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Thyrotropin Activates Mitogen-Activated Protein Kinase Pathway in FRTL-5 by a cAMP-Dependent Protein Kinase A-Independent Mechanism

LUISA IACOVELLI, LOREDANA CAPOBIANCO, LORENA SALVATORE, MICHELE SALLESE, GABRIEL MAXIMO D'ANCONA, and ANTONIO DE BLASI

Consorzio Mario Negri Sud, Istituto di Ricerche Farmacologiche "Mario Negri", Santa Maria Imbaro, Italy (L.S., M.S., G.M.D.); and Istituto Neurologico Mediterraneo Neuromed, Pozzilli, Italy (L.I., L.C., A.D.B.)

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ABSTRACT

The involvement of mitogen-activated protein (MAP) kinases in the mitogenic effect of thyrotropin (TSH) is not fully elucidated. In FRTL-5 cells, we found that the MAP kinase kinase (MEK) inhibitors UO126 and PD98059 substantially decreased TSH-induced DNA synthesis, indicating that MAP kinases are involved in the TSH-stimulated proliferative response. Accordingly, TSH, forskolin (FSK) and 8-bromo-cAMP induced a rapid (3 min) and transient activation of ERK1/2, as assessed by phosphorylation of myelin basic protein and ERK1/2. This effect was cAMP-dependent and protein kinase A (PKA)-independent. The activation of Rap1 and B-Raf was involved in the mechanism of MAP kinase stimulation by TSH. TSH induced rapid (3 min) GDP/GTP exchange and activation of Rap1. After a 3-min exposure to FSK, B-Raf was recruited to a vesicular compartment, where it colocalized with Rap1. Both activation of Rap1 and translocation of B-Raf were PKA-independent. The Rap1 dominant negative Rap1N17 significantly reduced TSH-stimulated but not insulin-like growth factor 1-stimulated ERK1/2 phosphorylation, whereas the Ras dominant negative RasN17 inhibited the effect of both agonists. In conclusion, our results document that TSH increases intracellular cAMP, which rapidly stimulates MAP kinase cascade independent of PKA. This novel mechanism could integrate other pathways involved in TSH-stimulated proliferative response.

The receptor for thyrotropin (TSH) belongs to the wide family of seven-membrane-spanning receptors that couples to the heterotrimeric G proteins. Binding of TSH to its receptor results in the activation of Gs and adenylyl cyclase and generation of the intracellular second messenger cAMP, which stimulates the cAMP-dependent protein kinase A (PKA). This cascade accounts for TSH-mediated regulation of function, differentiation, and proliferation of the thyroid gland (Porcellini et al., 1997). In the rat thyroid cell line FRTL-5 and in human and dog thyroid cells, direct activation of the cAMP pathway by forskolin (FSK), cholera toxin, or cAMP analogs is sufficient to reproduce all TSH-dependent effects (Ambesi-Impiombato et al., 1980; Dremier et al., 2000). The proliferation of FRTL-5 cells is also positively regulated by other growth factors, and a synergism between TSH and insulin-like growth factor-1 (IGF-1) in promoting cell growth has been demonstrated (Paschke and Ludgate, 1997).

The cAMP-dependent mechanisms involved in the mitogenic effect of TSH have not been completely elucidated. It was a common belief that PKA could mimic all the effects of TSH-induced cAMP increase in thyrocytes. Now it is becoming evident that PKA activation is not sufficient to account for the effects of cAMP on proliferation (Cass et al., 1999). In Wistar rat thyroid (WRT) cells, microinjection of the heat-stable PKA inhibitor (PKI) only partially inhibited TSH-induced DNA synthesis (Kupperman et al., 1993). In another study, overexpression of the catalytic subunit of PKA in dog thyrocytes was not sufficient to induce DNA synthesis (Dremier et al., 1997). Thus, TSH-induced cell proliferation requires, besides PKA activation, other cAMP-dependent pathways that do not involve PKA.

The possible role of MAP kinases (MAPK) in TSH-dependent cell proliferation in thyroid cells is another issue that was not fully addressed by previous investigations. In primary cultured human thyroid follicles, it was observed that

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ABBREVIATIONS: TSH, thyrotropin; PKA, protein kinase A; FSK, forskolin; IGF-1, insulin growth factor 1; WRT, Wistar rat thyroid; PKI, heat-stable PKA inhibitor; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; EPAC, exchange protein activated by cAMP; 8Br-cAMP, 8-bromo-cAMP; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; TKR, tyrosine kinase receptors.

TSH activated MAPK in a cAMP-independent manner (Saunier et al., 1995). Another study using primary cultured human thyroid cells showed that MAPK activation by H-Ras is necessary but not sufficient for proliferation (Gire et al., 1999). TSH failed to stimulate MAPK in WRT cells and in dog thyrocytes, leading the authors to conclude that MAPK cascade is not involved in TSH-stimulated proliferative response (Lamy et al., 1993; Miller et al., 1998). Rather, it was found that in WRT cells TSH inhibited Raf-1 and MAPK activation induced by serum after 15 to 30 min of stimulation (Al-Alawi et al., 1995).

The recent cloning of EPAC (de Rooij et al., 1998; Kawasaki et al., 1998) has highlighted a novel transduction pathway regulated by cAMP. EPAC is directly activated by cAMP; once activated, it promotes the GDP/GTP exchange on Rap1. These effects are PKA-independent. Rap1 is a Ras-like GTPase; its function in signal transduction is becoming increasingly important (Zwartkruis and Bos, 1999). Rap1 shares 50% sequence identity and basic biochemical properties with Ras. The two proteins have virtually identical effector domains and Rap1 associates in vitro with most of Ras effectors, such as B-Raf and Ral-GDS. It was proposed that the functional role of Rap1 is to antagonize Ras signaling pathways; however, the role of Rap1 in normal cells remains controversial (Zwartkruis et al., 1998). Rap1 is activated by stimulation of different receptors coupled either to G proteins or tyrosine kinases, such as thrombin receptor in platelets and fMLP and PAF receptors in neutrophils and nerve growth factor receptor in PC12 cells (Vossler et al., 1997; M'Rabet et al., 1998). The role of Rap1 in the regulation of cell proliferation is not well understood and seems to depend on the cell type. In NIH3T3 cells, Rap1 inhibits Ras-mediated cell proliferation (Kitayama et al., 1989), whereas in Swiss 3T3 cells, overexpression of Rap1b stimulates cell proliferation (Altschuler and Ribeiro-Neto, 1998).

In this article, we focus on cAMP-dependent mechanisms involved in the mitogenic effect of TSH. We demonstrate that the proliferation of FRTL-5 cells induced by TSH is mediated by an early stimulation of ERK1/2 MAPK. The molecular mechanism of ERK1/2 activation by TSH is cAMP-dependent but PKA-independent and involves the activation of Rap1 and B-Raf.

Experimental Procedures

Materials. TSH, 8Br-cAMP, Sepharose-conjugated Protein A, Hoechst 33258, and myelin basic protein (MBP) were from Sigma (St. Louis, MO), FSK was from RBI/Sigma (Natick, MA); H89, PD98059, Wortmannin, myristoylated PKI, IGF-1, and AG1478 were from Calbiochem (San Diego, CA), UO126 and polyclonal anti-phospho ERK1/2 was from Promega (Madison, WI), monoclonal anti-phospho ERK1/2 was from New England Biolabs (Beverly MA), and anti-Rap1, anti-B-Raf, anti-ERK1, anti-ERK2, anti-EPAC, and anti-p70s6K antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Rap1 was from Transduction Laboratories (Lexington, KY). Effectene transfection reagent was from QIAGEN (Chatsworth, CA)

Cell Culture and [^{3}H]Thymidine Uptake. FRTL-5 cells are a continuous line of rat thyroid cells and were grown essentially as already described (Iacovelli et al., 1996). PC12 cells were cultured in Dulbecco's modified Eagle's medium, plus 5% fetal calf serum and 5% horse serum. DNA synthesis in FRTL-5 cells was measured by [^{3}H]thymidine uptake essentially as described previously (Iacovelli et al., 1996), with minor modifications. Briefly, cells were plated in 96-well plates (10^4 cells/well) and allowed to grow for 4 days. After 48 h of starvation in Coon's modified F-12 medium plus 0.3% of bovine serum albumin and 20 mM glutamine, the cells were stimulated as appropriate for 30 h and a pulse of [^{3}H]thymidine was given in the last 6 to 8 h.

