RaLGDS Functions in Ras- and cAMP-mediated Growth Stimulation* 

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Thyroid-stimulating hormone stimulates proliferation through both the cAMP-dependent protein kinase and Ras but not through Raf-1 and mitogen-activated and extracellular signal-related kinase kinase. We now report that thyroid-stimulating hormone represses mitogen-activated protein kinase activity and that microinjection of an effector domain mutant Ha-Ras protein, Ras(12V,37G), defective in Raf-1 binding and mitogen-activated protein kinase activation, stimulates DNA synthesis in quiescent and thyroid-stimulating hormone-treated thyrocytes. A yeast two-hybrid screen identified RaLGDS as a Ras(12V,37G) binding protein and therefore a potential effector of Ras in these cells. Associations between Ras and RaLGDS were observed in extracts prepared from thyroid microinjection of a mutant RalA(28N) protein thought to sequester RaLGDS family members reduced DNA synthesis stimulated by Ras as well as cAMP-mediated DNA synthesis in two cell lines which respond to cAMP with mitogenesis. These results support the idea that RaLGDS may be an effector of Ras in cAMP-mediated growth stimulation.

The elucidation of the critical role played by Ras in growth control initiated an intensive effort to identify Ras binding effector molecules. The first Ras binding protein identified was p120GAP (1), which functions both as an effector and down-regulator of Ras. The best characterized Ras effector is the cytoplasmic serine/threonine protein kinase Raf-1 (2, 3). Direct interaction between the N-terminal domain of Raf-1 and the effector loop of Ras was first demonstrated using the yeast two-hybrid system (4, 5), while later studies reported the co-effector loop of Ras was first demonstrated using the yeast two-hybrid system (4, 5), while later studies reported the co-

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pressed and purified as described by the manufacturer. For injection studies, glutathione-Sepharose beads containing the fusion proteins were incubated with 1.5 units of thrombin for 16 h at 4 °C. Following centrifugation, the supernatant was incubated with 20 μl of p-amino-benzenzamidine-agarose beads for 30 min at 4 °C. Following centrifugation, the purified proteins were exchanged into injection buffer (20 mM Tris, pH 7.5, 20 mM NaCl, 2 mM MgCl2, 0.1 mM EDTA), concentrated, and aliquots frozen at −80 °C.

MAPK Activity—Immune complex kinase assays were performed as described previously (29). WRT cells were lysed in 50 mM HEPES, pH 7.4, 10 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2.5 μg/ml pepstatin, 150 mM NaCl, 500 μM sodium orthovanadate, 100 mM staurosporine, 50 mM β-glycerophosphate, 1% Nonidet P-40, and 500 μM Pefabloc. MAPK was immunoprecipitated using an anti-MAPK antibody (SC93 Santa Cruz). Immunoprecipitates were washed twice in 0.1 × phosphate-buffered saline and resuspended in 50 μl of kinase buffer (30 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 50 μM ATP, and 500 μM/ml myelin basic protein). 15 μl of [32P]ATP was added and the samples incubated at 30 °C for 10 min. The reactions were stopped by the addition of 5 × Laemmli sample buffer, boiled, and analyzed on 12% SDS-polyacrylamide gels. The gels were dried and subjected to autoradiography.

Construction of Thyroid Cell Library and Yeast Two-Hybrid Screen—Total cellular RNA was prepared from WRT cells using guanidinium hydrochloride and mRNA isolated using a Pharmacia oligo(dT) purification kit (Pharmacia Biotech Inc.). cDNA was synthesized using a Great Lengths cDNA synthesis kit (Clontech) and cloned into EcoRI-digested yeast two-hybrid vector pGADGH (Clontech) using EcoRI adapters. Mutant Ha-Ras(12V,37G) in the yeast two-hybrid vectors were fused to P3-X63-Ag8.653 cells to generate hybridomas following transformation with an expression vector encoding a portion of RalGDS as described previously (34). Spleens were fused to P3-X63-Ag8.653 cells to generate hybridomas following standard protocols (44).

