Functional Interaction between Goz and Rap1GAP Suggests a Novel Form of Cellular Cross-talk*

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Gz is a member of the G family of trimeric G proteins whose primary role in cell physiology is still unknown. In an ongoing effort to elucidate the cellular functions of Gz, the yeast two-hybrid system was employed to identify proteins that specifically interact with a mutationally activated form of Gz. One of the molecules uncovered in this screen was Rap1GAP, a previously identified protein that specifically stimulates GTP hydrolytic activity of the monomeric G protein Rap1 and thus is believed to function as a down-regulator of Rap1 signaling. Like Gz, the precise role of Rap1 in cell physiology is poorly understood. Biochemical analysis using purified recombinant proteins revealed that the physical interaction between Gz and Rap1GAP blocks the ability of RGSs (regulators of G protein signaling) to stimulate GTP hydrolysis of the α subunit, and also attenuates the ability of activated Gz to inhibit adenylyl cyclase. Structure-function analyses indicate that the first 74 amino-terminal residues of Rap1GAP, a region distinct from the catalytic core domain responsible for the GAP activity toward Rap1, is required for this interaction. Co-precipitation assays revealed that Gz, Rap1GAP, and Rap1 can form a stable complex. These data suggest that Rap1GAP acts as a signal integrator to somehow coordinate and/or integrate Gz signaling and Rap1 signaling in cells.

Trimeric guanine nucleotide binding regulatory proteins (G proteins) are key players in the transduction of a diverse array of extracellular signals to intracellular second messages (1–3). These G proteins consist of a GTP-binding α subunit and two additional subunits, termed β and γ, that are tightly associated with each other and function as a single βγ complex (1, 2). Based on structural homology between their α subunits, G proteins are divided into four groups, Gs, Gi, Gq, and G12. During G protein signaling, activation by an appropriately liganded receptor stimulates GTP binding to the α subunit and subsequent dissociation of α-GTP from the βγ complex to produce the active forms of the G proteins that transmit signals to downstream targets, termed effectors (3). In addition to receptors, G proteins, and effectors, a new family of proteins has recently been discovered that is important in signaling through trimeric G proteins. These proteins, termed RGSs (regulators of G protein signaling), act as negative regulators because of their abilities to stimulate GTP hydrolysis of G protein α subunits (2); in addition, some RGS proteins can apparently modulate other aspects of G protein signaling processes (4, 5).

Gz, a member of the Gz family, is a 41-kDa α subunit that has several unique, and presumably important, properties (6–8). First, its tissue distribution is quite restricted, being found primarily in brain, adrenal medulla, and platelets, while expression is virtually undetectable in other tissues (9, 10). Another property that distinguishes Gz from other members of the Gz family is its inability to serve as a substrate for pertussis toxin-catalyzed ADP-ribosylation (8, 10), making it a candidate for pertussis toxin-insensitive signaling processes. Additionally, the intrinsic rate of GTP hydrolysis by Gz is quite low compared with most other G protein α subunits (8), suggesting that RGSs or RGS-like molecules play important roles in regulation of Gz signaling. Finally, Gz is subjected to phosphorylation by protein kinase C; this phosphorylation occurs both in vitro and in vivo and the modification interferes with the interaction of Gz with both the βγ complex and a recently identified selective regulator, RGSZ1 (11–14).

While it is not yet clear which receptors are normally coupled to Gz, many receptors that couple to Gz proteins can also activate Gz signaling pathways if the receptors are overexpressed in cells (15, 16). Similarly, although the downstream effects of Gz activation are not well understood, activated Gz does possess an ability to inhibit some subtypes of adenylyl cyclase, a property shared with other members of the Gz family (15, 17). Also, stable expression of mutationally activated Gz can transform Swiss 3T3 and NIH3T3 cells by stimulating mitogenic pathways (18). Interestingly, this stimulation is apparently unrelated to the ability of Gz to inhibit adenylyl cyclase, suggesting that Gz signaling is coupled to additional signaling pathways (18).

