A GTPase-activating Protein for the G Protein Ga\textsubscript{z}

IDENTIFICATION, PURIFICATION, AND MECHANISM OF ACTION*

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A GTPase-activating protein (GAP) specific for Ga\textsubscript{z} was identified in brain, spleen, retina, platelet, C6 glioma cells, and several other tissues and cells. Ga\textsubscript{z} GAP from bovine brain is a membrane protein that is refractory to solubilization with most detergents but was solubilized with warm Triton X-100 and purified up to 50,000-fold. Activity is associated with at least two separate proteins of M\textsubscript{r} -22,000 and 28,000, both of which have similar specific activities. In an assay that measures the rate of hydrolysis of GTP pre-bound to detergent-soluble Ga\textsubscript{z}, the GAP accelerates hydrolysis over 200-fold, from 0.014 to 3 min\textsuperscript{-1} at 15 °C, or to 20 min\textsuperscript{-1} at 30 °C. It does not alter rates of nucleotide association or dissociation. When co-reconstituted into phospholipid vesicles with trimeric G\textsubscript{i} and m2 muscarinic receptor, G\textsubscript{z} GAP accelerates agonist-stimulated steady-state GTP hydrolysis as predicted by its effect on the hydrolytic reaction. In the single turnover assay, the K\textsubscript{m} of the GAP for Ga\textsubscript{z}-GTP is 2 nM. Its activity is inhibited by Ga\textsubscript{z}-guanosine 5'-O-thiotriphosphate (Ga\textsubscript{z}-GTP\textsubscript{S}) or by Ga\textsubscript{z}-GDP/AlF\textsubscript{4} with K\textsubscript{i} -1.5 ns for both species; Ga\textsubscript{o}-GDP does not inhibit. G protein \(\beta\gamma\) subunits inhibit G\textsubscript{z} GAP activity, apparently by forming a GTP-Ga\textsubscript{z}/G\textsubscript{z}-GDP complex that is a poor GAP substrate. G\textsubscript{z} GAP displays little GAP activity toward Ga\textsubscript{o1} or Ga\textsubscript{o2}, but its activity with Ga\textsubscript{z} is competitively inhibited by both Ga\textsubscript{o1} and Ga\textsubscript{o2} at nanomolar concentrations when they are bound to GTP\textsubscript{S} but not to GDP. Neither phospholipase C-\(\beta\) (a G\textsubscript{i} GAP) nor several adenyl cyclase isoforms display G\textsubscript{z} GAP activity.

G proteins mediate numerous cellular processes by traversing a cycle of GTP binding and hydrolysis. Bound GTP activates a G protein such that it can stimulate a downstream effector protein. Activation is terminated when the bound GTP is hydrolyzed to GDP, which does not activate. Each step of the cycle is controlled, such that both steady-state GTPase activity and the concentrations of the active and inactive forms are highly regulated.

Activation of heterotrimeric G proteins is promoted by seven-span cell-surface receptors that facilitate GDP release and GTP binding. Small monomeric G proteins (Ras, Rac, Rho, Arf, etc.) are activated by cytosolic proteins that similarly facilitate GTP binding.

In many cases, hydrolysis of bound GTP, the deactivation step, is accelerated by GTPase-activating proteins, or GAPs\textsuperscript{1} (1–4). GAPs appear to fulfill at least one of four definable roles. Some GAPs for monomeric signaling G proteins, such as Ras GAP, appear to attenuate G protein signal amplitude in response to inputs from inhibitory signaling pathways (1, 2, for review). GAPs for the monomeric G proteins involved in cytoplasmic vesicle trafficking are thought to act by terminating a G protein-dependent assembly or transit step. Some effector proteins that are regulated by heterotrimeric G proteins also act as GAPs for their G protein regulators. The GAP activities of these effectors, such as phospholipase C-\(\beta\) (5, 6) and the cyclic GMP phosphodiesterase \(\gamma\) subunit (7, 8), may allow effector-specific modulation of response times or may enhance the selectivity of receptor-G protein signaling (9). A fourth class of GAPs, also for the trimeric G proteins, includes members of the recently identified RGS protein family (4, 9–13). Little is known of the physiology of RGS proteins, but they can contribute to desensitization toward a prolonged signal (Sst2p in yeast; Refs. 10, 14, 15) or act as long-term attenuators of signal amplitude (Egl-10 protein in Caenorhabditis elegans; Ref. 9).