In Vitro Binding—GST fusion proteins encoding Ras(12V) or Rho(14V) were purified on glutathione-Sepharose. The proteins were eluted with 2 bed volumes of 5 mM glutathione in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM Pefabloc, 1 μM GTP or GDP for 10 min at 4 °C. The eluate was dialyzed against 50 mM Tris, pH 7.5, 20 mM KCl, 2 mM MgCl2, 7.5 mM EDTA, 1 mM dithiothreitol, 1 mM pefabloc, 1 μM GTP or GDP, concentrated, and protein determinations made. Nucleotide loading was performed in the same buffer supplemented with either 1 mM GTP or GDP for 30 min at 30 °C. The reaction was terminated by addition of 20 mM MgCl2 and cooling to 4 °C. WRT cell phase thyrocytes were lysed by addition of 100 μl of ice-cold lysis buffer (10 mM HEPES, 2 mM MgCl2, 30 mM NaCl, 0.5 mM NaVO4, 0.1 M NaF, 10 mM Na2P2O7, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 μg/ml pepstatin) per 100-mm dish. Approximately 20 μg of GST fusion protein was incubated with 1.2 μg of total cell protein for 1 h at 4 °C. Glutathione-Sepharose (250 μl) was added to each mixture and incubated for a further 60 min. The beads were washed with 3 × 1 ml ice-cold Tris-buffered saline (25 mM Tris, pH 7.5, 140 mM NaCl, 2 mM KCl), boiled in sample buffer, separated on an 8% SDS-polyacrylamide gel, and immunoblotted for Ras (SC29 Santa Cruz), GST (SC138 Santa Cruz), or RalGDS.

**FIG. 1. Effects of TSH on MAPK activity.** A, time course of the effects of TSH on MAPK. Immune complex kinase assays were performed on quiescent WRT cells subsequently stimulated with vehicle (PBS) (lane 1); TSH (10 mU/ml) for 5, 15, 30, 60, or 120 min (lanes 2–6, respectively); 12-O-tetradecanoylphorbol-13-acetate (200 ng/ml) for 15 min (lane 7); and 12-O-tetradecanoylphorbol-13-acetate for 15 min in a null kinase reaction performed for 0 min at 30 °C (lane 8). B, TSH reduces serum-stimulated MAPK activity. All treatments were for 15 min. Quiescent WRT cells were stimulated with vehicle (PBS) (lane 1), 20% fetal calf serum (lane 2), TSH (10 mU/ml) (lane 3), TSH (10 mU/ml) for 15 min followed by 20% fetal calf serum (lane 4), and 20% fetal calf serum in a mock immunoprecipitation reaction using rabbit IgG rather than the anti-extracellular signal-regulated protein kinase antibody (lane 5).
part and cellular Ras (data not shown). Unlike the Ras proteins, activated forms of Rho and Rac as well as GST alone (data not shown) failed to stimulate DNA synthesis, although they were purified in parallel with the Ras proteins. Like cellular Ras (21), Ras(12V,37G) retained its mitogenic activity in the presence of cAMP stimulated by TSH, consistent with its effects being independent of Raf-1.

Effects of TSH on MAPK Activity—In some cells, cAMP inhibits Raf activation without similar effects on MAPK activity (31, 32). Since TSH-stimulated DNA synthesis appears both Raf-1- and MEK-independent, the effects of TSH on MAPK activity were examined. Treatment with TSH for 5–120 min (Fig. 1A, lanes 2–6) failed to stimulate MAPK activity, although 12-0-tetradecanoylphorbol-13-acetate stimulated a dramatic increase in activity (lane 7). Similar to the effects of TSH, which elevates cAMP, forskolin and 8-Br-cAMP also failed to increase MAPK activity in these cells (data not shown). These results are in good agreement with those published earlier in canine thyrocytes (33). Not only did TSH fail to increase MAPK activity, pretreatment with TSH repressed serum-stimulated (Fig. 1B) and IGF-1-stimulated activity (data not shown). This data further supports our hypothesis that TSH acts through a novel pathway which is Ras-dependent but independent of Raf-1.