ERK1/2 in Vitro Assay. Quiescent FRTL-5 cells were stimulated at 37°C with appropriate stimuli. The cells were then rapidly washed with ice-cold PBS and solubilized in 500 μl of Triton X-100 lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 10 mM β-glycerophosphate) for 15 min. The lysates were cleared by centrifugation (10,000*g* for 10 min) and 300 to 350 μg of each sample were precleared with 60 μl of Protein A-Sepharose (1:1 slurry) for 30 min at 4°C. ERK1/2 were immunoprecipitated from the total lysates with 2 μg of anti-ERK1 antibody (sc-93; Santa Cruz Biotechnology) and 60 μl of Protein A Sepharose (1:1 slurry). The immunoprecipitates were washed 3 times with Triton X-100 lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 5 mM $MnCl_2$). The activity of ERK1/2 was determined by incubating the immune complexes at 30°C for 20 min with 50 μl of kinase buffer containing 1 mM DTT, 10 μg of MBP, 1 μM ATP, and 0.5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol). The reaction was stopped with Laemmli buffer, proteins were subjected to SDS-polyacrylamide gel electrophoresis (15% gel), and the phosphorylation of MBP was quantified by Instant Imager (Packard, Meriden, CT).

Western Blotting. For phospho-ERK immunoblots, 80 to 100 μg of cell lysates from the experiments of ERK1/2 activity assay were separated by SDS- polyacrylamide gel electrophoresis (12.5% acrylamide and 0.0625% bisacrylamide), blotted onto nitrocellulose or polyvinylidene difluoride membranes and probed using a commercial anti-phosphospecific antibody against phosphorylated ERK1/2.

Membranes were routinely stripped and reprobed with anti-ERK1/2 antibody (1:2000 dilution) to control equal protein loading. Monoclonal anti-phospho ERK1/2 antibody was used at 1:2000 dilution, and polyclonal anti-phospho ERK1/2 antibody was used at 1:5000 dilution. Anti-B-Raf antibody was used in immunoblot analysis at 1:500 dilution. Other Western blot analyses were performed using the antibodies according to the manufacturer's instructions. The immunoreactive bands were visualized either by enhanced chemiluminescence using horseradish peroxidase-linked secondary antibody or by using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Pull-Down Assay for the Determination of Rap1 and Ras Activation. Rap1 and Ras activation were assessed by pull-down assay as described previously (Seidel et al., 1999). GST-RalGDS-RBD and GST-Raf1-RBD were expressed in *Escherichia Coli* (strain BL21); after induction with isopropyl-1-thio-β-D-galactopyranoside, bacterial lysates were prepared as described previously (Sallese et al., 2000a). FRTL-5 cells were plated in 10-mm petri dishes and when 50% confluent were starved for 48 h from serum and hormones. Cells were stimulated in HBSS buffer containing 0.4% bovine serum albumin, 10 mM HEPES, and 0.5 mM 3-isobutyl-methylxanthine, pH 7.3. Incubations were followed by rapid rinsing with ice-cold PBS and addition of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, and 0.1% SDS) plus protease inhibitor cocktail and 100 μM Na_2Vo_3 to achieve cell lysis. Cell lysates were cleared by centrifugation (10,000*g* for 10 min, 4°C). Equal amounts of the resulting supernatants were incubated for 60 min with 30 μl of GST-RalGDS-RBD (1:1 slurry containing about 10 μg of immobilized GST fusion protein) to detect Rap1-GTP or with 30 μl of GST Raf1-RBD to detect Ras-GTP. Samples were washed 4 times with radioimmunoprecipitation assay buffer plus protease inhibitors and resuspended in Laemmli buffer. Proteins were separated by SDS electrophoresis, and blotted on polyvinylidene difluoride membranes. Precipitated Rap1-GTP and

Ras-GTP were revealed, respectively, by anti-Rap1 antibody (Santa Cruz Biotechnology) and by anti-Ras antibody (Transduction Labs).

FRTL-5 Cells Transient Transfection. FRTL-5 cells were plated in 60-mm petri dishes at a density of 10^6 cells/dish, the day before transfection. Cells were transfected using Effectene from QIAGEN according to the manufacturer's instructions, using 1 μg/dish of vector or of Rap1N17 (dominant negative form of Rap1) or RasN17 (dominant negative form of Ras) cDNAs. The transfection mixture was left onto the cells for 24 h and the experiments were performed on quiescent cells 72 h after transfections.

Apoptosis Estimation. FRTL-5 cells were seeded onto glass chamber slides and grown and stimulated as with [^{3}H]thymidine uptake. Cells were fixed in Camoy solution (acetic acid/methanol, 1:3) for 15 min and then air-dried. Fluorochrome Hoechst 33258 (Sigma) (1:1000 dilution in HBSS) was added for 30 min. The coverslips were washed four times with water, air-dried, and mounted on slides with Mowiol 4–88, and observed with a Zeiss Axiophot microscope (Carl Zeiss Inc., Jena, Germany).

Confocal Analysis of B-Raf and Rap1 and Immunofluorescence. Confocal analysis was performed as described previously (Sallese et al., 2000b). FRTL-5 cells were seeded on glass chamber slides and treated as with [^{3}H]thymidine uptake. After agonist stimulation, cells were fixed with 4% paraformaldehyde in PBS (0.1 M, pH 7.4) for 15 min at room temperature. The autofluorescence was quenched by incubation for 30 min in 50 mM NH_4Cl, 50 mM glycine in PBS and nonspecific interactions were blocked by treatment with blocking solution (0.05% saponin, 0.5% bovine serum albumin in PBS) for 30 min at room temperature. Cells were incubated overnight at 4°C with anti-B-Raf (C-19) (2 μg/ml) or anti-Rap 1 (4 μg/ml) polyclonal antibodies in blocking solution. In some experiments, we used the anti-Rap1 monoclonal antibody (3 μg/ml) from Transduction Laboratories. The chamber slides were then incubated with blocking solution containing Alexa-488 (Molecular Probes, Eugene, OR) (1:400) or Cy3 (Sigma) (1:400) anti-rabbit or anti-mouse IgGs for 1 h at room temperature. From the incubation with the secondary antibody, each incubation step was carried out in the dark and followed by careful washes with PBS (6 washes, 3 min each). After immunostaining, the coverslips were mounted on slides with Mowiol 4–88, and observed with a Zeiss Axiophot microscope. Localization of the Rap 1 and B-Raf antigens was assessed using a Zeiss LSM 510 Laser Scanning Microscope equipped with an Axiovert 100 M base port. Optical Z-sections from each experimental conditions were taken with 0.3-μm Z-steps from the top to the bottom of the cells. Image processing was performed on a Macintosh computer (Apple Computer, Cupertino, CA).

In colocalization experiments for Rap1 detection we used either the monoclonal anti-Rap1 antibody (Transduction Laboratory) or the polyclonal anti-Rap1 antibody (Santa Cruz Biotechnology) conjugated to the fluorochrome Alexa 594 (Protein labeling kit; Molecular Probes) and used at the concentration of 0.2 μg/ml. Colocalization of Rap1 and B-Raf antigens was assessed by INSIGHT PLUS laser scanning confocal microscope system (Meridian, Oketos, MI) equipped with an Olympus IMT-2 inverted microscope (Olympus, Tokyo, Japan). Ten groups of optical Z-section serial slices from each experiment were taken with 0.5-μm Z-steps from the top to the bottom of the specimen. Fluorescent images were recorded using a DAGE CCD camera (DAGE, Stamford, CT), and stored directly on computer. Merging of both immunofluorescence generated the colocalization maps by color. Colocalization was quantified by measuring the number of pixels labeled with the kinase, with the receptor and the pixels labeled by both antigens; the results are expressed as percentage of the pixels of each antigen.

Statistical Analysis. The statistical analysis was carried on experiments performed in duplicate or triplicate determinations, using Dunnet's analysis of variance.

