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TSH and cAMP Do Not Signal Mitogenesis through Ras Activation

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Ras activation by receptor tyrosine kinases or serpentine receptors is generally considered to be essential for G1 phase progression and mitogenesis. In the physiologically relevant model of primary dog thyrocytes, the accumulation of the GTP-bound form of Ras constituted an early convergence point of various mitogenic or comitogenic stimuli including EGF, HGF, phorbol esters, insulin and carbachol. By contrast, the basal level of GTP-Ras was slightly reduced by TSH and forskolin and did not increase during the TSH/cAMP-dependent progression into G1 phase. This rules out a role for the activation of Ras as a signal in the mitogenesis elicited by TSH via cAMP in these cells. © 2000 Academic Press

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The small G protein Ras integrates signals from transmembrane receptors, like tyrosine kinase receptors or receptors coupled to heterotrimeric G proteins and switches between active and inactive states, depending on whether GTP or GDP is bound. The actions of Ras may be mediated by multiple effectors, including Raf and the well characterized MAP kinases cascade, but also phosphatidylinositol 3-kinase (PI3K) and the guanine nucleotide exchange factors for Ral (1). In various systems, microinjection of Ras blocking antibodies or dominant negative Ras mutants indicates that Ras function is required throughout G1 phase for cyclin D1 expression, $p27^{kip1}$ downregulation, pRb phosphorylation and S phase initiation (2–6). More recently, a second major peak of Ras activation was described during mid-G1 phase in NIH 3T3 and HeLa cells (4, 7). Interestingly this cell cycle-dependent Ras activation is dissociated from MAP kinases activation. In synchronized HeLa cells, it is not secondary to tyrosine phosphorylation of Shc proteins and their binding to Grb2, but depends on new protein synthesis (7).

Dog thyroid epithelial cells in primary culture probably constitute the best characterized model of positive cell cycle regulation mediated by cAMP (8–11). As demonstrated in these cells in which cAMP is generated in response to TSH (12) and as confirmed in other cell systems (13–15), the cAMP-dependent mitogenesis is unique in that it is not associated with a stimulation of p42/p44 MAP kinases phosphorylation and activity. This suggests a lack of Ras activation in this pathway. However, Ras is suggested as an intermediary in the cAMP-dependent mitogenesis since microinjected dominant interfering mutants of Ras inhibit TSH/cAMP-stimulated DNA synthesis in the rat thyroid cell line WRT (16, 17). In this cell line, the lack of MAP kinases activation is ascribed to PKA inhibition of c-Raf (15) and redirection by cAMP of Ras signaling toward other effectors such as Ral GDS and PI3 kinase (17–20). Here we reevaluate the involvement of Ras in this pathway by directly assessing the content of Ras-GTP in normal dog thyrocytes. We compare the effect of TSH and forskolin, which enhance cAMP accumulation, of cAMP-independent mitogens including EGF, HGF and the phorbol ester TPA, and of insulin and carbachol which are permissive for cAMP-dependent mitogenesis ((21, 22); A.V.K., unpublished data).

MATERIALS AND METHODS

Primary cultures of dog thyroid follicular cells. Dog thyrocytes, seeded as follicles (2×10^4 cells/cm^2), were cultured in monolayer in the following mixture (23): DMEM + Ham's F12 medium + MCDB104 medium (2:1:1, by volume; GIBCO BRL, Paisley, Scotland), supplemented with ascorbic acid (40 μg/ml) and antibiotics. As indicated, bovine insulin (5 μg/ml) (Sigma Chemical Co., St. Louis, MO) was added or not to this medium since the seeding. The medium was changed every two days. At day 4, the cells were quiescent and

Abbreviations used: EGF, epidermal growth factor; HGF, hepatocyte growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TSH, thyroid stimulating hormone; MAP kinase, mitogens activated protein kinase, PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PI3K, phosphatidyl inositol 3 kinase; GST, glutathione *S*-transferase; RBD, ras binding domain; GEF, guanine exchange factor.