Identification of RalGDS as a Ras(37G) Binding Protein—To identify potential Ras effectors expressed in thyroid cells, a yeast two-hybrid screen was performed to identify Ras binding proteins. Ras(12V,37G) was used in this screen in order to identify proteins other than Raf-1 which bind to Ras. Approximately $2 \times 10^6$ clones were screened and a total of 4 positive clones were obtained that permitted growth under selective conditions and which expressed $\beta$-galactosidase (Fig. 2). The cDNA inserts from all 4 clones encoded a single sequence identical to the C-terminal Ras binding domain (RBD) of RalGDS (15–18, 34).

In some (25, 26) but not all (35) instances, expression of the RalGDS RBD or that from a RalGDS-related protein (RGL) interfered with Ras-mediated signaling. To test its effects on Ras-mediated signaling in thyroid cells, the RalGDS RBD was coinjected with Ras proteins and DNA synthesis examined. Injection of this peptide markedly reduced DNA synthesis stimulated by both Ras(12V) and Ras(12V,37G), suggesting that this domain binds Ras in living cells as it does in vitro (17) (Fig. 3A). Injection of the RalGDS RBD also abolished TSH-, cholera toxin-, and 8-Br-cAMP-stimulated DNA synthesis, confirming the importance of Ras in these signaling pathways (Fig. 3B). In contrast, this injected peptide had no effect on TSH- or 8-Br-cAMP-stimulated cAMP response element-regulated gene expression (Fig. 3C), results which are identical to

![Fig. 2. Specificity of the RalGDS and Ras(12V,37G) interaction. Cotransformation of pGADGH encoding RalGDS and pGBT9 encoding Ras(12V,37G) resulted in protein-protein interactions which permitted growth on selective medium (-His/-Trp/-Leu) (left) and stimulated \(\beta\)-galactosidase expression (right). Cotransformation of the Ras effector domain mutant with byr2 served as a positive control (22). Cotransformation of RalGDS with SNF served as a negative control. The -His/-Trp/-Leu plate was incubated at 30 °C for 2 days. \(\beta\)-Galactosidase expression was detected following incubation in the presence of 5-bromo-4-chloro-3-indoyl \(\beta\)-D-galactoside for 8 h at 30 °C.](image-url)
RalGDS and Growth Stimulation

FIG. 4. RalGDS binds Ras in vitro. Immobilized small G proteins were incubated with lysates prepared from exponentially growing thyroid cells. Following extensive washing, bound proteins were eluted and Western blotted with antibodies to RalGDS (A), mouse IgG (B), GST (C), and Ras (D). The positions of RalGDS and the GST-small G proteins are indicated. The numbers on the left represent the positions of the molecular size markers. Results shown are from one of four experiments which gave similar results.

those obtained following injection of a dominant interfering mutant Ras(17N) protein (20).

To assess whether RalGDS binds to Ras in thyroid cells, in vitro binding experiments were performed. Lysates from exponentially growing thyroid cells were incubated with immobilized GST fusion proteins encoding activated forms of Ras and Rho, washed, and associated proteins were analyzed by Western blotting with a RalGDS monoclonal antibody (Fig. 4A) or mouse IgG (Fig. 4B). RalGDS was detected only in lysates incubated with GTP-bound Ras and probed with the RalGDS antibody. Neither GDP-bound Ras nor GTP-bound Rho interacted detectably with RalGDS. Probing with anti-GST (Fig. 4C) and anti-Ras (Fig. 4D) antibodies revealed that the abundance of the immobilized GST fusion proteins was similar.

Ral Activity Is Required for Ras- and cAMP-stimulated DNA Synthesis—To assess whether RalGDS was required for Ras-stimulated DNA synthesis, a dominant interfering mutant RalA(28N) protein (19, 23) was utilized. Similar to dominant interfering Ras(17N), this mutant Ras protein exhibits a preferential affinity for GDP (36) and should therefore act to sequester molecules which bind to the GDP-bound form of RalA, including RalGDS and related molecules such as RGL (16). Injection of RalA(28N) protein reduced DNA synthesis stimulated by cellular, activated, and the effector domain Ras proteins in thyroid cells (Table II). Ras(12G) and Ras(12V,37G) appeared more sensitive to repression by RalA(28N) than was Ras(12V). The reason for this difference remains to be determined.