Results

ERK1/2 Inhibition Decreases TSH-Stimulated DNA Synthesis. To investigate the possible role of MAPK activation in the stimulation of FRTL-5 cell proliferation by TSH, quiescent cells were exposed to TSH, FSK, and 8Br-cAMP in the presence or in the absence of two different inhibitors of MAPK cascade, PD98059 and UO126, followed by measurement of DNA synthesis. [^{3}H]Thymidine uptake was increased up to 40-fold in TSH-stimulated cells. Incubation with PD98059 and UO126 strongly inhibited TSH-induced [^{3}H]thymidine uptake (Fig. 1, A and B). Similarly, PD98059 and UO126 inhibited [^{3}H]thymidine uptake induced by FSK and 8Br-cAMP, indicating that the effect of TSH was cAMP-mediated (Fig. 1A). The inhibition of TSH-stimulated [^{3}H]thymidine uptake by PD98059 and UO126 was dose-dependent and the relative IC_{50} values were, respectively, 15 and 8 μM (Fig. 1B). For FSK-stimulated [^{3}H]thymidine uptake PD98059 and UO126 IC_{50} values were 12 and 5 μM, respectively (data not shown). In the presence of 10 μM H89, which inhibits PKA, we found that TSH-stimulated [^{3}H]thymidine uptake was inhibited only partially ([^{3}H]thymidine uptake was 44 ± 5% of control values, $n = 7$), indicating that

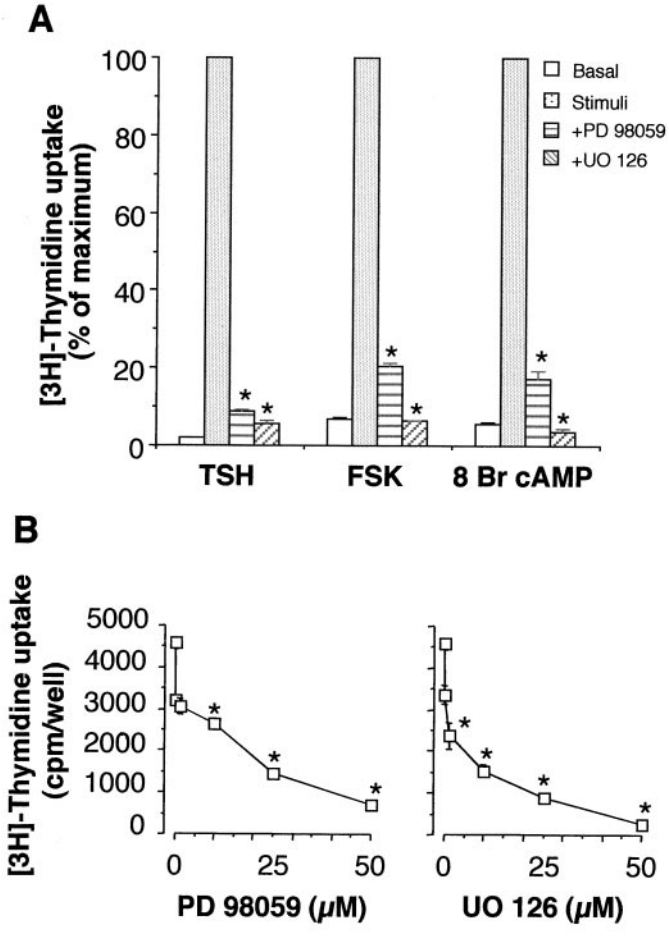

Fig. 1. TSH-induced DNA synthesis is blocked by MEK inhibitors. A, quiescent FRTL-5 cells were stimulated with TSH, FSK, and 8Br-cAMP (10 nM, 10 μM, and 1 mM, respectively) in the presence of PD98059 (50 μM) or UO126 (50 μM) and [^{3}H]thymidine uptake was measured. B, dose-dependent inhibition of TSH-stimulated [^{3}H]thymidine uptake by PD98059 or UO126. Data are mean ± S.E.M. of four to six experiments, each performed in triplicate. *$p < 0.01$; Dunnett's test.

TSH uses PKA-dependent and -independent mechanisms for proliferative response. To test the possibility that the inhibition of DNA synthesis by PD98059 and UO126 could be caused by induction of apoptosis, FRTL-5 cells were plated on glass chamber slides and grown as for [^{3}H]thymidine uptake experiments. After a 48-h starvation, the cells were stimulated with TSH (10 nM) or FSK (10 μM) in the absence or in the presence of different concentrations of PD98059 or UO126. The cells were then stained with Hoechst 33258 and the fraction of apoptotic cells in each sample was evaluated. At least 200 cells for each treatment were counted and the fractions of apoptotic cells was ~2 to 3% and did not differ significantly between cells stimulated with agonist and with agonist plus inhibitors (data not shown). The fraction of apoptotic cells treated with okadaic acid (250 nM), which was used as positive control, was ~50%.

Activation of ERK1/2 by cAMP. We assessed whether TSH- and cAMP-elevating agents were able to activate MAPK cascade. TSH induced ERK1/2 phosphorylation (assessed by immunoblot using anti-phospho ERK1/2) in a time-dependent manner (Fig. 2A). In parallel, we found an increase in ERK1/2 kinase activity, as assessed by in vitro kinase assay using MBP as ERK substrate (Fig. 2A). The activation of MAPK by TSH was rapid; it peaked at 3 min and returned to basal levels at 30 to 60 min (Fig. 2A). TSH induced ERK1/2 phosphorylation at physiological concentrations (Fig. 2B). At 10 nM TSH, which is the standard concentration used to stimulate DNA synthesis, MBP phosphorylation was increased up to 7-fold over basal.

TSH receptor can couple to different G proteins, including Gs, Gi, and Gq, and could therefore activate MAPK through different pathways. We found that direct stimulation of adenylyl cyclase by FSK or exposure to 8Br-cAMP for 3 min were able to activate MAPK, indicating that TSH activates this pathway through Gs (Fig. 3A).

In FRTL-5 cells, TSH is the principal activator of cell proliferation acting by PKA-dependent and -independent pathways. We investigated whether the TSH activation of MAPK cascade was mediated by PKA. Cells were exposed to FSK (10 μM) for 3 min in the absence or presence of H89 (10 μM) to inhibit PKA. H89 was preincubated 30 to 60 min before the addition of FSK for 3 min. FSK-induced MBP phosphorylation was not affected by H89 treatment, indicating that the FSK-stimulated MAPK pathway is PKA-independent (Fig. 3A). Consistently, TSH-induced ERK1/2 phosphorylation, as measured by immunoblot using anti-phospho ERK1/2 antibodies, was not affected by H89 (10 μM) and by the cell-permeable PKA inhibitor PKI (200 nM) (Fig. 3B). In some experiments, we noticed that H89 and PKI treatment induced a modest increase of ERK1/2 phosphorylation (Fig. 3B).

The activation of p70s6K by TSH, a cAMP- and PKA-dependent pathway that significantly contributes to the mitogenic effect of TSH (Cass and Meinkoth, 1998), was used as a positive control for H89 and PKI. The stimulation of p70s6K activity was revealed by mobility shift of the p70s6K in a Western blot. In FRTL-5 cells, p70s6K was stimulated by TSH and the mobility shift of p70s6K bands induced by TSH activation was prevented by mammalian target of rapamycin inhibitor rapamycin (not shown) and by 10 μM H89 and by 200 nM PKI, confirming the inhibition of PKA (Fig. 3C). Moreover, in PC12 cells, H89 (10 μM) treatment completely abolished KCl (60 mM)-induced MAPK activation (data not shown). In these cells, it has been documented that KCl increases cAMP levels and activates MAPK in a PKA-dependent manner (Grewal et al., 2000) further confirming that under our experimental conditions (30-min pretreatment plus 3-min treatment) H89 can inhibit PKA activity.

As expected, PD98059 (10 μM) and UO126 (10 μM) completely abolished cAMP-dependent MAPK stimulation (Fig. 3A). The phosphatidylinositol 3-kinase inhibitor wortmannin (100 nM) did not affect FSK-dependent MAPK stimulation, indicating that phosphatidylinositol 3-kinase is not involved in this pathway (Fig. 3A).