G protein GAP activity can potentially be used to identify and purify regulators of G protein function or to point to novel inputs to G protein signaling pathways. For GAPs that are also effectors, their identification can indicate what downstream signals the G protein mediates.

We began a search for new GAPs for heterotrimeric G proteins by looking for a GAP for G\textsubscript{z}, a pertussis toxin-insensitive member of the G\textsubscript{i} family that is abundant in brain, adrenal medulla, and platelets (16–19). There were three reasons for this choice. Although G\textsubscript{z} can mediate inhibition of adenylyl cyclase (20–22) and respond to receptors that regulate other G\textsubscript{i} effectors, their identification can indicate what downstream effectors, such as phospholipase C-\(\beta\) (a G\textsubscript{i} GAP) or several adenyl cyclase isoforms display G\textsubscript{z} GAP activity.

Experimental Procedures

Materials—Procedures for purification of Ga\textsubscript{z1} (20), Ga\textsubscript{o1} (24, 25), Ga\textsubscript{o2} (24, 26), Ga\textsubscript{z} (24), Ga\textsubscript{z1} (6), G\textsubscript{y} (6), m2 muscarinic cholinergic receptor (23), and phospholipase C-\(\beta\) (6) have been described. S9 membranes that contain recombinant adenyl cyclase isoforms (27) and purified Ga\textsubscript{z1} (20) were gifts from Drs. Carmen Dessauer and Tohru Kozasa (this department). Ga\textsubscript{z1}-agarose was prepared according to Mumby et al. (28) and phenyl-Sepharose was purchased from Pharmacia Biotech Inc.

1 The abbreviations used are: GAP, GTPase-activating protein; PMSF, phenylmethylsulfonyl fluoride; DT, dichotriothreitol; GTP-S, guanosine 5'-O-thiotriphosphate; CHAPS, 3-[3-cholamidopropyl]di-methylammonio]-1-propanesulfonic acid.
Cholic acid was purified as described (29), and other detergents were purchased from various suppliers. \([\gamma^{32}P]GTP\) was either purchased or synthesized (30) and purified as described (6).

Hydrolysis of \(G_g\)-bound \([\gamma^{32}P]GTP\) was bound to \(G_g\) by incubating 1% of total of \(G_g\), for 90 min at 30 °C in 20 mM NaHepes (pH 7.5), 3 mM MgCl\(_2\), 0.1% Triton X-100, 1 mM EDTA, 2 mM NaF, 1 mM cholate and washed with 5 volumes of the same buffer. GAP activity was eluted with a gradient of 0.25–500 mM NaCl in Buffer 2 and eluted as a broad peak. Active fractions were concentrated on an Amicon PM30 membrane and chromatographed on a column of Ultragel ACA-34 equilibrated with Buffer 2 plus 0.1 mM NaCl. A typical elution profile is shown in Fig. 2. The peak second of GAP activity was pooled in Buffer 2 plus 0.1 mM NaCl and eluted with a gradient of 0.25–0.55 mM NaCl in Buffer 2. Protein and GAP activity again eluted as a broad peak. Active fractions were concentrated on an Amicon PM30 membrane and chromatographed on a column of Ultragel ACA-34 equilibrated with Buffer 2 plus 0.1 mM NaCl.

For affinity chromatography, pooled Mono Q fractions were diluted 20-fold with Buffer 3 (25 mM NaHepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 0.25% cholate, 0.1 mM PMSF, 10 mM GDP, 50 mM AlCl\(_3\), 5 mM MgCl\(_2\), 10 mM NaF) and applied to a column of \(G_g\)-Sepharose that was equilibrated with Buffer 3. The column was washed with Buffer 3 plus 25 mM NaCl, and GAP activity was eluted with a gradient of 25–500 mM NaCl in Buffer 4 (Buffer 3 but containing 0.5% cholate and without AlCl\(_3\), MgCl\(_2\), or NaF). Peak fractions were pooled, concentrated by adsorption and elution from Q Sepharose as described above, and diluted 5-fold with Buffer 5 (20 mM NaHepes (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 1 mM NaCl, 0.1 mM PMSF). The pool was applied to a column of phenyl-Sepharose equilibrated with Buffer 5 plus 0.2% cholate and washed with 5 volumes of the same buffer. GAP activity was eluted with a discontinuous gradient of 0.2–1.0% cholate (0.5% steps) in Buffer 5 but without NaCl (see Fig. 3).