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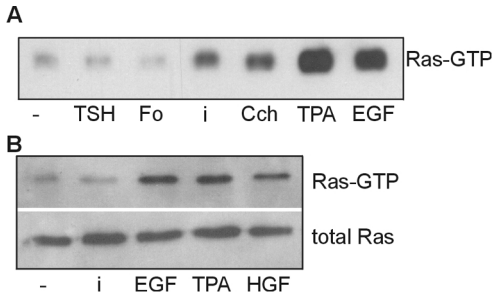

FIG. 1. Activation of Ras by the different agents after a stimulation of 5 min. Dog thyrocytes cultured in control medium were stimulated or not for 5 min with TSH, forskolin (Fo), insulin (i), carbachol (Cch), TPA, EGF, or HGF. Cells were lysed and Ras-GTP was isolated using GST-RBD prebound to glutathione-agarose beads. Ras was detected by Western blot analysis using an anti-Ras mouse monoclonal antibody. In B, the total amount of Ras contained in 20 μl of total lysate of the same experiment is also shown.

were treated with the following stimulants: bovine TSH (1 mU/ml, Sigma Chemical Co.), forskolin (10^{-5} M, Calbiochem), bovine insulin (5 μg/ml, Sigma Chemical Co.), murine EGF (100 ng/ml, Collaborative Research Inc.), carbachol (10^{-5} M, Sigma Chemical Co.), 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 100 ng/ml, Sigma Chemical Co.) and recombinant human HGF (40 ng/ml) (a kind gift of T. Nakamura, Osaka University Medical School).

Activated Ras assay. Ras activation was determined as previously described (24). Briefly, stimulated cells (from a 90-mm culture dish) were washed twice with ice-cold PBS, lysed and scraped in 800 μl of Ras-buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μM leupeptin, 60 μg/ml Pefabloc, 10 mM NaF and 1 mM Na_3VO_4) and the lysate was clarified by centrifugation. Supernatants were incubated for 35 min at 4°C with Ras binding domain of c-Raf-1 fused to GST (GST-RBD) pre-bound to glutathione-agarose beads (Pharmacia). After incubation, beads were washed four times in Ras-buffer and bound proteins were then eluted with SDS-Laemmli sample buffer. Samples were resolved by SDS–polyacrylamide gel electrophoresis (12%) followed by transfer to PVDF membranes and probed with an anti-Ras monoclonal antibody (Transductions Laboratories). A secondary antibody coupled to horseradish peroxidase (Amersham) was used for detection by enhanced chemiluminescence (ECL kit, Amersham). Aliquots (10%) of total cell lysate were also resolved for quantitation of total Ras.

All the results were reproduced at least three times in independent experiments.

RESULTS

Strong Activation of Ras by cAMP-Independent Mitogenic Treatments

EGF, HGF and TPA trigger mitogenesis in dog thyrocytes by cAMP-independent pathways and inhibit the expression of thyroid differentiation (23, 25, 26). It was previously shown that these three agents strongly activate p42 and p44 MAP kinases in these cells (12, 26). The activation of Ras was determined by pulling down the active GTP-bound Ras with the Raf-RBD and detecting it by western blotting using a Pan-Ras antibody. As shown in Figs. 1 and 2, a weak basal level of GTP-bound Ras was detected in cells maintained in control medium, i.e., in the absence of serum and insulin. TPA (100 ng/ml), EGF (100 ng/ml) (Figs. 1A and 1B) and HGF (40 ng/ml) (Fig. 1B) strongly increased the amount of GTP-bound Ras after a 5 min stimulation. As shown on Fig. 2, the strong effect of TPA and EGF persisted for at least 1 h. As the presence of insulin is required for the mitogenic effect of the different growth factors in thyrocytes (21, 23), Ras activation experiments for longer periods of stimulation were performed on cells cultured in control medium supplemented with insulin. After a 16-h stimulation by EGF, when most responding cells were in mid to late G1 phase, the presence of GTP-bound Ras was still increased but at a reduced level compared with a 5-min stimulation (Fig. 3A). Moderately elevated levels of GTP-bound Ras were also observed 4 h, 8 h or 12 h after EGF stimulation (data not shown). The Ras activation by EGF was thus strong during around 1 h, then persisted at a weaker level for at least 16 h.