As part of an ongoing effort to elucidate the signaling functions of Gz, we recently undertook a yeast two-hybrid screen to identify proteins that specifically interact with a mutationally activated form of Gz (14). In this report, we describe one of the molecules identified via this approach as Rap1GAP, a protein that had been previously identified as a specific activator of the GTP hydrolytic activity of the monomeric G protein Rap1 (19). The ubiquitous involvement of members of the Ras superfamily in mitogenic signaling pathways makes regulators of these proteins like Rap1GAP attractive candidates to act as signal integrators and/or coordinators with Gz. Interestingly, the expression pattern of Rap1GAP is similar to that mentioned above for Gz, with predominant expression being observed in brain among normal tissues examined (19). Characterization of

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§ The abbreviations used are: G protein, guanine nucleotide binding regulatory protein; HEK, human embryonic kidney; GTPyS, guanosine 5′-O-(thio)-triphosphate.
the interaction between Goa and Rap1GAP indicates that the active form of Goa specifically and functionally interacts with Rap1GAP and that the three proteins, i.e. Goa, Rap1GAP, and Rap1, can form a stable complex. These data point to an unanticipated cellular cross-talk between Ga and Rap1 signaling processes, and provide new insight into potential cellular functions of Ga.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human embryonic kidney (HEK) 293 cells and fall army worm (Spodoptera frugiperda, Sf9) cells were obtained from the American Type Culture Collection. The anti-Rap1GAP polyclonal antibody (20) and the anti-Goα polyclonal antibody (8) have been previously described. Anti-Rap1 polyclonal antibody was purchased from Transduction Laboratories. Radioactive compounds (γ-32P]GTP, [γ-35S]GTP·S, and [α-32P]-ATP) were purchased from NEN Life Science Products Inc.

**Plasmid Constructs**—Plasmids containing the cDNA of rat Goa (both wild type and the Q205L mutant), containing the Glu-Glu epitope at residues 3 to 8, were the gift of Henry Bayne (University of California at San Francisco) and have been described (21). Plasmids containing full-length Rap1GAP (designated pCAN-Rap1GAP) and fragments thereof were previously described (20). Other constructs used in yeast two-hybrid screen have also been described (14). The plasmid containing the cDNA of human Gaia in an SfiI-NcoI fragment was constructed by subcloning a pRSET-A vector (Invitrogen). The plasmid containing the cDNA of human Gaia·Tag was incubated with Gaia, Rap1GAP, and Rap1. The resulting Gaia·Tag was incubated with Goa, Rap1GAP, and Rap1, His-tagged Rap1, full-length Rap1GAP, and Gaia-GTP·S were first incubated in Buffer A containing 5 mM MgCl2, 400 μM AlCl3, 10 mM sodium fluoride, and 5 μM GDP or in Buffer A containing 5 mM MgCl2, 100 μM AlCl3, 10 mM sodium fluoride, and 5 μM GTP·S or in Buffer A containing 5 mM MgCl2, 100 μM GDP or in Buffer A containing 5 mM MgCl2 to decrease the nonspecific binding to Ni-NTA resin. Following dilution, the reactions were incubated with the Ni-NTA resin for 1 h at 4 °C. To assess complex formation between Goa, Rap1GAP, and Rap1, His-tagged Rap1, full-length Rap1GAP, and Gaia-GTP·S were first incubated in Buffer A containing 5 mM MgCl2, 400 μM AlCl3, 10 mM sodium fluoride, and 5 μM GTP·S at 30 °C for 15 min. Reaction mixtures were then diluted 5-fold with Buffer B containing 5 mM MgCl2 to decrease the nonspecific binding to Ni-NTA resin and the diluted reactions incubated with the Ni-NTA resin for 1 h at 4 °C. After the incubation, Ni-NTA resin was extensively washed. Resin then was boiled in SDS loading buffer to recover bound proteins and analyzed by SDS-polyacrylamide gel electrophoresis on 12% gels followed by Western blotting.