Other GTPase Assays—

\(G_g\) was reconstituted with \(G_g\)-by and m2 muscarinic cholinergic receptor into unilamellar phospholipid vesicles (phosphatidylserine:phosphatidylethanolamine:cholesterol hemisuccinate, 5:8:1) according to the method of Parker et al. (23). Trimeric \(G_g\) was prepared by mixing GDP-bound \(G_g\) with \(G_h\), \(\gamma (\alpha \beta Y = 0.4)\) before reconstitution (26). \(G_g\) was routinely quantitated according to the binding of 10 mM \([\alpha^{35}S]GTP\)S for 1.5 h at 30 °C (31). Binding of \([\gamma^{32}P]GTP\) and \([\alpha^{32}P]GTP\) to \(G_g\) was also measured by the nitrocellulose binding assay (31). Protein was measured by amido black binding (33).

Standard procedures were used for SDS-gel electrophoresis (34) and staining with Coomassie Blue or silver (35). For samples in which GAP activity was to be measured after electrophoresis, samples were denatured in sample buffer (34) that contained 1% SDS and 10 mM DTT. \(G_g\) GAP activity was extracted from slices of SDS-polyacrylamide gels and renatured by homogenizing the gel in 5–10 volumes of renaturation buffer (20 mM NaHepes (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 1% Triton X-100) and shaking overnight at 0 °C.

The concentration of free Mg\(^{2+}\) in assay buffers that contained significant concentrations of both EDTA and GTP was calibrated as described by Husken and Sherry (36).

**RESULTS**

Identification of \(G_g\)-GAP Activity—

We used the \(G_g\)-\([\gamma^{32}P]GTP\) complex as substrate to test for the presence of proteins that can increase the rate of hydrolysis of \(G_g\)-bound GTP. Purified \(G_g\) hydrolyzes bound GTP very slowly (16), such that \(G_g\)-\([\gamma^{32}P]GTP\) can be prepared and purified with good yield. About 25% of total \(G_g\) bound to \([\gamma^{32}P]GTP\) after gel filtration, with the rest bound to unlabeled GTP. Under standard assay conditions at 15 °C, GTP bound to \(G_g\) is hydrolyzed with a rate constant, \(k_{\text{hydrolysz}}\), of about 0.014 min\(^{-1}\), which corresponds to a \(t_{1/2}\) of about 50 min (Fig. 1).

Addition of a crude membrane fraction from bovine brain increased the rate of hydrolysis of \(G_g\)-bound GTP dramatically (Fig. 1A). Hydrolysis was a single component, first-order reaction over a 10-fold range of rates, and rate constants increased linearly with increasing amounts of membrane protein (Fig. 1B). These data indicate the existence of a GTPase-accelerating activity in bovine brain, i.e., a \(G_g\) GAP. Based on the linearity of GTP hydrolysis with added membrane protein, we defined a unit of \(G_g\) GAP activity as a 10-fold increment in the hydrolytic rate constant, \(k_{\text{hydrolyz}}\), of about 1 min\(^{-1}\). Both the free and bound GTP hydrolysis by \(G_g\) GAP were accelerated in this assay. The hydrolysis-accelerating activity is evidently that of a protein. In membranes, activity was destroyed by incubation with 6.7 µg/ml trypsin, 20 µg/ml chymotrypsin plus detergent or 0.5 mM N-ethylemaleimide. Added detergent markedly sensitized the GAP to proteolysis.

