G_{13}^t-dependent Activation of MAPK by Thyrotropin*

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Stimulation of the thyrotropin receptor (TSHR) activates G proteins of all four subfamilies (G_{sa}, G_{ia/o}, G_{q/11}, and G_{12/13}). Whereas G_{sa}/cAMP-dependent cellular responses upon TSHR stimulation are well established, other signaling pathways are less characterized. We evaluated TSH-elicited cellular responses in human follicular thyroid carcinoma cells stably expressing the TSHR and in primary, nonneoplastic human thyrocytes. In these cellular models, stimulation with TSH caused activation of p44/42 MAPK and subsequent induction of c-Fos. MAPK activation occurred independently of G_{sa}, G_{ia/o} and G_{q/11} signaling. Dominant negative constructs of G_{12} or G_{13} as well as shRNA-mediated suppression of G_{12} or G_{13} revealed that MAPK activation was dependent on G_{13}, but not on G_{12} signaling. Furthermore, G_{13} dependent transactivation of the epidermal growth factor receptor was necessary for MAPK activation in follicular carcinoma cells, whereas EGFR was not involved in MAPK activation in nonneoplastic primary thyrocytes. The use of bacterial inhibitors of monomeric GTPases revealed that MAPK activation proceeded independently of Rho proteins but was clathrin toxin B-sensitive, suggesting involvement of Cdc42 or Rac. Thus, our data shed new light on MAPK-independent TSHR signaling and identify the first G_{13} dependent TSHR signaling pathway in human thyrocytes.

The hypophysial glycoprotein hormone TSH\(^2\) plays an essential role in the regulation of metabolic functions, growth, and differentiation of thyroid cells (1–3). The complex physiological effects of TSH are mediated by the G protein-coupled TSH receptor (TSHR) (4). The human TSHR exhibits a remarkable promiscuity in G protein coupling, being capable of activating members of all four G protein subfamilies (5). Thus, a multitude of potential signaling pathways is initiated following TSH stimulation. The past decades have seen substantial progress in the characterization of TSHR-promoted G protein activation and TSH-induced generation of second messengers. However, the molecular effectors connecting upstream signaling events (i.e. G protein activation) and the biological effects of TSH have yet to be further delineated.

The best known signaling cascade initiated by the TSHR proceeds via activation of G_{sa} proteins and a subsequent increase in intracellular cAMP concentrations (6, 7). In human and dog thyrocytes, the TSHR also couples to G_{ia/o} (5, 8), partially opposing TSH-mediated cAMP formation. However, no TSH-regulated physiological processes that exclusively depend on the activation of G_{ia/o} proteins have been identified in thyroid cells so far. In contrast, several thyroid functions have been attributed to the activation of the G_{sa}/cAMP cascade. A TSH-dependent increase in intracellular cAMP concentrations is involved in such fundamental thyroid functions as iodide uptake and synthesis and secretion of thyroid hormone (3).

Additionally, TSH-dependent activation of the G_{sa}/cAMP pathway represents an important stimulus that induces growth of thyrocytes (9–12). However, this cAMP-dependent, proliferative effect of TSH does not induce dedifferentiation and, thus, does not favor malignant transformation of thyrocytes. Consequently, activating mutations of the TSHR or the G_{sa} protein leading to constitutively elevated cAMP levels are frequently found in benign thyroid adenomas but rarely in malignant thyroid tumors (13, 14). Accordingly, the role of TSH as a mitogenic signal in thyroid carcinoma is still not well defined. On the one hand, TSH has been considered as a potential proliferative stimulus of thyroid carcinomas \(\text{in vivo}\) (15); on the other hand, results obtained \(\text{in vitro}\) suggest that CAMP elevations may inhibit growth of thyroid tumor cells (16, 17). Therefore, the potential mitogenic action of TSH in thyroid carcinoma cells may be cAMP\(-\text{independent}\). A known TSH-dependent but cAMP-independent signaling pathway arises from coupling of the TSHR to G_{q/11} proteins, activation of phospholipase C\(_{a}\) and formation of inositol phosphates (5, 18, 19). Phospholipase C\(_{a}\)-dependent pathways have recently been shown to be required for iodine organization and thyroid hormone secretion and for the adaptive growth of the thyroid gland (20). Finally, G_{12/13} coupling of the TSHR has been shown in primary human thyrocytes and was suggested to be related to growth or differen-
tiation of thyrocytes (5). However, no TSHR-dependent molecular effector of G_{12/13} proteins in thyrocytes has been established to date.

TSHR signaling persistently regulates differentiation and proliferation of thyroid cells. Therefore, special attention has been paid to TSHR-mediated regulation of gene expression. With regard to G_{i/o}/cAMP-dependent downstream targets, a crucial role of the cAMP-response element-binding protein has been emphasized. In fact, expressing a dominant negative cAMP-response element-binding protein variant in murine thyroid glands resulted in a poorly developed thyroid gland and severe hypothyroidism (21). Furthermore, G_{i/o}-dependent signaling was discussed in conjunction with TSH-induced activation of c-Jun-terminal kinase, which could be blocked by pretreatment of thyroid cells with pertussis toxin (22). Additionally, the potential engagement of mitogen-activated protein kinases (MAPK), especially p44/42 MAPK, in TSH signaling has been evaluated in several studies. However, the results obtained are inconsistent, since three of six reports demonstrated TSH-mediated p44/42 MAPK activation (23–25) whereas no specific, TSH-promoted activation of p44/42 MAPK was observed in the remaining investigations (26–28). Based on this inconsistency, which might be due to different cellular model systems used in these studies, the importance of this pathway for TSH-dependent regulation of gene expression still remains under debate.

