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Activation of the Small G Protein Rap1 in Dog Thyroid Cells by Both cAMP-Dependent and -Independent Pathways

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Thyrotropin, through a cAMP-dependent pathway, stimulates function, differentiation, and proliferation of dog and human thyroid cells. Our previous findings suggested that, in addition to PKA activation, another cAMP-dependent mechanism is involved in TSH action. In this work, we assess whether the newly identified cAMP-Epac-Rap1 cascade is involved in TSH-cAMP-mediated effects in dog thyroid cells. We first demonstrate that TSH and forskolin strongly activate Rap1 in a PKA-independent manner. However, activation of Rap1 is not specific for TSH or cAMP. Indeed, carbachol, TPA, insulin, or EGF, which activate different cAMP-independent cascades, all independently activate Rap1. Rap1 is therefore a common step in all these cascades which exert various effects on proliferation, differentiation, and function of thyroid cells. Moreover, the microinjection of the Rap1 protein alone or in combination with the catalytic C subunit of PKA fails to induce proliferation or expression of thyroglobulin.

In dog and human thyroid cells, the pituitary hormone, thyrotropin (TSH) positively regulates function, expression of differentiation and proliferation through an increase of the intracellular cAMP level (1–3). Proliferation of the dog thyrocytes in primary culture is also stimulated by the epidermal growth factor (EGF) and hepatocyte growth factor (HGF) via the activation of their protein tyrosine kinase cascade and by phorbol esters, such as TPA, through the activation of PKC (3–5). In contrast to TSH, which induces differentiation, these other agents inhibit the expression of differentiation characteristics. IGF-1 or insulin at high concentration does not activate proliferation, but their action through the IGF-1 receptors is essential for the action of mitogens (6). Carbachol which raises the level of intracellular calcium and activates PKC, by activating phospholipase C, does not promote cell proliferation (7).

The cAMP-dependent mechanisms involved in the mitogenic and differentiating effects of TSH are still poorly understood. In contrast to stimuli acting via the cAMP-independent cascades, TSH does not lead to the activation of the Map kinases p42 and p44 (8), but activates PKA which itself phosphorylates the transcriptional factor CREB (9, 10). Recently we showed that activation of PKA was necessary but not sufficient to mimic the effects of cAMP on proliferation and on the expression of thyroglobulin, a thyroid specific gene. We suggested that, in addition to PKA activation, a cAMP-dependent but PKA-independent pathway might be involved in these effects (9). Interestingly, recently we have identified a novel protein directly activated by cAMP (11). This protein, Epac (exchange protein directly activated by cAMP; also called cAMP-GEF) is a guanine nucleotide exchange factor for the small GTPase Rap1 and abundantly present in many tissues, including thyroid tissue (12).

Rap1 is closely related to Ras, in particular within the effector domain; and, *in vitro*, Rap1 clearly associates with most of the Ras effector proteins such as B-Raf and the RalGEF family proteins (13). However, in intact cells, its mechanisms of action are poorly understood. Depending on the cell type, Rap1 has been found to be activated by various stimuli acting via different signaling pathways. In fact, in addition to cAMP-GEFs, several Rap1-specific GEFs are currently known, like the C3G protein which is associated with the Crk adaptor protein, an effector of various tyrosine kinase receptors, and the CalDAG-GEFI which is directly regulated by calcium and diacylglycerol binding. Thus Rap1 seems to hold a central position in the intracellular signaling pathway. Furthermore, Rap1 is also a substrate for PKA in several cell types, such as in human platelets (14) and in human neutrophils (15).

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The role of Rap1 in cell proliferation is not well established but seems to depend on the cell type. For instance, in NIH3T3 cells, Rap1 has been reported to antagonize Ras mediated proliferation (16), whereas in Swiss 3T3 cells, overexpression of wild-type Rap1b has been reported to stimulate cell proliferation (17).

The aim of the present work was to establish a putative link between Rap1 activation and proliferation of dog thyroid cells by determining whether Rap1 is activated in these cells upon stimulation by mitogenic and non mitogenic stimuli acting through cAMP-dependent and -independent pathways.

MATERIALS AND METHODS

Primary culture of dog thyroid cells. The cells were obtained from dog thyroid tissue as described previously (4). Briefly, the thyroid tissue was digested by collagenase (Type Ia; Sigma Chemical Co., St. Louis, MO), so that the resulting suspension consisted mainly of fragmented or intact follicles. These follicles were seeded in the following medium, which constitutes the control medium: Dulbecco's Modified Eagle's medium + Ham's F-12 medium + MCBD 104 medium (Gibco Labs, Paisley, UK, 2:1:1, by vol) with 2 mM sodium pyruvate, 40 μg/ml ascorbic acid; antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml) and fungizone (2.5 μg/ml). In one day, the follicles adhered to the petri dish and progressively developed as a monolayer.

