The dopamine D2S receptor (short isoform) couples to inhibitory Gq/11 proteins to inhibit thyrotropin-releasing hormone (TRH)-stimulated p42/p44 mitogen-activated protein kinase (ERK1/2) phosphorylation in GH4ZR7 rat pituitary cells, consistent with its actions to inhibit prolactin gene transcription and cell proliferation. However, the underlying mechanism is unclear. To identify novel Gq effectors, yeast two-hybrid screening of a GH4ZR7 cDNA library was done using constitutively active Goq/11-Q204L, and multiple clones of the RasGAP cDNA GAP1/IP4BP/RASA3 were identified. In yeast mating assay, RASA3 preferentially interacted with activated forms of Goq/11/12 proteins, but not with Goi. A direct interaction was indicated by in vitro pull-down assay, in which S-His-RASA3 preferentially bound guanosine 5’-O-(γ-thio)triphosphate-activated Goq and Goq compared with guanosine 5’-O-(β-thio)diphosphate-inactivated proteins. Similarly, in co-immunoprecipitation studies in HEK-293 cells, FLAG-tagged RASA3 preferentially interacted with activated mutants of Goq and Goq compared with wild type proteins. In GH4ZR7 cells, co-immunoprecipitation studies of endogenous proteins demonstrated a Goq/RASA3 complex that was induced upon TRH/D2S receptor co-activation. To address RASA3 function in dopamine D2S receptor-induced inhibition of ERK1/2 activity, endogenous RASA3 protein expression was suppressed (70% knockdown) in GH4ZR7 cells stably transfected with full-length antisense cDNA of RASA3. The selected antisense clones had similar levels of dopamine D2S receptor binding and D2S-induced inhibition of cAMP formation compared with parental GH4ZR7 cells. In these clones, D2S-mediated inhibition of TRH-induced phospho-ERK1/2 was reversed by 70–80% compared with parental GH4ZR7 cells. Our results provide a novel mechanism for dopamine D2S-induced inhibition of ERK1/2 and indicate that RASA3 links Gq11 proteins to inhibit Gq11-induced Ras/ERK1/2 activation.

Five dopamine receptor genes are known, and the dopamine-D2 receptor is among the most studied because of its involvement in mental and neurological disorders such as schizophrenia, addiction, and Parkinson disease. In addition, the dopamine-D2 receptor mediates inhibitory regulation of endocrine function, with a primary role in the pituitary to regulate prolactin synthesis, secretion, and lactotroph cell proliferation. For example, in homozygous mice deficient in the gene encoding the D2 receptor or lacking dopamine, pituitary adenoma and hyperprolactinemia occur with age (1–4). Conversely, mice lacking the gene encoding the dopamine transporter show dopamine hypersecretion that leads to pituitary hypotrophy (5). In addition, dopamine-D2 receptor agonists such as bromocriptine or cabergoline are used clinically to induce regression of pituitary adenomas (6–9). The dopamine D2 receptor gene contains an alternately spliced exon encoding 29 amino acids in the putative third intracellular loop to generate short (D2S) and long (D2L) forms of the receptor (10). Dopamine D2S receptors have been shown to have anti-proliferative actions in pituitary lactotrophs, whereas the D2L receptor did not appear to be anti-proliferative (11). However, the specific signaling mechanisms underlying dopamine D2S actions in pituitary cells remain to be fully elucidated.

GH4ZR7 cells are GH4Cl lactotroph cells stably transfected with the dopamine D2S receptor cDNA. These cells provide a useful model to study the signaling of the dopamine-D2S receptor in pituitary cells that retain differentiated properties of lactotrophs. These include expression of receptors that stimulate (thyrotropin-releasing hormone (TRH) receptors) or inhibit (somatostatin and muscarinic receptors) the synthesis and secretion of growth hormone and prolactin (PRL) (12). In GH4ZR7 cells, dopamine inhibits cAMP formation, PRL synthesis and secretion, and cell proliferation. Previously, a novel D2S receptor-mediated inhibition of TRH-induced ERK1/2 activation was identified in these cells (13, 14). Interestingly, D2L receptors did not couple to this pathway (15). Activation of ERK1/2 is implicated in TRH induced prolactin transcription (16), and its inhibition is a key pathway for dopamine-D2S-induced inhibition of prolactin transcription (17). This pathway is also observed in primary striatal cultures, suggesting a general role for this D2S receptor-mediated signaling pathway in neuroendocrine tissues (15).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

This work was supported by grants from the Canadian Institutes of Health and the Ontario Mental Health Research (to P. R. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The abbreviations used are: TRH, thyrotropin-releasing hormone; PRL, prolactin; ERK, extracellular signal-regulated kinase; GAP, GTPase activating protein; IP4, inositol 1,3,4,5-tetrakisphosphate; PH, pleckstrin homology; BTK, Bruton’s kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5′-O-(γ-thio)triphosphate; GDPβS, guanosine 5′-O-(β-thio)diphosphate; MEK, mitogen-activated protein kinase/ERK kinase; GST, glutathione S-transferase; Ni-NTA, nickel-nitrilotriacetic acid; RIPA, radioimmunoprecipitation assay.

The online version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

1 Supported by Ontario Graduate Scholarship.
2 Canadian Institutes of Health Research/Novartis Michael Smith Chair in Neurosciences. To whom correspondence should be addressed. Tel.: 613-562-5800, Ext. B307; Fax: 613-562-5403; E-mail: palbert@uottawa.ca.

