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Gβγ Dimers Released in Response to Thyrotropin Activate Phosphoinositide 3-Kinase and Regulate Gene Expression in Thyroid Cells

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Signaling by TSH through its receptor leads to the dissociation of trimeric G proteins into Gs and Gβγ. Gs activates adenylyl cyclase, which increases cAMP levels that induce several effects in the thyroid cell, including transcription of the sodium-iodide symporter (NIS) gene through a mechanism involving Pax8 binding to the NIS promoter. Much less is known about the function of Gβγ in thyroid differentiation, and therefore we studied their role in TSH signaling. Gβγ overexpression inhibits NIS promoter activation and reduces NIS protein accumulation in response to TSH and forskolin. Conversely, inhibition of Gβγ-dependent pathways increases NIS promoter activity elicited by TSH but does not modify forskolin-induced activation. Gβγ dimers are being released from the Gs subfamily of proteins, because cholera toxin mimics the effects elicited by TSH, whereas pertussis toxin has no effect on NIS promoter activity. We also found that TSH stimulates Akt phosphorylation in a phosphoinositide 3-kinase (PI3K)-dependent and cAMP-independent manner. This is mediated by Gβγ, because its overexpression or specific sequestration, respectively, increased or reduced phosphorylated Akt levels upon TSH stimulation. Gβγ sequestration increases NIS protein levels induced by TSH and Pax8 binding to the NIS promoter, which is also increased by PI3K inhibition. This is, at least in part, caused by Gβγ-mediated Pax8 exclusion from the nucleus that is attenuated when PI3K activity is blocked. These data unequivocally demonstrate that Gβγ released by TSH action stimulate PI3K, inhibiting NIS gene expression in a cAMP-independent manner due to a decrease in Pax8 binding to the NIS promoter. (Molecular Endocrinology 22: 1183–1199, 2008)
change factors (cAMP-GEFs or Epac) that function as exchange factors for the small GTPases Rap1 (14, 15), Rap2 (16), and Ras (17). GTP-bound Rap1 can activate the kinases Raf-1, B-Raf, and c-Raf, leading into ERK1/2 or p38MAPK pathways. Ras activation by cAMP-GEF is of particular relevance because Ras can activate other kinase cascades such as the phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase-1 pathway, and it has been demonstrated that Ras is required for TSH-induced mitogenesis in thyroid cells (18). In human and rat thyrocytes, TSH can also stimulate the Gq/phospholipase C cascade (19), and in dog and human thyrocytes, TSH also activates Gi, which partially opposes the stimulation through Gs and is not related to TSH-mediated proliferation (12).

Gβγ has been demonstrated to regulate more than 20 effectors including phospholipases (20), adenyl cyclases (21), ion channels (22), G protein-coupled receptor kinases (23), and PI3Ks (24). G protein-coupled receptors (GPCRs) regulate through βγ dimers intracellular signals involved in cell growth and differentiation in different cell types. Through βγ dimers, GPCRs can activate the MAPK pathway (25–27) and the PI3Ks, a family of lipid kinases involved in multiple biological processes including cell proliferation and survival, cytoskeletal remodeling, and membrane trafficking (28). In thyrocytes, PI3K has a central role in controlling both cell proliferation and differentiation. PI3K is activated in thyrocytes by many growth factors such as insulin/IGF-I, hepatocyte growth factor (HGF), or epidermal growth factor (EGF) (29). Treatment with PI3K inhibitors or the expression of a dominant-negative form of PI3K causes a G1 arrest of rat thyroid cells stimulated to proliferate with TSH (30, 31), and it has been reported that PI3K is required for thyrocytes to proliferate upon TSH stimulation (32). Concerning differentiation of thyroid cells, we have reported that activation of PI3K by IGF-I inhibits the expression of the NIS stimulated by TSH/cAMP (33). In thyroid cells, the role of Gβγ subunits in the transmission of intracellular signals induced by GPCRs remains essentially unknown. This, together with the fact that not all the actions of TSH/TSHR can be explained by cAMP increments, led us to study the involvement of Gβγ dimers in TSH-dependent signaling and the regulation of gene expression in thyroid cells. We have found that TSH activates a cAMP-independent signaling cascade that increases PI3K activity and regulates NIS gene expression.

RESULTS

Overexpression of Gβγ Dimers Inhibits NIS Transcription

Upon TSH binding to its cell surface GPCR, G proteins dissociate into Gα and Gβγ subunits. It is well known that the stimulatory Gα subunit (Gαs) activates adenyl cyclase, leading to an increase in cAMP levels that in turn induce several actions in the thyroid cell, including stimulation of NIS gene expression. The role of the Gβγ subunits released by TSH is not known, but one of the putative effectors of Gβγ, the lipid kinase PI3K, has been found by our group to decrease NIS gene expression (33). To initiate the study of Gβγ function in the thyroid cell, we analyzed the effect of βγ dimers on NIS transcription stimulated by TSH or forskolin, an activator of adenyl cyclase that increases cAMP levels. For this purpose, PCCi3 cells were transfected with the reporter vector pNIS-2.8 carrying luciferase driven by the NIS promoter and increasing amounts of the expression vectors for Gβ1 and Gγ2 subunits, FLAG-Gβ1 and HA-Gγ2. After transfection, cells were maintained for 3 d in starvation medium and then left untreated or treated for another 24 h with TSH or forskolin. Figure 1A shows that overexpression of Gβγ dimers significantly reduces NIS promoter activity stimulated by TSH and forskolin in a dose-dependent manner, indicating that Gβγ signaling interferes with cAMP induction of NIS gene transcription. Immunodetection of one of the tagged components of the βγ dimer, the FLAG-β1 protein, is shown. Due to the unspecificity of hemagglutinin (HA) antibodies, the expression of HA-γ2 was demonstrated by RT-PCR using RNA preparations from an experiment performed under the same conditions (data not shown). We also wanted to study whether Gβγ could regulate endogenous NIS gene expression in PCCi3 cells. For this purpose, PCCi3 cells were transfected either with 5 μg each of FLAG-β1 and HA-γ2 or with the same amount of the control vector pcDNA3.1. After 5 d in starvation medium, cells were left untreated or treated with TSH or forskolin for 24 h and then harvested for Western blot analysis of NIS protein levels. The results are shown in Fig. 1B. NIS protein expression induced by TSH or forskolin is clearly less in cells overexpressing Gβγ dimers, compared with cells transfected with the control vector. NIS expression levels were quantified with respect to actin expression from three independent experiments; these normalized levels are graphically shown below a representative Western blot and indicate that the overexpression of Gβγ dimers significantly decreases NIS protein levels induced by TSH (P < 0.01) and forskolin (P < 0.001).