In this study, we investigated the impact of TSH on MAPK activation in two cellular models of human thyroid cells: a follicular carcinoma cell line stably expressing TSHR and nonneoplastic primary human thyrocytes. In both cell systems, TSH promoted a substantial TSHR-dependent activation of the MAPK pathway, which proceeded independently of G_{s}, G_{i/o}, G_{q/11}, or G_{12} proteins. Instead, TSH-promoted MAPK signaling required the activation of G_{13}. In follicular carcinoma cells, EGF receptor (EGFR) transactivation by TSH was additionally involved in this signaling pathway. In primary thyrocytes, however, MAPK activation occurred independently of EGFR. In summary, we analyzed TSH-promoted signaling pathways that lead to the activation of p44/42 MAPK in two human cellular models and describe the first G_{13}-dependent cellular response elicited by TSH.

EXPERIMENTAL PROCEDURES

**Materials**—Cell culture materials were purchased from PAA (Coelbe, Germany). TSH used was a bovine TSH preparation from Sigma unless indicated otherwise. Cell-permeable myristoylated PKI 14-22 amide (myr-PKI), AG1478, AG1295, and AG1024 were from Calbiochem. Anti-phospho-EGFR Y845 antibody was from BIOSOURCE International (Camarillo, CA). Anti-EGFR, anti-p44/42 MAPK, and anti-c-Fos antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-p44/42 MAPK antibody was obtained from New England Biolabs (Beverly, MA). Neutralizing anti-HB-EGF antibodies were from R&D Systems (Minneapolis, MN). The TSH receptor-specific mouse antibody (clone 2C11) was from Serotec (Oxford, UK). The enhanced chemiluminescence system and Hybond C Extra membranes were purchased from Amersham Biosciences. Rotiblock and milk powder were from Carl Roth AG (Karlsruhe, Germany). Transfection was performed using Metafectene (Biontex, Martinsried, Germany) unless indicated otherwise. pcDNA3.1 plasmids were from Invitrogen. Expression plasmids harboring the cDNA sequence of the wild-type (WT) TSHR and TSHR-Y601H were constructed as described previously (29, 30). All other reagents were obtained from Sigma unless otherwise indicated.

**Cell Lines**—FTC-133 cells were a generous gift of Dr. P. E. Goretzki (Neuss, Germany). Primary human thyroid (PHT) cells were established from a thyroidecтомized patient with Grave disease. Thyroid tissue samples were dissected with scissors and digested with 1 mg/ml collagenase (Roche Applied Science) in Iscove’s modified Dulbecco’s medium with 1-glutamine (IMDM) for 2 h at 37 °C. Thereafter, cells were passed through a sieve and washed in IMDM containing 10% fetal calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin. Isolated cells were seeded in 25-cm² Falcon primary tissue culture flasks (BD Primaria) and passaged twice before being used for experiments. Both FTC-133 cells and PHT cells were grown in IMDM, additionally containing 10% (v/v) fetal calf serum, 5 μg/ml insulin, 5 μg/ml transferrin, 0.36 ng/ml hydrocortisone, 10 ng/ml liver cell growth factor (Gly-His-Lys), and 10 ng/ml somatostatin (5 H medium). To establish FTC-133 lines stably expressing WT TSHR or TSHR-Y601H, 2 × 10⁶ cells were seeded in a 10-cm dish and co-transfected with 2 μg of the respective plasmid DNA according to the manufacturer’s protocol. One day after transfection, the media were changed, and 3 days later selection with G418 (750 μg/ml) was initiated. Clones resistant to the antibiotic were isolated, grown, and functionally tested. For further investigations, FTC-133 WT TSHR and FTC-133 Y601H clones were chosen that express comparable receptor densities compared with FTC-133 cells stably expressing empty vector without TSHR.

**Transient Transfection with shRNA or Dominant Negative G_{12} and G_{13} Expression Vectors**—To investigate the role of G_{12} and G_{13} in TSHR-mediated MAPK activation, the RNA interference technique was used (31). For this purpose, shRNA was transiently expressed via a pSuper NeoGFP expression vector (32). shRNA targeting G_{12} and G_{13} were designed according to Reynolds et al. (33) with an additional testing of the three-dimensional structure of the mRNA target sequence to ensure optimal efficacy of RNA interference (34). As a control, shRNA against luciferase (Dharmacon, Lafayette, CO) was expressed. The DNA sequence was 5′-TTA AGA AGA TCC CCT TTA A-3′ for the G_{12}-specific construct and 5′-ATA AGA TGA TGT CGT TTG A-3′ for the G_{13}-specific construct. For transient transfection, metafectene was used according to the manufacturer’s protocol. Cells were seeded in 6-well dishes at a density of 2×10⁵ cells/well and incubated overnight. To ensure an extensive knockdown of the target genes, cells were transfected with 1 μg of RNA well on both day 2 and day 3. In each case, medium was replaced 6 h after transfection. After the second transfection, cells were incubated in serum-free medium for 18 h. On day 4, cells were stimulated to test for MAPK activation. To inhibit G_{12} and G_{13} signaling, constructs carrying cDNA for dominant negative variants G_{12}G228A and G_{13}G225A in a pcIS expression vector were evaluated.
used (generously provided by Dr. S. Offermanns, Heidelberg, Germany). Transient transfection of $\alpha_{12}$ or $\alpha_{13}$ constructs was performed using metfectene and 1 µg of the respective plasmids.

Quantification of TSH Receptor Expression by Cell Surface Enzyme-linked Immunosorbent Assay (ELISA)—Thyroid cells were seeded in a density of 2 $\times$ 10^4 cells/250 µl in a 48-well multititer plate. The next day, cells were incubated (1 h at 4 °C) with a TSHR-specific antibody (IgG concentration 5 µg/ml) that recognizes an extracellular portion of the receptor. Thereafter, cells were fixed with 4% formaldehyde for 15 min at room temperature. Cells were washed with phosphate-buffered saline and then incubated with a peroxidase-conjugated secondary antibody (goat, anti-mouse; Sigma) for 1 h at room temperature. To determine peroxidase activity, 100 µl of 3,3′,5,5′-tetramethyl benzidine solution (1-Step Turbo TMB-ELISA; Pierce) was added for 2 min. Then the reaction was stopped by adding 20 µl of 1 M sulfuric acid. Absorption was detected at 450 nm.