Weak Activation of Ras by Insulin and Carbachol in Dog Thyroid Cells

Insulin and carbachol used alone are not mitogenic factors in thyrocytes, but both increase the cell size (21). Insulin is permissive for the stimulation of DNA synthesis by cAMP, EGF and TPA, and carbachol reproduces this permissive effect on cAMP-dependent mitogenesis ((21–23); A.V.K., unpublished data). As compared to the strong effects of EGF and HGF, insulin, which also triggers a tyrosine kinase cascade, only increased weakly the content of GTP-bound Ras after times ranging from 5 min (Fig. 1A) to 1 h (Fig. 2). In some experiments, the effect of insulin on Ras activation was undetectable (Fig. 1B). Carbachol consistently

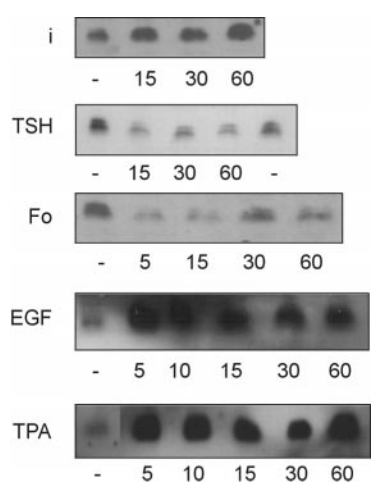

FIG. 2. Time courses of Ras activation by different agents. Thyrocytes cultured in control medium were stimulated or not by insulin (i), TSH, forskolin (Fo), EGF or TPA for times varying from 5 to 60 min. Ras-GTP was isolated using GST-RBD prebound to glutathione-agarose beads. Ras was detected by Western blot analysis using an anti-Ras mouse monoclonal antibody.

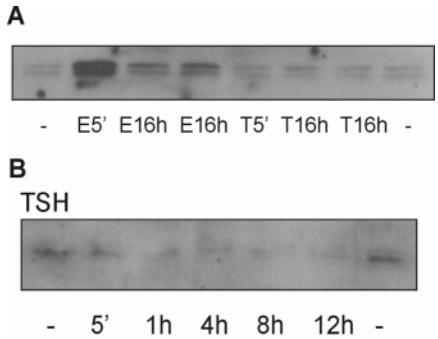

FIG. 3. Ras activation during G1 phase progression stimulated by TSH or EGF in the presence of insulin. (A) Thyrocytes cultured in control medium supplemented with insulin were stimulated either by EGF for 5 min (E5′) or 16 h (E16h) either by TSH for 5 min (T5′) or 16 h (T16h). (B) Time courses of Ras activation by TSH in thyrocytes cultured in control medium supplemented with insulin. The Ras activation assay was performed as described in the legend to Fig. 1. The total levels of cellular Ras remained constant during this period (data not shown).

stimulated the formation of GTP-bound Ras after 5 min, but more weakly than growth factors and TPA (Fig. 1A).

Lack of Ras Activation in the TSH/cAMP-Dependent Mitogenesis of Dog Thyroid Cells

In contrast to the stimulations by growth factors and TPA, the amount of GTP-bound Ras was not increased by TSH (1 mU/ml) after 5 min (Fig. 1A), 15 min, 30 min or 1 h (Fig. 2) of stimulation, but it was even weakly decreased by TSH compared to the control condition. Treatment of thyrocytes with forskolin (10^{-5} M), which is a direct activator of adenylyl cyclase, reproduced this inhibition (Figs. 1A and 2). As shown in Fig. 3, TSH also did not increase the content of GTP-Ras in cells cultured in the presence of insulin, i.e. in conditions that allow the progression of a majority of cells into the cell cycle. No stimulation was observed at various time points ranging from 5 min to 16 h covering the whole G0-S prereplicative phase (Figs. 3A and 3B).

DISCUSSION

Ras has long been thought to play an important role in the regulation of proliferation and differentiation of thyroid epithelial cells. Activating mutations of Ras genes are a very frequent early event in follicular thyroid tumorigenesis (27–29). Activated H-Ras induces a sustained proliferation in primary cultures of normal human thyrocytes (30), and it provokes both a loss of TSH-dependence for growth and a suppression of differentiation expression in rat thyroid cell lines (31, 32). However, Ras activation reflected by its GTP-loading in response to extracellular stimuli has never been investigated in thyroid cells.