3 The abbreviations used are: TRH, thyrotropin-releasing hormone; PRL, prolactin; ERK, extracellular signal-regulated kinase; GAP, GTPase activating protein; IP4, inositol 1,3,4,5-tetrakisphosphate; PH, pleckstrin homology; BTK, Bruton’s kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; GTPγS, guanosine 5′-O-(γ-thio)triphosphate; GDPβS, guanosine 5′-O-(β-thio)diphosphate; MEK, mitogen-activated protein kinase/ERK kinase; GST, glutathione S-transferase; Ni-NTA, nickel-nitrilotriacetic acid; RIPA, radioimmunoprecipitation assay.
The mechanism by which the dopamine-D2S receptor inhibits the Ras-ERK1/2 pathway has been partially characterized. Using a rescue strategy employing transfection of pertussis toxin-resistant \( \alpha_i \) proteins, we previously found that \( \alpha_i \) is implicated in dopamine D2S receptor-induced inhibition of ERK1/2 activation by TRH in GH4ZR7 pituitary cells (13). By contrast, blockade of G\( \beta\gamma \) signaling did not affect D2S-mediated inhibition of ERK1/2 activation, indicating that \( \alpha_i \), but not G\( \beta\gamma \) subunits, are required. We further found that the D2S receptor inhibited TRH-induced activation of MEK and c-Raf, suggesting that \( \alpha_i \) couples to inhibit this pathway upstream of c-Raf, possibly by inactivation of Ras. We hypothesized that a critical downstream effector may directly interact with \( \alpha_i \) to mediate dopamine D2S receptor inhibition of Ras-ERK1/2 signaling. To address this hypothesis we have used two-hybrid screening with a constitutively active mutant of \( \alpha_i \) as bait to probe a cDNA library from GH4 pituitary cells. In this screen we identified RASA3 as a novel \( \alpha_i \)-interacting protein and have validated the \( \alpha_i \)-RASA3 interaction in pituitary cells. Importantly we also demonstrate the requirement of RASA3 for D2S receptor signaling to inhibit ERK1/2 activation, indicating that RASA3 links \( \alpha_i \) proteins to inhibition of the Ras-ERK1/2 pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Apoporphine, puromycin, TRH, Sepharose G protein beads, anti-\( \beta \)-actin, anti-FLAG antibody, guanosine 5’-\( \gamma\)-thiotriphosphatetetrалithium salt, guanosine 5’-\( \beta\)-thio-diphosphate trilithium salt, and anti-FLAG M2 Affinity gel were from Sigma-Aldrich; polyvinylidene difluoride membrane was from PerkinElmer Life Sciences; enhanced chemiluminescence detection kits were from Roche Applied Sciences; sera and media were obtained from Wisent, Inc. (St-Bruno, Canada). Endonuclease were purchased from New England BioLabs, Inc. (Boston, MA); anti-G\( \alpha_i \), anti-G\( \alpha_G \), and anti-GST were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-phospho-p42/44 ERK1/2 antibody (T202/Y204) and anti-rabbit IgG, horseradish peroxidase-conjugate antibody were from Cell Signaling Technology, Inc. (Danvers, MA); S-protein horseradish peroxidase conjugate antibody was brought to neutral pH by using neutralization buffer (1 M NaH2PO4, 1 M NaCl, 0.5 M NaOH) and then eluted from the resin by adding elution buffer (0.1 M glycine/HCl, pH 2.5) and brief centrifugation. The antibody was brought to neutral pH by using neutralization buffer (1 M Tris/HCl, pH 9.0). Finally, it was concentrated by using Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA).

**Yeast Two-hybrid Screening**—Oligo(dT)-primed cDNA library from GH4ZR7 poly(A\(^+\)) RNA was constructed in yeast vector pGADT7 (Clontech), and over 80% of clones contained inserts with average sizes of 900-bp. The library was screened with constitutively active human G\( \alpha_i \) Q204L from UMR cDNA Resource Center (Rolla, MO) as bait. The yeast cells were grown at 30°C for 5–7 days. Transformants (10\(^6\)) were selected on SD-Leu-Trp-His-Ade plates and screened by 5-bromo-4-chloro-3-indolyl-\( \beta\)-d-galactopyranoside (X-gal) (Wisent, Canada) overlay assay. DNA from positive clones was extracted and sequenced.

**In Vitro Pull-down Assay and Western Blotting**—Bacterially expressed S-His\(_4\)-RASA3 (pET, Novagen) and GST-tagged G\( \alpha_i \) or G\( \alpha_G \) (pGEX; Amersham Biosciences) proteins were used as bait and prey, respectively. BL21 (DE3)-competent cells were transformed by either pET or pGEX vectors containing RASA3 or G\( \alpha_i \), respectively. They were induced with isopropyl-\( \beta\)-D-thiogalactoside for 3 h, harvested, and resuspended in lysis buffer (50 mM NaH2PO\(_4\), 300 mM NaCl, 10 mM imidazole), sonicated (6 \( \times \) 10 s) and finally centrifuged at 20,000 \( \times \) g for 30 min at 4°C. G\( \alpha_i \) and G\( \alpha_G \) supernatants were incubated with 100 \( \mu \)M GTPyS or GDP\(_\beta\)S (to activate or inactivate, respectively) and 10 mM MgCl\(_2\) at 30°C for 30 min on shaker. The reactions were terminated by transferring the samples on ice. Meanwhile, the RASA3 supernatant was incubated with Ni-NTA-agarose (Qiagen) on shaker for 1 h at 4°C and then washed three times with washing buffer containing 50 mM NaH2PO\(_4\), 300 mM NaCl, 20 mM imidazole. Then G\( \alpha_i \) or G\( \alpha_G \) supernatants were added and incubated with the resin on shaker for 1 h at 4°C. The washing steps were repeated. The
resin was incubated with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole) for 15 min on shaker at 4 °C and finally eluted. The proteins were resolved on an SDS-10% polyacrylamide gel that was transferred onto a polyvinylidene fluoride membrane and blocked at 22 °C for 1 h in 5% skimmed milk in Tris-buffered saline buffer. The membrane was blotted with anti-S antigen (Novagen) or anti-GST (Upstate) antibodies overnight at 4 °C, and then horseradish peroxidase-linked anti-mouse secondary antibody was added to detect anti-GST antibody. The membrane was incubated with chemiluminescence substrate (Roche Applied Science) and exposed to Kodak BioMax MR film.