Quantification of cAMP Formation or Levels of Total Inositol Phosphates—For functional assays, cultured FTC-133 cells were seeded in 12-well dishes (2 $\times$ 10^5 cells/well). One day later, cells were washed and growth-arrested by incubation in serum-free IMDM for 18 h. Labeling and assessment of total inositol phosphates (IP) or cAMP accumulation were performed in a modification of the original methods (35, 36), as described previously (37, 38).

Radiolabeled Ligand Binding Assay—For radiolabeled ligand binding studies, cells were harvested, and binding assays with membrane homogenates were performed as described previously (38). Briefly, cells were incubated for 1.5 h at 22 °C in 0.25 ml of a buffer consisting of 50 mM Tris (pH 7.4), 3 mM MgCl2, 1 mM EDTA, 0.1% bovine serum albumin, and 0.1 mg/ml bacitracin, supplemented with 40,000 cpm of 125I-labeled bovine TSH (54 µCi/µg; Brahms Diagnostika). Membranes and bound ligand were separated from unbound ligand by centrifugation (10,000 × g, 10 min) through a silicon oil layer. Nonspecific binding was defined as binding in the presence of 100 nM bovine TSH. Binding data were analyzed by a nonlinear least squares curve-fitting procedure.

Western Blot—For Western blot analysis, cells were grown in 6-well plates (3 $\times$ 10^5 cells/well) for 24 h. Cells were growth-arrested for 18 h by incubation in serum-free medium, stimulated as indicated, washed with ice-cold phosphate-buffered saline, and solubilized in 200 µl of lysis buffer (125 mM Tris-Cl, 2% (w/v) SDS, 10% (v/v) glycerol, and 50 µg/ml bromphenol blue). Proteins were separated by SDS-PAGE (9% gel) and electroblotted onto Hybond C Extra membranes. Blots were incubated in Rotiblock for 1 h to saturate nonspecific binding sites, washed in phosphate-buffered saline, and incubated with the respective primary antibody. Phosphorylated p44/42 MAPK was detected using a phosphospecific anti-p44/42 MAPK rabbit monoclonal antibody. Reblots as loading controls were performed using an anti-p44/42 MAPK mouse monoclonal antibody detecting total (phosphorylated and unphosphorylated) MAPK. For incubation with antisera against the different Gα proteins, nitrocellulose sheets were cut into smaller strips. Proteins were detected by chemiluminescence using peroxidase-conjugated secondary antibodies.

Quantitative Real Time PCR—Total RNA from cells was isolated using the TriFast reagent (Peqlab, Erlangen, Germany). First-strand synthesis was carried out with random hexamers (pdN6; Amersham Biosciences) as primers, using REVERTAID reverse transcriptase (MBI-Fermentas, Sankt Leon-Roth, Germany). Products were amplified using specific, intron-spanning primer pairs for regulatory PKA subunits RIα (Iα for and Iα rev), RIβ (Iβ for and Iβ rev), RIα (Iα for and Iα rev), and RIβ (Iβ for and Iβ rev), giving predicted product sizes of 86, 79, 107, and 79 bp, respectively, and β-actin primers (ACT for and ACT rev) as controls. Primer sequences were as follows: Iα for, 5′-GGC ATT CCT CAG GGA ATA CTT; Iα rev, 5′-CTG TAC GAG TGC CTG CCT TCT; Iβ for, 5′-GGT CCC CCA ATG AGG AGT A; Iβ rev, 5′-TTT ATG CCT GAC AGT GCA ATC TC; Iα for, 5′-AGT CTG GGG AAC TGC TGA GC; Iα rev, 5′-TCC AAA GTA CTG CCC CTT ATG; Iβ for, 5′-CTG CTA CCT CTC CTG GTG CT; Iβ rev, 5′-TTT TTT GGA TTT TTC TTC ACA; ACT for, 5′-GGC TAC AGC TTC ACC ACC AC; ACT rev, 5′-GGG TAC TTC GTG CGC TCA GGA GG. Real time PCR was done using the 2 $\times$ master mix from the Quantitec SYBR Green PCR kit (Qiagen, Hilden, Germany) containing a HotStar Taq polymerase, buffer, nucleotides, 2.5 mM MgCl2, and SYBR Green. Subsequently, 10 pmol of each primer pair and 0.2 µl from the first-strand synthesis were added to the reaction mixture, and PCR was carried out in a light cycler apparatus (Roche Applied Science) using the following conditions: 15 min of initial activation and 45 cycles of 12 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and 10 s at 80 °C each. Fluorescence intensities were recorded after the extension step at 80 °C after each cycle. All primers were tested by using diluted cDNA from the first-strand synthesis (10–1,000-fold) to confirm linearity of the reaction. Samples containing primer dimers were excluded by melting curve analysis and identification of the products by agarose gel electrophoresis. Crossing points were determined by the software program. The relative gene expression was quantified using the formula, 2$^{-}\Delta\Delta C_{t}$ (crossing point of β-actin − crossing point X) × 100 = percentage of reference gene expression.

Reproducibility of Results—All assays were independently performed at least three times unless otherwise indicated. In the case of immunoblots, one representative experiment is shown.