Release of \([\gamma^{32}P]\)orthophosphate in GAP assays such as shown
GTPase-activating Protein for Goα

**TABLE I**

**Distribution of Goα GAP activity**

| GAP activity | units/mg protein |
|--------------|-----------------|
| Cerebral cortex | 38 |
| Spleen | 12 |
| Retina | 10 |
| Lung | 2.1 |
| Platelet | 1.8 |
| Kidney, heart, liver, testis, pancreas, erythrocyte | 0.1–0.5 |
| Skeletal muscle | 0.04 |
| C6 glioma | 22–47 |
| CHO | 11 |
| Sf9, S49, Ins-1 | 0.2–0.65 |
| Other cell lines | 293, 8PP15, 7RP16, L, 1321N1, Cos, MDCK, NG108, MA104 | 2.5–6.5 |

**FIG. 1.** Goα GAP activity in bovine brain membranes. A, Goα-[γ-32P]GTP was incubated at 15°C with increasing amounts of bovine brain membranes (○, 5 μg; □, 10 μg; △, 15 μg; ◻, 30 μg; □, 15 μg of boiled membrane). Release of [γ-32P]orthophosphate was assayed at the times shown. Data are normalized to the total amount of Goα-[γ-32P]GTP at zero time (125 fmol) determined by nitrocellulose binding assay. At each time point, samples were also assayed for bound [γ-32P]GTP (not shown). The sum of [γ-32P]orthophosphate plus bound [γ-32P]GTP was constant over 120 min and not altered by the presence of the membranes. Hydrolysis was not altered by substitution of buffer for boiled membrane protein (not shown). In this early experiment, the assay buffer contained only 1 μM free Mg2+ and 0.1% Lubrol in place of 0.1% Triton X-100, resulting in the relatively low activities shown. B, each data set from A was well fit by a single component first-order reaction scheme (not shown). Values of the rate constant k_{3p} obtained from least-squares fits of these data are plotted against the amount of membrane protein added.

in Fig. 1 reflects only hydrolysis of [γ-32P]GTP bound to Goα rather than dissociation followed by hydrolysis catalyzed by other nucleoside triphosphatases in the membranes. Dissociation of GTP from Goα was unmeasurably slow, as estimated either by comparing the loss of bound [γ-32P]GTP with the appearance of [α-32P]orthophosphate or by monitoring the dissociation of [α-35P]GTP. The rate of formation of [α-35P]orthophosphate was equal both to the rate of loss of Goα-bound [γ-32P]GTP and to the rate of conversion of bound [α-35P]GTP to bound [α-35P]GDP (not shown), either with or without added membrane protein. Furthermore, the reaction is carried out in the presence of 5 mM free GTP to block any other nucleoside triphosphatase activity. In control experiments, no hydrolysis was observed when free [γ-32P]GTP was substituted for Goα-[γ-32P]GTP (not shown).

Homogenates of several mammalian tissues and cultured cells were screened for Goα GAP activity (Table I), and its distribution was found to be similar to that of Goα itself (16–19; confirmed qualitatively by anti-goα immunoblot during this study). Activity was highest in cerebral cortex, although there was considerable activity in membranes of spleen and retina. Peripheral fat, lung, platelets, and testis also displayed readily measurable activity. Several other tissues displayed low activity, which may represent contamination by adipose, neuronal, or vascular tissue or the action of other GAPs with low activity toward Goα. We do not know how many proteins in these tissues display Goα GAP activity. Among the cultured cells tested, C6 glioma cells displayed activity similar to that of brain, 20–50 units/mg depending upon the source of the cells and the culture conditions. Several other cell lines displayed more modest activities. Goα GAP activity was low in S49 murine lymphoma cells and was barely detected in Sf9 cells. Because activity was highest in brain and bovine brain is readily obtainable, we concentrated on this source and have not attempted to determine the multiplicity of Goα GAPs in other tissues.

**Purification of Goα GAP**—There is no soluble Goα GAP activity detectable in brain homogenates (<2%). Goα GAP behaves as an integral membrane protein and is difficult to solubilize. It is not extracted by washing at high or low ionic strength and is not solubilized by many detergents. At 0°C, neither cholate, deoxycholate, Lubrol PX, Triton X-100, CHAPS, digitonin, nor several other detergents solubilized any Goα GAP activity. Small and irreproducible amounts of activity were solubilized by decyl maltoside and lauroyl succrose. Fortunately, incubation of membranes with 2% Triton X-100 at 30°C released considerable Goα GAP activity into a 200,000 × g supernatant. This apparently soluble GAP was still badly aggregated, however, and substantial increases in specific activity were not obtained by chromatography in multiple systems. Two sequential rounds of anion exchange chromatography, which included transfer from Triton X-100 to cholate, provided little purification and substantial loss of activity (Table II) but did allow subsequent purification. Although about half of the Goα GAP activity remained aggregated, the other half behaved as an apparently monodisperse species of reasonable molecular size and was thus purified about 20-fold by gel filtration in cholate (Fig. 2). This soluble material was used for further purification.