Co-immunoprecipitation Assay—The pcDNA3-FLAG-RASA3 construct was transiently co-transfected with wild type or constitutively active form of either Gαᵣ or Gαᵢ in pcDNA3 vector into HEK-293 cells by the calcium phosphate co-precipitation method. Forty-eight h after transfection, the cells were scraped in RIPA lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1%(w/v) SDS, 5 mM EDTA, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 10 mM NaF, 10 mM Na₂PPi), left at 4 °C on shaker for 3 h, and lysed further by passing through 25-gauge needle. The cell lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatants were incubated with ~40 µl of protein G-Sepharose, Fast Flow (Sigma) at 4 °C for an hour in a preclear phase. They were centrifuged again, and the supernatants were incubated with ~40 µl of anti-FLAG M2-agarose affinity gel (Sigma) on a shaker at 4 °C overnight. The gel was washed three times with RIPA lysis buffer, resuspended in 2× SDS-loading buffer, and boiled for 5 min to elute the proteins. The eluted supernatants were resolved on an SDS-10% polyacrylamide gel and analyzed by Western blotting. For co-immunoprecipitation of endogenous RASA3 and Gαᵢ, GH4ZR7 cells were treated with apomorphine and/or TRH for 15 min. The cells were scraped in RIPA lysis buffer and lysed as mentioned above. After preclear phase, the supernatants were incubated with 4 µl of anti-Gαᵢ antibody at 4 °C overnight. The next day, ~40 µl of protein G-Sepharose, Fast Flow (Sigma) was added to each tube and incubated at 4 °C for an hour. Washing and elution were as described above.

Stable Transfection—GH4ZR7 cells were co-transfected with plasmids for antisense RASA3 (5 µg) and pGK-puro (0.5 µg)/10-mm dish using Lipofectamine (Invitrogen) (1.5×, w/v). The transfected cells were cultured in Ham’s F-10 + 8% fetal bovine serum containing puromycin (20 µg/ml) for 3–4 weeks. Antibiotic-resistant clones were picked (34 clones) and tested for expression of the corresponding RASA3 protein by Western blot analysis.

Ligand Binding—Dopamine-D2 receptor density was measured by specific binding of the antagonist [³H]spiperone. The cell membranes were prepared from 15-cm dishes by replacing the medium with hypotonic buffer (15 mmol/liter Tris/HisCl, pH 7.4, 2.5 mmol/liter MgCl₂, 0.2 mmol/liter EDTA). The cells were scraped from the plate and centrifuged at 500 × g for 15 min at 4 °C, and the pellet was resuspended in cold TME buffer (75 mmol/liter Tris, pH 7.4, 12.5 mmol/liter MgCl₂, 1 mmol/liter EDTA). The cells were lysed further by passing gently through a 25-gauge needle and centrifuged at 12,000 × g for 30 min at 4 °C and resuspended in TME. For binding assay, aliquots of 100 µg/tube membrane preparation were added to triplicate tubes containing 0.2 ml of TME + 0.1% ascorbic acid with 9000 cpm of [³H]spiperone ± 10⁻⁶ M apomorphine. After 30 min of incubation at room temperature, the reactions were terminated by the addition of 1 ml of ice-cold 50 mM Tris, pH 7.4. The samples were filtered through GF/C glass microfiber filters (Whatman, Clifton, NJ) and washed three times with 3 ml of ice-cold 50 mM Tris, pH 7.4. The filters were then combined with 3 ml of scintillation fluid (InterSciences Inc., Markham, ON), and radioactivity was detected using the Packard TRI-CARB 2100TR scintillation counter (PerkinElmer Life Sciences). The receptor density was normalized to protein concentration determined by Bradford assay.

Measurement of Phospho-ERK1/2 in GH4ZR7 Cells—Equal numbers of cells (3 × 10⁶ cells/well) were plated in six-well plates. At 80% confluence, the cells were placed in serum-free Ham’s F-10 medium (1 h, 37 °C). The cells were treated with the indicated drugs at 37 °C, and after the indicated time the plates were transferred on ice and washed two times with cold phosphate-buffered saline. The cells were lysed in 100 µl of lysis buffer containing equal volumes of 2.5× SDS loading and RIPA buffers, stored on ice, sonicated for 6 s, and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant (30 µl) was heated (100 °C, 5 min) and rapidly cooled on ice. The samples were centrifuged 30 s and were separated by SDS-PAGE and subjected to Western blot analysis. Phosphorylation was detected using (1:1000) anti-phospho-p42/44 ERK1/2 (ERK1/2). The corresponding bands for ERK1 and ERK2 were digitally quantified using Adobe Photoshop. The results were normalized to β-actin control.