The various agents used in this study were added to the medium at day 4 of the culture: bovine TSH (1 mU/ml; Sigma Chemical Co., St. Louis; MO), forskolin (10^{-5} M; Calbiochem–Behring Corp., La Jolla, CA), human recombinant EGF (50 ng/ml; Collaborative Research Inc., Bedford, MA) bovine insulin (5 μg/ml; Sigma Chemical Co.), carbachol (10^{-5} M; Sigma Chemical) and phorbol ester 12-*O*-tetradecanoylphorbol (TPA; 100 ng/ml; Sigma Chemical).

Rap1 activation assays. Rap1 activation was determined as previously described (18). Briefly, following growth factor stimulation, cells (from a 90-mm culture dish) were washed twice with ice-cold PBS and lysed in 1 ml of Ral-buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μM leupeptin, 10 mM NaF and 1 mM Na_3VO_4). Lysates were clarified by centrifugation and supernatants were incubated for 45 min at 4°C with 15 μg of (GST)-RalGDS-RBD precoupled to glutathione-agarose beads (7 μl packed beads). After incubation, beads were washed four times in Ral buffer and then resuspended in SDS–Laemmli sample buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (15%) followed by transfer to PVDF membranes and probed with a monoclonal antibody against Rap1 (Transduction Laboratories, Lexington, KY). Isolated proteins were detected by ECL. All GTPase activation assays were performed at least twice.

Mesasurement of cell proliferation. The cells in 35-mm petri dishes were maintained until day 4 of the culture in the control medium supplemented with insulin (5 μg/ml). Cells were then stimulated by various mitogenic agents and DNA synthesis or, more precisely, the fraction of cells entering into DNA synthesis, was estimated after 48 h of stimulation by the frequency of ^{3}H thymidine-labeled nuclei, as estimated by autoradiography. The cells were incubated for the last 24 h in the complete medium containing 3×10^{-5} M thymidine, 10^{-4} M deoxycytidine, and [^{3}H]-thymidine 10 mCi/l. After removal of the medium, the cells were fixed with methanol and extensively washed. Autoradiography was performed, as described previously (1), directly in the petri dishes. The cells were stained with toluidine blue.

Microinjection of Rap1 and PKA proteins into thyroid cells. Microinjection experiments into thyroid cells were performed as described previously (9). Cells were cultured in the control medium supplemented with insulin until day 4 of the culture. At this time, cells were quiescent and spread enough to be injected. As the capacity to proliferate and to express thyroglobulin (Tg) can differ for each patch of cells derived from one follicle, we divided them in two halves and microinjected only one part of both. By this way, we could compare the effects caused by microinjection in cells originating from the same follicle. For each condition, a minimum of three follicles, in which on average 100 cells were injected, were considered. The catalytic C subunit of PKA (4.8 mg/ml) and the wild-type Rap1 proteins (0.4 mg/ml) were microinjected into cytoplasm of cells using the Eppendorf micromanipulator (5242)-injector (5170) system mounted on a Zeiss inverted phase microscope (axiovert 35M). Glass capillaries (type GC120TF-10) were pulled with a P-87 horizontal pulley (Sutter Instrument Co., Novato, CA) so that the tips diameter was less than 1 μm.

The catalytic (C) subunit of bovine heart was prepared and purified as described elsewhere (19). Purified C subunit was dialyzed in Intracellular Buffer (48 mM K_2HPO_4, 14 mM NaH_2PO_4, 4.5 mM KH_2PO_4, pH 7.2) supplemented with 1 mM glutathione, 0.3 mM ATP, and 3 mM $MgSO_4$. The concentration of purified C subunit was about 90 μM.

The Rap1B protein was made as a GST fusion protein made in pGEX4T3 vector. This plasmid was introduced in bacteria and protein expression was induced with 30 μM IPTG at 25°C for 18 h. Bacteria were lysed by sonication in Resuspension Buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 10 mM 2-ME, 10 μg/ml leupeptin). Lysate was clarified and incubated with glutathione-Sepharose beads (Pharmacia) for 30 min at 4°C in GSH Wash Buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM benzamidine, 10 mM 2-ME, 0.02% Brij35, 10 mM $MgCl_2$). Beads were washed four times with GSH Wash Buffer. The GST-Rap1B was eluted from beads in 50 mM Tris, pH 8, containing 5 mM glutathione. Protein was concentrated and dialyzed in Intracellular Buffer. The final concentration of purified GST-Rap1B was 0.4 mg/ml.