The extent of ERK1/2 activation by TSH (10 nM), as measured by MBP phosphorylation, was comparable with that of IGF-1 (100 ng/ml) (Fig. 4A). MAPK activation by cAMP

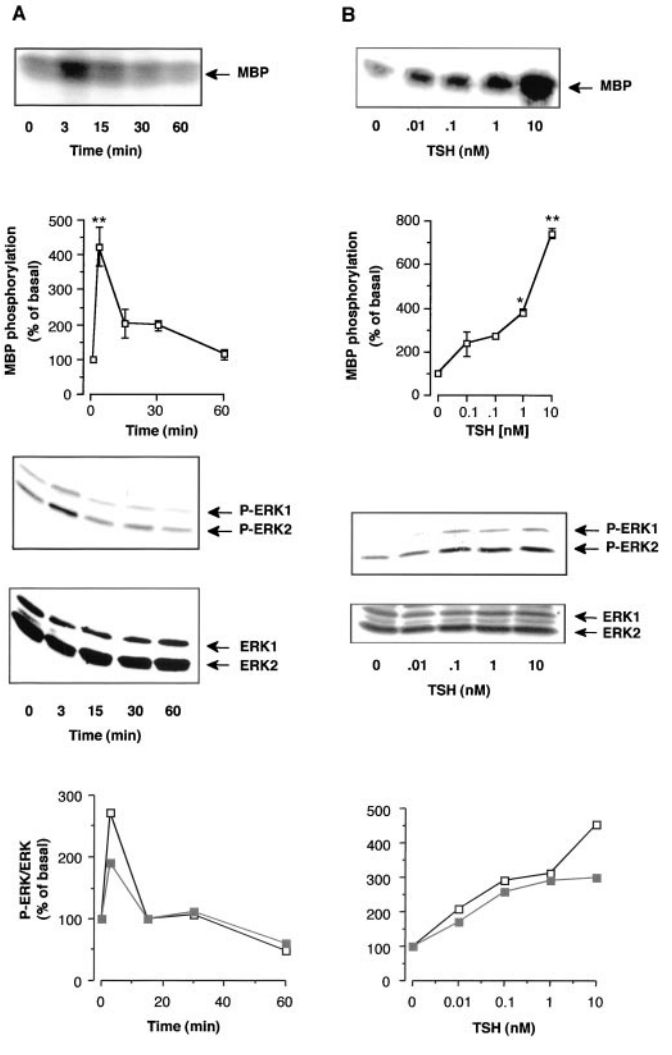

Fig. 2. Activation of ERK1/2 by TSH. A, time course of ERK1/2 stimulation by TSH (10 nM) in FRTL-5. MAPK activity was measured as MBP phosphorylation by immunoprecipitated ERK (top). Means ± S.E.M. of three MBP phosphorylation experiments are presented in the middle panel. Phosphorylation of ERK1/2 was assessed in similar experiments using anti-phospho ERK antibodies (P-ERK1/2). B, dose-dependent stimulation of ERK1/2 by TSH. FRTL-5 were stimulated for 3 min. The representative MBP phosphorylation experiments and phospho-ERK immunoblots were repeated at least three times with similar results. In A and B, the lower panels represent the Western blots identifying the levels of ERK1 and ERK2 as controls for protein loading and the quantification of these experiments, calculated as P-ERK/ERK independently for ERK1 (□) and ERK2 (■). (*$p < 0.01$; **$p < 0.001$; Dunnett's test)

seems to be a general response in thyroid cells, in that it was observed also in another thyroid cell line. We found that FSK treatment (3 min) of PcCl3 thyroid cells increased ERK1/2 phosphorylation by 2-fold, as assessed by Western blot using anti-phospho ERK antibody (data not shown).

One mechanism by which several G protein-coupled receptors can activate MAPK cascade is the transactivation of tyrosine kinase receptors (TKR) (Daub et al., 1997). FRTL-5 cells express different TKRs, including IGF-1 receptor, which, together with the TSH receptor, contributes to induce cell proliferation. We tested whether the TSH activation of MAPK cascade could be mediated by the *trans*-activation of a TKR, such as IGF-1. The tyrosine kinase inhibitor AG1478 (Daub et al., 1997) did not affect the ability of TSH and FSK to stimulate MAPK, indicating that TSH receptor did not *trans*-activate a TKR to activate MAPK cascade (Fig. 4B). IGF-1-stimulated MAPK activation was substantially inhibited by AG1478 (Fig. 4C). In experiments of [^{3}H]thymidine uptake, we also found that TSH-induced DNA synthesis was not affected by AG1418 (data not shown).

Expression of Rap1-Related Signaling Proteins in FRTL-5. The results presented so far document that TSH induced a rapid and transient activation of MAPK cascade by a cAMP-dependent, PKA-independent mechanism. We investigated whether the newly identified cAMP-EPAC-Rap1 cascade (de Rooij et al., 1998) could be involved in this process and we assessed whether this pathway is activated by TSH in FRTL-5. We first analyzed the expression of the proteins that could be involved in the cAMP-EPAC-Rap1 signaling cascade. Rap1 is highly expressed in FRTL-5, as documented by immunoblot (Fig. 5). Rap1 was 7- to 10-fold more abundant in

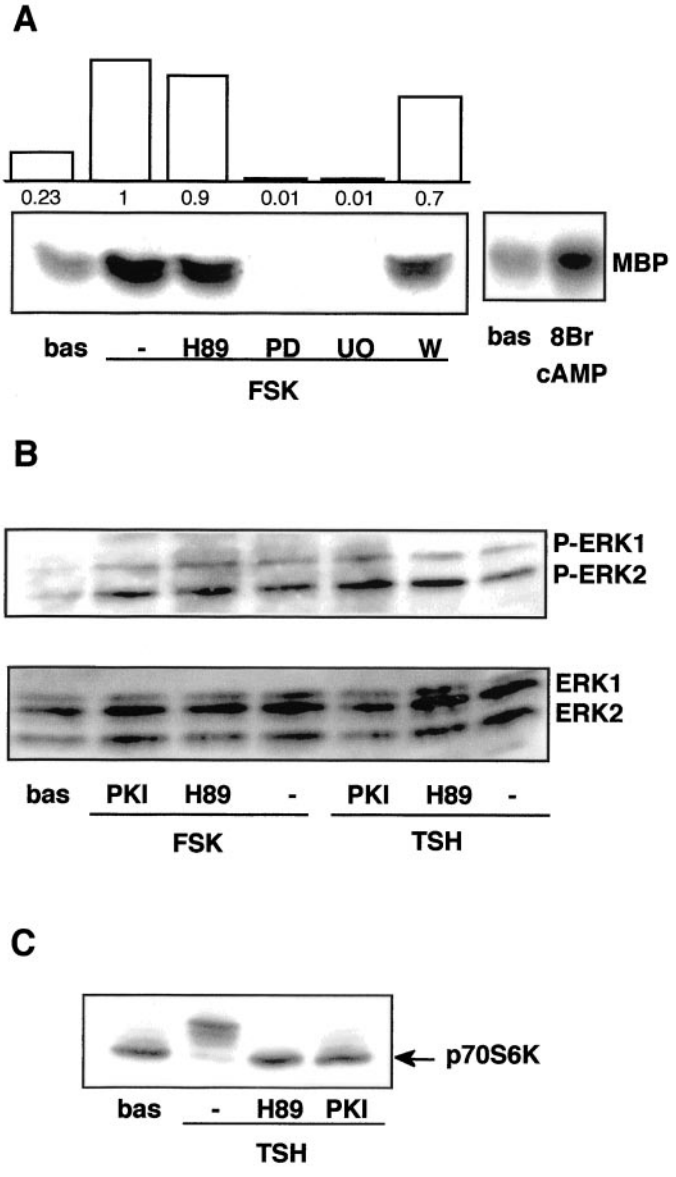

Fig. 3. Activation of ERK1/2 by different agents in FRTL-5. A, FRTL-5 cells were untreated (bas) or treated with 8Br-cAMP (1 mM) or with FSK (10 μM) for 3 min in the absence or in the presence of 10 μM H89, 10 μM PD98059 (PD), 10 μM UO126 (UO), or 100 nM wortmannin (W). ERK1/2 kinase activity was measured as MBP phosphorylation. The quantification of the experiment presented (by NIH image) is reported at the top (numbers represent arbitrary units). B, FRTL-5 cells were treated with TSH (10 nM) or FSK (10 μM) for 3 min in the absence or in the presence of H89 (10 μM) or cell-permeable PKI (200 nM). ERK1/2 phosphorylation was assessed by anti-phospho ERK1/2 antibodies. Immunoblot of total ERK1/2 is also shown. C, TSH-stimulated p70s6K mobility shift is blocked by H89 (10 μM) and PKI (200 nM). FRTL-5 cells were treated with TSH (10 nM for 30 min) total cell lysates (80 μg) were analyzed by immunoblot using an anti-p70s6K antibody. Activation of p70s6K results in multisite phosphorylation, evident as a mobility shift in the gel. Inhibitors were preincubated for 30 to 60 min before TSH treatment. These experiments were repeated at least three times with similar results.

