Modulation of Rap Activity by Direct Interaction of Gαo with Rap1 GTPase-activating Protein*

(Received for publication, May 26, 1999, and in revised form, June 11, 1999)

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We used the yeast two-hybrid system to identify proteins that interact directly with Gαo. Mutant-activated Gαo was used as the bait to screen a cDNA library from chick dorsal root ganglion neurons. We found that Gαo interacted with several proteins including Gz-GTPase-activating protein (Gz-GAP), a new RGS protein (RGS-17), a novel protein of unknown function (IP6), and Rap1GAP. This study focuses on Rap1GAP, which selectively interacts with Gαo and Gαq but not with Gαs or Gα12. Rap1GAP interacts more avidly with the unactivated Gαo as compared with the mutant (Q205L)-activated Gαo. When expressed in HEK-293 cells, unactivated Gαo co-immunoprecipitates with the Rap1GAP. Expression of chick Rap1GAP in PC-12 cells inhibited activation of Rap1 by forskolin. When unactivated Gαo was expressed, the amount of activated Rap1 was greatly increased. This effect was not observed with the Q205L-Gαo. Expression of unactivated Gαo stimulated MAP-kinase (MAPK1/2) activity in a Rap1GAP-dependent manner. These results identify a novel function of Gαo, which in its resting state can sequester Rap1GAP thereby regulating Rap1 activity and consequently gating signal flow from Rap1 to MAPK1/2. Thus, activation of Gαo could modulate the Rap1 effects on a variety of cellular functions.

Heterotrimeric G proteins function as signal transducers for receptors for a large number of hormones, neurotransmitters, autocrine and paracrine factors, cytokines, and sensory signals. Both the Gα (1) and the Gβγ subunits (2) are capable of transferring receptor signals to effectors. There are four families of Gα subunits (3). Direct effectors for the Gαq (4) and Gα12 (5) family proteins have been well characterized. Effectors for the Gαo family are less well defined. Gαo, the visual G protein, activates the GMP phosphodiesterase (1), and Gαi inhibits adenylyl cyclases (6) directly (7). However, direct effectors for Gαi, an abundant G protein in the brain (8, 9), have not yet been identified. Gαo has been implicated in receptor-mediated inhibition of Ca2+ channels in chick dorsal root ganglion neurons (10). Hence, it appeared feasible that this system could be used to identify proteins that directly interact with Gαo. We used the yeast two-hybrid system to identify potential Gαo effectors. For this purpose, we screened the chick dorsal root ganglion cDNA library with the mutationally (Q205L)-activated form of Gαo. In this article we present data indicating that the inactive form of Gαo preferentially interacts with Rap1GAP1 and thus regulates the activity of the small G protein Rap.

EXPERIMENTAL PROCEDURES

Materials—The cDNA synthesis system was from Life Technologies, Inc. Anti-Gαo and anti-MAPK2 antibodies were from Santa Cruz Biotechnology, Anti-M2-FLAG antibody was from Sigma, anti-Gα12,α6 antibody was from Upstate Biotechnology, Inc., and phospho-specific and total MAPK antibodies were from New England Biolabs. Most bio-chemicals were from Sigma, and cell culture supplies were from Life Technologies, Inc. All restriction enzymes were from New England Biolabs. Yeast culture media and amino acids were from CLONTECH. DNA plasmid preparation reagents and Effectene and Superfect transfection reagents were from Qiagen, Inc. ECL reagents were from Amersham Pharmacia Biotech. All other reagents were of the highest grade available.

