrocellulose membrane. RET/PTC1 and RET/PTC3 were detected using a rabbit polyclonal anti-RET (Santa Cruz Biotechnology, Inc.) and a horseradish peroxidase-conjugated goat antirabbit IgG. B, RET/PTC3 was immunoprecipitated from total cell extract prepared from RET/PTC3–5 cells incubated with or without 1.0 μg/ml of doxycycline for 24 h using a rabbit anti-RET IgG. The resulting immunoprecipitate was then incubated with ATP γ^{32}P as described in *Materials and Methods*. The kinase reactions were then subjected to SDS-PAGE and autophosphorylation of RET/PTC3 protein was detected using phosphoimager. C, One hundred micrograms of protein from total cell extracts prepared from RET/PTC3–5 cells incubated with or without 1.0 μg/ml of doxycycline for 24 h were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Phospho-PLCγ and PLCγ were detected using a rabbit anti-phospho-PLCγ and rabbit) or rabbit anti-PLC, respectively and an horseradish peroxidase-conjugated goat antirabbit IgG.

in vitro kinase assays (Fig. 2B), and activation of phospholipase C (PLC) γ by Western blotting with phospho-specific antibodies (Fig. 2C). Taken together, these findings corroborated appropriate conditional induction of expression and functional activation of RET/PTC oncoproteins in these cell lines, which were thus considered suitable for biological studies.

Effects of Acute Activation of RET/PTC on PCCL3 Cell Growth

We next explored the effects of acute activation of either RET/PTC1 or RET/PTC3 on thyroid cell growth. PC-rtTA cells, which express only the tetracycline-dependent transactivator protein, behave in all respects like parental PCCL3 cells and were used as negative controls. As shown in Fig. 3, PC-rtTA cells were dependent on the presence of TSH for growth, which was not affected by incubation with doxycycline. Expression of either RET/PTC1 (PTC1–21 and PTC1–31 cells) or RET/PTC3 (PTC3–5 cells) by addition of doxycycline did not confer cells with the ability to grow in the absence of TSH. Moreover, TSH-induced growth was partially impaired by expression of RET/PTC1, and particularly of RET/PTC3.

Effects of RET/PTC Expression on DNA Synthesis and Apoptosis

RET/PTC has been well established to function as an oncoprotein at early stages of papillary thyroid cancer development. The fact that acute RET/PTC activation did not stimulate PCCL3 cell growth was therefore unexpected. We next examined whether expression of the RET chimeric proteins affected DNA synthesis as determined by incorporation of [^{3}H]thymidine (Fig. 4). When grown in the absence of TSH, both RET/PTC1 and RET/PTC3 stimulated DNA synthesis (Fig. 4A). Conversely, both RET oncoproteins inhibited DNA synthesis in cells grown in the presence of TSH (Fig. 4B). This latter effect, as will be shown in greater detail below, is due in all likelihood to interference by RET/PTC of TSH-mediated signaling at several levels.

Thus, despite inducing a significant increase in DNA synthesis, RET/PTC1 and 3 do not stimulate cell growth in the absence of TSH. We next explored whether this could be accounted for by concomitant activation of cell death. As shown in Fig. 5, doxycycline-induced expression of RET/PTC3 was associated with induction of apoptosis as determined by cell detachment or Apo-BrdU assays. Acute activation of RET/PTC1 had similar effects on thyroid cell apoptosis, although of a lesser magnitude (not shown).

Effects of RET/PTC Mutants on DNA Synthesis and Apoptosis

As RET/PTC3 activation appears to induce both cell division as well as apoptosis, we proceeded to examine whether the signaling pathways responsible for these outcomes were distinct from each other. For this purpose, we examined the functional consequences of acute activation of RET/PTC mutants that are unable to associate with either PLCγ or Shc, respectively. We report elsewhere the development and characterization of PCCL3 cells with doxycycline-inducible expression of RET/PTC3^{Y541F}, and of RET/PTC2-PDZ (Knauf, J. A., M. Croyle, E. Kimura, and J. A. Fagin,

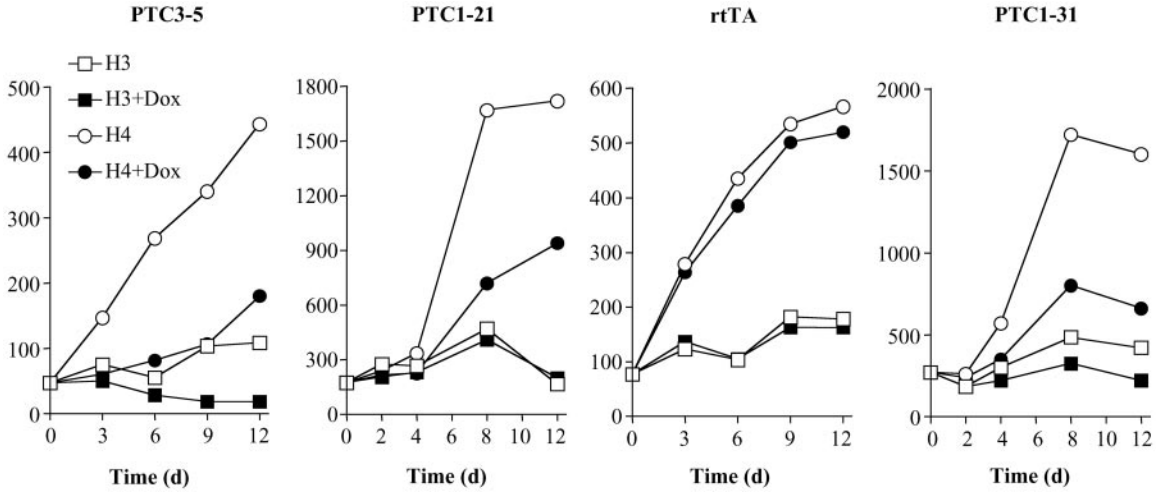

Fig. 3. Effects of RET/PTC Expression on Growth of PCCL3 Cells

Growth curves of PCCL3 cells with doxycycline inducible expression of RET/PTC1 or RET/PTC3. Data illustrate results of one experiment performed in triplicate. Similar data were obtained in a second independent experiment. *Squares* and *circles* represent cell counts with (H4) or without (H3) TSH, respectively. *Open* and *closed symbols* represent cell counts in the absence or presence of 1.0 μg/ml of doxycycline, respectively.