The second series of co-precipitation assays was designed to assess molecular interactions between the relevant proteins in intact cells. HEK293 cells were harvested 48 h after transfection and lysed in 20 mM Tris-Cl, pH 7.7, 1 mM EDTA, 1 mM diethiothreitol, 0.1% Nonidet P-40, and a mixture of protease inhibitors (22). Cytosolic fractions were prepared by centrifugation of cell extracts at 30,000 × g for 1 h. Immobilized Rap1GAP antibody was prepared by incubation of the affinity purified antiserum with Protein A-Sepharose resin (Amersham Pharmacia Biotech) at 4 °C for 12 h. Rap1GAP was immunoprecipitated from the protein G column fractions by addition of the immobilized antiserum followed by incubation at 4 °C for another 2 h. Resin then was collected, washed, and analyzed as described above.

**RESULTS**

To identify proteins involved in Goa signaling pathways, a yeast two-hybrid screen was performed in which the mutationally activated Q205L variant of Goa was used as "bait" to screen a human brain cDNA library. Because an active form of Goa was used in this screen, the expected interactors included regulators of Goa GTP hydrolysis and/or downstream effectors. This expectation was validated by our previously reported finding that one of the molecules identified in this screen was a novel Rgs protein, RGSZ1, a selective regulator of the Goa GTPase reaction (14). In addition to RGSZ1, among more than 100 total positive clones identified, 24 sequences encoded a previously identified protein termed Rap1GAP, a GTPase activating protein for the monomeric GTPase protein Rap1.

The initial examination of the specificity of the interaction between the active form of Goa and Rap1GAP was performed through the use of a two-hybrid counter-screen. In this counter-screen, the Rap1GAP cDNA was co-transformed in the yeast reporter system together with the mutationally activated forms of several other G protein α subunits, including two other Gα
family members (Go1s and Go12) and at least one member from each of the remaining three G protein families (Table I). No interaction could be detected in this counter-screen between Rap1GAP and any other G protein α subunit with the exception of Gαo2, for which a very weak interaction was observed. To eliminate the possibility that the inability to detect the interaction between Rap1GAP and the G protein α subunits was due to lack of expression of the α subunits, an additional counter-screen between all the α subunits and the aforementioned RGSZ1 was performed. Interactions between RGSZ1 and Gαs and Gαo2, as well as Gαz, were detected in the system (Table I), indicating that at least Gαs and Gαo2 were expressed in functional forms in yeast cells.

The observed interaction between the active form of Gαz and Rap1GAP, and the accumulating literature indicating that both Gα and Rap1 were involved in signaling pathways influencing cell proliferation and differentiation (18, 29, 30), prompted us to initiate an investigation of the biochemical properties and functional significance of their interaction. In the first series of experiments, the interaction between Gαz and Rap1GAP was further evaluated both in vitro, using purified recombinant proteins, and in the context of a cell expressing both proteins. In the in vitro experiments, interaction between purified full-length Rap1GAP containing an appended NH2-terminal His-tag and recombinant Gαz, purified from a bacterial expression system, was examined in co-precipitation assays. Interaction of activated forms of Gαz, i.e. the GTPγS-bound form or that activated by aluminum fluoride, with Rap1GAP was readily detected, while interaction with GDP-bound form of Gαz could not be detected (Fig. 1A). To investigate the interaction between Gαz and Rap1GAP in a cellular environment, co-precipitation experiments were performed using extracts from HEK293 cells expressing the proteins. Cells were transfected with Gαz (either the Q205L constitutive-active mutant or the wild-type form) in both the absence and presence of co-transfection with full-length Rap1GAP. Cells were lysed 48 h post-transfection and cytosolic fractions isolated. A polyclonal antibody against Rap1GAP was then used to immunoprecipitate Rap1GAP along with associated proteins from this cytosolic fraction. The results of this experiment showed that the mutationally activated form of Gαz, but not wild-type Gαz, could be co-immunoprecipitated with Rap1GAP (Fig. 1B), even though the wild-type protein was expressed at an equal level in the cells (not shown). These results, together with the in vitro co-precipitation results, confirmed that the activated form of Gαz specifically interacts with Rap1GAP.