RESULTS

RASA3, a Novel Gaᵢ-interacting Protein—To identify novel Gαᵢ effectors involved in Gαᵢ-induced inhibition of ERK1/2 activation in pituitary cells, we constructed an oligo(dt)-primed cDNA library from GH4ZR7 pituitary cell poly(A⁺) RNA for yeast two-hybrid screening with constitutively active Gαᵢ, Q204L as bait. In two sets of screening we identified six of six positive/10⁶ and four of four positive/10⁶ clones that encoded 834-amino acid rat RASA3 cDNA (AB020479), also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY also known as Ras p21 protein activator 3, 35910 JOURNAL OF BIOLOGICAL CHEMISTRY
GAP activity against Rap, Ras, and R-Ras (19, 20). RASA3 is associated with the plasma membrane via PH domain-PIP2 interaction and activated by inositol 1,3,4,5-tetrakisphosphate (IP4) binding to the BTK domain (21, 22). As a RasGAP protein, RASA3 could directly couple G\(_{\text{G}}\)/H9251 proteins to inhibition of Ras activation, and hence its role was examined.

**RASA3 Expression in GH4ZR7 cells**—Because commercially available anti-peptide antibodies against RASA3 lacked the sensitivity or specificity to detect endogenous RASA3 (data not shown), a rabbit polyclonal antibody was raised against full-length recombinant rat RASA3, named D40. By Western blot analysis, the D40 antibody detected purified recombinant RASA3 in GH4ZR7 cells.

**RASA3 Mediates D2S-induced ERK1/2 Inhibition**

**FIGURE 1.** RASA3, a G\(_{\text{G}}\)/H9251-QL-interacting clone from yeast two-hybrid screen of GH4ZR7 cDNA library. A, amino acid sequences of rat, mouse, and human RASA3 (GenBank\textsuperscript{TM} accession numbers NP_113762, NM_009025, and NM_007368, respectively) were aligned with G\(_{\text{G}}\)/H9251-interacting clone using the DNAStar MegAlign program; the boxed letters represent amino acid differences between sequences. B, percentage of amino acid identity between the new G\(_{\text{G}}\)/H9251 interactor and rat, mouse, and human RASA3. C, schematic representation of functional domains of RASA3.
RASA3 Mediates D2S-induced ERK1/2 Inhibition

FIGURE 2. Presence of RASA3 in GH4ZR7 cells detected using a polyclonal anti-RASA3 antibody. A, concentrated and purified D40 (full-length RASA3 antibody) detected the endogenous RASA3 protein in GH4ZR7 cell lysate (50 μg/lane) with 1/500 and 1/1000 dilution, whereas 1/1000 dilution of preimmune serum (Preimm) failed to recognize the protein. Nsp, nonspecific band detected by preimmune serum. B, affinity-purified D40 was used to detect endogenous level of RASA3 (arrow) in GH4ZR7 or BDYII cells and two derivative stable cell lines of overexpressed or depleted RASA3. GH4Z7R, GH4C1 cells stably transfected with D2S; RASA3-AS30, GH4ZR7 stably transfected with antisense RASA3; BDYII, Balb-c/3T3 cells stably transfected with D2S; BDYII-21, BDYII cells stably transfected with sense RASA3. The same total protein concentration (50 μg/lane) was loaded in each well.

RASA3 (data not shown) and endogenous RASA3 (96 kDa) in GH4ZR7 cell extracts, whereas preimmune serum failed to detect RASA3, although some nonspecific products were present (Fig. 2A). Furthermore, in GH4ZR7 cells depleted of RASA3 using antisense construct (RASA3-AS30 clone, see below), a reduced level of RASA3 was detected by the purified D40 antibody, whereas in Balb-D2S fibroblast cells (BDYII) stably transfected with sense RASA3 (BDYII-21), the antibody detected an increase in RASA3 protein (Fig. 2B). These experiments validated the specificity of the D40 antibody for the detection of endogenous rat RASA3 in GH4ZR7 cells.

Ga_i/RASA3 Interactions—Ga_i/RASA3-Ga_i interactions were quantified using the yeast mating/β-galactosidase assay (Fig. 3). Both wild type and constitutively active Ga_i proteins (Ga_i1, Ga_i2, Ga_i3, Ga_o, and Ga_o) interacted with RASA3. By contrast, Ga_o and Ga_o were unable to interact with RASA3 (i.e. no colonies were obtained), suggesting that RASA3 preferentially interacts with Ga_o family proteins. The preferential interaction of RASA3 with activated Ga_o family proteins suggests a role for RASA3 in Ga_o signaling.

To verify the RASA3-Ga_i interaction, in vitro pull-down assays using bacterially expressed S-His_o-RASA3 and GST-tagged Ga_o2 or Ga_o3 proteins were done. Ni-NTA-agarose bound to S-His_o-RASA3 with no contaminating GST-tagged proteins. On the other hand, in the presence of S-His_o-RASA3, both GDPβS-inactivated and GTPγS-activated Ga_o3 and Ga_o2 bound to RASA3 (Fig. 4), consistent with interactions observed in yeast mating assays. The interaction with RASA3 was stronger for activated than nonactivated G proteins.