RESULTS

We first determined whether Rap1 was activated in dog thyrocytes stimulated by TSH (1 mU/ml) or by forskolin (10^{-5} M), a direct activator of adenylyl cyclase. The activation of Rap1 was determined by isolating the active GTP-bound Rap1 with the RalGDS-RBD and further detected by Western blotting. As shown in Fig. 1, the basal level of GTP-bound Rap1 detected in cells maintained in control medium, which did not contain serum nor insulin, was barely detectable. The amount of GTP-bound Rap1 was increased after 5 and 15 min of stimulation by TSH (Fig. 1A). A similar increase was also observed in thyrocytes treated with forskolin, indicating that TSH activated Rap1 through a cAMP-dependent mechanism. As shown by kinetic activation experiments (Fig. 1B), Rap1 activation in response to TSH persisted for at least 5 h. We then analyzed a possible link between Rap1 activation and TSH-induced cell proliferation, by determining the minimal concentration of TSH required to activate Rap1. Although the GTP-bound Rap1 level was barely detectable until 0.033 mU/ml of TSH, 0.1 mU/ml of TSH was sufficient to induce a strong activation, similar to the one observed at higher concentration (Fig. 2A). In parallel, the induction of cell proliferation was evaluated by the number of cells

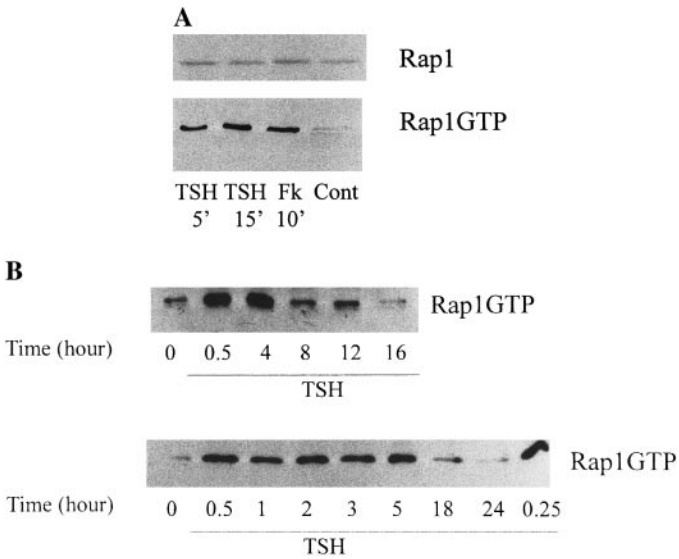

FIG. 1. Activation of Rap1 by mitogenic stimuli acting through cAMP. Stimulated cells were lysed and Rap1 GTP was isolated using GST-RalGDS-RBD. Rap1 was detected by Western blot analysis using a monoclonal antibody against Rap1. (A) Stimulation by TSH (1 mU/ml) for 5 or 15 min, or by forskolin (10^{-5} M). The upper panel represents the total amount of Rap1 contained in 20 μl of total lysate. (B) Kinetics of Rap1 activation by TSH (1 mU/ml).