RESULTS

TSH Promotes p44/42 MAPK Activation in Follicular Carcinoma Cells Stably Expressing the TSHR and in Thyrocytes Endogenously Expressing TSHR—We first investigated whether TSH is able to activate the MAPK signaling pathway in a human thyroid carcinoma cell line. As a cellular model, we used FTC-133 cells, which are derived from human thyroid cancer cells but do not express a functional TSHR (39). Thus, we generated a FTC-133 cell line stably expressing the human TSHR (FTC-133 WT TSHR) and compared this new cell line with FTC-133 cells that only expressed the empty vector. In the latter cell line (FTC-133), TSH had no effect on the MAPK signaling pathway (Fig. 1, A and E), excluding the possibility that TSH used in this study contained unknown factors that activate MAPK in a
TSHR-independent mechanism, as described previously (40). In contrast, testing FTC-133 WT TSHR cells, we found a concentration-dependent increase in MAPK phosphorylation following stimulation with TSH (Fig. 1, B and E). Regarding kinetics, MAPK activation was detectable within 1–5 min following stimulation with 100 mIU/ml of TSH and reached a peak after 5–10 min. After 30 min of hormone stimulation, the MAPK response decreased and faded completely after 120 min (Fig. 1C). In a second cellular model, PHT cells, analogous results were obtained (Fig. 1, D and E). These cells were derived from a patient with Grave disease and thus are nonneoplastic cells. In PHT cells, treatment with 100 mIU/ml of TSH for 5 min also caused stimulation of MAPK, which returned to basal levels within 30 min (Fig. 1D). Next, we analyzed the molecular mechanism underlying TSH-promoted activation of the MAPK signaling pathway.

TSH Induces c-Fos in FTC-133 WT TSHR and PHT Cells—Induction of c-Fos expression represents a typical response following nuclear translocation of MAPK. Thus, we evaluated whether TSH led to the induction of this immediate early gene. Stimulation of FTC-133 WT TSHR cells with 100 mIU/ml of

FIGURE 1. Phosphorylation of p44/42 MAPK in FTC-133, FTC-133 WT TSHR, or PHT cells after stimulation with TSH. Serum-starved FTC-133 (A) or FTC-133 WT TSHR (B) cells were treated for 5 min with various concentrations of TSH. Furthermore, serum-starved FTC-133 WT TSHR (C) or PHT (D) cells were treated with 100 milliunits/ml of TSH or 10 ng/ml EGF for the indicated time. Western blot analysis using whole cell lysates was performed with an anti-phospho (P)-p44/42 MAPK antibody (upper panels). The same membrane was stripped and reprobed with an antibody for unphosphorylated p42 MAPK (lower panels) as a control for protein loading. Three independent experiments were carried out, and representative immunoblots are shown. Furthermore, the effect of TSH stimulation (100 mIU/ml, 5 min) on phosphorylation of p44/42 MAPK has been evaluated densitometrically (E). Ratios of bands with phosphospecific versus nonphosphospecific antibody were determined, and values of unstimulated samples have been set to 100%. Means are given ± S.D.

FIGURE 2. TSH-promoted induction of c-Fos expression. Serum-starved FTC-133 WT TSHR (A) or PHT cells (B) were treated with 100 milliunits/ml TSH for the indicated time. Additionally, the effect of pretreatment with MEK inhibitor PD98058 (10 μM, 30 min prior to TSH) is shown (A). Representative immunoblots of whole cell lysates with an anti-phospho-c-Fos antibody (upper panels) or a p42 MAPK antibody (lower panels) as a measure of protein loading are shown. Each experiment was carried out four times. Furthermore, the effect of TSH stimulation on induction of c-Fos was evaluated densitometrically (C). Ratios of bands with c-Fos-specific versus nonphosphospecific MAPK antibody were determined, and values of unstimulated samples have been set to 100%. Means are given ± S.D.
**G**$_{13}$-dependent MAPK Activation by TSH

TSH led to a pronounced induction of c-Fos within 2 h (Fig. 2A), which could be blocked by inhibition of mitogen-activated protein kinase kinase with 10 µM PD98058, suggesting that MAPK was necessary for TSH-mediated c-Fos induction. Analogous results were found for PHT cells (Fig. 2B). Since PHT cells endogenously express TSHR, both MAPK activation and downstream effects (i.e. c-Fos induction) do not result from overexpression of signaling components but may represent physiologic responses induced by TSHR activation.

**TSH-mediated p44/42 MAPK Activation in FTC-133 WT TSHR and PHT Cells Is Independent of G$_s$ and G$_{i/o}$ Activation**—Most known TSHR-mediated cellular responses are dependent on the activation of the classical G$_s$ pathway, leading to an increase in intracellular cAMP concentrations. Thus, we tested whether this was also the case for the TSH-promoted activation of the MAPK pathway in the investigated cellular models. In principle, elevated cAMP concentrations can either activate or inhibit MAPK activity (41) via cAMP-dependent protein kinase (PKA), depending on the signaling context and the regulatory PKA subunits involved in this process. Furthermore, PKA-independent activation of MAPK can also occur via exchange proteins activated by cAMP, so-called EPACs (42). In FTC-133 WT TSHR cells, direct stimulation of adenyl cyclase (100 µM forskolin, 1 h) or PKA (2.5–10 µM 8-bromo-cAMP; 30 min) decreased basal MAPK activity (Fig. 3, A and B), indicating an inhibition of the MAPK pathway by PKA in these cells. In line with these results, pretreatment of FTC-133 WT TSHR cells with a phosphodiesterase inhibitor (10 µM IBMX; 30 min) prevented TSH-promoted MAPK activation (Fig. 3A), whereas PKA-inhibitors (10 µM H89 or 1 µM myr-PKI; 30 min) enhanced TSH-induced MAPK phosphorylation (Fig. 3C). Thus, G$_s$ signaling does not activate but rather inhibits MAPK signaling in FTC-133 WT TSHR cells and, therefore, cannot account for the TSH-promoted MAPK activation observed in these cells. Similarly, in PHT cells, pretreatment with IBMX blocked TSH-induced MAPK activation (Fig. 3D). Furthermore, activation of adenyl cyclase by forskolin inhibited basal MAPK activation in PHT cells as well (data not shown), whereas inhibition of PKA by H89 led to increased basal and TSH-promoted MAPK activation in PHT cells (data not shown) in analogy to the findings obtained in FTC-133 WT TSHR cells. Next, we tested whether TSH-induced MAPK stimulation requires the activation of G$_{i/o}$ proteins. Treatment of neither FTC-133 WT TSHR nor PHT cells with 100 ng/ml pertussis toxin overnight did affect TSH-mediated or GPCR-independent, EGF-promoted (10 ng/ml, 5 min) stimulation of MAPK (Fig. 3, E and F). In FTC-133 WT TSHR cells, MAPK activation by TSH was comparable in its magnitude with MAPK activation by EGF, whereas TSH-promoted MAPK activation in PHT cells proved to be weaker than EGF-induced stimulation. Taken together, these findings indicate that MAPK activation by TSH occurred neither via a G$_s$- nor via a G$_{i/o}$-dependent signaling pathway.