Because TSH-stimulated DNA synthesis is Raf-1-, MEK-, and MAPK-independent, we assessed whether RalA(28N) would reduce it. Microinjection of RalA(28N) markedly reduced TSH-stimulated DNA synthesis (Fig. 5, a and b), cholera toxin-stimulated, and 8-Br-cAMP-stimulated DNA synthesis, all mitogens which act through elevations in cAMP, to near background levels (Table III). In contrast, DNA synthesis stimulated by IGF-1 (Fig. 5, c and d) or fetal calf serum (FCS) was not reduced by injection of RalA(28N). Similarly, injection of RalA(28N) did not reduce FCS-stimulated DNA synthesis in NIH3T3 or REF52 fibroblasts, pathways which are partially Ras-dependent but independent of cAMP (data not shown).

These results suggested that Ral activity is critical for cAMP-mediated DNA synthesis. To examine this further, we extended our analysis to proliferating rat Schwann cells, which respond to cAMP with mitogenesis (28, 37, 38). Injection of RalA(28N) profoundly reduced DNA synthesis stimulated by cholera toxin and 8-Br-cAMP in these cells (Table III). Because RalA(28N) is thought to act by sequestering Ras exchange factors, including RalGDS (39), the repressive effects of RalA(28N) on cAMP- and Ras-mediated DNA synthesis suggest that a member or members of the RalGDS family play an essential role in Ras-dependent, cAMP-mediated mitogenic signaling pathways.

DISCUSSION

TSH stimulates proliferation through a cAMP-dependent pathway which requires Ras activity (20). Although Ras-dependent, TSH does not utilize Raf-1 or MEK in mitogenic signaling (21). We now demonstrate that TSH fails to stimulate MAPK activity and represses serum-stimulated activity. Consistent with these results, microinjection of a Ras effector domain mutant Ras(12V,37G), defective in Raf-1 interaction, stimulated DNA synthesis in quiescent and TSH-treated thyrocytes. Based on these results, this mutant Ras protein was
used in a yeast two-hybrid screen of a thyroid cell library. This screen identified RalGDS as a Ras(12V,37G) binding protein and therefore a potential effector of Ras. Similar results were recently reported by White et al., who found that Ras(12V,37G) binds RalGDS expressed in a fibroblast library (18). RalGDS was first isolated from a mouse cell library using sequences derived from yeast Ras guanine nucleotide dissociation stimulator proteins as probes (34). Although its repeated identification as a Ras binding protein (15–17) suggests that RalGDS acts in Ras-dependent signaling pathways, its biological roles remain unknown. Kikuchi et al. reported that protein kinase A enhanced Ras association with RalGDS by decreasing Ras–Raf-1 interaction (24). Because TSH-stimulated DNA synthesis is both protein kinase A- and Ras-dependent, we examined whether RalGDS played a role in mitogenic signaling initiated by TSH.

In vitro binding data revealed that Ras binds to RalGDS in thyroid cell extracts. Consistently, microinjection of the RalGDS RBDD reduced Ras-stimulated DNA synthesis, suggesting that this domain also binds to Ras in living cells. As expected, injection of the RalGDS RBDD also reduced TSH- and cAMP-stimulated DNA synthesis in these cells, confirming the importance of Ras in this signaling pathway. A mutant RalA(28N) protein exhibiting a preferential affinity for GDP (36) analogous to the dominant interfering Ras(17N) mutant was used to examine whether RalGDS or a related activity was required for TSH-stimulated mitogenesis. RalA(28N) has been used previously to document a requirement for Ras activity in Src-stimulated phospholipase D activation (23) and in Ras-stimulated transformation (19). Microinjection of RalA(28N) dramatically reduced DNA synthesis stimulated by TSH, cholera toxin, and 8-Br-cAMP, all mitogens which act through elevations in cAMP. The level of DNA synthesis in quiescent thyrocytes varies between 0 and 30% depending upon how long the cells have been in culture. In most experiments, injection of RalA(28N) very nearly abolished DNA synthesis stimulated by agents which act through CAMP. In contrast, IGF-1- and FCS-stimulated DNA synthesis was little affected by injection of RalA(28N). These results suggest that a greater proportion of cAMP-mediated mitogenic signaling proceeds through a RalGDS-sensitive pathway in these cells than does signaling stimulated by IGF-1 or FCS. This would be expected given that IGF-1 and FCS do not elevate cAMP and therefore cannot transduce Ras-dependent signals through Raf-1 and the MAPK cascade. Unlike TSH, which fails to stimulate MAPK activity in either rat (Fig. 2) or canine thyrocytes (33), both IGF-1 and FCS increased MAPK activity in thyroid cells. Pretreatment with TSH abolished this activation, demonstrating that TSH interferes with signaling through the Raf-MAPK cascade and suggesting that TSH channels its Ras-mediated signals to a novel pathway involving RalGDS or a related molecule.