submitted). We also confirmed the inability of RET/PTC3^{Y541F} to associate with or phosphorylate PLCγ, whereas the interaction with Shc is unaffected. Conversely, RET/PTC2-PDZ associates with and activates PLCγ, but not Shc-Ras (22). As shown in Fig. 6, doxycycline-induced expression of either of the RET/PTC mutants evoked similar effects on growth both in the presence and absence of TSH as we observed with wild-type RET/PTC (Fig. 4). Similarly, acute expression of the RET/PTC mutants also induced apoptosis. This indicates that direct activation of PLCγ or Shc-Ras-MEK-MAPK by RET/PTC is not required for the RET/PTC-induced effects on growth and apoptosis.

Effects of RET/PTC Expression on TSH-R mRNA Levels

We next explored possible mechanisms by which RET/PTC oncoproteins interfere with TSH-mediated cell growth. Santoro *et al.* (20) previously reported that TSH-R mRNA levels were markedly decreased in RET/PTC1-expressing thyroid cell lines. We show in Fig. 7 that acute expression of RET/PTC3 decreases TSH-R mRNA abundance by 77%, consistent with inhibition of TSH action through lower expression levels of this GPCR.

Effects of RET/PTC Expression on Adenylyl Cyclase

We next determined the impact of RET/PTC expression on adenylyl cyclase activity. Predictably, acute activation of RET/PTC3 decreased TSH-induced cAMP levels by about 50%. However, expression of the RET oncoprotein also reduced forskolin- and cholera toxin-stimulated cAMP levels (Fig. 8A). Similarly, RET/PTC dampened the modest induction of cAMP by pertussis toxin (PT) and markedly impaired the combined effects of TSH and PT on adenylyl cyclase activity (Fig. 8B). In addition, Western blots demonstrated that RET/PTC expression did not alter expression of G proteins (data not shown). These data suggest that RET/PTC may block adenylyl cyclase activity directly. Expression of either RET/PTC3^{Y541F} or RET/PTC2-PDZ did not impair TSH-induced cAMP production (Fig. 8C), suggesting that RET/PTC inhibition of adenylyl cyclase requires association of RET/PTC with both PLCγ and Shc.

Effects of RET/PTC Expression on cAMP-Induced DNA Synthesis

We next examined whether RET/PTC3 had effects on cAMP-induced DNA synthesis. As shown in Fig. 9, activation of expression of either of the RET/PTC isoforms 24 h before the addition of cAMP blunted induction of DNA synthesis, consistent with an effect of the oncoprotein on pathways distal to adenylate cyclase activation. Activation of RET/PTC expression did not interfere with cAMP response element binding protein (CREB) phosphorylation by cAMP, suggesting that the effects of RET/PTC on cAMP-induced DNA synthesis are likely to be mediated through a PKA-independent pathway (not shown).

DISCUSSION

An initial objective of this report was to explore the mechanisms of thyroid tumor initiation by RET/PTC. Previous studies by Santoro and colleagues showed

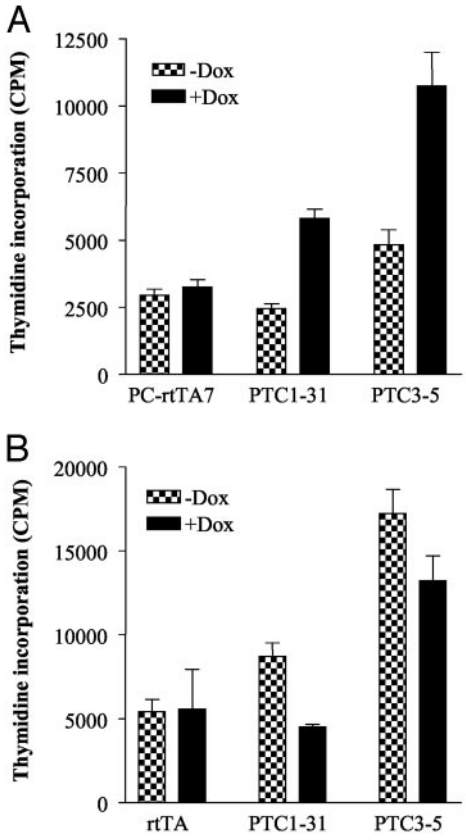

Fig. 4. Effects of RET/PTC Expression on DNA Synthesis in PCCL3 Cells

Cells were incubated in the absence of TSH for 48 h and then doxycycline was added for 24 h. Cells were then incubated with [^{3}H]thymidine for 24 h in the absence (A) or presence of TSH (B). *Bars* represent the average ^{3}H incorporation from a single experiment performed in quadruplicate. Similar results were obtained in three separate experiments.

that expression of RET/PTC1 in PCCL3 cells was associated with TSH-independent growth stimulation (20). These studies were performed in clonal lines derived from PCCL3 cells after selection after stable infection with retroviral vectors encoding RET/PTC1, and it is conceivable that secondary genetic or epigenetic events may have enabled these clones to gain a selective advantage. In our model, we activated RET/PTC1 or RET/PTC3 using a tetracycline-inducible expression system to explore very early events after oncoprotein activation. Several independent clones were studied and showed no evidence of growth after acute expression of either RET/PTC1 or RET/PTC3 in the absence of TSH. This is consistent with the notion that tumor initiation by this oncoprotein introduces a fairly weak growth stimulus and is not sufficient to induce growth in a TSH-independent manner. Interestingly, despite the lack of growth stimulation, both RET/PTC isoforms induced DNA synthesis. However, RET/PTC 1 and in particular RET/PTC3 concomitantly activated cell death, which presumably accounts for the absence of any significant effects on growth.