After gel filtration, Goα GAP activity was appropriately purified by affinity chromatography on Goα-agarose. GAP activity bound to the column when the covalently coupled Goα was activated with either GTP-S or with GDP/AlF4 but not when the Goα was in the nonactivated, GDP-bound form (not shown). For purification, peak fractions from gel filtration were applied to Al3+/F−-activated Goα-agarose, and after extensive washing, GAP activity was eluted by removal of Al3+, F−, and Mg2+ and increasing the concentration of detergent and salt.

Further purification of Goα GAP was achieved by phenyl-Sepharose chromatography (Fig. 3). Goα GAP activity was consistently eluted from phenyl-Sepharose in two peaks. This was true for multiple elution protocols that included increasing the
TABLE II  
Purification of Ga2 GAP from bovine brain  

| Total protein | Specific activity | Purification | Yield |
|---------------|------------------|--------------|-------|
| mg            | unit/mg          | .fold        | %     |
| Triton X-100 extract | 13,500          | 35           | (1.2) | (100) |
| DEAE-Sepharose | 7,500            | 50           | 1.5   | 80    |
| Q-Sepharose   | 1,000            | 130          | 3.7   | 27    |
| Ultrogel Aa-34 | 60              | 1,000        | 29    | 12.5  |
| Ga2-agarose   | 0.6              | 35,000       | 1,000 | 4.4   |
| Phenyl-Sepharose | 0.045          | 280,000      | 8,000 | 2.7   |

The first peak is at the void volume. About 50–75% of Ga2 GAP activity results from monomeric proteins in the size range 22–28 kDa, but the number of species remains uncertain.

**Mechanism of Action of Ga2 GAP**—A single GAP molecule can turn over multiple molecules of Ga2-GTP (Fig. 5). Its behavior is most readily analyzed when it is considered as an enzyme that acts upon the substrate Ga2-GTP and converts it to the products Ga2-GDP plus orthophosphate. Its Ka for Ga2-GTP is about 2 nM, which represents sufficiently high affinity binding to be physiologically reasonable. The maximum GAP-stimu-
lated hydrolysis rate can be estimated two ways. Because velocity increases linearly with the amount of GAP at low GAP concentrations (Figs. 1 and 5B), the maximum GAP-stimulated hydrolytic rate constant ($k_{gap}$) can be calculated by dividing $V_{max}$ by the molar concentration of GAP, which is calculated according to its estimated purity and approximate molecular weight. This calculation yields a $k_{gap}$ of 3 min$^{-1}$ at 15°C. The other estimate of $k_{gap}$ derives from a titration of GAP when the concentration of G$\alpha$z-GTP is maintained at or above the $K_m$ (Fig. 5B). The maximum in such an experiment, 1.8 min$^{-1}$, can be corrected for the subsaturating concentration of G$\alpha$z-GTP to yield a true maximum $k_{gap}$ of 3.1 min$^{-1}$. Thus, both determinations of $k_{gap}$ are about 3 min$^{-1}$, more than a 200-fold stimulation over $k_{hydrol}$ for G$\alpha$z-GTP.

We estimate that $k_{gap}$ at 30°C is over 20 min$^{-1}$, which corresponds to an average lifetime for activated G$\alpha$z of $\sim$2 s. Thus, because $k_{gap}$ is fast and because G$\alpha$z GAP has a high affinity for its G$\alpha$z-GTP substrate ($K_m$ ~2 nM), its action is sufficient to allow G$\alpha$z-mediated signal transduction with physiologically appropriate rates.

G$\alpha$z can hydrolyze bound GTP at very low concentrations of free Mg$^{2+}$, and its intrinsic $k_{hydrol}$ is independent of the concentration of Mg$^{2+}$ up to 10 mM (Fig. 6). In contrast, G$\alpha$z GAP activity displays a marked Mg$^{2+}$ optimum at ~1 mM. Although the GAP is active over a wide range of Mg$^{2+}$ concentrations, stimulation is ~4-fold higher at the optimum. Neither Ca$^{2+}$ nor Mn$^{2+}$ exerted a unique regulatory effect on G$\alpha$z GAP, although either can replace Mg$^{2+}$ over approximately the same range of concentrations (not shown).