**Expression of PKA Regulatory Subunit RIIβ Is Low in FTC-133 WT TSHR and PHT Cells**—Since the expression levels of the diverse regulatory PKA subunits have been shown to determine whether PKA activation leads to MAPK stimulation or not, we tested the relative mRNA levels of the regulatory subunits RIIα, RIIβ, RIIo, and RIIβ in FTC-133 WT TSHR and PHT cells by quantitative reverse transcription-PCR. Both in FTC-133 WT TSHR and PHT cells, we observed predominant expression of regulatory subunits RIIo and RIIβ as well as RIIα but very low expression levels of RIIβ (Fig. 4). The expression of the latter isoform in fibroblasts has been shown to be a prereq-
TSHR on the plasma membrane was determined by cell surface ELISA using a monoclonal antibody directed against the N terminus of the TSHR. Binding of the TSHR-specific antibody was demonstrated by a peroxidase-conjugated secondary antibody. As a control, a peroxidase reaction without incubation of the first antibody is shown. Means of two independent experiments performed in quadruplicates are given. Error bars, S.D.

FIGURE 4. mRNA levels of PKA regulatory subunits R1α, R1β, RIIα, and RIIβ. Transcript levels of various PKA subunits were quantified in total RNA pools by quantitative real time reverse transcription-PCR with primer pairs listed under “Experimental Procedures.” Values of the regulatory subunits are presented as percentage of β-actin expression. Means of four independent experiments are given. Error bars, S.D.

FIGURE 5. Protein expression of TSHR in FTC-133 WT TSHR, FTC-133 Y601H, and PHT cells. Expression of TSHR on the plasma membrane was determined by cell surface ELISA using a monoclonal antibody directed against the N terminus of the TSHR. Binding of the TSHR-specific antibody was demonstrated by a peroxidase-conjugated secondary antibody. As a control, a peroxidase reaction without incubation of the first antibody is shown. Means of two independent experiments performed in quadruplicates are given. Error bars, S.D.

G₁₃-dependent MAPK Activation by TSH

Next, we tested whether stimulation of FTC-133 Y601H cells by TSH activated MAPK in the absence of phospholipase C activation. Remarkably, TSH stimulation of FTC-133 Y601H cells led to MAPK activation with kinetics comparable with those seen in FTC-133 WT TSHR cells (Fig. 6D). Furthermore, the specific protein kinase C inhibitor GF109208X (10 μM, 30 min) decreased TSH-promoted MAPK activation neither in FTC-133 WT TSHR cells (Fig. 6C) nor in FTC-133 Y601H cells (Fig. 6E). Analogous results were found in PHT cells; both inhibition of phospholipase C by U73122 (10 μM, 30 min) and inhibition of protein kinase C did not prevent activation of MAPK by TSH (Fig. 6F). To summarize, results obtained so far indicate that TSH-induced MAPK activation in FTC-133 WT TSHR and PHT cells requires neither G₁₂/₁₃ nor G₁₃-dependent signaling.

p44/42 MAPK Activation Depends on G₁₃ Signaling in FTC-133 WT TSHR and PHT Cells—Since G₁₂, G₁₃, and G₁₃ signaling pathways did not account for TSHR-mediated MAPK activation, G₁₂/₁₃-dependent signaling remained as a possible mechanism for G protein–dependent activation of MAPK. Thus, to evaluate the role of G₁₂/₁₃ signaling in the TSHR-mediated response, we used dominant negative mutants of Gα₁₂ (Gα₁₂-G228A) and...
G\textsubscript{13}\textsubscript{dependent MAPK Activation by TSH

![Graph and Table]

FIGURE 6. Role of G\textsubscript{13} signaling for TSH-promoted p44/42 MAPK activation. To check for ligand affinity of the TSHR variants, TSH-binding properties of WT TSHR [\(\text{WT}
\)] and TSHR-Y601H [\(\text{Y601H}
\)] were monitored by radiolabeled ligand binding assays with total membrane preparations (A). For the functional characterization of WT TSHR and TSHR-Y601H upon expression in the cell line FTC-133, the cells were seeded in 12-well plates (\(2 \times 10^5\text{/well}
\)). One day before labeling, cells were washed and maintained in medium containing 0.5% fetal calf serum. Subsequently, cells were incubated with 100 milliunits/ml bovine TSH, and intracellular cAMP (B) or IP level (C) was determined and presented as the mean \pm S.D. of at least three independent experiments performed in triplicates. Furthermore, Western blot analysis using whole cell lysates were performed with an anti-phospho-p44/42 MAPK antibody (upper panels). The same membrane was stripped and reprobed with an antibody for unphosphorylated p42 MAPK (lower panels) as a control for protein loading. D, serum-starved FTC-133 Y601H cells treated with 100 milliunits/ml TSH for 5–120 min. E, FTC-133 Y601H cells were pretreated with H89 or GF109208X (10\(\mu M\)) for 30 min and stimulated with TSH (100 milliunits/ml) for an additional 5 min. PHT cells were treated with 10\(\mu M\) U73122 or GF109208X prior to the stimulation with 100 mIU/ml TSH for 5 min (F). Three independent experiments were carried out, and representative immunoblots are shown.