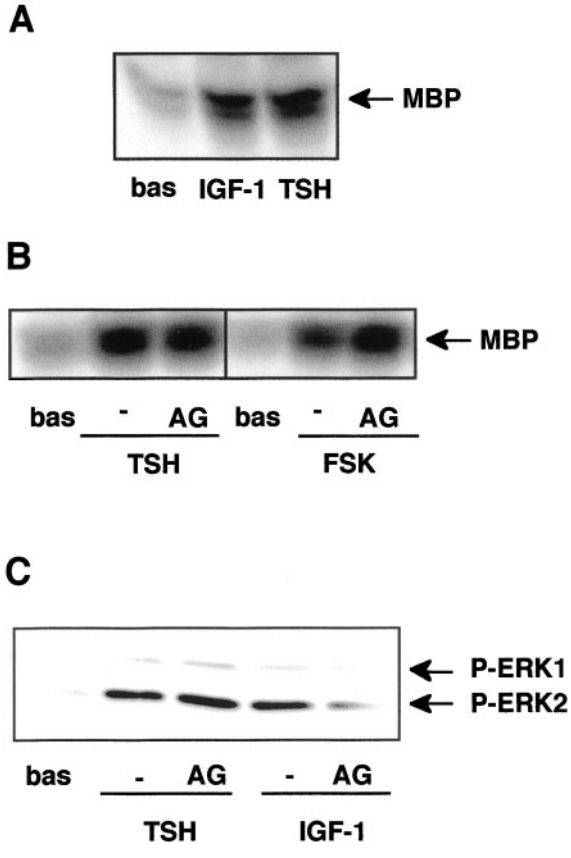

Fig. 4. A, FRTL-5 cells were treated with TSH (10 nM) or IGF-1 (100 ng/ml) and ERK1/2 kinase activity was measured as MBP phosphorylation. B, cells were treated with TSH (10 nM) or FSK (10 μM) in the absence or in the presence of 100 nM AG1478 (AG, preincubated for 60 min), and ERK1/2 kinase activity was measured as MBP phosphorylation. The experiments shown are representative of two to three similar experiments. C, phosphorylation of ERK1/2 (assessed by anti-phospho ERK1/2 antibody) in cells untreated (bas) or treated with TSH or IGF-1 alone or plus AG1478. This experiment is representative of two similar experiments.

FRTL-5 than in PC12 cells (Fig. 5), in which the role of Rap1 in mediating proliferative signaling has been documented (Vossler et al., 1997). B-Raf was expressed at relatively high levels in FRTL-5, as assessed by immunoblot (Fig. 5). Although the anti-B-Raf antibody used could recognize both the 68- and 95-kDa isoforms of B-Raf, only the 95-kDa band was detected in FRTL-5, similar to what was observed in PC12 cells (Vossler et al., 1997). It should be noted that the 95-kDa form, but not the 68-kDa form, can be activated by growth factors (Vossler et al., 1997). The cAMP-sensitive GTP exchange factor EPAC was also highly expressed in FRTL-5 cells, as documented by immunoblot (Fig. 5).

Activation of Rap1 by cAMP. The activation of Rap1 was assessed by measuring the GTP-bound form of this protein by a pull-down assay (Franke et al., 1997; Seidel et al., 1999). Rap1-GTP (i.e., activated Rap1) from cell lysates was specifically bound to a GST-RalGDS-RBD fusion protein precoupled to glutathione-agarose beads and subsequently visualized by Western blotting. TSH (10 nM) and FSK (10 μM) clearly increased the amount of endogenous Rap1-GTP and the PKA-inhibitor H89 did not inhibit the activation of Rap1 by TSH and FSK, indicating that this is a cAMP-dependent, PKA-independent effect (Fig. 6A). This effect was rapid with maximal activation observed after 3 min of treatment (Fig. 6B).

We also observed that FSK treatment induced a mobility shift of the Rap1 band at 30 min (Fig. 6C). This mobility shift was already found in platelets (Franke et al., 1997) and in thyroid cells (Dremier et al., 2000; Tsygankova et al., 2001) and was explained as Rap1 phosphorylation by PKA. Accordingly, we found that H89 treatment substantially prevented the occurrence of the Rap1 mobility shift (data not shown), confirming that also in FRTL-5 cells cAMP increase induced a PKA-dependent phosphorylation of Rap1. The two effects of cAMP on Rap1 observed in FRTL-5 (i.e., Rap1 activation, assessed by GST-RalGDS-RBD binding and phosphorylation, assessed as mobility shift) are totally dissociated in a time course experiment. Binding to GTP is rapid (maximal at 3 min), whereas PKA phosphorylation is detectable after 30 min. Because the activation of MAPK by cAMP-elevating agents was observed after 3 min of treatment, this effect is temporally related to activation (i.e., GTP-binding) of Rap1

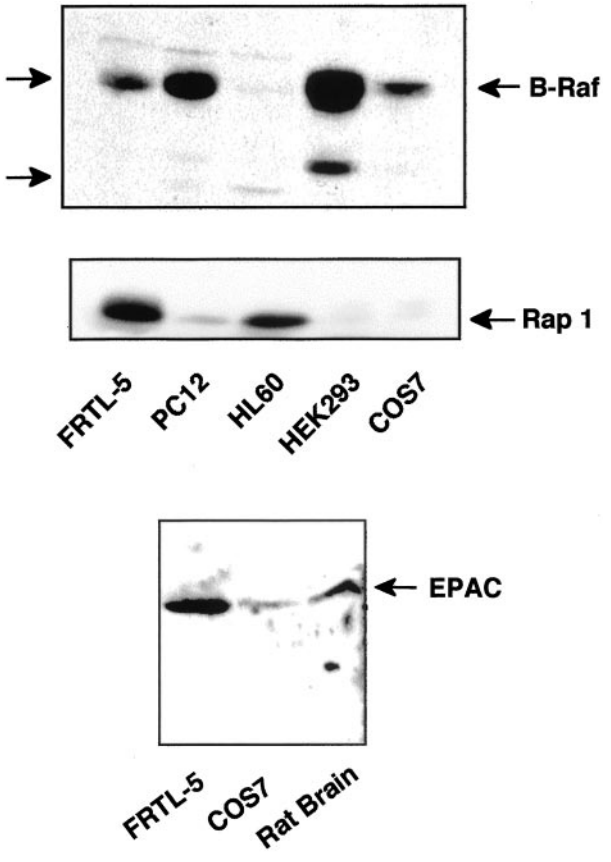

Fig. 5. Expression of Rap1, B-Raf, and EPAC determined by immunoblot in different cell lines. For B-Raf, the arrows indicate the 68- and 95-kDa isoforms. In the immunoblots of Rap1 and B-Raf shown, the same membrane was probed with anti-Rap1 or anti-B-Raf antibodies. The immunoblots shown are representative of two to three similar experiments.