Yeast Two-hybrid Screening—A directional oligo(dT)-primed cDNA library was constructed from 12-day embryonic chick dorsal root ganglion mRNA. cDNA was synthesized using a cDNA synthesis system and ligated into the GAL4 DNA-activation domain plasmid pPC86 using the Sall/No1 restriction sites (11). Plasmid DNA was isolated from the unamplified library using a Qiagen Plasmid Maxi Kit. Q205L-Gαo was cloned into the Sall/No1 restriction sites of the GAL4 DNA-binding domain plasmid pPC97-cycloheximide (11). The Q205L-Gαo-BD plasmid and the library were co-transformed into the yeast strain MaV203, plated on selective media lacking leucine, tryptophan, and histidine and containing 25 μM 3-aminotriazole, and incubated at 30 °C for 3 days. His+ colonies were then tested for β-galactosidase activity using a filter lift assay. Plasmid DNA was then isolated from positive yeast clones and reintroduced into MaV203 yeast expressing the GAL4 DNA-binding domain in-frame with either the wild type or Q205L-Gαo cDNA.Positive clones were then sequenced, and BLAST analysis was performed using GenBankTM. The Gαq, Gαo, and Gα12 cDNAs encoding both the wild type and the constitutively activated mutants were subcloned into the pPC97-cycloheximide plasmid.

CRPG Assay—The MaV203 yeast strain was co-transformed with the GAL4 DNA-binding domain and GAL4 DNA activation domain plasmids as indicated and incubated on selective media lacking tryptophan and leucine for 3 days at 30 °C. Clones were grown for 24 h in selective liquid media and then inoculated into complete media and grown to an A600 of 1.5. Yeast were lysed with glass beads and incubated in a CRPG assay buffer (100 mM HEPES, pH 7.3, 154 mM NaCl, 4.5 mM 1-aspartate, 1% bovine serum albumin, and 0.05% Tween 20) containing 2 mM CPG (Roche Molecular Biochemicals) for 2–4 h. The reaction was stopped by the addition of ZnCl2 to 1 mM, and the absorbance was measured at 574 nm.

Co-immunoprecipitations—HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum

* This work was supported by National Institutes of Health Grants GM-54508 and DK-38671 (to R. l.) and CA-72971 (to P. J. S. S.). 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 1734 solely to indicate this fact.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AF151967, AF151968, AF151734, and AF151966.

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Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 31, Issue of July 30, pp. 21507–21510, 1999

The abbreviations used are: Rap1GAP, Rap1 GTPase-activating protein; CRPG, chlorphenol red-β-D-galactopyranoside; HRP, horseradish peroxidase; MPO, myeloperoxidase; PGE2, 1(p-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HRPP and MPO, horseradish peroxidase and myeloperoxidase, respectively; SBR, suff- fered saline; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; RGS, regulators of G protein signaling; RalGDS, Ral GDP dissociation stimulator.

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and antibodies. Cells were transfected using Effectene and harvested after 48 h in Buffer A (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 6 mM MgCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). One mg of whole cell lysate was pre-cleared for 1 h with protein G-agarose and antibodies. Immunoprecipitations were performed overnight with 5 μg of M2-FLAG monoclonal antibody on 4 °C incubation with 25 μl of protein G-agarose. Samples were washed three times with phosphate-buffered saline (PBS) containing protease inhibitors and then resolved using SDS-PAGE. After transfer to nitrocellulose, the membranes were blocked in 5% nonfat dry milk, immunoblotted with anti-M2-FLAG monoclonal antibody at 4 °C followed by a 4-h incubation with 1:10,000 dilution of HRP-conjugated secondary antibody (1:10,000). Proteins were visualized using anti-FLAG and anti-Gαo antibodies.

**RESULTS**

Using the yeast two-hybrid system, we screened the chick DRG library with Q205L-Gαo, as the bait. Initial screens dorsal root ganglion positive interacting clones. Further analysis of the top positive clones allowed us to identify four interacting proteins in the initial screens. These proteins all listed in Table I. The sequences for all of these proteins have been submitted to GenBank™ and the accession numbers are listed in Table I. The first protein identified was Gz-GAP, an RGS that has been characterized by other groups to selectively regulate the GTPase activity of Gz (13, 14). We also identified the cDNA clone for a hitherto unrecognized RGS protein. We have named this protein RGS-17. Furthermore, we identified a pro-
as a substrate (n = 5) overnight and assayed for yeast strain MaV203. Clones were grown in selective liquid media.