It should be noted that due to the long half-life of the NIS protein (34), cells must be kept in starvation medium for at least 5 d. Because TSH elicits survival signals in the cell and a prolonged deprivation of the hormone could render the cells apoptotic, we performed a cell cycle profile (data not shown) of cells maintained in starvation medium for up to 6 d to confirm that the results obtained are not due to a high rate of apoptotic cells under the conditions used in these experiments. The data obtained indicate that the percentage of apoptotic cells deprived of TSH for 6 d (sub-G1 = 2.91%) is similar to that of cells growing in control medium containing TSH and serum (sub-G1 = 0.20%). These data confirm that the experimental ap-
TSH Induces the Release of G\(\beta\gamma\) Dimers that Inhibit NIS But Not Tg Expression

Next we wanted to study whether endogenous G\(\beta\gamma\) dimers released in response to TSH could also regulate NIS transcription. To this end, we inhibited G\(\beta\gamma\) signaling by using two different G\(\beta\gamma\) scavengers, CD8-\(\beta\)ARK and pcCIS\(\alpha\)T-1 (G\(\alpha\)T). CD8-\(\beta\)ARK is a chimeric molecule containing the extracellular and transmembrane domains of CD8 fused to the carboxyl-terminal domain of \(\beta\)-adrenergic kinase (\(\beta\)ARK), which includes the high-affinity \(\beta\)-binding region of the kinase and thus acts as a G\(\beta\gamma\) scavenger (35). The \(\beta\)ARK peptide specifically inhibits G\(\beta\gamma\) signaling and is a useful tool to distinguish between G\(\alpha\) and G\(\beta\gamma\)-dependent pathways (36). G\(\alpha\)T is a vector that expresses the G protein \(\alpha\)-subunit of transducin, which also acts as a \(\beta\gamma\) scavenger. We discard the possibility of using RNA interference to deplete G\(\beta\) and G\(\gamma\) subunits expression because it has been reported that complete loss of expression of G\(\beta\gamma\) dimers also abolishes G\(\alpha\)-mediated pathways (37). Furthermore, the G\(\beta\) and G\(\gamma\) isoforms expressed in the thyroid and the specific forms released by TSH binding to its receptor are currently unknown. Expression of CD8-\(\beta\)ARK produced a dose-

Fig. 1. Regulation of NIS Gene Expression by G\(\beta\gamma\)

A, PCCl3 cells were transfected with 5 \(\mu\)g pNIS-2.8, 0.5 \(\mu\)g pRL-TK, and 0, 1, 3, or 5 \(\mu\)g each of FLAG-G\(\beta\)1 and HA-G\(\gamma\)2 and control vector pcDNA3.1 to keep the total amount of transfected DNA constant. After transfection, cells were maintained for 48 h in starvation medium and then left untreated (−) or treated with TSH or forskolin (Forsk) for 24 h. Cells were then harvested for luciferase and Renilla activity determinations. Relative luciferase activity is the light units normalized to Renilla activity derived from transfected pRL-TK to correct for transfection efficiency. NIS promoter activity is expressed as the fold induction over the basal levels (equal to 1) of untreated cells not transfected with FLAG-G\(\beta\)1 and HA-G\(\gamma\)2. The data represent the mean ± SD (n = 3) of one experiment, which is representative of at least three independent experiments. Statistical significance was evaluated by a two-tailed t test. Differences vs. cells not transfected with FLAG-G\(\beta\)1 and HA-G\(\gamma\)2 under the same treatment were considered significant at \(P < 0.05\); *, \(P < 0.05\); **, \(P = 0.01–0.001\). The same protein extracts were used for detection by Western blot analysis of FLAG-G\(\beta\)1 and actin shown below. B, PCCl3 cells transfected with 10 \(\mu\)g of pcDNA3.1 or 5 \(\mu\)g each of FLAG-\(\beta\)1 and HA-\(\gamma\)2 expression vectors. After transfection, cells were maintained for 5 d in starvation medium and were then left untreated (−) or treated with TSH or forskolin for 24 h. Cells were then harvested and total protein extracted for immunoblotting with anti-NIS antibody. Anti-Flag antibody was used to control transfection efficiency and anti-actin antibody as a loading control. The panel shows a representative Western blot, and the graphic below is the quantification of NIS protein levels normalized to the loading control. NIS relative protein levels are expressed as the fold induction over the basal levels (equal to 1). Values represent the average of three different experiments ± SD. Statistical significance was evaluated by a two-tailed t test. Differences vs. cells transfected with the control vector under the same treatment were considered significant at \(P < 0.05\); **, \(P = 0.01–0.001\); ***, \(P < 0.001\).

The approach is reliable and that apoptosis does not appreciably affect the results obtained.

**TSH Induces the Release of G\(\beta\gamma\) Dimers that Inhibit NIS But Not Tg Expression**

Next we wanted to study whether endogenous G\(\beta\gamma\) dimers released in response to TSH could also regulate NIS transcription. To this end, we inhibited G\(\beta\gamma\) signaling by using two different G\(\beta\gamma\) scavengers, CD8-\(\beta\)ARK and pcCIS\(\alpha\)T-1 (G\(\alpha\)T). CD8-\(\beta\)ARK is a chimeric molecule containing the extracellular and transmembrane domains of CD8 fused to the carboxyl-terminal domain of \(\beta\)-adrenergic kinase (\(\beta\)ARK), which includes
dependent increase in the stimulation of NIS transcription by TSH; however, CD8-βARK expression did not produce any significant change in the stimulation by forskolin (Fig. 2A, left and middle panels). The same results were obtained when GaT was transfected and cells were treated with TSH (Fig. 2A, right panel) or forskolin (not shown). These findings indicate that Gβγ subunits released after TSH treatment inhibit NIS transcription. As expected, this effect was not observed when cells were treated with forskolin, which directly activates adenyl cyclase and cAMP production but does not induce Gβγ release. Although it does not affect the interpretation of the results, it is interesting to note that we observe an increase in CD8-βARK expression levels when the cells are stimulated with TSH or forskolin; this is possibly due to the differences between the expression machinery of quiescent and active cells (38) (Fig. 2A, lower panels). Because it has been reported that the TSHR is able to couple to members of the four G protein families, it is interesting to determine from which G protein subtype the Gβγ dimers that decrease NIS expression are being released. TSH transmits its signal into the cell mainly through Gs proteins (1), but it has been reported that high nonphysiological doses of TSH can activate the Gq/phospholipase C pathway in rat (39) and human thyroid (40) cells. Despite this high concentration of hormone, the activation of this pathway is always very weak (1, 39). Because we are using hormone concentrations at physiological levels, the Gq family of proteins can be ruled out as a potential Gβγ donor in our experiments.