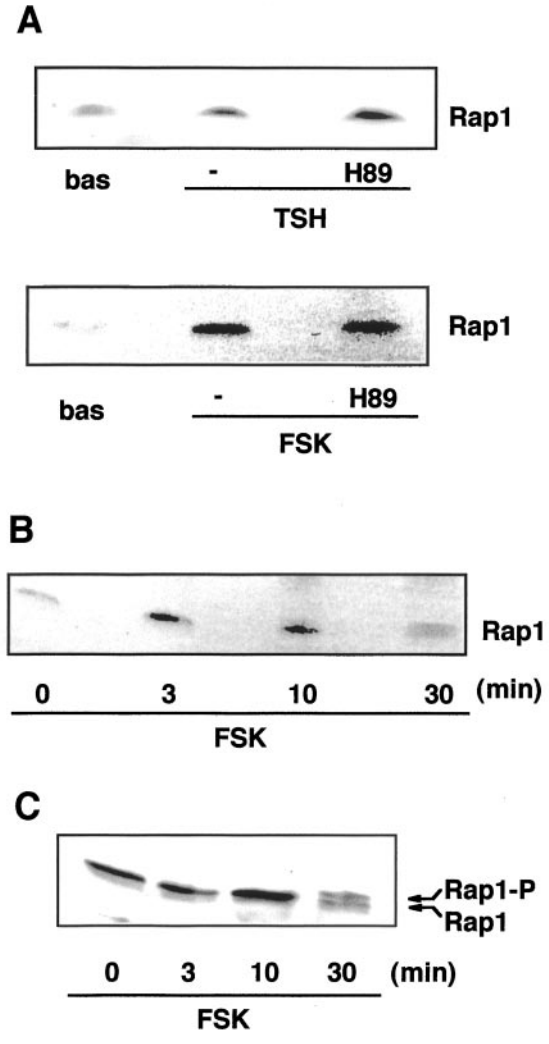

Fig. 6. Activation of Rap1 by TSH. A, FRTL-5 were untreated (bas) or stimulated with TSH (10 nM) or FSK (10 μM) for 3 min, in the presence, where indicated, of H89 (10 μM), which was preincubated for 60 min. B, FRTL-5 were treated with FSK for the indicated times. In A and B, after incubation cells were lysed, clarified by centrifugation, and 500 μg of proteins from each total cell lysate were incubated with GST-RalGDS-RBD conjugated to glutathione agarose beads (to pull down GTP-Rap1) and activated Rap1 was identified by immunoblot using a polyclonal antibody. The experiments shown are representative of three similar experiments. C, FRTL-5 were treated with FSK and 80 μg of proteins from each total cell lysate were used for immunoblot to detect the total amount of Rap1. The lower mobility Rap1 band observed after 30 min of FSK treatment, which represents phosphorylated Rap1, is indicated (Rap1-P).

rather than to PKA-dependent phosphorylation, which is observed after 30 min of treatment. This is in agreement with the hypothesis that MAPK activation by cAMP is a PKA-independent phenomenon.

Activation of B-Raf by cAMP. To further investigate the mechanism of cAMP activation of MAP kinases, we sought to assess whether B-Raf, which is one of the Rap1 effectors, could be involved in the pathway of MAPK activation by cAMP in FRTL-5 cells. To test whether B-Raf is activated by cAMP in FRTL-5, we analyzed FSK-stimulated translocation of B-Raf and the colocalization with Rap1 by immunofluorescence analysis, using confocal microscopy.

In resting conditions, B-Raf immunoflurescence is predominantly detectable in the cytosol with intense perinuclear staining. After short-term (3-min) exposure to FSK, B-Raf immunostaining was mostly punctated, indicating a redistribution to membranes, in vesicular compartments (Fig. 7). This translocation was not prevented by H89 preincubation, thus demonstrating that this is a PKA-independent effect. After 30 min of FSK stimulation, the immunofluorescence of B-Raf was diffused in the cytosol (Fig. 7), as in basal conditions. In parallel experiments, Rap1 immunofluorescence was associated to vesicles both in resting and in stimulated conditions and H89 pretreatment did not affect the Rap1 localization (Fig. 7). This localization was similar to that observed in neuronal cells (York et al., 2000). Confocal microscopy analysis showed that, after FSK stimulation for 3 min, B-Raf significantly colocalized with Rap1 in the same vesicles (Fig. 8). We performed a quantitative analysis using NIH Image analysis program (http://rsb.info.nih-.gov/nih-image), which gives an estimation of colocalized staining. In untreated FRTL-5 cells, 12 ± 1.9% (n = 8) of the B-Raf and 21 ± 9.1% (n = 8) of the Rap1 staining were reciprocally colocalized. After a 3-min exposure to FSK, the level of colocalization increased to 56 ± 4.6% (n = 8) for B-Raf and to 73 ± 8.3 (n = 8) for Rap1 indicating that the FSK-induced redistribution of B-Raf resulted in increased colocalization with Rap1.

The Expression of Rap1 Dominant Negative Mutant Inhibits TSH-Stimulated MAPK Activation. To obtain direct evidence of the involvement of Rap1 in the pathway mediating TSH-stimulated MAPK activation, we used the Rap1N17 mutant, which acts as a Rap1 dominant negative (Buscà et al., 2000). In FRTL-5 cells transiently transfected with Rap1N17, TSH-stimulated ERK1/2 activation was reduced by 45 ± 4.2%, compared with the vector-transfected cells. In parallel samples, the activation of MAPK induced by IGF-1 was not affected by Rap1N17 (87 ± 2.3% of control values) (Fig. 9A).

Involvement of Ras in TSH-Stimulated MAPK Activation. Previous studies demonstrated that in thyroid cells, exposure to TSH induced a rapid activation of Ras (Tsygankova et al., 2000). This effect is mediated by cAMP and, depending on cell types and experimental conditions, may or may not involve the activation of PKA. Because we have shown that in FRTL-5 cells TSH stimulated the rapid activation of MAPK by a PKA-independent mechanism, we investigated whether, in addition to Rap1, Ras activation could also be involved in the TSH-stimulated MAPK activation, using the Ras dominant negative mutant RasN17 to address this issue. In FRTL-5 cells transiently transfected with RasN17 cDNA, TSH-stimulated ERK1/2 activation was reduced by 53 ± 5.7%, compared with vector-transfected cells. In parallel samples, the activation of MAPK induced by IGF-1 was also reduced by 61 ± 4%, in the presence of RasN17, compared with vector-transfected cells (Fig. 9A).

The rapid activation of Ras by TSH was determined by pull-down assay using GST-Raf1 RBD. We could show that TSH rapidly (3 min) stimulates the activation of Ras (Fig.