The RASA3-Ga_o interaction was further tested by co-transfection and co-immunoprecipitation of FLAG-tagged RASA3 and wild type or constitutively active Ga_o2 and Ga_o3 proteins in HEK-293 cells (Fig. 5). Transfected FLAG-RASA3 was successfully immunoprecipitated by anti-FLAG M2 Affinity gel, with no FLAG-RASA3 detected in nontransfected cells. Immunoprecipitation of FLAG-RASA3 co-immunoprecipitated both wild type or constitutively active forms of Ga_o2 and Ga_o, with no Ga_o protein present in immunoprecipitate from nontransfected cells. Interestingly, much more of the constitutively active forms of Ga_i (Ga_o-QL and Ga_o-QL) were bound to RASA3, consistent with an increased association of active Ga_i with RASA3.

Because Ga_o3 is implicated in D2S signaling to ERK1/2, the interaction between endogenous Ga_o3 and RASA3 was assessed in GH4ZR7 cells. GH4ZR7 cells were untreated or treated with apomorphine (1 μM) to activate dopamine D2S receptor signaling, and cell lysates were prepared. The Ga_o3 proteins were immunoprecipitated using anti-Ga_o3 antibody, and endogenous proteins were detected using anti-Ga_o3 and the D40 anti-RASA3 antibody (Fig. 6). When cell extracts were run on columns without anti-Ga_o3 antibody, no Ga_o3 or RASA3 was detected. By contrast when Ga_o3 was immunoprecipitated, both endogenous Ga_o3 and RASA3 were present in immunoprecipitates from untreated and treated GH4ZR7 cells. These results show that a weak interaction is present between Ga_o3 and RASA3 in unstimulated, as well as D2S-stimulated cells. No detectable difference in RASA3-Ga_o3 interaction upon D2S receptor stimulation raised the possibility of a synergistic effect of TRH and D2S pathways on RASA3-Ga_o3 interaction. The cells were treated with TRH alone or TRH with apomorphine, and the co-immunoprecipitation was repeated as described...

FIGURE 3. Specificity of RASA3-Ga_i interaction. Interactions between RASA3 and the indicated wild type or constitutively active mutant (*) Ga_i proteins were examined by yeast mating assay. No colonies were obtained for Ga_o (not shown). Quantitative β-galactosidase assay was performed on cell lysates of colonies from mating, and the data were normalized as percentages of the positive control (pCL1) from the same experiment, presented as means ± S.E. (n = 4–5).
RASA3 Mediates D2S-induced ERK1/2 Inhibition

Inhibition of TRH-induced ERK1/2 phosphorylation by dopamine-D2S receptor signaling was examined in these clones (Fig. 9). Basal phosphorylation of ERK1/2 was undetectable and was not altered by the dopamine agonist apomorphine. TRH greatly increased ERK1/2 phosphorylation, and this effect was completely blocked by co-activation of dopamine-D2S receptors using apomorphine. Apomorphine-induced inhibition of TRH action was greatly suppressed in RASA3-AS30 and AS20 clones (Fig. 9, A and B, respectively), by 70–80% compared with maximal D2S-induced inhibition in GH4ZR7 cells (Fig. 9C). Importantly, the AS20 and AS30 clones displayed similar levels of dopamine D2 receptor density compared with parental GH4ZR7 cells (83 ± 8, 97 ± 4, and 115 ± 8 fmol/mg, respectively). Similarly, D2S-inhibited induction of forskolin (1 μM)-induced cAMP formation was similar (87.8 ± 3.6, 84.5 ± 3.1, and 95.1 ± 0.3% in AS20, AS30, and GH4ZR7 cells, respectively). This indicates that the reduced D2S receptor signaling to ERK1/2 was not due to fewer D2S receptors or a general impairment of D2S signaling in the RASA3 antisense clones. The pronounced reduction of D2S-mediated inhibition of ERK1/2 phosphorylation in GH4ZR7 clones depleted of RASA3 indicates a critical role for RASA3 in mediating D2S receptor signaling to inhibit ERK1/2 activation.

**DISCUSSION**

**RASA3, a Link between Go and Ras Signaling**—Our results delineate the obligatory role of a novel Go target, RASA3, in signaling of the dopamine-D2S receptor to inhibit ERK1/2 activation in GH4ZR7 pituitary cells. RASA3 was the predominant cDNA clone identified in the two-hybrid screen with constitutively active Go13-QL mutant, and its interaction with activated Goα proteins was validated by several different approaches. First, yeast mating assay verified that RASA3 can interact with both wild type and constitutively active forms of Goα13/2 proteins (Fig. 3). Second, purified recombinant RASA3 directly and preferentially interacted with GTPγS-activated versus GDPβS-inactivated Goα13 and Goα2 in in vitro pull-down assay (Fig. 4). Third, transfected FLAG-RASA3 immunoprecipitated both wild type and constitutively active mutants of Goα13 and Goα2, with greater interaction with the activated forms (Fig. 5). Finally, we found that endogenous RASA3 and Goα13 interact, because anti-Goα13 immunoprecipitated RASA3 (Fig. 6). Despite the evidence of preferential interaction of RASA3 with activated Goα proteins, we did not observe an increase in endogenous RASA3-Goα13 interaction upon stimulation with D2 agonist apomorphine alone (Fig. 6). However, concurrent treatment with TRH and apomorphine induced a pronounced increase in

---

**FIGURE 4. Direct interaction between RASA3 and Goα2 and Goα3, in vitro.** For in vitro pull-down assay, bacterially expressed GST, GST-Goα2, or Goα3 fusion protein was incubated with S-His6-RASA3 protein, and the His-tag was pulled down using Ni-NTA beads. The proteins were resolved by SDS-PAGE and immunoblotted using anti-S (1/5000) or anti-GST (1/1000) antibodies. To inactivate or activate Gα proteins, we did not observe an increase in endogenous RASA3-Gα13 complex, whereas TRH alone, like apomorphine alone (Fig. 6), failed to increase the basal binding level (arrow). The slight shift in migration of RASA3 in the TRH/apomorphine-treated sample may reflect a post-translational modification, such as phosphorylation.