Coupling of the TSHR to Gi proteins has also been reported (11). Inhibition of Gi signaling using pertussis toxin (PTX) increases cAMP accumulation in response to TSH in human thyroid slices (10) but not in rat PCCl3 (41) or FRTL-5 (42) cells.

![Graph](image)

**Fig. 2.** Effects of Gβγ Sequestration and PTX or CTX Treatment on NIS Gene Expression

A, PCCl3 cells were transfected with 5 μg pNIS-2.8, 0.5 μg pRL-TK, and 0, 1, 3, or 5 μg CD8-βARK or pCISGnT1 and control vector CD8 to keep the total amount of transfected DNA constant. After transfection, cells were maintained for 48 h in starvation medium and left untreated (−) or treated with TSH or forskolin (Forsk) for 24 h. Cells were then harvested for luciferase and Renilla activity assays; NIS promoter activity was determined as described in the legend of Fig. 1A. Statistical significance was evaluated by a two-tailed t test. Differences vs. cells transfected with the control vector under the same treatment were considered significant at P < 0.05; ns, not significant (P > 0.05); **, P = 0.01–0.001; ***, P < 0.001. The same protein extracts were used for Western blot detection of CD8-βARK and actin shown below. B, PCCl3 cells were transfected with 5 μg pNIS-2.8, 0.5 μg pRL-TK (both panels), and 5 μg CD8-βARK or CD8 control vector (right panel). After transfection, cells were maintained for 48 h in starvation medium and treated with TSH or CTX (right panel) for 24 h. PTX was added 24 h before TSH treatment (left panel). Cells were then harvested for luciferase and Renilla activity assays; NIS promoter activity was determined as described in the legend of Fig. 1A. Statistical significance was evaluated by a two-tailed t test. Differences vs. cells in the absence of PTX (left panel) or vs. cells transfected with the control vector under the same treatment (right panel) were considered significant at P < 0.05; ns, not significant (P > 0.05); **, P = 0.01–0.001; ***, P < 0.001.
If G\(\beta\gamma\) dimers released in response to TSH are coming from the activation of Gi, there should be an increase in the activity of the NIS promoter when Gi coupling to the TSHR is blocked using PTX. We show in Fig. 2B, left panel, that TSH promotes an increase in the activity of the NIS promoter, but there is no further significant increase when the cells are stimulated in the presence of PTX. These data indicate that, at least in these cells, TSH is not promoting the activation of Gi proteins at the concentrations used.

We have also performed an experiment using cholera toxin (CTX) as a source of G\(\beta\gamma\) dimers specifically released from Gs proteins. Treatment of PCCl3 cells with CTX elicits an increase in the activity of the NIS promoter to a similar extent as TSH. Moreover, CTX treatment of PCCl3 cells transfected with \(\beta\)ARK promotes a further and significant increase (\(P = 0.01–0.001\)) in the activity of the NIS promoter in the same way as in cells stimulated with TSH (Fig. 2B, right panel). Together, these results show that G\(\beta\gamma\) dimers that decrease NIS promoter activation in response to TSH are released upon Gs protein activation.

To analyze how G\(\beta\gamma\) sequestration influences endogenous NIS protein expression, PCCl3 cells were transfected with CD8-\(\beta\)ARK or a vector carrying CD8 only. After 5 d in starvation medium, cells were left untreated or treated with TSH or forskolin for 24 h and then harvested for Western blot analysis. Figure 3A shows that induction of NIS protein levels by TSH in cells expressing CD8-\(\beta\)ARK is significantly (\(P = 0.01–0.001\)) higher than in cells treated with the control CD8 vector. This difference was not found when cells were treated with forskolin; i.e. NIS protein levels were similar in cells transfected with CD8-\(\beta\)ARK and with the CD8-vector. Different results were obtained when Tg protein levels were analyzed. Tg protein expression was induced by TSH and forskolin to the same extent, and both effects were not significantly altered by CD8-\(\beta\)ARK transfection. The induction levels were quantified with respect to actin or Sp1 expression and are shown below a representative Western blot. We also analyzed the activity of the Tg promoter in cells expressing CD8-\(\beta\)ARK. The results shown in Fig. 3B indicate that G\(\beta\gamma\) subunits released by TSH do not significantly inhibit Tg transcription.

Thus, G\(\beta\gamma\) dimers released in response to TSH inhibit NIS gene expression but do not interfere with Tg expression, demonstrating that signaling pathways...
downstream from Gβγ differentially regulate thyroid gene expression. This effect was not due to variations in cAMP levels, because overexpression or sequestration of Gβγ dimers did not have any apparent effect on the fold induction of cAMP in response to TSH (Fig. 4A). The absence of apparent changes in cAMP induction levels was also demonstrated transfecting PCCl3 cells with a luciferase reporter construct containing three cAMP response elements (CRE) in tandem (Fig. 4B).

TSH Induces Akt Phosphorylation through PI3K

PI3K has been reported to be one of the main targets of Gβγ signaling initiated by GPCRs. Because we found in a previous study that PI3K down-regulates NIS gene expression (33), we decided to study whether PI3K is an effector of Gβγ subunits in thyroid cells, able to inhibit NIS expression. First of all, we analyzed PI3K activation by TSH, forskolin, and IGF-I in PCCl3 cells. To this end, we analyzed by Western blot the phosphorylation level of the PI3K target Akt. Figure 5A shows that TSH induces Akt phosphorylation in a time-dependent manner, with a maximum stimulation between 30 min and 1 h. Forskolin also increased phosphorylated Akt (pAkt) levels although to a lesser extent than TSH. pAkt levels after 15 min of IGF-I treatment are shown as a positive control and indicate that Akt phosphorylation in response to TSH, although clearly detectable, is not as high as the strong signal elicited by IGF-I. Induction of Akt phosphorylation by either TSH or forskolin, at short times (30 min) or for a longer period of 24 h, was inhibited by LY294002 (LY) (Fig. 5B), indicating that PI3K is mediating Akt phosphorylation in response to TSH and forskolin. Total Akt levels were not altered among the different experimental conditions, indicating that changes in the phosphorylation status of Akt are not due to an increase in total Akt protein.