Here we use dog thyroid cells in primary culture, a model close to physiology and similar, in terms of mitogenic regulation, to the human thyroid cell (9). We show that Ras activation is an early point of convergence of mitogenic pathways elicited by growth factors (EGF, HGF) and by phorbol ester TPA, but also of the permissive agents, insulin which supports the induction of DNA synthesis by cAMP, EGF and TPA, and carbachol which more specifically reproduces this permissive action on cAMP-dependent mitogenesis. So far as it has been studied, there is a good qualitative correlation between the effects of the various agents on the activation of Ras and MAP kinases in dog thyrocytes: the strongest stimulus is TPA, EGF and HGF have a strong effect, and the effects of carbachol and insulin are weaker ((12), Vandeput, unpublished data). Both variables thus reflect the activity of the Ras-Raf-MAPK canonical pathway in these cells. By contrast, the strong activation of Ras by TPA does not lead to a stimulation of PI3 kinase and PKB activities, which are totally unresponsive to TPA in dog thyrocytes (33).

The strong activation of Ras by growth factors and TPA, which induce mitogenesis and reversibly inhibit the expression of differentiation in dog thyrocytes is consistent with a role of Ras in these cell processes, in agreement with previous analyses of ectopically expressed oncogenic Ras in rat thyroid cell lines. However, in dog thyrocytes, normal Ras activation by extracellular stimuli is not sufficient to trigger mitogenesis as all its activators except HGF, fail to trigger it by themselves.

TPA, acting through PKC, was first described to activate MAP kinases by phosphorylating and activating Raf independently of Ras activity (34). However, several recent studies described a Ras activation by TPA ascribed to PKC (35, 36). This mechanism of Ras activation appears to be cell type specific since no Ras activation by TPA was shown in NIH 3T3 ((37); confirmed by the Ras-GTP pull-down assay, de Rooij, Zwartkruis, and Bos, personal communication). Carbachol, which raises the level of intracellular calcium and activates PKC by activating phospholipase C (38), also activates Ras in thyrocytes but more weakly than TPA. TPA and carbachol could also act on Ras activity through a direct activation of specialized Ras-specific GEFs sensitive to calcium or diacylglycerol (39).

In contrast to what is observed in the growth factor and phorbol ester signaling pathways, we show here that TSH and cAMP, which induce both proliferation and differentiation expression (9), do not activate Ras in dog thyrocytes. We also did not observe an increase of the GTP-loading of Ras during G1 phase progression stimulated by TSH in the presence of insulin, at variance with the G1 phase activation of Ras reported using the same assay from NIH-3T3 and HeLa cells (7) which was claimed not to lead to MAP kinase activation. TSH even partially inhibited the basal GTP-

loading of Ras. This unexpected effect is cAMP-dependent since it is mimicked by forskolin, but the mechanism is unknown. This lack of Ras activation explains the lack of MAP kinases activation in the cAMP-dependent pathway, which therefore does not result from the uncoupling by cAMP of c-Raf from Ras. The fact that MAP kinases phosphorylation and nuclear translocation induced by EGF are not affected by forskolin in dog thyrocytes (40), at variance with other systems (41, 42) including WRT thyroid cells (15), also make such uncoupling unlikely. Through the MAP kinases cascade and other effector pathways, Ras is considered to be crucial for cell cycle progression by exerting several essential functions including stabilization of c-Myc protein (43), induction of cyclin D1 and down-regulation of $p27^{kip1}$ (3). The lack of Ras activation thus contributes to explain unique characteristics of the cAMP-dependent cell cycle in dog thyrocytes, such as the particuliary transient induction of c-Myc (44), the lack of cyclin D1 induction (11) and the stimulation rather than inhibition of $p27^{kip1}$ accumulation by TSH (45).

The lack of Ras stimulation by TSH has to be reconciled with previous studies suggesting a role of Ras in the cAMP-dependent mitogenic pathway in thyroid cells. Indeed, microinjected dominant interfering mutant of Ras inhibits TSH-stimulated DNA synthesis in WRT cells, which has been interpreted as an evidence that Ras is an intermediary of the cAMP-dependent pathway (16) whereas it only suggests a requirement for ras activity (17). We have consistently observed a low basal level of Ras-GTP in the present experiments. In dog thyrocytes, PD098059, which specifically inhibits p42/p44 MAP kinases, also inhibits DNA synthesis triggered by TSH in the presence of insulin, even though TSH does not activate MAP kinases (Vandeput, unpublished data). This may suggest a requirement for a basal activity of MAP kinases and thus perhaps of Ras as one condition permitting the cAMP-dependent mitogenesis. However, the present results demonstrate that Ras activation does not contribute as a *signal* in the still enigmatic mechanism by which TSH can trigger mitogenesis through cAMP.

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