Having confirmed that activated Gαz and Rap1GAP could indeed form a complex, experiments were designed to determine whether the interaction had functional consequences in terms of measurable activities of Gαz. Rap1GAP binding to Gαz did not enhance the single turnover GTPase activity of the G protein (Fig. 2C and results not shown). Because RGS proteins also bind selectively to active forms of a number of G protein α subunits (2, 5), including Gαz, the possibility that the interaction between Gαz and Rap1GAP would interfere with the ability of RGS proteins to act on Gαz was then examined. Two RGS proteins, RGS10 and RGSZ1, were chosen to test this hypothesis; RGS10 acts on Gαz as well as several other members of the G family (26), while RGSZ1 action is quite selective for Gαz (14, 31). To assess whether the interactions of Rap1GAP and the RGS proteins with Gαz were mutually exclusive, purified recombinant proteins produced in bacteria were used in single-turnover GTPase assays. Briefly, Gαz was loaded with [γ-32P]GTP and the GTP-bound form of Gαz was isolated by gel filtration. Both basal and RGS-stimulated GTP hydrolysis by Gαz was then determined in the presence or absence of Rap1GAP. Stimulation of GTP hydrolysis of Gαz by both RGS10 and RGSZ1 was readily observed in these assays (Fig. 2). Rap1GAP itself did not affect the basal GTP hydrolysis rate of Gαz (data not shown), but the presence of Rap1GAP mark-
RGS10 concentration was 10 nM. The ability of Rap1GAP to attenuate RGS-mediated stimulation of \( \alpha_z \) GTPase was concentration-dependent and nearly complete (Fig. 2). Additionally, the effect was quite specific for \( \alpha_z \), as Rap1GAP did not affect the ability of RGS10 to stimulate \( \alpha_{\gamma_2} \) GTPase activity (Fig. 2C), even though a weak interaction between Rap1GAP and \( \alpha_{\gamma_2} \) had been detected in the two-hybrid system (Table I).

We also sought to determine whether the interaction of Rap1GAP with \( \alpha_z \) could interfere with the G protein's ability to regulate effector molecules. The only characterized activity of \( \alpha_z \) in terms of modulation of effector activity is that the active form of \( \alpha_z \) can inhibit some subtypes of adenylyl cyclase (15). To examine whether Rap1GAP binding to activated \( \alpha_z \) could influence this activity of the G protein, studies were performed with adenylyl cyclase type V (AC-V), which is subject to inhibition by activated \( \alpha_z \) (15). AC-V was expressed in Sf9 cells and the membrane fraction containing the protein prepared. This recombinant AC-V preparation was then activated with either the GTP\(\gamma\)S-bound form of \( \alpha_z \) or forskolin, and the influence of \( \alpha_z \) in the presence or absence of Rap1GAP was determined. As previously reported (15), inhibition of AC-V activity by the GTP\(\gamma\)S-activated form of \( \alpha_z \) could be readily observed (Fig. 3A). Addition of purified recombinant Rap1GAP attenuated the inhibition by activated \( \alpha_z \), and this attenuation increased with the increasing concentration of Rap1GAP (Fig. 3A). Similar results were obtained when forskolin-activated AC-V was used (Fig. 3B). While the effect was somewhat modest, the specificity of the interaction was confirmed by the finding that Rap1GAP had no effect on the ability of the GTP\(\gamma\)S-bound form of \( \alpha_z \) to inhibit adenylyl cyclase under the same conditions (data not shown).