It has been suggested that some of the effects observed in thyroid cells in response to TSH, such as PI3K/Akt activation, could be due to growth factor contamination of the TSH preparation. Experiments shown up to now in the present study were performed using bovine pituitary TSH. To rule out the possibility of growth factor contamination, we analyzed the effect of human recombinant TSH (hrTSH) both on NIS transcription (Fig. 5C) and on Akt phosphorylation (Fig. 5D). NIS promoter activity was similar in cells treated with hrTSH and in cells treated with bovine TSH. In addition, hrTSH also stimulates Akt phosphorylation in a time-dependent manner. We confirmed that the increase in pAkt levels induced by hrTSH is due to PI3K activation using two different PI3K inhibitors: LY294002 (LY), at concentrations ranging from 1–20 μM, and wortmannin (Wort), at concentrations from 25–100 nM. Western blot analyses of pAkt and total Akt levels are shown in Fig. 5E.

![Image](318x383 to 526x709)

Fig. 4. Effect of Gβγ on TSH-Induced cAMP Production

A, PCCl3 cells were transfected with 5 μg pcDNA3.1, 3 or 5 μg CD8-βARK, or 3 μg of both FLAG-Gβ1 and HA-Gγ2 expression vectors. Transfected cells were maintained for 48 h in starvation medium and treated with 0.5 mM IBMX for 30 min, followed by 1 nM TSH treatment for 1 h. Cells were then harvested for determination of cAMP levels. cAMP induction in response to TSH is shown as the fold induction over basal levels (equal to 1) of starved cells (−). The data represent the mean ± SD (n = 3) of one experiment, which is representative of three independent experiments. Statistical significance was evaluated by a two-tailed t test. Differences of TSH-treated, expression vector-transfected cells vs. cells transfected with the control vector and treated with TSH were not significant (ns, P > 0.05). B, PCCl3 cells were transfected with 5 μg 3XCRE-LUC reporter, 0.5 μg pRL-TK, 5 μg pcDNA3.1, and 3 or 5 μg CD8-βARK or 5 μg of both FLAG-Gβ1 and HA-Gγ2 expression vectors. After transfection, cells were maintained for 48 h in starvation medium and were then left untreated or treated with TSH for 24 h. Cells were then harvested for luciferase and Renilla activity assays. Relative luciferase activity is the value of light units normalized to Renilla activity derived from transfected pRL-TK to correct for transfection efficiency. 3XCRE-LUC activity is expressed as the fold induction over basal levels (equal to 1) of starved cells (−). The data represent the mean ± SD (n = 3) of one experiment, which is representative of three independent experiments. Statistical significance was evaluated by a two-tailed t test. Differences vs. cells transfected with the control vector were not significant (ns, P > 0.05).
In view of these results, it can be concluded that TSH stimulates Akt phosphorylation in PCCl3 cells through PI3K activation.

TSH Induces Akt Phosphorylation in a cAMP-Independent Manner

Stimulation of PI3K by TSH in thyroid cells and the involvement of cAMP in this signaling pathway are controversial. The results reported up to date show that PI3K activation by TSH depends on the thyroid cell type studied. When this pathway is active, it has been suggested that cAMP is mediating the stimulation of PI3K by TSH. In the present study, we wanted to analyze whether cAMP is mediating the signal initiated by TSH to activate PI3K in PCCl3 cells. For this purpose, we used two different inhibitors that act at different levels in the cAMP signaling pathway. The first one, H89, inhibits the activity of PKA, one of the most important targets of cAMP. We have analyzed the effect of H89 on the phosphorylation of Akt by TSH in PCCl3 cells. The results are shown in Fig. 6A. Pretreatment with H89 increases basal pAkt levels as reported (43) and does not significantly affect TSH and forskolin stimulation of Akt phosphorylation. The induction levels were quantified with respect to total Akt expression and are shown below a representative Western blot.

The second inhibitor used was adenosine 3',5'-cyclic monophosphorothioate, Rp-isomer, triethylammo-
nium salt (Rp-cAMP), a specific competitor of cAMP for its binding site that avoids cAMP activation of the different cAMP targets, including PKA. Cells were treated with 10 and 30 μM Rp-cAMP and then treated with TSH for 30 min. The presence of Rp-cAMP does not prevent stimulation of Akt phosphorylation by hrTSH at the different concentrations used (Fig. 6B). To demonstrate that Rp-cAMP is active in these conditions, we analyzed the phosphorylation state of CRE-binding protein (CREB), a transcription factor that is phosphorylated in response to cAMP increases. The results show that both TSH- and forskolin-induced CREB phosphorylation is strongly diminished in the presence of 30 μM Rp-cAMP (Fig. 6, B and C, respectively), indicating that Rp-cAMP at this concentration is inhibiting cAMP signaling. Taken together, these results demonstrate that cAMP does not completely mediate TSH-dependent stimulation of Akt phosphorylation in PCC13 cells.

**Gβγ Subunits Released by TSH Action Stimulate Akt Phosphorylation**

The results reported above indicate that TSH can activate the PI3K pathway in a cAMP-independent manner. In view of these findings, the question arises whether PI3K is an effector of Gβγ dimers released by the action of TSH in PCC13 cells. To answer that question, we analyzed the effect of βγ overexpression or sequestration on pAkt levels induced by TSH, forskolin, or IGF-I. Figure 7A shows that overexpression of βγ subunits induces phosphorylation of Akt. On the other hand, transfection of CD8-βARK inhibits Akt phosphorylation induced by TSH (Fig. 7B). As expected, this effect was not observed in cells treated with forskolin or after treatment with IGF-I, a growth factor that mediates PI3K/Akt activation through its binding to tyrosine kinase receptors not coupled to G proteins (Fig. 7B). In conclusion, these results indicate that TSH can stimulate Akt phosphorylation through the release of Gβγ dimers.