To determine whether the apparent requirement for Raf activity extended to other cells in which cAMP stimulates mitogenesis, RalA(28N) was injected into secondary rat Schwann cells. In the presence of serum, these cells cycle only very slowly. Addition of cholera toxin, 8-Br-cAMP, or forskolin stimulates a dramatic increase in DNA synthesis (28, 37, 38). Similar to the effects observed in rat thyrocytes, injection of RalA(28N) profoundly reduced both cholera toxin- and 8-Br-cAMP-stimulated DNA synthesis in secondary Schwann cells. These results support the hypothesis that RalGDS plays an important role in cAMP-stimulated mitogenesis.

Although in vitro RalGDS catalyzes nucleotide exchange on Raf, its effectors in vivo are unknown. By analogy with Ras, it is possible that recruitment of RalGDS to the plasma membrane through association with Ras activates Raf. However, microinjection of RalA or a GTPase-defective mutant RalA(72L) protein failed to stimulate DNA synthesis in quiescent thyrocytes. This may indicate that other factors, i.e. cAMP or Ras, are required for Raf to exert its mitogenic effects. This would be consistent with recent results demonstrating that RalA(72L) failed to stimulate phospholipase D activity, although it potentiated this activity in src-transformed cells (23), and that it failed to stimulate focus formation although it enhanced Ras-mediated transformation (19). If Raf is the effector of RalGDS, then other small G proteins are likely to function in this signaling pathway. Three laboratories have identified Raf binding proteins which exhibit characteristics expected for a Raf effector and cdc42GAP activity (40–42). It is also conceivable that there are targets of RalGDS other than Raf and Ras. In fibroblasts, RalGDS but not Raf collaborated with Ras in focus-forming assays (18). In further support of this idea, microinjection of RafA protein repressed TSH-stimulated DNA synthesis, perhaps indicating that RafA interferes with RalGDS-mediated signaling to another effector. Attempts to isolate full length RalGDS protein for microinjection studies have not yet been successful. Since thyrocytes arrest in G1 following intranuclear DNA injections and exhibit very low transfection efficiencies in transient assays, it will be necessary to generate stable lines overexpressing RalGDS and Raf in order to determine whether they exert similar effects on thyrocyte growth control.

In addition to Ha- and K-Ras, RalGDS also binds Rap1A (19), R-Ras (43), and TC21 (35), although binding to these molecules does not stimulate RalGDS activity in vitro (19). Several lines of evidence support the idea that TSH transduces its mitogenic signals through Ras, rather than one of these other proteins, to RalGDS or a related protein. First, purified Ras protein binds to RalGDS in thyroid cell extracts. Second, microinjection of the RalGDS RBDD reduced DNA synthesis stimulated by Ras and TSH, suggesting that this domain binds to Ras in living cells. Third, similar to the effects of TSH, overexpression of Ras stimulates DNA synthesis in thyroid cells. It remains to be determined whether any of the other small G proteins which bind RalGDS exhibit similar effects. Additional support for signaling from Ras to RalGDS has been reported. In Xenopus...
ococytes, expression of the Ras-interacting domain of RGL interfered with Ras-mediated maturation (25). In NIH3T3 cells, this domain reduced Ras-mediated transformation (26), although in another study, expression of the RBD of RalGDS reportedly had no inhibitory effect on Ras- or TC21-stimulated transformation (35). Last, the observation that protein kinase A stimulates Ras-RalGDS association (24) further supports the hypothesis that Ras is capable of signaling through RalGDS and that this signaling pathway might predominate in the presence of cAMP.

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