**FIGURE 5. Preferential interaction between RASA3 and constitutively active Goα13 and Goα2.** HEK293 cells were transiently co-transfected with pcDNA3FLAG-RASA3 with pCDNA3-Goα13 (A) or pcDNA3-Goα2 (B) (wild type or constitutively active mutant). For co-immunoprecipitation (IP), cell lysate was incubated with anti-FLAG M2-agarose affinity beads, eluted, and resolved by SDS-PAGE. The membranes were blotted with anti-FLAG antibody (1/1000) or specific anti-Goα13 (1/1000) or anti-Goα2 (1/500) antibodies. Note that RASA3 interacted with both Goα13 and Goα2 wild type and constitutively active forms, but the latter activated forms showed the stronger interaction.

---

**TABLE**

| S-His-RASA3 | GST tag | Gai3-GDPS | Gai3-GTPyS | Gai2-GDPS | Gai2-GTPyS |
|-------------|---------|------------|------------|-----------|------------|
| +           | +       | +          | +          | +         | +          |

---

Above (Fig. 7), TRH with apomorphine induced a robust increase in RASA3-Gα3 complex, whereas TRH alone, like apomorphine alone (Fig. 6), failed to increase the basal binding level (arrow). The slight shift in migration of RASA3 in the TRH/apomorphine-treated sample may reflect a post-translational modification, such as phosphorylation.

**Functional Role of RASA3 in ERK1/2 Pathway**—To address RASA3 function in D2S-induced signaling to inhibit ERK1/2 activation, GH4ZR7 cells were stably transfected with a full-length antisense RASA3 construct. As shown in Fig. 8, several GH4ZR7 clones stably transfected with AS-RASA3 show significant knockdown of RASA3 determined using D40 antibody in Western blot analysis. Clones RASA3-AS30 and RASA3-AS20 displayed the greatest knockdown of RASA3 protein levels (to about 30%) and were examined further for signaling to ERK1/2 compared with parental GH4ZR7 cells.

---

**FIGURE 5. Preferential interaction between RASA3 and constitutively active Goα13 and Goα2.** HEK293 cells were transiently co-transfected with pcDNA3FLAG-RASA3 with pCDNA3-Goα13 (A) or pcDNA3-Goα2 (B) (wild type or constitutively active mutant). For co-immunoprecipitation (IP), cell lysate was incubated with anti-FLAG M2-agarose affinity beads, eluted, and resolved by SDS-PAGE. The membranes were blotted with anti-FLAG antibody (1/1000) or specific anti-Goα13 (1/1000) or anti-Goα2 (1/500) antibodies. Note that RASA3 interacted with both Goα13 and Goα2 wild type and constitutively active forms, but the latter activated forms showed the stronger interaction.

---

**TABLE**

| S-His-RASA3 | GST tag | Gai3-GDPS | Gai3-GTPyS | Gai2-GDPS | Gai2-GTPyS |
|-------------|---------|------------|------------|-----------|------------|
| +           | +       | +          | +          | +         | +          |
RASA3 Mediates D2S-induced ERK1/2 Inhibition

TRH signals via $G_{\alpha_i}$ to stimulate PLC, generating inositol 1,4,5-trisphosphate and IP4 from PIP2 (phosphatidylinositol 4,5-bisphosphate) and phosphatidylinositol 3,4,5-trisphosphate. Because RASA3 is localized to the membrane via interaction with PI(4,5)P2, the breakdown of PI(4,5)P2 and the generation of IP4, which competes with PI(4,5)P2, may release RASA3 from the membrane. Activated $G_{\alpha_i}$ appears to preferentially bind to the released RASA3 and may recruit RASA3 to the membrane to bind Ras and inhibit its signaling.

To more directly address the role of RASA3 in D2 receptor signaling, depletion of this protein was done using antisense cDNA constructs (Fig. 8). We have found that stable transfection of full-length antisense constructs in GH4 cells provides excellent specificity for knockdown individual $G_{\alpha_i}$ subunits, which are highly (90% amino acid identity) conserved (23–25). Depletion of RASA3 in two independent clones strongly blocked dopamine D2S-induced inhibition of TRH-induced ERK1/2 activation (Fig. 9), which clearly displays the critical role of RASA3 in this pathway. Furthermore, suppression of RASA3 had no effect on basal or TRH-induced ERK1/2 activity in antisense clones (Fig. 9). Together these data support a critical role for RASA3 in mediating D2S receptor signaling via $G_{\alpha_i}$ to inhibit TRH-induced ERK1/2 activation.