**Gβγ Release Induced by TSH Reduces Pax8 Binding to the NIS Promoter**

Our data clearly show that Gβγ dimers repress NIS promoter activity, and therefore, we explored the mechanism involved in this inhibition. We focused our attention on the transcription factor Pax8 because it is the main mediator of NIS transcription (6). First we determined Pax8 protein levels in PCC13 cells transfected with the CD8 expression vector, as a control, or with the CD8-βARK expression vector. As shown in Fig. 8A, TSH (P = 0.01–0.001) and forskolin (P < 0.001) treatment significantly increased Pax8 protein levels compared with untreated cells. Sequestration of Gβγ dimers by expression of CD8-βARK did not sig-

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**Fig. 6. Role of cAMP and PKA in TSH- and Forskolin-Induced Akt Phosphorylation**

PCC13 cells were maintained for 72 h in starvation medium and then left untreated or treated with TSH or forskolin (Forsk) for 30 min. A, The PKA inhibitor H89 was added at 10 μM 1 h before TSH or forskolin treatment. The panel shows a representative Western blot, and the graphic below shows the quantification of pAkt protein levels normalized to Akt1/2 protein levels. The pAkt relative protein levels are expressed as the fold induction over basal levels (equal to 1) of starved cells (−). Values represent the average of three different experiments ± SD. Statistical significance was evaluated by a two-tailed t test. Differences between similarly treated cells in the absence or presence of H89 were considered significant at P < 0.05; ns, not significant (P > 0.05); *, P < 0.05. B, Different concentrations of the cAMP competitor Rp-cAMP were added 1 h before hrTSH treatment for 30 min. C, Rp-cAMP (30 μM) was added 1 h before treatment with 1 μM forskolin for 30 min. The immunoblots were probed with anti-pAkt, Akt1/2, pCREB, and CREB1 as indicated.
significantly affect Pax8 protein levels compared with the cells transfected with the control vector.

Because G\(\beta\gamma\) dimers sequestration did not modify Pax8 protein levels, we determined whether under these conditions Pax8 binding to its site within the NUE in response to TSH or forskolin is changed. To this end, PCCl3 cells were transfected with the CD8 or the CD8-\(\beta\)ARK expression vectors, maintained for 3 d in starvation medium, and were then left untreated or treated with TSH or forskolin (Forsk) for 30 min (A and B) or with IGF-I for 15 min (B). Cells were then harvested for total protein extraction and \(\alpha\)-K detection by Western blot. Membranes were hybridized with anti-FLAG (A) or anti-CD8 (B) antibodies to show FLAG-G\(\beta\)1 and CD8-\(\beta\)ARK expression, respectively. Anti-Akt1/2 detection was used as a loading control. Western blots representative of at least three independent experiments are shown.

**DISCUSSION**

In the present study, we demonstrate a role for G\(\beta\gamma\) dimers in TSH-dependent signaling in thyroid cells. G\(\beta\gamma\) dimers released after TSH treatment lead to PI3K/Akt activation and regulate NIS gene expression in PCCl3 cells. In a previous study (33), we reported an increase of TSH-dependent induction of NIS gene expression in the presence of the PI3K inhibitor LY294002. Those results suggested the existence of a pathway from TSH to PI3K that inhibits NIS gene expression. Here, we confirm this suggestion specifically at the transcriptional level, affecting Pax8 binding to the NUE. This effect occurs only when cells are treated with TSH but not with forskolin, which does not induce G\(\beta\gamma\) release, and is reverted when PI3K is inhibited. This confirms that G\(\beta\gamma\) dimers released upon TSH binding to its receptor are mediating a decrease of Pax8 binding to the NIS promoter that is transmitted by the PI3K pathway.

The effect of G\(\beta\gamma\) dimers on Pax8 localization was studied by immunofluorescence (Fig. 9). In cells cultured in the absence of TSH for 2 d, Pax8 is barely expressed. When these cells are treated with TSH for 24 h, Pax8 expression is increased and is predominantly located in the nucleus and not observed in the cytoplasm. The transfection with G\(\beta\)1 and G\(\gamma\)2 subunits expression vectors induces a significant exclusion of Pax8 from the nucleus to the cytoplasm, an effect that was impaired by LY294002 pretreatment. G\(\beta\gamma\) sequestration by \(\beta\)ARK transfection retains Pax8 into the nucleus.

**Fig. 7. G\(\beta\gamma\) Effect on Akt Phosphorylation**
PCCl3 cells were transfected with 5 \(\mu\)g each of FLAG-G\(\beta\)1 and HA-G\(\gamma\)2 or with 10 \(\mu\)g pcDNA3.1 (A) or with 5 \(\mu\)g CD8-\(\beta\)ARK or CD8 control vector (B). After transfection, cells were maintained for 48 h in starvation medium and were then left untreated or treated with TSH or forskolin (Forsk) for 30 min (A and B) or with IGF-I for 15 min (B). Cells were then harvested for total protein extraction and \(\alpha\)-K detection by Western blot. Membranes were hybridized with anti-FLAG (A) or anti-CD8 (B) antibodies to show FLAG-G\(\beta\)1 and CD8-\(\beta\)ARK expression, respectively. Anti-Akt1/2 detection was used as a loading control. Western blots representative of at least three independent experiments are shown.
impairing Gβγ dimers signaling by using two different
Gβγ scavengers, CD8-βARK and GaT. Expression of
these molecules results in a further activation of the
NIS promoter elicited by TSH demonstrating unequiv-
ocally a role of Gβγ.

Most of our study has been performed with the spe-
cific scavenger CD8-βARK, a construct that expresses a
chimeric protein containing not only the C-terminal do-
main of the βARK1 protein, where the Gβγ binding do-
main is located but also the extracellular and transmem-
brane domains of the CD8 protein that is targeted to the
membrane where it is able to interact with and sequester
Gβγ dimers, making it an effective and specific scaven-
ger (35). It has been clearly established that the C-termi-
nal βARK peptide discriminates between Gβγ- and Gα
mediated pathways (35, 36, 44, 45). The specificity of the
GaT as a Gβγ scavenger has also been previously tested
(44–48).
der a confocal microscope using a mounted on Vectashield containing DAPI and observed un-
secondary antibodies immunostaining. Cells were then CD8 antibodies followed by Alexa 488 and TexasRed 546
itor was added 1 h before TSH addition. After treatment, cells untreated or treated with TSH for 24 h. LY294002 (LY) inhib-
10

The data are the mean ± SD of three independent experi-
ments. A 0- to 50-μm scale is shown in the first picture of the third column. Bottom panel, Quan-
titative analysis (%) of successfully transfected cells that present a consistent Pax8 cytoplasmic localization. Five differ-
ent fields were randomly chosen from each experiment. The data are the mean ± SD of three independent experi-
ments.