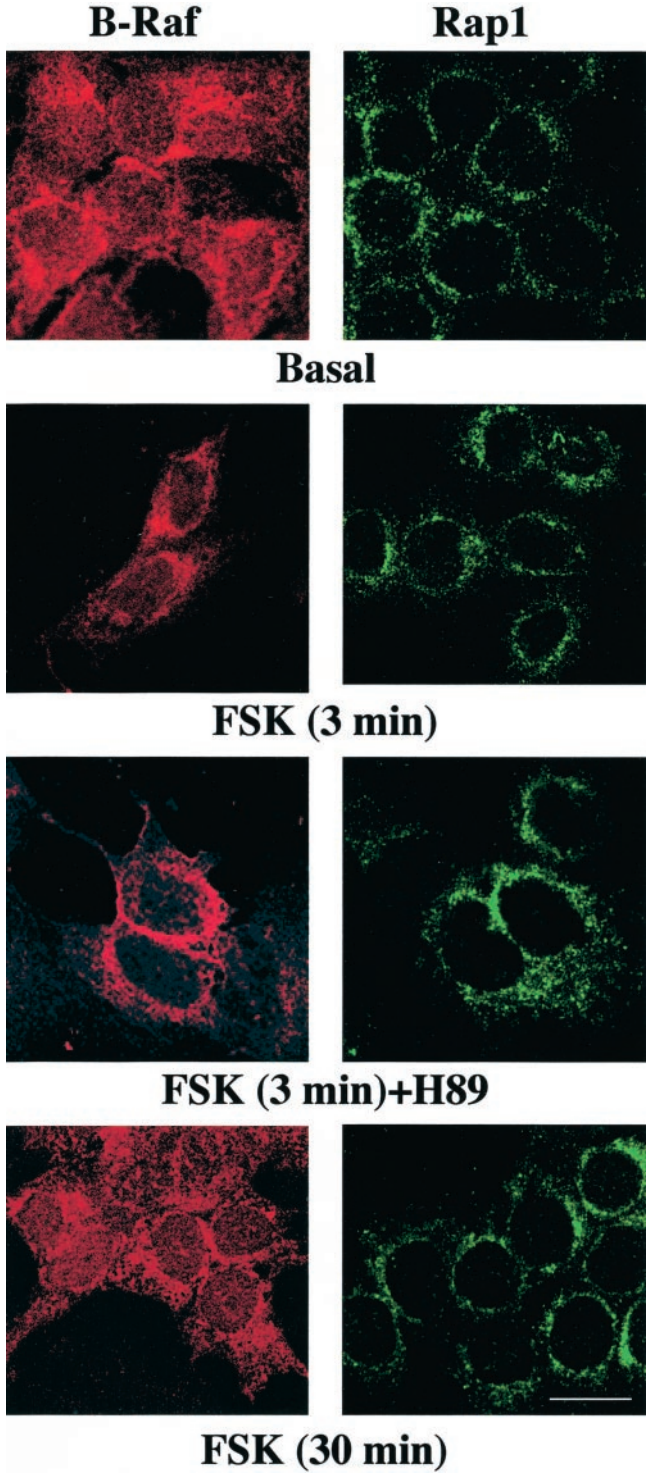

Fig. 7. Translocation of B-Raf by FSK in FRTL-5. The fluorescence imaging of B-Raf (in red) and Rap1 (in green) using confocal microscopy is shown. FRTL-5 cells were untreated (Basal) or treated with FSK (10 μM) for the indicated times. Where indicated, H89 (10 μM) was added to inhibit PKA. The experiment shown was repeated four times with similar results. Bar, 5 μm.

9B), thus confirming previous findings by other groups (Tsygankova et al., 2000).

Discussion

This study describes a novel mechanism by which TSH stimulates cell proliferation in the FRTL-5 rat thyroid cells. We show that TSH rapidly stimulates MAPK cascade through a cAMP-dependent, PKA-independent mechanism. The activation of MAPK by TSH peaked at 3 min and was transient; it returned to basal levels in 30 min. We found that the activation of MAPK by TSH was independent from PKA activation, because H89 and the cell-permeable PKI did not

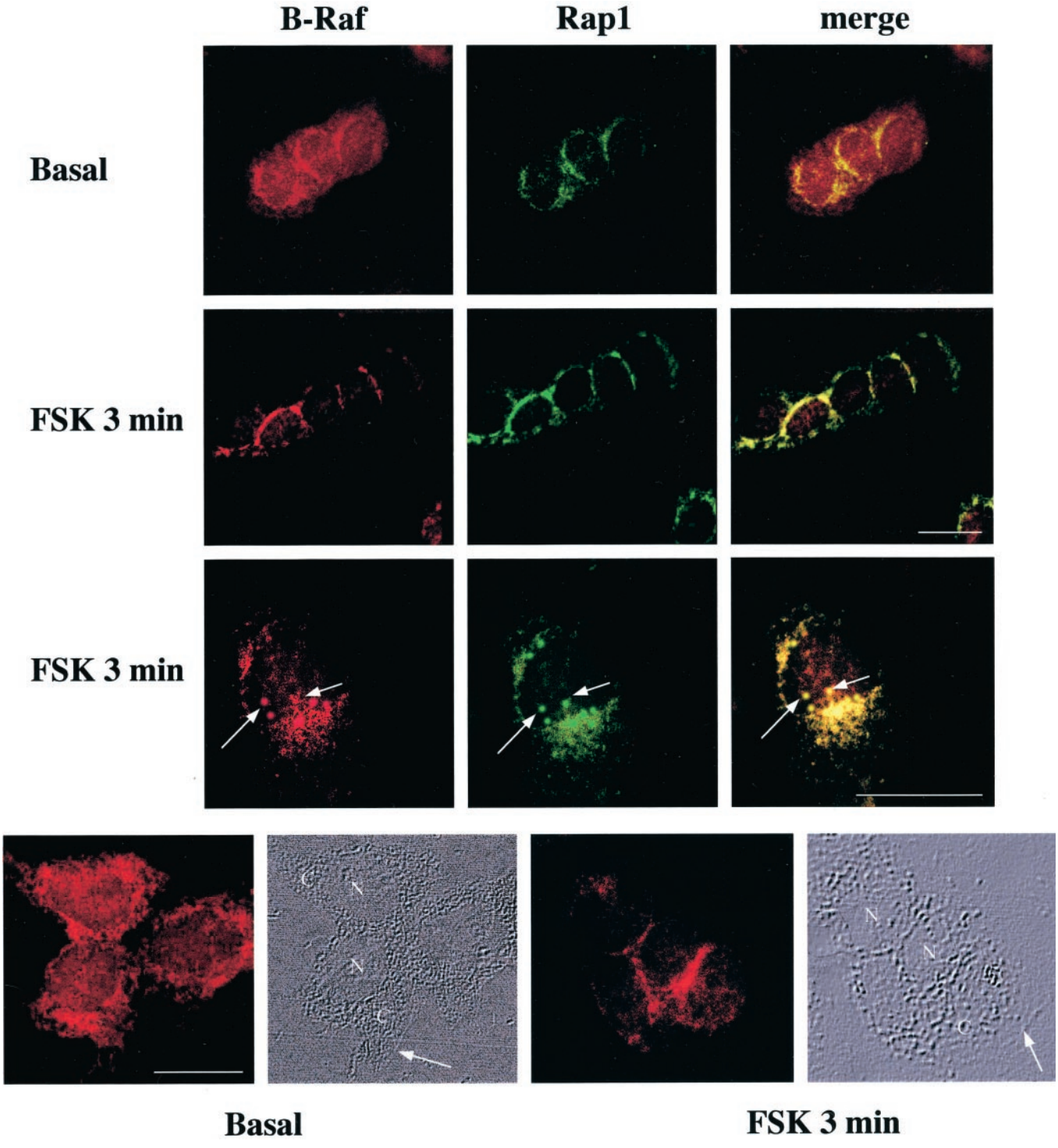

Fig. 8. Colocalization of B-Raf and Rap1 after FSK treatment. FRTL-5 cells were either untreated (Basal) or exposed to 10 μM FSK for 3 min and double-stained with anti-Rap1 and anti-B-Raf antibodies to visualize B-Raf (stained in red), Rap1 (stained in green), or both simultaneously (stained in yellow; merge). For 3 min of FSK treatment, panels showing a higher magnification are presented and the arrows indicate some vesicles where B-Raf and Rap1 are colocalized. The lower panels show B-Raf immunofluorescence and the corresponding bright field images of basal and treated cells. Nuclei (N), cytosol (C), and plasma membranes (arrows) are indicated in the bright field images. The experiments shown were repeated three times with similar results. Bar, 5 μm.

modify this response. The activation of MAPK cascade seemed to play an important role in TSH-stimulated proliferative response, in that the MEK inhibitors UO126 and PD98059 were able to abolish TSH-induced DNA synthesis. A number of previous investigations failed to demonstrate the involvement of MAP kinase activation in TSH-induced thyroid cell proliferation (Lamy et al., 1993; Al-Alawi et al., 1995; Miller et al., 1998). The apparent discrepancy between our results and those from previous studies reporting that TSH does not stimulate MAPK cascade is probably explained by the fact that TSH-stimulated MAPK activation is fast and rapidly reversible [i.e., it can only be observed after short term (<5 min) exposure to TSH].