Having verified the binding of Rap1GAP to activated \( \alpha_z \) and determined that there were indeed functional consequences to the interaction of the two molecules in terms of \( \alpha_z \) activity, we next sought to determine whether Rap1 binding to Rap1GAP could influence the latter's ability to interact with \( \alpha_z \). In initial experiments, very little influence of Rap1 binding to Rap1GAP on interactions with \( \alpha_z \), or vice versa, was detected (results not shown). While the failure to observe “communication” between Rap1 and \( \alpha_z \) interactions with Rap1GAP was initially disappointing, the data obtained did suggest that Rap1GAP contained distinct binding sites for Rap1 and \( \alpha_z \), otherwise at least competition between the two molecules would have been observed. Hence, we performed a preliminary structure-function study to obtain information on the region of Rap1GAP responsible for conferring the ability to interact with \( \alpha_z \). Prior to this work, the only known activity of Rap1GAP was its ability to stimulate GTP hydrolysis of Rap1; the region responsible for this activity, termed the “core domain,” had been localized to residues 75 to 416 in the primary sequence (19). To determine whether this or other region(s) of Rap1GAP were required for the interaction with \( \alpha_z \), a series of NH2-terminal and COOH-terminal truncations of Rap1GAP, produced as recombinant proteins in bacteria, were examined for their abilities to interact with activated \( \alpha_z \). A Rap1GAP fragment in which the COOH-terminal 221 amino acids were deleted retained both the abilities to block RGS action on \( \alpha_z \) and to function as a GAP for Rap1 (Fig. 4). However, deletion of the NH2-terminal 74 residues resulted in a Rap1GAP fragment that had completely lost its ability to compete for RGS action on \( \alpha_z \), even though, as previously reported (19), it retained ability to act as a GAP for Rap1. As expected from these results, deletion of both the NH2-terminal 74 and COOH-terminal 221 residues produced a fragment with full GAP activity toward Rap1 but no ability to block the RGS action on \( \alpha_z \). While we
have not identified a NH2-terminal fragment that interacts only with activated Ga, these data indicate that the binding domains for Ga and Rap1 are dissociable in the Rap1GAP structure.

The finding that distinct domains of Rap1GAP are required for interactions with the two distinct GTP-binding proteins provides strong support for a model in which Rap1GAP functions somehow to integrate Ga and Rap1 signaling pathways. Furthermore, these findings suggested that it should be possible to assemble a ternary complex that contained all three proteins, i.e. Rap1GAP, Ga, and Rap1. To test this hypothesis, it was necessary to develop a method to detect association between Rap1GAP and Rap1 that did not rely on a transient catalytic read-out, i.e. GTP hydrolysis by Rap1. The assay developed was based on a previous report that Rap1 and Rap1GAP form a stable complex when Rap1 is activated by aluminum fluoride (32). To confirm this finding, a co-precipitation assay was performed in which His-tagged Rap1GAP was co-precipitated together with Rap1GAP in this assay (Fig. 5A).

The confirmation that an interaction, sufficiently stable for co-precipitation, could be induced between Rap1 and Rap1GAP allowed a variation of this assay designed to detect ternary complex formation to be performed. His-Rap1, untagged Rap1GAP, and Ga-GTP (which exhibited stronger interaction with Rap1GAP than Ga-GDP-AlF4, see Fig. 1A), were incubated together and Ni-NTA resin added to precipitate Rap1-Rap1GAP complex. Since His-Rap1 was the "handle" in this experiment, co-precipitation of Ga would indicate that both Ga and His-Rap1 were simultaneously bound to Rap1GAP. Indeed, only in the presence of both Rap1 and Rap1GAP could Ga be precipitated with the Ni-NTA resin (Fig. 5B). Increasing the amount of Rap1GAP in the assay resulted in increased precipitation of Ga, indicating that Rap1GAP was the limiting protein in this system and acted as a bridge to bring Ga and Rap1 together (Fig. 5B). These results suggest that the three proteins can form a stable complex, and that this ternary complex might perform unique function(s) in the cell.

**DISCUSSION**

Stable expression of constitutively active Ga can result in transformation of Swiss 3T3 and NIH3T3 cells (18); this study provided the initial link between Ga signaling and mitogenic pathways. Because of the universal involvement of monomeric GTP-binding proteins in Ras superfamily in mitogenic signaling pathways (33), regulators of these proteins would be attrac-
G Protein Interaction with Rap1GAP

While the data indicating a functional consequence of Rap1GAP binding on Gα functions are quite compelling, it is less clear whether the Gα-Rap1GAP interaction also affects the ability of Rap1GAP to modulate Rap1 signaling properties. What is clear from both the structure-function analysis and co-precipitation experiments is that the abilities of Rap1GAP to both bind active forms of Gα and stimulate Rap1GTP hydrolysis are dissociable. Although our preliminary experiments indicate that the Gα binding to Rap1GAP does not modify the GAP activity of Rap1GAP toward Rap1 (data not shown), an alternative mode of cross-regulation may still exist in which Gα affects Rap1 signaling by recruiting its regulator, Rap1GAP, from cytosol to plasma membrane. Through such a “colocalization-type” mechanism, relocalization of Rap1GAP to some specific subcellular location might result in simultaneous modulation of both Gz and Rap1 signaling processes.