Considerable progress has been made in identifying signaling pathways activated by RET that may be responsible for its biological effects (23–27). Deletions or substitutions of specific tyrosine residues in the intracellular domain of RET have been performed to explore the relative contribution of the distinct signaling effectors to which they couple to the transforming effects of the oncoprotein. Y1062 plays an essential role in RET signaling because its mutation impairs transformation of fibroblasts mediated by oncogenic mutants such as RET/MEN2A, RET/MEN2B, and RET/PTC2 (22, 28, 29). De Vita *et al.* (30) reported that mutation of Y1062 abolished the ability of RET/MEN2A to promote neuronal differentiation in PC12 cells. Y1062 appears to be involved in complex associations with several docking proteins, such as Shc (31, 32) and FRS-2 (33), both ultimately resulting in Ras activation, and IRS-1 (30). RET/PTC2, which like all other RET/PTC chimeric proteins lacks a transmembrane domain, is recruited to the cell surface in part by interaction with the protein Enigma, also through tyrosine 1062. Unlike Shc and the other effectors, the interaction of Y1062 with Enigma is not affected by Y1062 phosphorylation, but nevertheless this association is required for mitogenesis in fibroblasts (22). Thus, mutation of this single tyrosine residue, in addition to blocking interaction with Shc-Ras and perhaps other signaling effectors, may interfere more broadly with the function of the oncoprotein through mislocalization away from the cell surface. Because of this concern, we explored the role of Y1062 by using a truncated fragment of RET/PTC2 lacking the C terminal 22 residues, including the tyrosine corresponding to Y1062 of wild-type RET. This fragment was fused to an N-terminal PDZ domain, which interacts with Enigma and allows appropriate membrane localization (22). We show elsewhere that after doxycycline-induced expression of RET/PTC2-PDZ (C'574-PDZ) in PCCL3 cells, the mutant oncoprotein does not associate with Shc, yet retains its ability to interact and activate other effectors such as PLCγ (Knauf, J. A., M. Croyle, E. Kimura, and J. A. Fagin, submitted). Here we show that acute activation of RET/PTC2-PDZ recapitulates in all respects the effects of intact forms of RET/PTC on growth and apoptosis. Thus, direct interaction with Ras through Shc is not required for either of these outcomes. This does not exclude a role for effectors downstream of Ras on this process because MAPK, for example, can be activated through alternative routes. Thus, cAMP can activate MAPK in a PKA-independent manner via Rap1 (34), and protein kinase C (PKC) ϵ has been shown to activate Raf independent of Ras (35). We previously reported that acute activation of oncogenic mutants of H-Ras or of its effector MEKI resulted in a high rate of cell death in PCCL3 cells (36). In this model, H-Ras activation is associated with sustained high intensity activation of MAPK, whereas RET/PTC oncoproteins evoke more transient and less intense activation of this effector (Basu, S., and J. A. Fagin, unpublished).

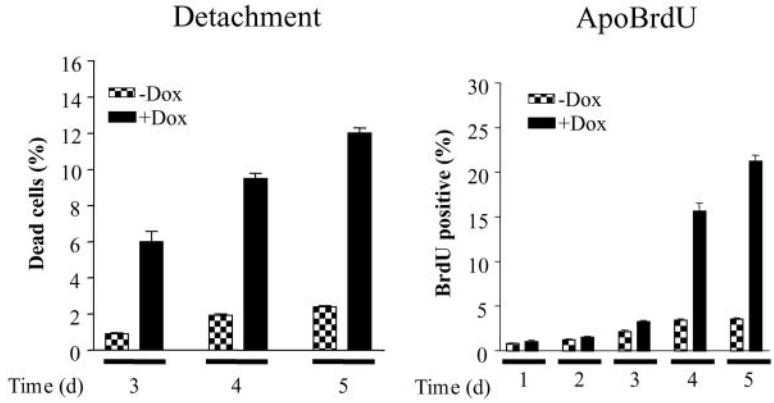

Fig. 5. Effects of RET/PTC3 Expression on Apoptosis of PCCL3 Cells

Cells were incubated in the absence of TSH for 48 h and then doxycycline (1.0 μg/ml) added for 1–5 d. At the indicated times apoptosis was measured by either cell detachment or by Apo-BrdU assay (PharMingen, San Diego, CA). *Bars* represent either the average percent of dead cells (detachment) or the average percent of BrdU-positive cells (Apo-BrdU).

Similarly, the experiments with the RET/PTC3^{Y541F} mutant, which cannot associate directly with PLCγ but signals appropriately through Shc and other effectors, indicate that direct activation of PLCγ is not required for the effects on growth or apoptosis. This was of interest because of the observation that PKCϵ, which is selectively activated by RET/PTC via PLCγ and then down-regulated, may play a role in controlling cell survival in thyroid cells (37). Again, these experiments do not exclude participation of PKCϵ in this process because PKC isozymes can also be activated through alternative pathways. For instance, phosphorylation of PKC isozymes by the phosphatidylinositol 3-kinase-dependent kinase, PDK1 is required for activation (38, 39). Taken together, these data indicate that upstream interference of activation of Shc and PLCγ by RET/PTC is not sufficient to block either cell proliferation or apoptosis.