G$\alpha$z GAP binds tightly to G$\alpha$z, but only in its GTP-activated form. Both G$\alpha$z-GTP$^\gamma$S and G$\alpha$z-GDP/AIF$_4^-$ thus inhibited G$\alpha$z GAP activity, with a $K_i$ of ~1.5 nM for either nucleotide (Fig. 7). This value of $K_i$ is similar to the $K_m$ for the substrate G$\alpha$z-GTP, which suggests that G$\alpha$z-GTP, G$\alpha$z-GTP$^\gamma$S, and G$\alpha$z-GDP/AIF$_4^-$ all bind the GAP at a common site and with similar affinities. G$\alpha$z-GDP did not inhibit at concentrations up to 20 nM. Note that in the experiment of Fig. 7, G$\alpha$z-GDP/AIF$_4^-$ was formed in the reaction mixture by the addition of Al$^{3+}$ plus F$^-$, which appear to inhibit GAP activity by themselves. This inhibition results from the presence of G$\alpha$z-GDP in the preparation of G$\alpha$z-GTP substrate, such that about 6 nM G$\alpha$z-GDP/AIF$_4^-$ is present in the assay even when no excess G$\alpha$z-GDP was added. Selectivity of the GAP for the active conformation of G$\alpha$z is confirmed by the selective binding of the GAP to G$\alpha$z-agarose when it is activated by either GTP$^\gamma$S or GDP/AIF$_4^-$. 

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of partially purified G$\alpha$z GAP. A sample of the second peak from phenyl-Sepharose chromatography (fraction 6 in Fig. 3) was diluted into SDS sample buffer and electrophoresed on a 15% polyacrylamide gel. The gel lane was sliced, and protein was eluted from each slice as described under "Experimental Procedures." A, G$\alpha$z GAP activity was assayed in the eluate from each slice. B, an aliquot of each eluate (and of phenyl-Sepharose fraction 6, denoted c) was denatured in SDS and electrophoresed again. The gel was silver-stained. The molecular mass markers were carbonic anhydrase (29 kDa) and β-lactoglobulin (18.4 kDa).

**Fig. 5.** GAP activity at increasing concentrations of G$\alpha$z GAP and of G$\alpha$z-[γ-32P]GTP. A, GAP activity was assayed at the concentrations of G$\alpha$z-[γ-32P]GTP shown on the abscissa, as determined in a [γ-32P]GTP binding assay. The concentration of GAP was 30 pm (7.5 ng/ml, ~10% pure; $M_r$ ~25,000; purified through affinity chromatography). Inset, Hanes replot. B, GAP activity was assayed at the GAP concentrations shown on the abscissa using 2.5 nM G$\alpha$z-[γ-32P]GTP as substrate. Assay times in both experiments were adjusted to obtain accurate measurements of either the initial reaction rate or of the first-order rate constant $k_{app}$.
GTPase-activating Protein for Goα

Inhibition of Goα GAP by GTPγS-bound Go subunits

Assays contained 1.35 nm Goα-[γ-32P]GTP and 15.6 nm competing α subunits purified from the sources shown. Concentrations of competing Go subunits were measured according to bound [35S]GTPγS. E. coli, myr, co-expressed in E. coli with yeast N-myristoyltransferase (25). 100% activity was 60 milliunits. GDP-bound forms of these Go subunits did not inhibit (not shown).

| Goα | GAP activity % |
|-----|---------------|
| None | (100)         |
| Goα, (Sf9) | 15           |
| Goα, (brain) | 30           |
| Goα, (Escherichia coli) | 85           |
| Goα1, (E. coli, myr) | 59           |
| Goα1, (E. coli) | 88           |
| Goα2, (Sf9) | 93           |
| Goα2, (E. coli) | 99           |
| Goα12, (Sf9) | 100          |

The following small molecules had no effect upon Goα GAP activity: inositol trisphosphate, cyclic AMP, cyclic GMP, GTP, GTPγ-S, and ATP (not shown).