There may be a biological precedence to the ability of a GAP for a monomeric G protein to influence signaling by a trimeric G protein. In Drosophila development, a protein termed the neurofibromatosis type 1 protein (NF1), which can function as a Ras-specific GAP, is required for the activation of adenylyl cyclase in response to the agonist PACAP-38 (38, 39). The phenotype derived from loss of NF1 expression can be rescued by elevating cAMP, but not by manipulating Ras signaling (39).

Therefore, both NF1 and the events leading to cAMP elevation, e.g. activation of Gz or an attenuation of a Gi input, seem to be somehow linked in a signaling network. Additionally, it has been reported recently that activated Gα12 can directly interact with, and stimulate the GAP activity of, a specific type of GAP that acts on Ras, termed RasGAP1m (40). In yet other studies, interaction of activated G12 with an exchange factor for the monomeric G protein Rho stimulated the exchange activity of the molecule (42, 43). Together, these and current studies support the notion that complex cellular communication exists between monomeric and heterotrimeric G proteins through regulators of the monomeric G proteins. Elucidating the mechanisms and functional consequences of these communication networks should prove an exciting challenge for the future.

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FIG. 5. Rap1GAP, Gαz, and Rap1 form a ternary complex. Purified recombinant proteins produced from bacteria were used in this in vitro co-precipitation assay as described under “Experimental Procedures.” Arrows point to the positions of protein bands in Western blots detected with specific antibodies against the indicated proteins. A, demonstration of Rap1-Rap1GAP complex formation. His-tagged Rap1GAP and associated molecules were precipitated by Ni-NTA resin. Rap1GAP concentration was 200 nM. Rap1 forms were as indicated; Gαz concentration was 200 nM. B, demonstration of ternary complex formation between Rap1, Rap1GAP, and Gαz. Concentration of each protein was specified in the chart. GTPγS, Gαz-GTPγS; AIFγ, Rap1-GDP-AIFγ.

Analysis of the functional consequences of Rap1GAP binding to Gαz revealed that the binding both blocked the ability of RGS proteins to stimulate GTP hydrolysis of Gαz and attenuated the ability of activated Gαz to inhibit adenylyl cyclase activity. Furthermore, the binary complex of Gαz and Rap1GAP retained the ability to interact with the active form of Rap1, allowing formation of a ternary complex that contained all three proteins. Structural analyses demonstrate that heterotrimeric G proteins possess distinct domains whose conformations are altered upon activation (35, 36); these so-called “switch regions” have been implicated in both RGS and effector interactions of activated Gα subunits (37, 38). Presumably, Rap1GAP interacts with one or more of the switch regions of Gαz and competes for access of RGS proteins. Similarly, interaction of Rap1GAP with one or more of these regions of Gαz would also account for the observed attenuation of the ability of activated Gαz to inhibit adenylyl cyclase, although this latter effect was not as pronounced as the inhibition of RGS access to the protein.

Prior to this study, the only known function of Rap1GAP was its GAP activity toward Rap1 that presumably serves to down-regulate signaling through Rap1 (19). However, as with most GAPs for monomeric G proteins, only a relatively small segment of Rap1GAP is required for the GAP activity. Specifically, the catalytic domain of Rap1GAP had been identified as contained within the region from residue 75 to 416 of the primary structure (20), while no function had been identified as being associated with the NH2-terminal or COOH-terminal regions of the protein. The finding that the NH2-terminal domain of Rap1GAP is required for the protein’s ability to bind activated Gαz provides a function within its NH2-terminal domain that is distinct from that of its core domain. These results thus reveal Rap1GAP as a possible signal integrator between Gz signaling and Rap1 signaling.

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