This effect is not observed in cells treated with the adenylly cyclase activator forskolin, indicating that the Gβγ dimers coupled to the TSHR are activating signal-
ing pathways leading to NIS gene repression. Although overexpressed Gβγ could bind to Gαs, thereby inactivating it and decreasing cAMP levels, we believe that the effects observed are not due to a decrease in cAMP because this inhibitory effect was also observed in forskolin-treated cells; forskolin acts downstream of Gαs and directly activates adenylly cyclase. Thus, in-
activation of Gαs subunits by Gβγ overexpression should not interfere with forskolin induction of cAMP elevation. In addition, the induction levels of cAMP in response to TSH are the same in cells overexpressing Gβγ as in cells expressing the scavenger βARK. Thus, we can conclude that Gβγ inhibition of TSH-induced NIS transcription is due to a direct action of Gβγ and not to decreased cAMP levels.

Although much work has been done to study the signaling pathways involved in thyroid cell prolifera-
tion, little is known concerning signaling cascades that regulate thyroid cell differentiation other than the cAMP/PKA pathway. Other factors such as insulin/IGF-I have been reported to increase TSH stimulation of Tg, TPO, and TSHR expression (49–51), but as we reported recently, NIS gene expression is inhibited by insulin/IGF-I, and this effect is mediated in part by PI3K (33). Thus, PI3K appears to regulate Tg and NIS gene expression in different ways.

Gβγ dimers have been reported to regulate a large number of effectors, including PI3Ks (24). PI3Ks con-
stitute a large family of lipid kinases that have been divided into three classes. They phosphorylate the D3 position of the inositol ring of phosphoinositides, generating phospholipids that are involved in cellular func-
tions including chemotaxis, differentiation, glucose homeostasis, proliferation, survival, and trafficking (28, 52). These lipid kinases are under tight control of cell surface receptors, including receptor tyrosine kinases and GPCRs (53). The most studied PI3Ks are those belonging to class I. Class IA enzymes consist of a p110 catalytic subunit complexed to a regulatory sub-
unit, p85, and signal downstream of tyrosine kinases and Ras, although an activation of p110β by GPCRs through Gβγ dimers has been reported (54). Class IB PI3K consists of the p110γ catalytic subunit complexed to the p101 regulatory subunit and signals downstream of GPCRs and Ras. GPCRs activate PI3Kγ and PI3Kβ through direct interaction with Gβγ. The results we present here indicate that TSH can activate the PI3K/Akt pathway through Gβγ dimers and that this pathway is interfering with the activation of NIS gene expression in thyroid cells by cAMP. In the thyroid cell, PI3K plays a critical role in controlling cell proliferation (30, 31). Several growth factors have been shown to activate the PI3K pathway (29), and inhibition of PI3K activity leads to a G0/G1 arrest of thyroid cells (30, 31). Depending on the cell type, opposing results have been reported concerning the activation of PI3K/Akt pathway by TSH (29). It has been suggested that PI3K/Akt activation by TSH could be due to contam-
inants of the TSH preparation. To address this ques-
tion we used hrTSH and we observed that hrTSH induces Akt phosphorylation in a PI3K-dependent manner. In addition, Akt phosphorylation induced by TSH was abolished when cells were incubated in the presence of an anti-TSHR antibody (data not shown). Taken together, these data unequivocally demonstrate that TSH increases Akt phosphorylation at 10–15 min,
reaching its maximal effect at 30-60 min. At earlier time points, almost undetectable changes can be observed in our experimental conditions (data not shown), which considerably differ from those used by Lou et al. (55). These authors observed a decrease in Akt phosphorylation after TSH treatment, but cells were cultured in the presence of insulin, which strongly activates the PI3K pathway. Furthermore, cell cultures were deprived of serum and TSH for only 16 h, whereas our starvation period is much longer (2–3 d), and therefore, basal pAkt levels are lower. Thus, the induction of Akt phosphorylation observed in our experiments is due only to TSH action. This activation does not appear to be mediated by PKA and cAMP signaling, because pretreatment with H89 (an inhibitor of PKA activity) or Rp-cAMP (an analog competitor of cAMP that inhibits signaling downstream of cAMP, including PKA) does not affect TSH induction of Akt phosphorylation. We found that forskolin is also able to induce PI3K-dependent Akt phosphorylation but to a much lesser extent than TSH. In view of these results, we cannot rule out the existence of a cAMP-PKA/PI3K pathway in PCCl3 cells, as has been demonstrated in FRTL5 cells (56), but it does not seem to be the only signaling pathway promoting stimulation of PI3K by TSH. No doubt, both PKA and PI3K are playing crucial roles in the regulation of thyroid cell proliferation and differentiation, and the reported opposite effects of PKA on PI3K activity reflect the complexity of the signaling cascades and the processes these kinases are controlling. Much work needs to be done to unravel whether this complex regulation is explained by different PKA and PI3K isoforms. Thus, our results describe a new signaling pathway leading to PI3K activation in response to TSH, involving Gβγ dimers and occurring in a cAMP-independent manner. This new pathway regulates thyroid differentiation and thus thyroid function.

To further address the mechanism of inhibition of NIS gene expression by Gβγ, we analyzed the levels of Pax8 and its binding to the rat NIS promoter. Pax8 is the main transcription factor regulating NIS gene expression. It has been demonstrated that Pax8 binding to this region is required for the TSH/cAMP induction of NIS transcription. Here we show that TSH induction of Pax8 binding to the NIS promoter increases when Gβγ signaling is inhibited by overexpression of Gβγ scavengers. On the other hand, Gβγ sequestration does not affect the binding of Pax8 induced by forskolin, reflecting the results obtained when promoter activity was analyzed. The amount of Pax8 bound to the NIS promoter in cells treated with TSH also increases when PI3K is inhibited by LY294002, indicating that signals leading to PI3K activation diminish Pax8 binding activity to the NIS promoter. We can conclude that the release of Gβγ dimers induced by TSH activates PI3K in PCCl3 cells and down-regulates NIS transcription by decreasing Pax8 binding to the NIS promoter.