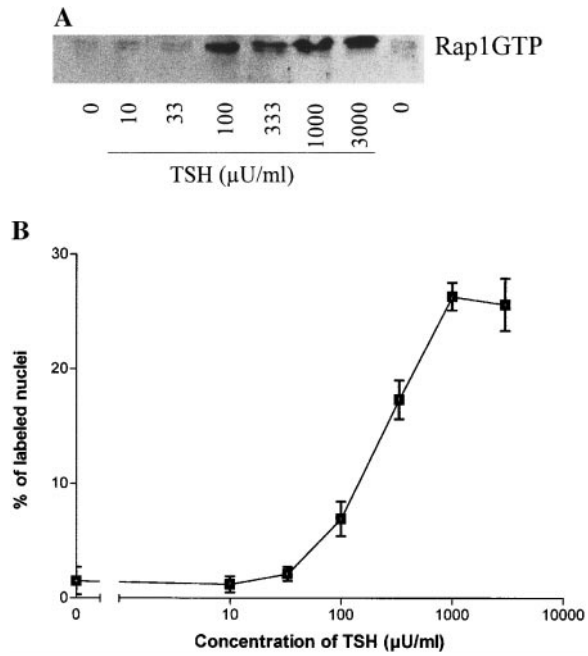

FIG. 2. Concentration-dependent effects of TSH on Rap1 activation (A) and DNA synthesis (B). Thyrocytes were cultured in control medium supplemented with insulin until day 4. At this day, cells were stimulated by increasing concentrations of TSH (0 to 3 mU/ml) for 10 min for Rap1 activation assay (A) and for 48 h for proliferation assay (B). (A) Rap1 GTP was isolated and detected as described in Fig. 1. (B) The percentage of cells entering in DNA synthesis was determined by [^{3}H]-thymidine incorporation into their nuclei.

entering into DNA synthesis, i.e., incorporation of [^{3}H]-thymidine in their DNA (Fig. 2B). It was also at 0.1 mU/ml that TSH started to stimulate the induction of DNA synthesis. Thus the same concentration of TSH is active on both phenomena.

As Rap1 is known to be phosphorylated by PKA, we examined whether the cAMP effect on Rap activation was PKA dependent by treating the cells with the specific PKA inhibitor H89 at 10^{-5} M or 5×10^{-5} M. In dog thyroid cells, we have previously shown that TSH induces, in a PKA-dependent manner, the retraction of the cell membrane. At both concentrations, H89 blocked the cAMP-induced cytoplasmic retractions, confirming that PKA action was abolished in the treated cells (data not shown). However, as shown in Fig. 3, H89 did not affect the level of active Rap1 in control or cAMP-stimulated cells, indicating that the cAMP activation of Rap1 did not require PKA activity.

We then investigated whether Rap1 was activated in dog thyroid cells upon stimulation by agents acting via cAMP-independent pathways, such as EGF (50 ng/ml), TPA (100 ng/ml) insulin (5 μg/ml) and carbachol (10^{-5} M). As shown in Fig. 4, the level of active GTP-bound Rap1 was increased by all the agents tested with a similar intensity to that observed in response to TSH or forskolin. Moreover, no significant differences were revealed between the kinetics of Rap1 activation by the various agents. These data indicate that Rap1 activation represents a common event of the cAMP-dependent and -independent signaling pathways. The fact that Rap1 was activated by EGF, TPA, insulin and carbachol, which by themselves are not mitogenic for thyroid cells, suggests that its activation is not sufficient to promote the proliferation of these cells.

Since the presence of insulin is required for mitogens to promote dog thyrocytes proliferation, we also examined whether Rap1 was more activated when insulin was added to the medium either at the beginning of the culture, or simultaneously with the mitogenic agents. The effects on Rap1 activation in response to TSH, EGF or TPA were similar to those observed in the absence of insulin (data not shown).

In Swiss3T3 cells, which, like thyrocytes, proliferate in response to cAMP as well, the overexpression of the

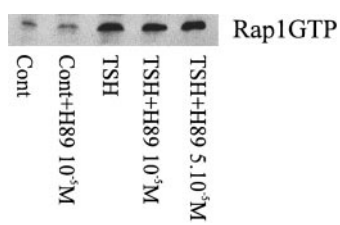

FIG. 3. Effect of the H89 PKA inhibitor on Rap1 activation. Thyroid cells were pretreated or not for 1 h with H89 (10^{-5} or 5×10^{-5} M), and then stimulated for 10 min by TSH (1 mU/ml). The Rap1 activation assay was performed as described in the legend to Fig. 1.