The preferential coupling of the dopamine D2S receptor via $G_{\alpha_i}$ to inhibit ERK1/2 activation was suggested by previous studies of G protein specificity in GH4ZR7 cells using a rescue strategy with stable transfection of pertussis toxin-insensitive $G_{\alpha}$ subunit cDNAs (13). Using this approach, we showed that the dopamine-D2S receptor signals through different subsets of

![Image of Western blot](image_url)

**FIGURE 6. Interaction between endogenous RASA3 and $G_{\alpha_i}$ in GH4ZR7 cells.** For assay, the cells were placed in serum-free medium for 1 h and then incubated or not with apomorphine (1 μM) for 15 min followed by cell lysis. The cell lysate was incubated with anti-$G_{\alpha_i}$ antibody and protein G-Sepharose, eluted, and resolved by SDS-PAGE. The membranes were blotted with either D40 anti-RASA3 antibody (1/1000) or anti-$G_{\alpha_i}$ antibody (1/1000). Note that in both unstimulated, as well as apomorphine-stimulated cells RASA3 interact with $G_{\alpha_i}$, but not in the absence of anti-$G_{\alpha_i}$ antibody (negative control). IP, immunoprecipitation.

**FIGURE 7. Preferential interaction between endogenous RASA3 and $G_{\alpha_i}$ in GH4ZR7 cells in the presence of both TRH and apomorphine.** For assay, the cells were starved with serum-free medium for 1 h and then incubated or not with apomorphine (1 μM) and/or TRH (1 μM) for 10 min followed by cell lysis. The cell lysate was incubated with anti-$G_{\alpha_i}$ antibody and protein G-Sepharose, eluted, and resolved by SDS-PAGE. The membranes were blotted with either D40 anti-RASA3 antibody (1/1000) or anti-$G_{\alpha_i}$ antibody (1/1000). The arrow indicates immunoprecipitated RASA3. IP, immunoprecipitation.

RASA3-$G_{\alpha_i}$ interaction (Fig. 7), whereas TRH or apomorphine alone had no effect. Thus, TRH signaling potentiates RASA3 interaction with receptor-activated $G_{\alpha_i}$.
Produced inhibition of basal ERK1/2 activity is mediated through Gi/Go proteins to inhibit cAMP formation (Gi2) also found in GH4ZR7 cells that dopamine-D2S-induced coupling of the D2S receptor to Gi/Go proteins to inhibit cAMP formation (Gi2) of D2S receptor for pertussis toxin-insensitive Gi2 interact with RASA3 indicates that both Gi2 and Gi3- to inhibit Ras-ERK1/2 signaling, although via different G protein pathways. Liu et al. (14) also found in GH4ZR7 cells that dopamine-D2S-induced inhibition of basal ERK1/2 activity is mediated through Gi2O, but not Gi12. In our studies, the dopamine-D2S receptor inhibited both TRH-stimulated c-Raf and basal B-Raf activity (TRH did not stimulate B-Raf) (13). Although RASA3 has dual activity against both Ras and Rap proteins (20, 27), TRH failed to increase B-Raf activity, ruling out inhibition of Rap activation as a mechanism of D2S-induced inhibition of TRH action. Although D2S signaling did reduce basal B-Raf activity, this more likely involves B-Raf inhibition by Gαs-induced mobilization Rap1GAP to inhibit Rap1-mediated B-Raf-ERK1/2 signaling (26).

We have recently shown that somatostatin and muscarinic receptors also mediate inhibition of TRH-induced ERK1/2 activation (15), consistent with previous findings that these receptors are able to couple via Gαs in GH4 cells (25, 28). However, not all Gαs-coupled receptors coupled to this pathway, because dopamine-D2L receptors failed to inhibit TRH-induced ERK1/2 activation (15). The lack of coupling of the D2L receptor may reflect inefficient coupling of this receptor to Gαs in these cells, but this has not been tested. Thus, receptors that couple to Gαs would be predicted to mediate this signaling to inhibit ERK1/2 in cells that express RASA3.

RASA3: Structure, Function, and Regulation—RASA3 is a member of the GAP1 family of RasGAP proteins, based on their sequence homology, particularly in the RasGAP domain. The GAP1 proteins all have similar primary structure containing C2, RasGAP, PH, and BTK domains (Fig. 1), although based on the secondary structure of p120GAP, it is believed that protein is folded so that both the N-terminal C2 domains and the C-terminal PH/Btk domains are in close proximity (29). GAP1m is the most closely related variant of RASA3, with similar C2 domains that lack key residues required for Ca2+ binding; thus these two proteins are not regulated by [Ca2+]i, unlike CAPRI and RASAL (18, 21, 29 –32). Interestingly, although not regulated by calcium, Gα12 has been shown to bind directly to GAP1m via its PH/Btk domain, leading to its activation (33). Our identification of the Gαi-RASA3 interaction is the first evidence that RasGAPs are connected to Gαi/i3 protein signaling. In yeast mating assays, the C-terminal portion of RASA3 containing PH/Btk domains did not interact with Gαi3 (data not shown), suggesting that the interaction domain may be larger or different from that of GAP1m and Gα12. Thus, both GAP1m and RASA3 link to G proteins to inhibit Ras-ERK1/2 signaling, although via different G protein pathways.