**Fig. 1. Interaction of Rap1GAP with Go subunits.** a, the MaV203 yeast strain was co-transformed with the indicated GAL4 DNA-binding domain and activation domain constructs. Yeast were then assayed for β-galactosidase activity using a standard filter lift assay using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as a substrate (n = 3). *wt*, wild type; *o*, Q205L-Go, as indicated. Cells were treated with NGF for 10 min as indicated, and lysates were prepared as described under “Experimental Procedures.” Following immunoprecipitation with anti-FLAG (M2) antibody, the immunoprecipitates were separated by SDS-PAGE, immunoblotted with phospho-MAPK (New England BioLabs), and visualized by ECL. The position of phosphorylated MAPK2 (pMAPK2) is indicated.

**Fig. 2. Co-immunoprecipitation of Rap1GAP and wild type Go proteins.** HEK-293 cells were transfected with the indicated expression constructs, and total cell lysates prepared. One mg of lysate was immunoprecipitated with the M2-FLAG antibody and resolved using SDS-PAGE and immunoblotted with the Go (xw) antibody, the immunoprecipitates were separated by SDS-PAGE, immunoblotted with anti-FLAG (M2) antibody, and visualized by ECL. The position of MAPK2 is indicated.

**Fig. 3. Go wild type, but not Go(Q205L), activates Rap1 in PC-12 cells.** Western blot showing the levels of activated MAPK2 as measured by phospho-MAPK immunoblotting. PC-12 cells were transfected with FLAG-MAPK2 and either Rap1GAP, Go, or Q205L-Go as indicated. Cells were treated with forskolin for 10 min as indicated, and lysates were prepared as described under “Experimental Procedures.” Following immunoprecipitation with anti-FLAG (M2) antibody, the immunoprecipitates were separated by SDS-PAGE, immunoblotted with anti-FLAG (M2) antibody, and visualized by ECL. The position of MAPK2 is indicated.

**Fig. 4. Go wild type, but not Go(Q205L), activates MAPK2 in PC-12 cells.** Top, Western blot showing the levels of activated MAPK2 as measured by phospho-MAPK immunoblotting. PC-12 cells were transfected with FLAG-MAPK2 and either Rap1GAP, Go, or Q205L-Go as indicated. Cells were treated with NGF for 10 min as indicated, and lysates were prepared as described under “Experimental Procedures.” Following immunoprecipitation with anti-FLAG (M2) antibody, the immunoprecipitates were separated by SDS-PAGE, immunoblotted with anti-FLAG (M2) antibody, and visualized by ECL. The position of MAPK2 is indicated.

Because Gα binds the transfected as well as the endogenous Rap1GAP inactivating it, thus allowing Rap1 activation. The addition of forskolin did not yield any further activation (Fig. 3, lane 6), which suggests that endogenous Rap1GAP may be essential to regulate Rap1 activity. In contrast, when Q205L-Go was used, activated Rap1 was inhibited under basal conditions and was not stimulated even by incubation with forskolin (Fig. 3, lanes 7 and 8). This finding may result from the inhibition of adenyl cyclase by the activated form of Go, or some other unrecognized Go signaling pathway. The experiments shown in Fig. 3 indicate that inactive but not activated Gα can facilitate the activation of Rap1 by sequestering Rap1GAP.

Because it is known that Rap1 regulates the activity of B-Raf and that B-Raf can regulate the activation state of MAPK1/2 (16), we next examined the effect of both wild type Gα and Q205L-Gα on MAPK1/2 signaling. For this determination, PC-12 cells were transfected with FLAG-tagged MAPK2 with and without Rap1GAP, Go, or Q205L-Go, and then exposed to NGF to activate MAPK. When cells were treated with NGF, activation of MAPK2 was observed (Fig. 4, lanes 1 and 2) as had previously been demonstrated (17). When Rap1GAP was expressed, stimulation of MAPK2 by NGF was not observed (Fig. 4, lanes 7 and 8), indicating that the activation of MAPK2 is dependent on Rap1. When wild type Go was expressed, there was a basal activation state of MAPK2 that could not be potentiated by NGF treatment (Fig. 4, lanes 3 and 4). However, when Q205L-Gα was expressed, MAPK2 activation by NGF was inhibited (Fig. 4, lanes 5 and 6), which is in agreement with the activation state of Rap1 (see Fig. 3). These data support the idea that unactivated Gα but not activated Gα, is able to sequestrate Rap1GAP, thus regulating Rap1 signaling leading to an increase in the activated state of MAPK2.