One well-known feature of papillary thyroid carcinomas is that they exhibit decreased expression of thyroid-specific genes and lower radioiodine uptake compared with normal thyroid tissue. Santoro *et al.* (20) previously reported that expression of RET/PTC1 in PCCL3 cells is associated with lower abundance of thyroglobulin, thyroid peroxidase, and TSH-R mRNA levels. This implies that at least some of the effects of the oncoprotein on differentiation may be accounted for by lower abundance of TSH-R and consequent decreased responsiveness to its ligand. Here, using an acute model of RET/PTC1 and RET/PTC3 activation, we confirm this observation. However, in addition to the likely interference with the TSH-mediated signal at this step, we also show that acute expression of RET/PTC interrupts signal transmission at distal sites along this pathway. Thus, neither forskolin nor cholera toxin evoked appropriate activation of adenylyl cyclase after RET/PTC expression. This cannot be accounted for simply by changes in the abundance of G proteins, or of key adenylyl cyclase isoforms (not shown). The identity of adenylyl cyclase isoforms in other cell types has been shown to be instrumental in modulating proliferative responses because they differ in their response to upstream signal inputs (40). Vanvooren *et al.* (41) have shown that dog and human thyrocytes express primarily adenylyl cyclases ACIII, ACVI, and ACIX . Of these, ACVI is known to be inhibited by low (μM) concentrations of Ca^{2+} and is also expressed in rat thyroid cells. Moreover, treatment of dog thyroid cells with thapsigargin in the presence of extracellular calcium markedly inhibits forskolin-induced increases in cAMP levels (41). Treatment with the calcium ionophore A 23–187 evoked similar responses (42). A reasonable hypothesis is that RET/PTC may inhibit adenylyl cyclase activity primarily through intracellular Ca^{2+}-mediated inhibition of ACVI. Other effectors engaged by RET/PTC may also play a role in modulating adenylyl cyclase activity. Receptor TKs are thought to modulate adenylyl cyclase acting via serine phosphorylation, although the precise effectors involved may vary (43). IGF-I has been shown to enhance adenylyl cyclase VI activity in human embryonic kidney cells through p74^{raf1} (43). In PC12 cells, PKC inhibits ACVI activity, likely through either PKC ϵ or δ (44). The fact that RET/PTC1 and 3 selectively activate PKCϵ makes this isozyme an interesting candidate for the effects we report here. Recently, adenylyl cyclase has been termed a "coincidence detector" because it integrates concurrent signal inputs from several pathways (45). The observation that neither RET/PTC3^{Y541F} nor RET/PTC2-PDZ inhibited TSH-induced cAMP levels suggests that the interaction of RET/PTC with both Shc and PLCγ are required for this effect. Our data point to critical effects on RET/PTC presumably acting directly on adenylyl cyclase, but the precise biochemical mechanism will require further study.

In addition to its effects on adenylyl cyclase activity, RET/PTC also acts at a more distal step along this signal transduction cascade, as evidenced by lower cAMP-induced DNA synthesis 48 h after RET/PTC activation. Evidence that RET/PTC does not impede CREB