\(\text{G}_{13}\) (\(\text{G}_{13}\)-G225A) (45) transiently expressed in FTC-133 WT TSHR cells. Notably, the constructs exhibited different effects on TSHR signaling; expression of the \(\text{G}_{13}\)-G225A construct inhibited MAPK activation by TSH (Fig. 7A, right), whereas the respective \(\text{G}_{12}\) construct did not (Fig. 7A, left). The effect of \(\text{G}_{13}\)-G225A expression on MAPK activity was due to a specific inhibition of the TSH-promoted pathway, since MAPK activation by EGF in the same cells was unaffected (Fig. 7A, right). Therefore, it appears that in FTC-133 WT TSHR cells, TSH-promoted MAPK activation is dependent on \(\text{G}_{13}\). To substantiate this finding, we used an additional, independent approach and investigated the contribution of \(\text{G}_{12}\) and \(\text{G}_{13}\) proteins to TSHR signaling by RNA interference. For this purpose, plasmid DNAs that encoded shRNA sequences specific for \(\text{G}_{12}\) or \(\text{G}_{13}\) were introduced into FTC-133 WT TSHR and PHT cells. The specificity of the shRNA used is shown in Fig. 7B, demonstrating that transfection of cells with the respective constructs solely reduced the expression of the corresponding G protein. Although shRNA against \(\text{G}_{12}\) or luciferase did not inhibit MAPK activation by TSH, shRNA against \(\text{G}_{13}\) blocked the TSH-induced p44/42 MAPK activation in FTC-133 WT TSHR (Fig. 7C, left) as well as in PHT cells (Fig. 7C, right). In conclusion, two independent approaches, dominant negative mutants and specific shRNA, demonstrate that \(\text{G}_{13}\), but not \(\text{G}_{12}\) is involved in TSH-promoted MAPK signaling in two different cell lines.

Transactivation of the EGF R Is Necessary for TSHR-mediated p44/42 MAPK Activation in FTC-133 WT TSHR, but Not in PHT Cells—RTKs (e.g. the receptors for EGF, platelet-derived growth factor, or insulin-like growth factor) are often activated following stimulation of GPCRs and then transfer signals from GPCRs to MAPK, a process termed receptor transactivation. Thus, we tested whether the TSH-promoted signaling cascade leading to MAPK activation requires RTK transactivation. Using a blocker of the EGFR, AG1478 (100 \(\text{nm}\), 30 min), a pronounced inhibitory effect on TSH-induced MAPK activation was observed in FTC-133 WT TSHR cells, comparable with the effect of the mitogen-activated protein kinase kinase inhibitor PD98059 (10 \(\mu M\), 30 min) (Fig. 8A), suggesting that EGFR signaling was necessary for TSH-promoted MAPK activation. In a well established pathway of EGFR transactivation, agonist-promoted GPCR signaling induces the shedding of membrane-associated heparin-binding EGF (HB-EGF), which subsequently acts as an auto- or paracrine stimulator of the EGFR. Accordingly, we found that heparin (100 \(\mu M\)) blocked TSH-induced MAPK stimulation (Fig. 8B), most probably based on its neutralizing effects on HB-EGF. To exclude nonspecific effects of heparin, we also treated FTC-133 WT TSHR cells with an antibody (10 ng/ml) that specifically neutralized HB-EGF activity. Pretreatment of the cells with this antibody markedly inhibited TSH-induced MAPK activation (Fig. 8C), suggesting that HB-EGF was required for TSH-promoted activation of MAPK. A well known mechanism leading to GPCR-induced release of HB-EGF is mediated by matrix metalloproteinases (46, 47). Consequently, we found a marked inhibition of the MAPK response to TSH with broad-spectrum matrix metalloproteinase inhibitors, such as marimastat (data not shown) or GI 129471 (Fig. 8D). To further elucidate the relationship between TSHR and EGFR signaling in FTC-133 WT TSHR cells, we analyzed the involvement of \(\text{G}_{13}\) in TSHR-promoted EGFR transactivation by directly monitoring EGFR phosphorylation. TSH-promoted EGFR phosphorylation was observed in FTC-133 WT TSHR cells and was absent after co-expression of a dominant negative \(\text{G}_{13}\) mutant yet remained unaltered after expression
promoted MAPK activation in both cell types, divergent signaling pathways connect G_{13} with this downstream effector.

*p44/42 MAPK Activation by TSH in FTC-133 WT TSHR and PHT Cells Is Clostridium difficile Toxin B-sensitive*—The best characterized downstream targets of G_{13} proteins are monomeric GTPases belonging to the Rho family. Thus, we used various clostridial toxins known to inhibit monomeric GTPases to further analyze G_{13}-dependent activation of MAPK. First, we tested the effect of toxin B and its variant, TcdB 1470, both derived from *C. difficile.* Toxin B inhibits several GTPases, including Rho, Rac, and Cdc42, whereas TcdB 1470 lacks the inhibitory effect on Rho proteins (48). Surprisingly, pretreatment of FTC-133 WT TSHR and PHT cells with both toxin variants inhibited TSH-dependent stimulation of MAPK (Fig. 9, A and C). Since TcdB 1470 does not inhibit Rho activity, but still decreased MAPK activation, a contribution of Rho proteins to TSH-mediated MAPK activation is unlikely. Therefore, MAPK activation appears to be mediated by other toxin B-sensitive GTPases, such as Rac or Cdc42. To further exclude an involvement of Rho in TSH-promoted MAPK activation, we used C2IN-C3, a toxin with C3-transferase activity, thus acting as a specific Rho inhibitor. Since C2IN-C3 does not enter the cell, it was co-administered with C2Ila, the transport unit of C2-toxin, to ensure intracellular delivery (49, 50). Treatment of FTC-133 WT TSHR or PHT cells with C2IN-C3 plus C2Ila did not inhibit stimulation of MAPK by TSH (Fig. 9, B and D), providing further evidence that Rho activity is not required for TSH-induced MAPK activation. Thus, we suggest Rac and/or Cdc42 as potential downstream effectors of G_{13} proteins when activated by the TSHR.