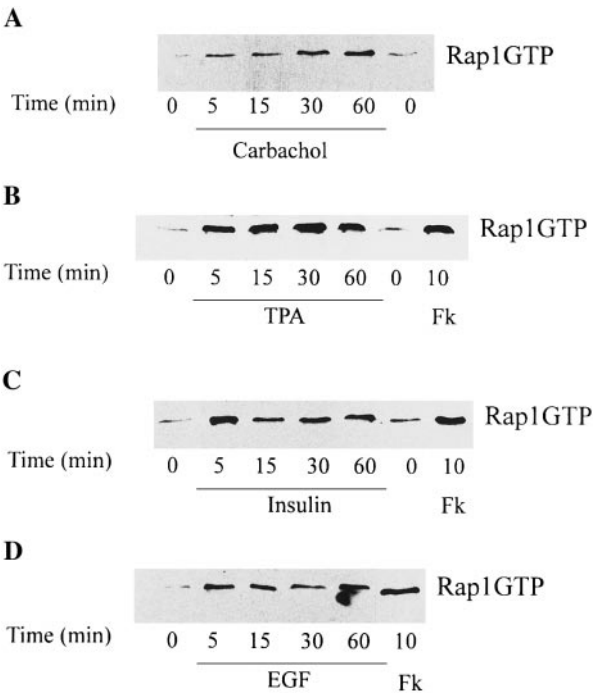

FIG. 4. Rap1 activation by various stimuli acting through cAMP-independent pathways. Thyroid cells were maintained in control medium without insulin until day 4. Cells were then stimulated for the indicated times by A, carbachol (10^{-5} M); B, TPA (100 ng/ml); C, insulin (5 μg/ml); or D, EGF (50 ng/ml). For each experiment, stimulation by forskolin was made as a positive control. Rap1 GTP was isolated and detected as described in the legend to Fig. 1.

wild-type Rap1 protein promotes cell proliferation (17). In dog thyroid cells, we have previously reported that the activation of PKA was not sufficient to mimic the cAMP effect on proliferation and thyroglobulin expression (9). To assess if Rap1 might be sufficient to promote thyroid cell proliferation, we performed several experiments of microinjection of the wild-type Rap1 protein alone or in combination with the catalytic C subunit of PKA into dog thyroid cells. The effects of these microinjections were analyzed on both DNA synthesis and thyroglobulin expression. However, we never observed any stimulatory effect in those experiments, neither on DNA synthesis, nor on thyroglobulin expression (data not shown).

DISCUSSION

The small G protein Rap1 is known to be activated by a variety of stimuli (acting through a variety of signaling pathways) in various cell types, namely by stimuli acting through rising in intracellular cAMP (18, 20). The recently described direct activation by cyclic AMP of the Rap1 guanylnucleotide exchange factor Epac raises the question of the possible role of this new mechanism of action of cyclic AMP in thyroid cells. Epac has indeed been shown to be abundantly expressed in human and dog thyroid cells (12, and our unpublished results). The function of this cascade is demonstrated by the activation by TSH and forskolin of Rap1 in intact cells. The absence of inhibition of this effect by the PKA inhibitor H89 is compatible with a direct, non PKA-dependent, effect of cyclic AMP.

However, activation of Rap1 is not specific for TSH or cyclic AMP. Indeed, carbachol which activates phospholipase C and consequently increases intracellular Ca^{2+} levels and stimulates protein kinase C, TPA which directly activates protein kinase C, and insulin and EGF which activate different tyrosine protein kinases cascades, all independently activate Rap1. Rap1 activation is therefore a common step in all these cascades which have very different effects on the cells (2, 3): TSH and cyclic AMP stimulate thyroid cell function, proliferation and differentiation, while carbachol activates some functions (H_2O_2 generation, thyroid hormone synthesis, etc.) but little affects proliferation or differentiation (21). EGF and phorbol esters activate the proliferation of the cells but repress the differentiation characteristics. Insulin has no mitogenic effect per se but it required for the mitogenic action of EGF, TPA, TSH and forskolin. Rap1 activation is therefore neither the specific common feature of the mitogenic cascades nor a specific pathway activated by cAMP. Therefore, although Rap1 activation may participate in or be necessary for the mitogenic action of TSH, forskolin, EGF and TPA, for the permissive action of insulin, or for the functional effects of cyclic AMP or carbachol, it is not sufficient for any of these actions. The negative results of experiments in which wild type Rap1 alone or in combination with the catalytic C subunit of PKA, was microinjected in the cells support the fact that Rap1 is not sufficient to induce mitogenesis or even to complement PKA in inducing it. Rather, Rap1 activation, as cFos induction in the same cells, seems to be a common step of the cascades activated by exogenous stimuli (22). As for cFos induction it is therefore probably a general feature and perhaps a necessary step in any type of thyroid cell stimulation or arousal.

Although Rap1 is able, *in vitro,* to associate with various Ras effector proteins, such as Raf and Ral GDS proteins, how it functions in intact cells is not clear. In some cell types, for instance in PC12, it has been reported that Rap1 activates MAP kinase through B-Raf (23, 24). However, in dog thyroid cells, we previously showed that, contrary to the agents acting through cAMP-independent cascades, TSH does not stimulate the MAP kinases p42 and p44 (12). As Rap1 is now found to be activated by TSH, our results suggest that, in our cells, Rap1 does not trigger the activation of the B-Raf-MAP kinases pathway.

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