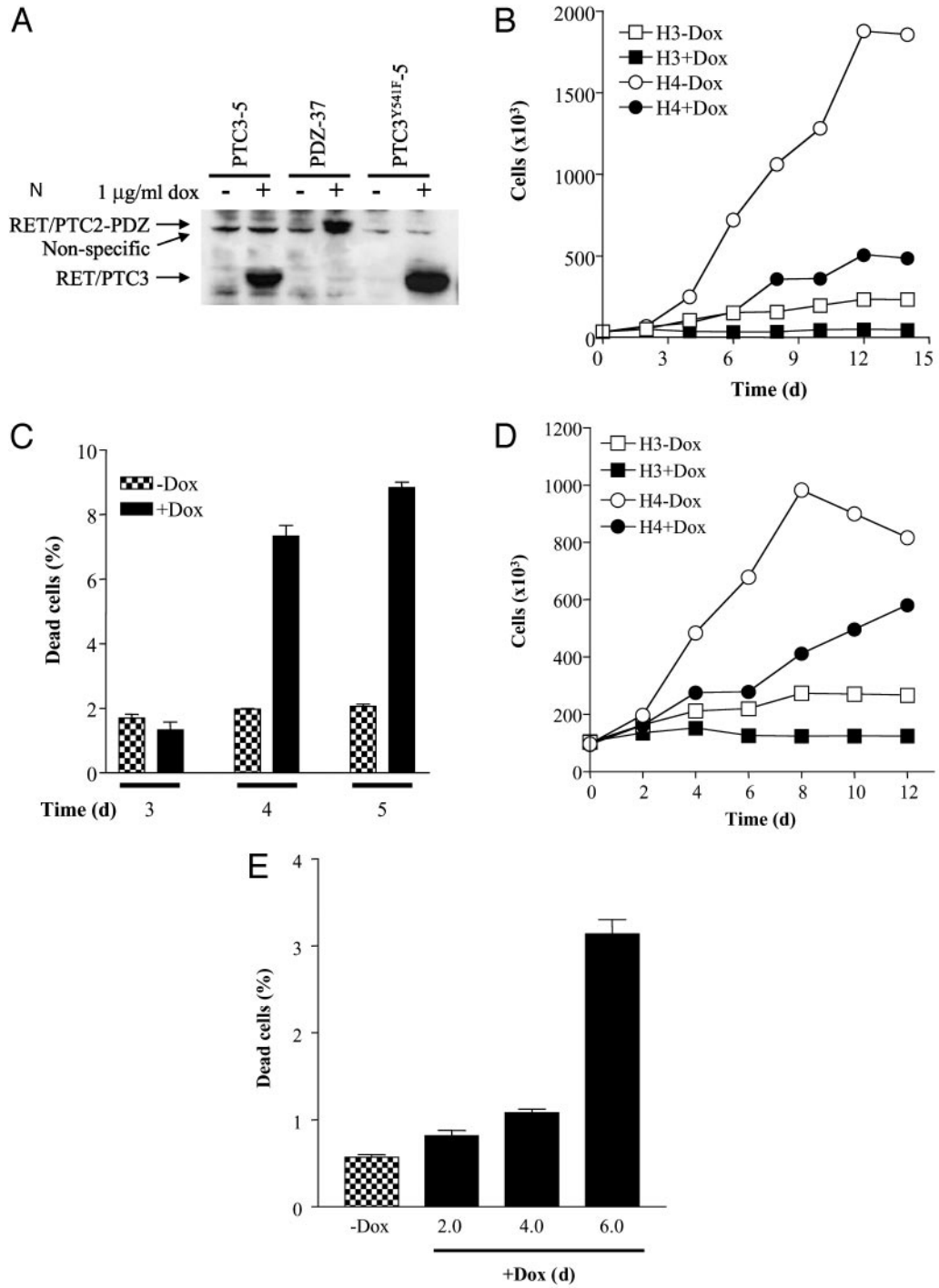

Fig. 6. Effects of RET/PTC Mutants on Growth and Apoptosis of PCCL3 Cells

A, Fifty micrograms of protein of total cell extracts from the indicated cell lines treated with doxycycline for 48 h were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a mouse anti-Ret IgG and a horseradish peroxidase-conjugated goat antimouse IgG. Growth curves of PCCL3 cells with doxycycline inducible expression of RET/PTC3^{Y541F} (B) or RET/PTC2-PDZ (D). Data illustrate results of one experiment performed in triplicate, which was similar to those obtained in a second independent experiment. *Squares* and *circles* represent cell counts with (H4) or without (H3) TSH, respectively. *Open* and *closed symbols* represent cell counts in the absence or presence of 1.0 μg/ml of doxycycline, respectively. RET/PTC3^{Y541F} (C) or RET/PTC2-PDZ (E) cells were incubated in the absence of TSH for 48 h and then doxycycline (1.0 μg/ml) added for 3–5 d. At the indicated times after the addition of doxycycline, cell death was measured by cell detachment. *Bars* represent the average percent of dead cells from a single experiment performed in triplicate. Similar results were obtained in two additional experiments.

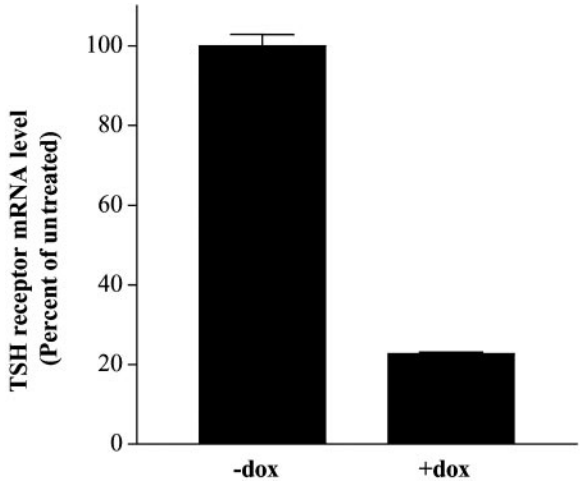

Fig. 7. Effects of RET/PTC Expression on TSH-R mRNA Levels in PCCL3 Cells

Cells were incubated in the absence of TSH for 48 h and then doxycycline added for 24 h. Cells were then incubated with TSH for 48 h, RNA extracted, and cDNA prepared as described in *Materials and Methods*. The cDNA was used as a template in real-time PCR, which were performed as described to determine the relative expression of the TSH-R. *Bars* represent the average normalized TSH-R expression of a single experiment preformed in triplicate.

phosphorylation points to a PKA-independent mode of action. Recently, Ribeiro-Neto *et al.* (34) reported that PKA activation of Rap1b is required for TSH-induced mitogenic activity in thyroid follicular cells. The manner in which RET/PTC signaling intersects with effectors distal to Rap1b may help explain these changes.

In conclusion, this study extends previous information on the mechanism of the tumor initiation by RET/PTC in several ways. First, we demonstrate that expression of this oncoprotein is not sufficient to promote cell growth because it simultaneously activates both DNA synthesis and apoptosis. This likely creates a new homeostatic state, in which cells may become susceptible to secondary genetic or epigenetic changes that may disable growth inhibitory or antiapoptotic signaling effectors. We also demonstrate that simple deletion of two of the key signaling pathways activated by RET/PTC is not sufficient to alter the phenotype. Finally, we show for the first time that RET/PTC interferes with thyroid differentiation at steps distal to the TSH-R, predicting that simple restoration of TSH-R expression may not be sufficient to normalize TSH responsiveness in tumors with constitutive activation of RET.

MATERIALS AND METHODS

Cell Culture

PCCL3 cells and their derivatives were maintained in H4 complete medium consisting of Coon's medium/F12 high zinc supplemented with 5% fetal bovine serum, 0.3 mg/ml L-glutamine, 1 mIU/ml TSH, 10 μg/ml insulin, 5 μg/ml apotransferrin, 10 nM hydrocortisone, and penicillin/streptomycin. H3 complete medium was identical to H4 complete medium but without addition of TSH.

Cell Transfections

We used the expression system developed by Bujard and colleagues (46, 47) to deliver doxycycline-inducible expression based on the high specificity of the *Escherichia coli* tet repressor-operator-doxycycline interaction. The generation of PCCL3 lines with constitutive expression of the transactivator rtTA, conferring cells with high doxycycline-inducible expression of reporter gene constructs under the control of a tet-operator were previously described (36). One of these lines (PC-rtTA7) was used for secondary transfections with tet-inducible expression vectors for RET/PTC isoforms.