Selection of Goα GAP—The selectivity of brain Goα GAP among different Go subunits was tested initially by comparing their abilities to compete with Goα-GTP in the standard assay (Fig. 7, Table III). Both myristoylated, recombinant Goα, and bovine brain Goα, inhibited competitively, Goα-GTPγ-S with a Ki of −5 nm (Fig. 7) and Goα1-GTPγ-S with a Ki of about 20 nm.

Their GDP-bound forms did not inhibit (not shown). Inhibition by Goα or Goα1 required that they be myristoylated; nonmyristoylated Go subunits inhibited weakly or not at all. Based on these data, we measured the ability of the Goα GAP to accelerate hydrolysis of Goα-GTP and Goα2-GTP, using the single turnover assay of Higashijima et al. (26) to accommodate the faster basal hydrolytic rates of these Go subunits. Although both Goα and Goα1 hydrolyze bound GTP much faster than does Goα, the relative effect of Goα GAP on both Goα subunits was minimal when compared with Goα2 (Table IV): 30% and 7% compared with more than a 30-fold effect on Goα. The GTPγS-bound forms of Goα1, Goαγ, and Goα12 did not compete significantly in the Goα GAP assay (Table III), and their activities as GAP substrates were not tested.

Regulation of Goα GAP by G Protein βγ Subunits—Although G protein βγ subunits had little if any effect on the rate of hydrolysis of Goα-GTP, Gβγ inhibited Goα GAP activity up to 80% (Fig. 8). Inhibition was most marked at low concentrations of Goα, and is caused by an increase in Km of at least 5-fold (Fig. 8A). No effect of Gβγ on Vmax was detected, but we were unable to achieve saturation with Goα-GTP at high Gβγ concentrations, and we may have failed to observe a slight decrease in Vmax. Goα GAP was inhibited approximately equally by Gβ1γ2, Gβ2γ2, and Gβ2γ6 (not shown). The increase in Km caused by Gβγ apparently reflects formation of the GTP-bound Goαβγ heterotrimer. The IC50 for Gβ1γ2 (~400 nm, Fig. 8B) agrees well with its affinity for GTP-bound Goα1 (37), and we have found no evidence for Gβγ binding directly to the GAP (which would yield classical competitive inhibition). Goα2βγ- GTP may be a low affinity (high Km) GAP substrate or it may simply block GAP binding to Goα2-GTP. These alternatives are potentially distinguishable according to the dependence of the apparent Km on the concentration of Gβγ, but we have been unable to determine Km accurately over a high enough range of Gβγ concentrations to answer this question.

In addition to inhibiting the GAP, Gβγ decreased the rate of dissociation of GTP, but not GTP, from Goα, as is true for other Go subunits (38). We observed no other effects of Gβγ in this system.

Goα GAP Activity During Receptor-stimulated Steady-state GTP Hydrolysis—To study the effect of Goα GAP on the receptor-stimulated steady-state GTPase cycle, we co-reconstituted Goα GAP with m2 muscarinic cholinergic receptor and heterotrimeric Go into unilamellar phospholipid vesicles. When the muscarinic agonist carbacol was added to promote receptor-catalyzed exchange (23), Goα GAP increased the steady-state GTPase rate by about 2.5-fold (Fig. 9A). This effect seems small in comparison to the 200-fold maximum effect of the GAP on
hydrolysis of preformed \( \beta \gamma \)-GTP, but the steady-state concentration of \( \beta \gamma \)-GTP in the vesicles is in significant molar excess over that of the GAP. The effect of the GAP on the steady-state GTPase rate is consistent with its observed activity in the single turnover assay. The effect of GAP on steady-state GTPase rates is evidently exerted only at the hydrolytic step. In the absence of agonist, where steady-state GTPase activity is limited by the GDP/GTP exchange rate, GAP had no effect, and GAP also had no effect on the rates of nucleotide binding (Fig. 9B) or release (not shown).