**DISCUSSION**

The TSHR exhibits the almost unique propensity to activate members of four different G protein subfamilies (being G_s, G_{12/13}, G_{q/11}, and G_{12/13}) in a given cell (5, 8, 19). This intriguing variety of signaling cascades activated by the same receptor raises the difficulty of assigning a distinct signaling pathway to a single G protein. Thus, besides signaling cascades stimulated via the well-established G_s coupling of the TSHR, (3, 20), little information

![Image](Image 49x362 to 408x733)

**FIGURE 7. G_{12} and G_{13} dependence of TSH-mediated p44/42 MAPK activation.** FTC-133 WT TSHR cells were transiently transfected with dominant negative mutants of G_{12} (G_{12,G228A}) and G_{13} (G_{13,G225A}) (A). Western blots of whole cell lysates were probed with an anti-phospho (P)-p44/42 MAPK antibody (upper panel) or a p42 MAPK antibody (lower panel). Representative immunoblots of three independent experiments are shown. Furthermore, shRNAs specific for G_{12} or G_{13} as a control for firefly luciferase were expressed in FTC-133 WT TSHR cells (B). Samples of whole cell lysates were probed using anti G_{12} (top) or G_{13} (bottom) antibodies. The same membrane that was probed with the anti-G_{12} antibody was stripped and reprobed with the G_{13} antibody to exclude effects by different protein loading. Representative immunoblots of three independent experiments are shown. C, TSH-promoted activation of p44/42 MAPK was investigated in FTC-133 WT TSHR (left) or PHT cells (right) expressing shRNAs against G_{12}, G_{13}, or firefly luciferase. Cells were treated with 100 milliunits/ml TSH for 5 min. Samples were probed with an anti-phospho-p44/42 MAPK antibody (top) or a p42 MAPK antibody (bottom). Representative immunoblots of three independent experiments are shown.

of a mutant blocking G_{12} activity (Fig. 8E), suggesting that G_{13} activation is a prerequisite for EGFR transactivation. Summarizing these results, TSH-promoted MAPK activation in FTC-133 WT TSHR cells required the transactivation of the EGFR mediated by matrix metalloprotease-catalyzed mobilization of HB-EGF and was dependent on G_{13} signaling. Surprisingly, in PHT cells, inhibitors of the EGFR (AG1478) (Fig. 8F), the platelet-derived growth factor receptor (AG1296), or the insulin-like growth factor receptor (AG1024) (data not shown) did not affect at all TSH-promoted MAPK activation. Although PHT cells showed much lower EGFR expression levels as compared with FTC-133 cells (Fig. 8G), EGF induced a comparable phosphorylation of MAPK in both cell lines (Figs. 3 and 8). Thus, we conclude that a reduced responsiveness of PHT cells to EGF is not responsible for the lack of receptor transactivation, suggesting that, despite the common role of G_{13} observed in TSH-dependent MAPK activation.
G₁₃-dependent MAPK Activation by TSH

**FIGURE 8.** EGFR-dependent activation of p44/42 MAPK by TSH. Western blot analysis using whole cell lysates were performed with an anti-phospho (P)-p44/42 MAPK antibody (top). The same membrane was stripped and reprobed with an antibody against unphosphorylated p42 MAPK (lower panels) as a control for protein loading. In A, results obtained with serum-starved FTC-133 WT TSHR cells pretreated for 30 min with a 100 nm concentration of the tyrosine kinase inhibitor AG1478 or with 10 µM of MEK inhibitor PD98059 as shown. To test the effects of heparin, FTC-133 WT TSHR cells were pretreated for 30 min with 100 µg/ml heparin (B). Furthermore, a neutralizing antibody against HB-EGF was used in a concentration of 10 ng/ml, and cells were preincubated with the antibody for 30 min (C). The involvement of matrix metalloproteinases was investigated by pretreating cells for 30 min with a 10 µM concentration of the broad spectrum inhibitor GI 129471 (D). Furthermore, FTC-133 WT TSHR cells or cells transiently transfected with dominant negative mutants of Ga₁₃ (Ga₁₃-G228A) or Ga₁₂ (Ga₁₂-G225A) were treated with 100 milliunits/ml TSH for 5 min (E). Samples of whole cell lysates were probed using a phospho-EGFR-specific antibody (E, top) or an anti-EGFR antibody (E, bottom). F, results obtained in serum-starved PHT cells pretreated for 30 min with a 100 nm concentration of the tyrosine kinase inhibitor AG1478, stimulated with TSH (100 milliunits/ml) or EGF (10 ng/ml) (upper panels), anti-phospho-p44/42 MAPK, lower panels, p42 MAPK antibody). Additionally, expression of the EGF receptor in FTC-133 WT TSHR and PHT cells was compared (G) using an anti-EGFR antibody (G, top) or an anti-p42 MAPK antibody (G, bottom) as a control for protein loading. Representative immunoblots of three independent experiments are shown.

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exists on TSHR-dependent signaling pathways that are exclusively activated by one of the other G proteins. In the present investigation, we delineate a novel signaling pathway of the TSHR that leads in two cellular models to a G₁₃-dependent activation of p44/42 MAPK by a G₁₃-dependent mechanism. Thus, we identify the first G₁₃-dependent cellular response of TSHR stimulation.