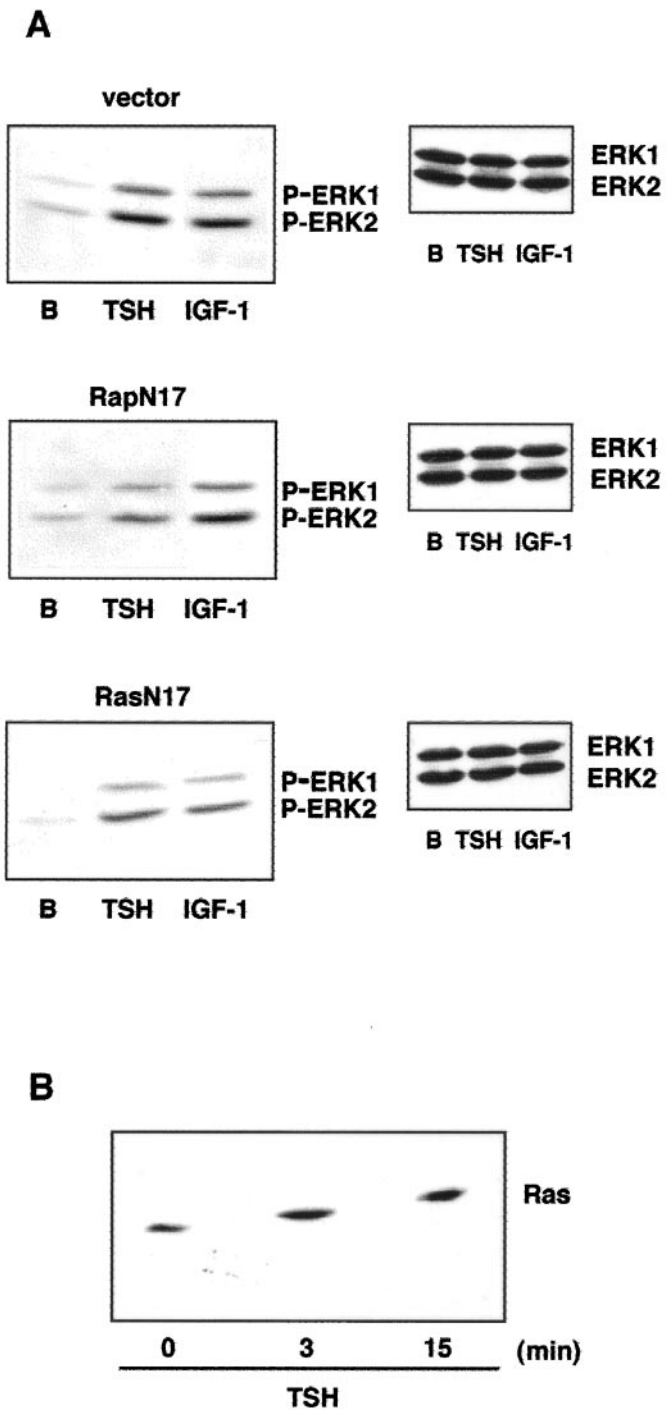

Fig. 9. Rap1 and Ras mediate the TSH-stimulated activation of ERK1/2. A, an empty vector (vector) and the dominant negative forms of Rap1 (Rap1N17) or Ras (RasN17) were transiently transfected in FRTL-5 cells and ERK1/2 phosphorylation was assessed by anti-phospho-ERK antibody in untreated cells (lane B) or cells treated with TSH or IGF-1. Total ERK1/2 levels are determined in the same experiments and are shown on the right. B, activation of Ras, induced by TSH treatment for the indicated times, was assessed by pull-down assay using GST-Raf1-RBD as a binding protein for GTP-bound Ras. The experiments shown are representative of two to three similar experiments.

The small G-protein Rap1 and B-Raf are involved in this signaling cascade. Rap1 is expressed at high levels in FRTL-5 cells, and this suggests that this small G protein may have a major role for signaling in these cells. In FRTL-5 cells, TSH stimulated GDP/GTP exchange, and hence the activation of Rap1, in agreement with what is observed in other thyroid cell models (Dremier et al., 2000; Tsygankova et al., 2001). Consistent with the effect on MAPK, the activation of Rap1 by TSH (or FSK) was rapid (the peak of the effect was at 3 min) and was cAMP-dependent and PKA-independent. In addition, we could demonstrate that transfection of Rap1 dominant negative Rap1N17 significantly inhibited TSH-stimulated MAPK activation without any effect on IGF-1–stimulated MAPK, indicating that Rap1 mediates TSH-dependent MAPK activation. The rapid activation of Rap1 by TSH could probably be mediated by the Rap1-specific nucleotide exchange factor EPAC that is directly activated by cAMP (de Rooji et al., 1998). EPAC is expressed at high levels in thyroid cells (Kawasaki et al., 1998; this study) and the possible role of the EPAC/Rap1 complex in thyroid cell replication has been postulated (de Rooij et al., 1998).

B-Raf also seemed to play a role in the TSH-dependent MAPK activating cascade. After FSK stimulation for 3 min, B-Raf seemed to be translocated to a vesicular compartment, where it is colocalized with Rap1 and is probably activated by Rap1 (Vossler et al., 1997). Based on these findings, we propose the following model: TSH-stimulated intracellular cAMP binds and activates EPAC, which stimulates GDP/GTP exchange and hence the activation of Rap1. GTP-bound Rap1 activates B-Raf, which in turn could activate MEK and hence ERK1/2. These events are rapid (3 min) and are stimulated by cAMP in a PKA-independent manner.

We show that Ras is also involved the rapid TSH-dependent activation of MAPK observed in FRTL-5, as demonstrated by the inhibition of this effect by the RasN17 dominant negative mutant. Unlike Rap1N17, however, RasN17 also inhibited IGF-1–stimulated MAPK activation, indicating that Ras is involved in both G protein-coupled receptor- and TRK-induced MAPK signaling cascade.

It should be emphasized that the molecular mechanisms driving these intracellular events are strictly cell type-dependent (Zwartkruis and Bos, 1999). Similar to what we observed in thyroid cells, a signaling cascade involving Rap1/B-Raf -mediated MAPK activation by cAMP was recently described in PC12 cells (Vossler et al., 1997) but, unlike what we found in FRTL-5, these events in PC12 cells are mediated by PKA. By contrast, a recent study in melanocytes reported that MAPK are stimulated by cAMP through a PKA-independent mechanism, which involves Ras, but not Rap1, activation of B-Raf (Buscà et al., 2000).

In thyroid cells, the TSH-stimulated increase of cAMP activates different pathways that are either PKA-dependent or PKA-independent. The TSH-stimulated proliferative pathway described here, which leads to the activation of ERK1/2,

represents one of these signaling cascades. Rap1 and Ras are both involved in our model indicating that TSH utilizes these two small GTPases for rapid activation of ERK1/2. This activation is early and transient and probably requires low cAMP concentration.

Prolonged TSH stimulation induces further cAMP accumulation and hence the activation of PKA. It is likely that Rap1 and Ras, that mediate the rapid PKA-independent signaling cascade stimulated by TSH, are in turn selectively regulated by TSH-activated PKA. For example, the analysis of different Ras effectors in WRT thyroid cells indicated that the cross talk between Ras and PKA discriminates between distinct Ras effector pathways (Miller et al., 1998). As far as Rap1 is concerned, in agreement with a recent report (Franke et al., 1997), we found that after 30 min of exposure to cAMP-elevating agents Rap1 was phosphorylated by PKA, indicating a biphasic effect of cAMP on Rap1 with a rapid (3-min) activation followed by a delayed (30-min) PKA-dependent phosphorylation. PKA phosphorylation prolonged Rap1 activation (Tsygankova et al., 2001) and this effect could account for the enhanced cAMP-dependent MAPK activation that we observed in FRTL-5 cells exposed to H89 and PKI.

PKA can also activate other pathways that contribute to thyroid cell proliferation and differentiation. For example, in agreement with previous reports (Cass and Meinkoth, 1998) we found that exposure to TSH for at least 20 to 30 min activated p70s6k in a PKA-dependent manner. Treatment with the mammalian target of rapamycin inhibitor rapamycin substantially inhibited cAMP-stimulated [^{3}H]thymidine uptake, suggesting a key role of this pathway in TSH-dependent proliferative response of FRTL-5 (L. Iacovelli, unpublished observations). In addition, Pomerance et al. (2000) showed that, in FRTL-5 cells, TSH stimulation activates p38-MAPK in a PKA-dependent manner. This effect, which was insensitive to PKC and p70s6k inhibitors, was mediated by Rac1 but not by Ras (Pomerance et al., 2000). Further investigations will shed light on the reciprocal interplay and cross talk among all these pathways.

In conclusion, this study support the idea that TSH stimulates multiple independent mechanisms that act in an integrated manner to lead to thyroid cell proliferation and differentiation.

Acknowledgments

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Address correspondence to: Dr. Antonio De Blasi, INM Neuromed IRCCS, località Caramelle, 86077, Pozzilli (IS), Italy. E-mail: deblasi@neuromed.it