The mechanism involved in the regulation of Pax8 binding to NIS promoter is still not well defined. Pax8 is a phosphoprotein whose phosphorylation seems to be not dependent on cAMP pathway activation (57). However, the Pax8 activation domain is dependent upon the catalytic subunit of the PKA and it has been proposed that this dependence involves a hypothetical adaptor that is targeted by PKA and interacts with the activating domain of Pax8 (58). Concerning the regulation of Pax8 binding by other kinases, such as PI3K, the only data reported so far are the ones described here. We think that the situation could be similar to the one reported for cAMP/PKA, because Pax8 phosphorylation seems to be independent of PI3K activation (unpublished data). In fact, our preliminary results suggest that the inhibition of Pax8 binding to the NIS promoter by PI3K/Akt is mediated by the interaction of Pax8 with the transcription factor FoxO1, a forkhead factor activated by the IGF-I/PI3K/Akt pathway (59). These preliminary results raise interesting questions regarding the regulation of Pax8 activity. Along with this line, we report here that Pax8 is excluded from the nucleus when Gβγ dimers are over-expressed, and this effect is partly impaired when PI3K is inhibited. This is an interesting observation that remains to be studied in detail, although we hypothesize that FoxO1 could also be involved, because it has been described that FoxO1 is excluded from the nucleus after phosphorylation by PI3K (60). The new pathway described here does not seem to be involved in the regulation of expression of Tg, another thyroid differentiation marker. Like NIS expression, Tg gene expression is stimulated by TSH/cAMP, but contrary to what happens for NIS, Pax8 is not the main transcription factor regulating the expression of Tg. Moreover, IGF-I differentially regulates TSH-induced Tg (49) and NIS (33) expression. Thus, it would be expected that a decrease in Pax8 DNA binding activity and/or PI3K activation would not affect the expression of the Tg gene.

In view of the results presented here, we can conclude that TSH activates the PI3K/Akt pathway not only in a cAMP-dependent manner, as was reported previously (30, 61), but also by a cAMP-independent mechanism that involves Gβγ subunits. We demonstrate that Gβγ subunits induce Akt phosphorylation and interfere with NIS gene expression induced by TSH and forskolin. Down-regulation of NIS gene expression by Gβγ dimers occurs at the transcriptional level, because Gβγ dimers reduce the ability of Pax8 to bind the NIS promoter and induce the exclusion of Pax8 from the nucleus. These results demonstrate a functional role for Gβγ dimers in thyroid cell differentiation, and more studies are needed to clarify the mechanism underlying the inhibition of Pax8 DNA binding and its exclusion from the nucleus. On the other hand, the diversity of signaling pathways activating PI3K in the thyroid cell (summarized in Fig. 10) suggests the involvement of different PI3K subtypes. Future studies will be focused on the identification of
MATERIALS AND METHODS

Materials

hrTSH was kindly provided by Genzyme (Madrid, Spain). Tissue culture media, bovine TSH, BSA, anti-Flag antibody, PTX, and bovine insulin were purchased from Sigma Chemical Co. (St. Louis, MO). IGF-I was obtained from Peprotech (Rocky Hill, NJ). Forskolin, H89, Rp-cAMP, LY294002, wortmannin, 3-isobutyl-1-methylxanthine (IBMX), anti-CREB-1, and anti-pCREB antibodies were purchased from Calbiochem (La Jolla, CA). Donor calf serum and DMEM were obtained from Life Technologies, Inc. (Gaithersburg, MD). cAMP, enzyme immunoassay Biotrak (EIA) System (dual range) was from Amersham, General Electric Healthcare (Buckinghamshire, UK). The dual-luciferase reporter assay system and the products for the RT-PCR were purchased from Promega Corp. (Madison, WI). Restriction enzymes were obtained from Invitrogen (Paisley, UK); streptavidin-horseradish peroxidase conjugate, anti-Akt1/2, anti-CD8α, anti-Sp1, and anti-actin antibodies, and Luminol detection reagent were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-pAkt (pSer472/473/474) was from Pharmingen (San Diego, CA). Anti-Tg antibody was from Dako (Glostrup, Denmark), and anti-Pax8 antibody was from BioPat (Piedimonte Matese, Italy). Vectashield mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, Inc. (Burlingame, CA). Anti-NIS antibody was a generous gift from Dr. N. Carrasco (Albert Einstein College of Medicine, Bronx, NY). CTX was kindly provided by Dr. M. J. Toro (Universidad de Alcalá de Henares, Madrid, Spain).

Plasmids

pRL-TK, which contains a cDNA coding for Renilla, was used to monitor transfection efficiency. The reporter constructs used were: pNIS-2.B-Luc, which contains a 2.854-bp DNA fragment of the rat NIS promoter (33); hTGenh/prm-Luc, which contains the human Tg promoter/enhancer (64), and 3xCRE-Luc, which contains three CREs in tandem (65). N-terminal FLAG-tagged human G-protein γ1 subunit and N-terminal HA-tagged human G-protein γ2 subunit, cloned into pcDNA3.1+, were obtained from the Guthrie cDNA Resource Center (Sayre, PA). pCISGpT-1, an expression vector for the γ-subunit of bovine transducin, CD8-ARK, an expression vector containing the extracellular and transmembrane domain of the CD8 lymphocyte-specific receptor and the carboxyl-terminal fragment of human γARK1 (35), were kindly provided by Dr. F. Mayor, and Dr. C. Murga (Centro de Biología Molecular, Consejo Superior de Investigaciones Científicas, Madrid, Spain). The same vector containing the extracellular and the transmembrane domain of the CD8 lymphocyte-specific receptor was used as the control vector in transfection experiments.

Cell Culture

PCC13 cells are a continuous line of thyroid follicular cells derived from Fischer rats that constitute a model system with which to study differentiation and growth regulation in a thyroid epithelial cell setting. These cells express the thyroid-specific genes Tg, TPO, and NIS as well as the thyroid-specific transcription factors TTF1, FoxE1, and Pax8 (66). They were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% donor calf serum and a six-hormone mixture [1 nM TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 mM hydrocortisone, and 10 ng/ml glycyl-L-histidyl-L-lysine acetate] (35, 66). The effect of hormones and growth factors was studied by starving near-confluent cells for TSH and insulin in the presence of 0.2% BSA (starvation medium, indicated as – in the figures) from 2–6 d.

Ligands were added to the culture medium at the following final concentrations: 1 nM TSH, 100 ng/ml IGF-I, and 10 μM forskolin (unless otherwise indicated). hrTSH was used at concentrations ranging from 0.1–1 μg/ml. The inhibitors H89, LY294002, and wortmannin and the cAMP competitor Rp-
cAMP were added to the cells 1 h before hormone addition at the concentrations and times indicated in the experiments described in Results. PTX (100 ng/ml) was added 24 h before treatment, and CTX was added for 24 h at a final concentration of 200 ng/ml.