The RET/PTC1 and RET/PTC3 cDNAs were a generous gift from Drs. Sissy Jhiang (Ohio State University) and Massimo Santoro (University of Naples), respectively. The cDNAs were subcloned into the pUHG 10–3 vector downstream of the heptamerized tetO and a minimal cytomegalovirus promoter. The RET/PTC1 cDNA was removed from the pBluescript vector using *Sac*II and *Eco*RV. The fragment was gel purified and then subcloned into the pUHG10–3 vector downstream of the heptamerized tetO and a minimal cytomegalovirus promoter, which had been cut with *Bam*HI, the overhang filled in with Klenow, and then digested with *Sac*II. The RET/PTC3 cDNA was removed from the pcDNA3 vector with *Hin*dIII and *Eco*RV and the overhangs filled in with Klenow. The gel-purified fragment was then ligated into the pUHG10–3 vector, which had been cut with *Hin*dIII and the overhangs filled in with Klenow. The RET/PTC2-PDZ mutant [C'574-PDZ; Susan Taylor, University of California, San Diego; (22)] was removed from the pcDNA3 vector with *Hin*dIII and *Xba*I. The overhang created by *Hin*dIII was filled in with Klenow and the gel-purified fragment subcloned into the pUHG10–3 vector, which had been cut with *Sac*II, overhang blunted with T4 polymerase, and then cut by *Xba*I. The RET/PTC3^{Y541F} mutant was created by site-directed mutagenesis of the RET/PTC3 pUHG10–3 vector as previously described (Knauf, J. A., M. Croyle, E. Kimura, and J. A. Fagin, submitted). The different constructs were then cotransfected with thymidine kinase-hygro, which contains the hygromycin resistance gene under the control of a minimal TK promoter, into the previously described cell line, PC-rtTA7, using the LipofectAMINE reagent (Life Technologies, Inc., Rockville, MD).

Northern and Western Blot Analyses

RNA was isolated using TRI reagent as directed by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). Northern blots containing 20 μg of total RNA isolated from cells treated with and without doxycycline for the indicated time were performed as described (36, 48), and hybridized with full-length human [^{32}P]deoxy-CTP-labeled RET/PTC3. Western blots of total cell lysates were performed as previously described (37), and probed with the indicated antibodies. Rabbit anti-RET and rabbit anti-PLCγ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal mouse anti-RET IgG was a gift from Dr. Yuri Nikiforov (University of Cincinnati). Rabbit anti-phospho-PLCγ and rabbit anti-phospho-CREB were purchased from Cell Signaling (Beverly, MA). Rabbit IgGs against the following proteins were purchased from Calbiochem (San Diego, CA): anti-CREB, anti-$G_i\alpha1/G_i\alpha2$, anti-$G_i\alpha3/G_0\alpha$, anti-$G_i\alpha3$, anti-$G_s\alpha$, and anti-Gβ IgGs.