RASA3 is regulated by a number of PLC-generated second messengers, in addition to its regulation by Gαi proteins. The PH/Btk domain of RASA3 binds to phosphatidylinositol 3,4,5-trisphosphate and PI(3,4,5)P3, resulting in constitutive association with the plasma membrane (21, 29). Among GAP1 family members, RASA3 is uniquely regulated by IP4, produced from inositol 1,4,5-trisphosphate and PI(3,4,5)P3, resulting in constitutive association with the plasma membrane (21, 29). Among GAP1 family members, RASA3 is uniquely regulated by IP4, produced from inositol 1,4,5-trisphosphate and PI(3,4,5)P3, resulting in constitutive association with the plasma membrane (21, 29).
may alter RASA3 structure to promote the binding of D2S-activated Ga\textsubscript{i3}. Although PLC activation appears to displace RASA3 from the membrane, activation of Ga\textsubscript{i3} may recruit RASA3 to the membrane to inhibit Ras signaling.

Roles of D2S Receptor-Ga\textsubscript{i3}-RASA3 Signaling—Several lines of evidence indicate that dopamine-D2 receptors negatively regulate the proliferation and differentiation of lactotrophs in vitro, as well as inhibiting PRL synthesis and secretion (37, 38). However, the specific signaling pathways involved in D2 receptor actions on cell proliferation and PRL synthesis have not been clarified.

In GH4 cells, TRH induces ERK1/2 activation in part via protein kinase C (34, 35), which mediates an increase in PRL transcription (16, 36), and inhibition of ERK1/2 is required for dopamine-D2S receptor mediated inhibition of PRL gene transcription (14, 17). Because inhibition of RASA3 largely prevented apomorphine-induced inhibition of TRH-mediated ERK1/2 activation (Fig. 9), it is likely that this pathway is critical to inhibit TRH-induced PRL transcription. However, it remains unclear whether this pathway also inhibits basal PRL synthesis, because we were unable to detect basal ERK1/2 phosphorylation under our conditions. As discussed above, because RASA3 inactivates both Ras and Rap, it is possible that it mediates inhibition of basal ERK1/2 activity. In addition, although dopamine D2 receptor activation inhibits cell proliferation, the role of inhibition of ERK1/2 activation remains unclear, because multiple G proteins (and presumably alternate signaling pathways) are involved in inhibition of cell proliferation (28).

Because we found in GH4ZR7 cells that D2S signaling to ERK1/2 is inhibited upon depletion of RASA3, it is possible that other cell types that express RASA3 can mediate inhibitory Ga\textsubscript{i3} signaling to ERK1/2. RASA3 RNA is expressed in different human tissues including brain, skeletal muscles, spleen, peripheral blood leukocytes, and platelets, suggesting roles in all of these tissues (39, 40). In the brain, immunoreactivity for RASA3 is highest in the CA1 of the hippocampus, amygdala, cerebellum, and pyriform cortex (41). As discussed above, other Ga\textsubscript{i3} coupled receptors have the potential to couple to RASA3 to inhibit ERK1/2 activation, and the role of RASA3 may depend on the receptors present. More recently we have shown in primary rat striatal cultures that the D2-selective agonist quinpirole inhibited potassium-stimulated ERK1/2 activation (15). However, the role of RASA3 in inhibitory regulation of ERK1/2 activation in striatal cells remains to be elucidated. The widespread distribution of RASA3 and Ga\textsubscript{i3} proteins suggests an important role for this coupling in a variety of physiological processes involving regulation of ERK1/2 signaling. For example, if Ga\textsubscript{i3}-RASA3 signaling is important in vivo, the development of pharmacological compounds could provide novel anti-proliferative agents by activating RASA3 to inhibit cell proliferation.

Conclusion—In this study we identified a novel Ga\textsubscript{i3}-interacting protein, RASA3, and determined the importance of Gi-RASA3 coupling in the negative regulation of Ras-ERK1/2 activation by the D2S receptor. Our results indicate that the D2S receptor couples via Ga\textsubscript{i3}-RASA3 to inhibit TRH-induced Ras-ERK1/2 activation in pituitary cells, suggesting that RASA3 may be a general mediator of Ga\textsubscript{i3}-induced inhibition of ERK1/2 activation.
32. Liu, Q., Walker, S. A., Gao, D., Taylor, J. A., Dai, Y. F., Arkell, R. S., Bootman, M. D., Roderick, H. L., Cullen, P. J., and Lockyer, P. J. (2005) J. Cell Biol. 170, 183–190
33. Jiang, Y., Ma, W., Wan, Y., Kozasa, T., Hattori, S., and Huang, X. Y. (1998) Nature 395, 808–813
34. Jones, B. W., Song, G. J., Greuber, E. K., and Hinkle, P. M. (2007) J. Biol. Chem. 282, 12893–12906
35. Gutkind, J. S. (2000) Sci. STKE 2000, 1–13
36. Smith, J., Yu, R., and Hinkle, P. M. (2001) Mol. Endocrinol. 15, 1539–1548
37. Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000) Physiol. Rev. 80, 1523–1631
38. Ben-Jonathan, N., and Hnasko, R. (2001) Endocr. Rev. 22, 724–763
39. Lockyer, P. J., Vanlingen, S., Reynolds, J. S., McNulty, T. J., Irvine, R. F., Parys, J. B., and Cullen, P. J. (1999) Biochem. Biophys. Res. Commun. 255, 421–426
40. McNulty, T. J., Letcher, A. J., Dawson, A. P., and Irvine, R. F. (2001) Cell Signal. 13, 877–886
41. Signore, A. P., O’Rourke, F., Lu, X., Feinstein, M. B., and Yeh, H. H. (1999) J. Neurosci. Res. 55, 321–328

RASA3 Mediates D2S-induced ERK1/2 Inhibition