Immunofluorescence Assay

Cells were seeded on coverslips and transfected with the plasmids indicated in Fig. 9. After a 2-d starvation period, cells were stimulated with TSH for 24 h. The PI3K inhibitor LY294002 was added at a final concentration of 10 μM, 1 h before the hormone treatment. The coverslips were washed three times and fixed in 70% methanol at −20 C for 10 min, washed again, blocked with PBS containing 5% donor calf serum and 0.05% Tween 20 for 1 h at room temperature, incubated with anti-Pax8 and anti-FLAG or anti-CD8 for 1 h at RT, washed three times in PBS-Tween 20 for 5 min, incubated for 1 h at room temperature with the secondary antibodies Alexa 488 and TexasRed 546, washed three times with PBS-Tween, and mounted on Vectashield containing DAPI (Vector). Cells were observed under a confocal microscope using ×63 magnification under an oil immersion objective (Leica Corp., Deerfield, IL). A 0- to 50-μm scale is shown in the first picture of the third column. The quantification of the experiments is represented as the percentage of successfully transfected cells that present a clear (>30%) Pax8 cytoplasmic localization. Five different fields were randomly chosen from each experiment. The data are the mean ± SD of three independent experiments. In all the immunofluorescence studies, the control of cells transfected with Gβγ or βARK was respectively performed with anti-FLAG or anti-CD8 antibodies.

cAMP Assays

The Biotrak cAMP competitive enzyme immunoassay system was used following the manufacturer’s instructions. Briefly, CDB-βARK- or Gβγ/Gγ2-transfected PCC13 cells were grown in p60 plates (6 × 10^5 cells), maintained in starvation medium for 2 d, and then treated with 0.5 mM IBMX to inhibit cAMP degradation, followed by 1 mM TSH treatment for 1 h. Cells were then lysed, the lysate was applied to a donkey antirabbit antibody (1:1000) and incubated with anti-cAMP antiserum for 2 h at 4 C, after which samples were incubated in a cAMP-peroxidase-conjugated antibody (1 h at 4 C) and washed four times with washing buffer. The enzyme substrate was added immediately afterward to all wells and incubated (1 h at room temperature). Before OD determination in a plate reader at 450 nm, the reaction was terminated by adding 1 μl H_2SO_4 to each well. In parallel, a standard curve was prepared with cAMP concentrations ranging from 12.5–3200 fmol/well. Each value represents the mean ± SD of three different experiments.

Flow Cytometry Assay

PCC13 cells were seeded at a density of 6 × 10^5 cells per 60-mm-diameter tissue culture dish and maintained in the presence of a six-hormone mixture including TSH, insulin, and 5% serum (control) for 2, 4, or 6 d. After treatment, cells were trypsinized and collected, washed three times in cold PBS, and fixed with ethanol at −20 C overnight. After three washes with cold PBS, cells were stained with propidium iodide (15 μg/ml). At least 10,000 cells were analyzed per sample, and cell cycle distribution was quantified using a BD Biosciences (San Jose, CA) fluorescence-activated cell analyzer. Data were analyzed with Cell Quest Pro software.

Protein Extraction and Western Blot Analysis

Whole-cell extracts were obtained by resuspending the cell pellet in a buffer containing 50 mM HEPES (pH 7.0), 2 mM MgCl_2, 250 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2M Na_3VO_4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. If protein extrac- 
s were used for luciferase assays, cell pellets were resuspended in passive lysis buffer from the dual-luciferase reporter assay system. Protein concentration was determined according to Bradford (67) with the Bio-Rad Laboratories, Inc. (Hercules, CA), protein assay kit. Total cell extract proteins (30 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked in PBS-T buffer (PBS plus 0.1% Tween 20, pH 7.5) containing 5% nonfat milk. After incubation with antibodies in PBS-T containing 5% nonfat milk, membranes were washed four times with PBS-T buffer and incubated with streptavidin-horseradish peroxidase conjugate, followed by four washes of 10 min each with PBS-T buffer. Immunoreactive bands were visualized with the Luminol Western blot detection reagent (Santa Cruz Biotechnology). Protein expression levels were quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). The protein of interest was quantified and normalized in all cases to its loading control.

The graphic below each Western blot assay shows the average of at least three different experiments. Data were analyzed with GraphPad Prism (Intuitive Software for Science, San Diego). Relative protein expression is the mean ± SD. Statistical significance was determined by t test analysis (two-tailed), and differences were considered significant at P < 0.05.

EMSA

Nuclear extracts from PCC13 cells were prepared following the procedure described by Andrews and Faller (68). Protein concentration was measured as described above using BSA as a standard. An oligonucleotide probe corresponding to the Pax8-binding site in the rat NIS promoter (site PB within the NUE) (6) was labeled with [γ^32P]ATP by polynucleotide kinase. Nuclear extracts (7 μg) from PCC13 cells transfected and treated as indicated in the corresponding figures were incubated with the labeled probe. Binding reactions were preincubated for 15 min on ice with an excess of cold Pax8 DNA-binding site oligonucleotide or unrelated DNA for competition experiments. When required, an anti-Pax8 antibody directed against the Pax8 DNA-binding site was used. Samples were electrophoresed on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA. Gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

Transfections

PCC13 cells were plated at a density of 6 × 10^5 cells per 60-mm-diameter tissue culture dish 48 h before transfection. Transfections were performed by calcium phosphate coprecipitation as described previously (69). Twenty-four hours after transfection, culture medium was changed to starvation medium, and cells were maintained in this medium for 48–72 h or for 5 d for NIS and Tg detection. After this time, cells were treated with the different hormones for the times indicated in each experiment. For NIS, Tg, and CRE promoter activity analyses, cells were transfected with 5 μg pNIS-2.8, 3 μg hTGenh/prm-Luc, or 5 μg 3XCRE-Luc. To correct for transfection efficiency, 0.5 μg of the Renilla-encoding pRL-TK reporter plasmid was co-transfected. Protein expression levels were determined using a Luminol Western blot detection reagent (Santa Cruz Biotechnology). Protein expression levels were quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). The protein of interest was quantified and normalized in all cases to its loading control.
vector was added in all cases. Different amounts of FLAG- 
Gβ1, HA-Gγ2, and CD8-βARK, ranging from 1–5 μg, were 
transfected as indicated. After 48 h, cells were harvested, 
lysed, and analyzed for luciferase and Renilla activities. 
The promoter activity in cells transfected with the expression 
vector was determined as the ratio between luciferase and Renilla, relative to the ratio obtained in cells transfected with the corresponding control vector. The results shown are the average ± SD of three different experiments performed in triplicate, and data were analyzed with GraphPad Prism (Intuitive Software for Science, San Diego, CA). Statistical sign- 
ificance was determined by t test analysis (two-tailed), and differences were considered significant at P < 0.05.

Western blots were done where indicated to determine the levels of the transfected expression vectors.

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