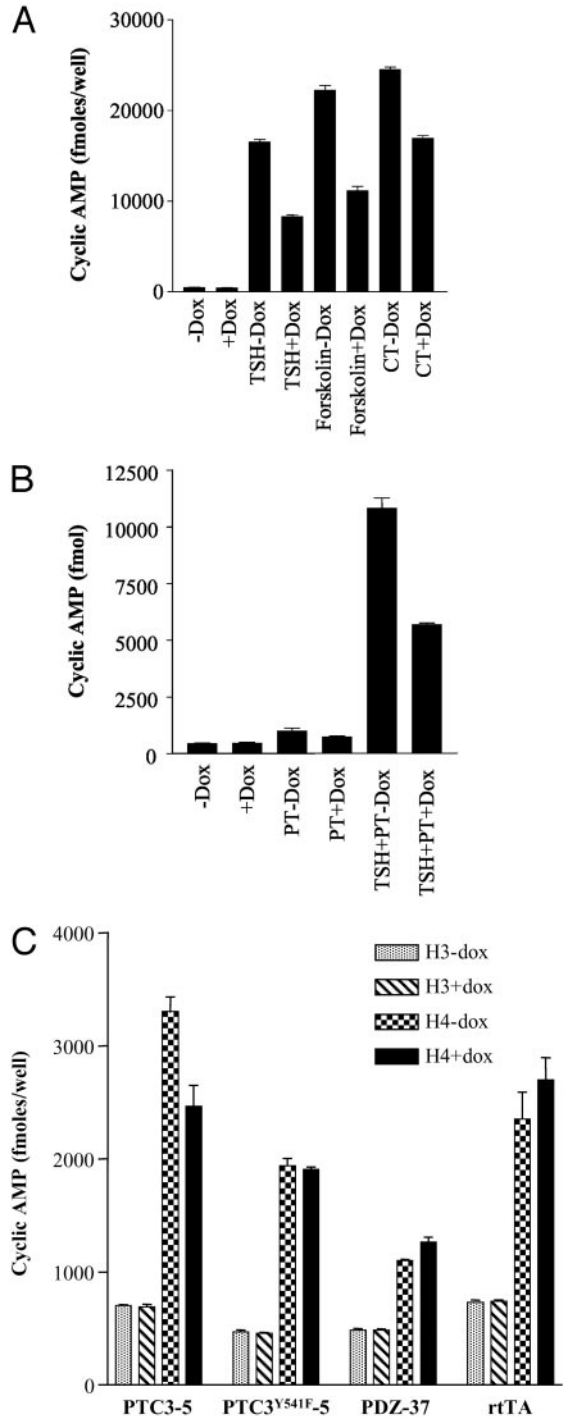

Fig. 8. Effects of RET/PTC Expression on cAMP Levels in PCCL3 Cells

Cells were incubated in the absence of TSH for 48 h and then doxycycline added for 24 h. A, Cells were then incubated with TSH (1 mIU/ml) or forskolin (25 μM) for 45 min or cholera toxin (2 μg/ml) for 24 h in the presence of 1 μM IBMX. B, Cells were incubated with or without TSH (1 mIU/ml) and with or without PT (2 μg/ml) for 45 min in the presence of 1 μM IBMX. Cell lysates were prepared in the presence of 1 μM IBMX and cAMP levels determined. C, The indicated cell lines were incubated with (H4) or without (H3) 1 mIU/ml TSH for 45 min in the presence of 1 μM IBMX. Cell lysates were prepared in the presence of 1 μM IBMX and cAMP levels determined. *Bars* represent the mean cAMP levels from a single experiment performed in triplicate. Similar results were obtained in at least two additional independent experiments.

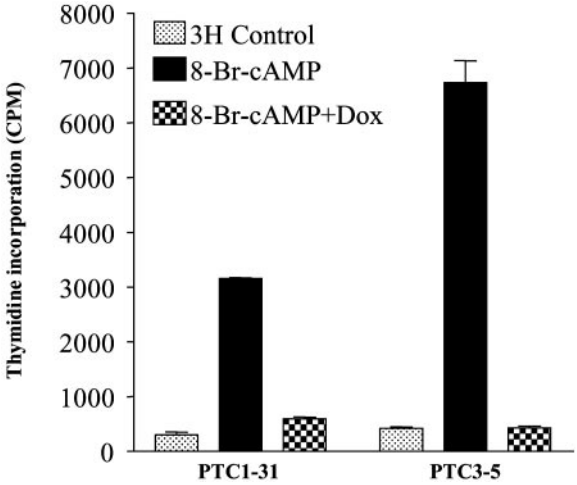

Fig. 9. Effects of RET/PTC Expression on cAMP Stimulated DNA Synthesis in PCCL3 Cells

Cells were incubated in the absence of TSH for 48 h and then doxycycline added for 24 h. Cells were then incubated with [^{3}H]thymidine for 24 h in the absence or presence of 1 mM 8-Br-cAMP. *Bars* represent the average ^{3}H incorporation from a single experiment performed in quadruplicate. Similar results were obtained in an additional independent experiment.

Dimerization of RET/PTC

Cell lysates from either RET/PTC1 or RET/PTC3-expressing cells treated with and without doxycycline (1 μg/ml) for 2 d were incubated with 0.02% glutaraldehyde at 95 C for 0, 1, 2, 4, 8, and 16 min to induce cross-linking. The samples were then resolved by SDS-PAGE (7.5%) and the presence of RET/PTC monomer and dimer assessed by immunoblotting with rabbit anti-RET antibody (Santa Cruz Biotechnology, Inc.).

Assays for RET/PTC Activity

RET/PTC in vitro kinase assay. RET/PTC3–5 cells were incubated with or without doxycycline for 24 h. Cells were then lysed in RIPA buffer [20 mM Tris pH 7.5; 150 mM NaCl; 5 mM EDTA; 1 mM EGTA; 5 mM NaF; 1% Nonidet P-40; 1% Tween 20; 1 mM sodium orthovanadate; protease inhibitor cocktail (Sigma, St. Louis, MO), 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol (DTT)] and RET/PTC3 immunoprecipitated from the cell lysate with rabbit anti-RET IgG. After washing the immunoprecipitate three times with RIPA buffer, it was resuspended in kinase reaction buffer (50 mM HEPES, pH 7.2; 5–8 μCi ^{32}P-γATP; 20 mM $MnCl_2$; 5 mM $MgCl_2$; 1 mM sodium vanadate; and 0.5 mM DTT) and incubated at 30 C for 30 min. The kinase reaction was terminated by the addition of stop buffer (10 mM $NaPO_4$, pH 7.0; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 1 mM sodium vanadate; 1 mM ATP; 5 mM EDTA; and 5 μg/ml aprotinin) and the immunoprecipitate washed three times with kinase buffer without ^{32}P-γATP. Samples were then subjected to SDS-

PAGE, transferred to nitrocelloluse, and autophosphorylation of RET/PTC detected using a phosphorimager (Amersham Biosciences, Sunnyvale, CA).

Phosphorylation of PLCγ

RET/PTC3–5 cells were incubated with or without doxycycline for 24 h and cells lysed in RIPA buffer. One hundred micrograms of protein lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either rabbit anti-PLCγ IgG (Santa Cruz Biotechnology, Inc.) or rabbit anti-phospho(Tyr783) PLCγ (Cell Signaling).

Growth Curves

Cells (5×10^4) were plated in six-well plates and incubated for 72 h without TSH (*i.e.* H3 media). Cells were then changed to either H3 or H4 medium in the presence or absence of doxycycline (1 μg/ml) for up to 16 d, with media changes every 2 d. At the indicated times, the cells were washed with PBS, removed from the wells by trypsinization, recovered by centrifugation, resuspended in PBS, and counted using a Z1 Coulter counter.

DNA Synthesis

Cells were plated at a density of 5×10^4 per well in 24-well plates, allowed to attach, and the medium replaced with H3 medium. Cells were then incubated for 3 d, and medium replaced with H3 medium with or without TSH (1 mIU/ml), forskolin (25 μM), or 8-bromo (Br)-cAMP (1 mM) in the presence or absence of 1 μg/ml doxycycline. Cell were incubated for 24 h and then 1 μCi/well of [^{3}H]thymidine was added. After 24 h cells were harvested to quantitate the [^{3}H]thymidine DNA incorporation. Briefly, wells were washed twice with PBS, once with 10% trichloroacetic acid, and once with 100% ethanol. After air-drying, 0.3 ml of 1 N NaOH were added to each well and the plates incubated overnight at 37 C. The content of each well was transferred into a scintillation vial containing 0.3 ml of 5 N HCl and 5 ml of scintillation cocktail and radioactivity counted using a Wallace 1410 liquid scintillation counter.