**DISCUSSION**

The studies described here show that Gα, in its unactivated state can selectively interact with Rap1GAP. The region of Rap1GAP involved in interaction with Gα is currently not known. However, the N terminus of Rap1GAP is highly conserved between the chicken and human sequence, and in fact all of the independent two-hybrid clones for chicken Rap1GAP contained the entire N terminus. This finding is intriguing because the N-terminal 35 amino acids of Rap1GAP are highly similar to regions conserved in two other Go interacting proteins: LGN, a Go interacting protein (18), and PCP-2, a
guanine nucleotide exchange factor for Gαo (19). This similarity suggests that the N terminus of Rap1GAP may be the region that interacts with Gαo, and that this conserved domain may be used by proteins to interact specifically with Gα family subunits.

This interaction between Gαo and Rap1GAP results in the sequestration of Rap1GAP such that the levels of activated Rap1 increases. Thus, one might envisage that activation of Gαo would lead to the inhibition of Rap and consequently of Rap-mediated signaling. Thus, Gαo like Gαi would be able to negatively modulate signaling by the cAMP pathway, but for Gαo this would occur at the level of Rap. In contrast to our current canonical model of G protein regulation of intracellular signaling, we propose that Gαo does so in a hitherto unrecognized manner. Rather than the activated form of Gαo binding to and regulating an effector, it is the inactive form that binds an inhibitory protein (Rap1GAP). Activation of Gαo would release Rap1GAP, which then would be free to inhibit the activity of Rap. Although this mechanism is quite the opposite of the manner in which other heterotrimeric G protein subunits regulate signal flow, it is not entirely implausible. First, Gαo is the most abundant of the G protein α subunits and is particularly abundant in the brain, as is Rap1GAP (15); hence, it is possible that there is enough Gαo such that part of it can be used to sequester Rap1GAP. Second, Gαo is most often coupled to inhibitory receptors such as the α2-adrenergic (20) and opiate receptors (21) in the brain. Activation of these receptors results in the inhibition of cAMP signaling, because cAMP is capable of activating Rap via both protein kinase A and the newly discovered exchange factors that directly bind cAMP (22, 23). Interestingly, forskolin’s activation of Rap1, but not that of Gαo, was blocked by protein kinase A inhibitor (PKI; data not shown). Gαo may be able to antagonize cAMP-dependent gene expression, especially signals routed through the Rap1 → B-Raf → MAPK1/2 pathway (24), in neurons by interacting with Rap1GAP. The experiments shown in Fig. 4 support such a mechanism of regulation. Thus Gαo-coupled receptors could gate signal flow through the Rap1 to B-Raf pathway (25, 26). The physiological significance remains to be determined.

This interaction of Gαo with Rap1GAP is not likely to explain all of the biological actions of Gαo. Activated Gαo has been shown to trigger neurite outgrowth in neuronal cells (27). Although activated Rap1 can induce neurites in PC-12 cells (16), Rap1 activation was not detected in this study using activated Gαo. Therefore, it does not appear feasible that the effects on Rap will explain how activated Gαo stimulates neurite outgrowth nor its reported regulation of tyrosine kinases in chick dorsal root ganglion neurons (10). Other studies in our laboratory indicate that in NIH-3T3 cells, Q205L-Gαo activates Stat-3 via Src.2 The relevance of this pathway in neuronal cells is currently not known. The search for direct effectors regulated by activated Gαo continues.

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