For GPCR, many mechanisms that activate the Ras/Raf/mitogen-activated protein kinase kinase/MAPK pathway have been described (51–53). For example, cAMP-dependent MAPK stimulation via EPACs, exchange proteins activated by cAMP (54), has been characterized as a possible pathway (42). In fact, Iacovelli et al. (24) found a cAMP-dependent p44/42 MAPK activation in human thyrocytes. In contrast, in the present study, activation of cAMP-dependent pathways inhibited basal and TSHR-dependent p44/42 MAPK activation in FTC-133 WT TSHR and PHT cells. Hence, we suggest that G₁₃-promoted cAMP accumulation does not account for the TSH-promoted MAPK activation observed in human thyroid cells used in the present study. cAMP-dependent inhibition of MAPK has been shown to involve the activation of PKA. In line with this observation, inhibition of PKA increased basal MAPK activation in FTC-133 WT TSHR cells. Since TSH-induced activation of p44/42 MAPK in FTC-133 and PHT cells was also pertussis toxin-insensitive, we further exclude an involvement of G₁₁, proteins in this pathway.

TSH-mediated p44/42 MAPK activation has been demonstrated in several previous studies (23–25). Surprisingly, other reports questioned such a TSH-promoted activation of p44/42 MAPK (26–28). This discrepancy could be explained by the different cellular models used. For example, it appears that TSH-promoted activation of p44/42 MAPK is not detectable in dog thyrocytes (27, 28), whereas TSH activated MAPK in studies in human thyrocytes (27, 28), whereas TSH activates MAPK in studies in human thyrocytes (27, 28). Interestingly, in contrast to the human TSHR that activates G₁₁ proteins, the dog TSHR failed to do so (8). Thus, an involvement of G₁₁ signaling in MAPK activation appeared to be likely (23), since it would explain the differences observed in canine versus human thyrocytes. However, using chemical inhibitors of G₁₁-dependent pathways and a TSHR variant, TSHR Y601H, with disrupted coupling to the G₁₁/phospholipase C system (29), we could still observe TSH-promoted MAPK activation, excluding a contribution of G₁₁ proteins to this signaling pathway in human thyrocytes. Interestingly, in accordance with our results, a previous report provided intriguing data indicating that TSH-promoted activation of the MAPK pathway in astrocytes does not involve G₁, G₁₁, and G₁₁ proteins (55). These data directed our attention toward a putative role of the G₁₂/₁₃ coupling in TSH-promoted
Using dominant negative mutants and shRNAs, we show that stimulation of MAPK was dependent on the activation of G13, but not on G12. It is tempting to speculate that the TSH-promoted and G13-dependent activation of MAPK shown herein may represent a Gs/cAMP-independent signaling pathway of the TSHR involved in the regulation of cellular proliferation.

Furthermore, we identified the EGFR as a downstream target of the TSHR in FTC-133 WT TSHR but not in PHT cells. Transactivation of RTKs has already been shown for a number of GPCRs (for a review, see Ref. 53). TSHR signaling did not directly activate EGFR, because neutralizing antibodies against HB-EGF blocked TSH-induced stimulation of MAPK. As expected, HB-EGF liberation was blocked by matrix metalloprotease inhibitors, which have been shown to link GPCR-promoted signaling with EGFR activation by a triple-membrane-passing mechanism. Since this phenomenon was observed in the carcinoma cell line FTC-133 WT TSHR, which recombiantly expressed the TSHR but not in nonneoplastic HPT cells, the physiological significance of EGFR transactivation for TSH-promoted signaling has to be further defined in other cell systems that endogenously express the TSHR. At least for the luteinizing hormone and FSH receptors that are closely related to TSHR, transactivation of EGFR-like receptors has been already demonstrated (56–58).

There is evidence that the EGFR can activate G13 in a GPCR-independent manner (59), provoking the question of whether G13 proteins are located up- or downstream of EGFR in TSH-promoted MAPK activation in FTC-133 WT TSHR cells. EGF-promoted activation of MAPK in these cells was unaffected by the expression of dominant negative G13, whereas the activation of EGFR by TSH was blocked by dominant negative G13. Therefore, we conclude that G13 is upstream of EGFR in the TSHR-mediated MAPK pathway and, thus, most probably acts as a linker between the TSHR and matrix metalloprotease. Interestingly, dominant negative G12 mutants failed to inhibit the EGFR via matrix metalloprotease, providing further evidence for the unique role of G13 in this pathway. The specific role of G13 in EGFR transactivation confirms the previous finding that cytoskeletal reorganization upon stimulation of LPA, B2, and 5HT2C receptors occurred via G13-dependent transactivation of the EGFR (45, 60).

It is remarkable that stimulation of RTKs (e.g. by EGF or insulin-like growth factor) in thyrocytes is considered to be a proliferative signal, which concomitantly induces a loss of differentiation, whereas activation of the TSHR preserves cellular differentiation, suggesting a model in which stimulation of RTKs and TSHR engage two independent parallel signaling pathways (28). The data presented here indicate the possibility that activation of EGFR may, at least in some types of TSH-expressing cells, occur downstream of the TSHR as a sequential signaling event. In this context, it is tempting to speculate that TSHR-dependent activation of EGFR could represent a signaling pathway correlated with a more dedifferentiated phenotype (e.g. in follicular carcinoma), whereas parallel signaling of TSHR and EGFR is realized in more differentiated cells, such as in PHT cells. However, further investigations in thyroid cells that endogenously express the TSHR but exhibit distinct grades of differentiation are required to obtain a more detailed picture of the interplay of TSHR and EGFR in normal and neoplastic thyroid cells.

Finally, to identify downstream signaling molecules involved in TSH-promoted and G13-mediated activation of MAPK, we used small-GTPase-modifying toxins, since it is well-established that G13 regulates RhoGEF proteins, leading to the activation of monomeric GTPases, such as Rho proteins. Interestingly, clostridial toxin B, which specifically inhibits Rho, Rac, and possibly Cdc42 activity, blunted TSH-induced MAPK acti-
G$_{\text{13}}$-dependent MAPK Activation by TSH

In conclusion, we report here that TSH-promotes MAPK activation in human thyroid cells via a $G_{\text{13}}$-dependent mechanism. This so far unknown signaling pathway paradigmatically reveals the first $G_{\text{13}}$-dependent signaling pathway of TSH.

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