Cell Detachment Assay

Cells were incubated in H3 medium for 3 d and then medium changed to H3 with or without 1.0 μg/ml of doxycycline. At the indicated times, the medium was removed and the detached cells collected by centrifugation. The cell pellet was resuspended in PBS and counted utilizing a hemocytometer. Attached cells were washed with PBS, removed from the plates by trypsinization, recovered by centrifugation, and resuspended in PBS. The attached cell fraction was stained with trypan blue, and the percentage of alive and dead cells counted utilizing a hemocytometer. The overall number of dead cells was calculated by combining the number of cells in the detached fraction and the number of trypan blue-stained cells in the attached fraction.

Apo-BrdU Assay

Apo-BrdU assays were performed after the manufacturer's recommended procedure (PharMingen, San Diego, CA). Briefly, cells were incubated with 1% paraformaldehyde, fixed in 70% ethanol and then stored at −20 C until assayed. For the DNA labeling procedure, cells were washed and resuspended in a DNA labeling solution containing terminal deoxynucleotide transferase and Br-deoxyuridine triphosphatase and incubated at 37 C for 60 min. After labeling, cells were washed and stained with fluorescein-labeled anti-BrdU antibody for 30 min, and then treated with propidium iodide and ribonuclease A. Apo-BrdU-positive cells were evaluated by fluorescence-activated cell sorting analysis.

Real-Time RT-PCR for TSH-R mRNA

Cells were harvested at the indicated time and RNA isolated using TRI reagent as directed by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). One microgram of total RNA was reverse transcribed with 200 U of Superscript reverse transcriptase (Life Technologies, Inc., Grand Island, NY) in the presence of 2.5 μM of random 9-mer primers, 20 μM deoxynucleotide triphosphate for 60 min at 37 C, followed by a 5-min heat inactivation at 95 C. Two microliters of the cDNA reaction mixture were then used as a template in a PCR containing 1 μM of the primer pairs for amplification of either β-actin ($^{5'}$ctgaaccctaaggccaaccgtg$^{3'}$ and $^{5'}$ggcatacagggacagcacagcc$^{3'}$) or TSH-R ($^{5'}$caaagatgcctttggaggag$^{3'}$ and $^{5'}$agctctttgaggtgctccag$^{3'}$). PCR amplifications were performed using the Fast-Start DNA Master SYBR Green real-time PCR kit as directed by manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). The amplification conditions were optimized for the LightCycler instrument (Cepheid, Sunnyvale, CA) and subsequent PCR products were demonstrated to be a single PCR product by melting curve and electrophoretic analysis. To avoid potential problems from contaminating genomic DNA, PCR primers were designed to span a large intron whose location was determined by blasting the rat TSH-R sequence against the NCBI rat genome database. This was further verified by the lack of a signal when reactions were performed in the absence of reverse transcriptase. The cycle threshold (CT) value, which was determined using the second derivative, was used to calculate the normalized expression of the TSH-R mRNA using the *Q-Gene* program (49).

Cellular cAMP Levels

Cells were plated into 48-well plates, allowed to attach, and then medium replaced with H3 medium. Cells were incubated for 3 d and medium changed to H3 with or without 1 μg/ml doxycycline. Cells were incubated for 24 h and then treated with or without 1 mIU/ml TSH, 25 μM forskolin, 2.0 μg/ml PT, or 2.0 μg/ml cholera toxin in the presence of 1 μM isobutylmethylxanthine (IBMX) for the indicated times. Cells were harvested and solubilized in precooled *N*-propanol for 3 h. Aliquots were drawn into glass tubes, dried overnight, and resuspended in assay buffer for measurement of intracellular cAMP levels by RIA, after the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). cAMP levels were expressed as fmol/well.

Effects of RET/PTC3 on G Protein Subunit Levels

RET/PTC3 cells were grown in H4 medium until confluent and then medium was replaced with H3 medium. Cells were incubated for 3 d, medium replaced with H3 or H4 medium with or without 1 μg/ml doxycycline, and incubated for 24 h. Cells were then washed with 1× PBS and lysed in 300 μl of triple detergent buffer (50 mM Tris, pH 8; 150 mM NaCl; 100 μg/ml phenylmethylsulfonyl fluoride; 0.1% sodium dodecyl sulfate; 1% Nonidet P-40; 0.25% deoxycholate) containing the protease inhibitors tosyl phenylalanyl chloromethylketone and tosyl lysyl chloromethylketone. Fifty micrograms protein lysate were then subjected to SDS-PAGE and Western blotted with antibodies to the G protein subunits $G_i\alpha1$, G_iA2, $G_i\alpha3$, $G\beta$, $G_s\alpha$, and $G_o\alpha$.

Effect of RET/PTC3 on 8-Br-cAMP-Induced CREB Phosphorylation

RET/PTC3 cells were grown in H4 medium until confluent and then medium changed to H3 medium. Cells were incubated for 2 d and then medium replaced with fresh H3 medium with or without 1 μg/ml doxycycline. Cells were incubated for 24 h and then treated with or without 1.0 mM Br-cAMP for 0–6 min. Cells were then washed with 1$\times$ PBS and lysed in 300 μl of triple detergent buffer containing the protease inhibitors tosyl phenylalanyl chloromethylketone and tosyl lysyl chloromethylketone. Fifty micrograms of protein lysate were then subjected to SDS-PAGE and Western blotting for either phospho-CREB or total CREB.

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