Consistent with its effect on hydrolysis of bound GTP, \( \beta \gamma \) GAP decreased the steady-state concentration of the active \( \beta \gamma \)-GTP complex during stimulation by agonist. When assayed at either 5 or 15 min in the system shown in Fig. 9A, the addition of \( \beta \gamma \) GAP decreased the concentration of \( \beta \gamma \)-GTP by about 30%. Both of these sample times were after steady-state was reached, as indicated by the constant concentration of bound GTP in this interval and by the completion of agonist-stimulated GTP\(\gamma\)S binding in about 2 min (Fig. 9B). Simple kinetic models predict that the relative effect of GAP on the accumulation of \( \beta \gamma \)-GTP would be greater in the absence of agonist, but we were unable to measure accurately the small amount of binding of \( \gamma \)-GTP binding that occurred without agonist.

The results of the experiments shown in Fig. 9 indicate that purified \( \beta \gamma \) GAP can reassociate with membrane lipids and regulate \( \beta \gamma \) appropriately in (or on the surface of) a phospholipid bilayer. The addition of detergent-soluble \( \beta \gamma \) GAP to preformed vesicles had no effect on the steady-state GTPase rate (not shown), which is consistent with the idea that the GAP is an integral membrane protein.

**DISCUSSION**

\( \beta \gamma \) GAP hydrolyzes bound GTP (deactivates) extremely slowly. Although it is activated at a normal rate in response to receptors (23), its active state decays with an average lifetime of about 7 min at physiological temperature (16). With these kinetics, it would be hard to understand how \( \beta \gamma \) can mediate signaling responses in a reasonable way, although it clearly does.

The data presented here describe the identification of a GAP for \( \beta \gamma \) in brain membranes, its purification, and mechanistic behavior. Similar activity was identified in membranes of several other tissues and cultured cells. By accelerating GTP hydrolysis, a \( \beta \gamma \) GAP reconciles aberrant deactivation kinetics with normal signaling functions. However, its precise role in signaling physiology remains unclear. \( \beta \gamma \) GAP may be a \( \beta \gamma \)-regulated effector protein, in analogy with phospholipase C-\( \beta \) and cyclic GMP phosphodiesterase. These effectors are both regulated by G proteins and have GAP activity specific for their G protein regulators, \( \beta \gamma \) and \( \delta \) (5, 7, 8). The low \( K_0 \) of the \( \beta \gamma \) GAP, about 2 nM, is in the same range of affinities as that displayed by \( \beta \gamma \) GAP, \( \beta \gamma \), or \( \beta \gamma \) for their effectors (5, 6, 39, 40), and its selectivity for the activated form of \( \beta \gamma \) is also consistent with this role. Alternatively, the GAP may be a negative regulatory component of the \( \beta \gamma \) pathway, involved either in desensitization or in mediating negative input from another signaling pathway. The model for such regulation could be either the GAPs for \( \beta \gamma \) and related small, monomeric G proteins (2) or the RGS proteins, a large family of related proteins that inhibit signaling (4, 9, 10) and whose prototypes are GAPs (12). Whether the cerebral \( \beta \gamma \) GAP is an effector or a modulator of inhibition will probably be elucidated when its cDNA can be used to manipulate its expression in cells.

**Purification**—\( \beta \gamma \) GAP was initially purified about 12,000-fold according to the specific activity of phenyl-Sepharose fractions, and the specific activity of the purest fractions from SDS gels is about 4-fold higher (Fig. 4). \( \beta \gamma \) GAP is thus a rare protein in brain, its richest source, and is perhaps expressed in

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**TABLE IV**

| Substrate       | \( k_{\text{hydro}} \) (min\(^{-1}\)) | \( -\text{GAP} \) | \( +\text{GAP} \) | Fold stimulation |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| \( \gamma \)-GTP | 0.135           | 0.455           | 33.7            |
| \( \gamma \)-GTP | 0.488           | 0.635           | 1.3             |
| \( \gamma \)-GTP | 1.10            | 1.18            | 1.07            |

**FIG. 8. Inhibition of \( \beta \gamma \) GAP activity by \( \beta \gamma \) subunits.** A, the activity of 40 pm \( \beta \gamma \) GAP was assayed at the concentrations of \( \gamma \)-GTP shown on the abscissa either in the presence (△) or absence (●) of 1.2 \( \mu \)M \( \beta \) and \( \gamma \). B, GAP activity was assayed at 2.0 nm \( \gamma \)-GTP in the presence of the concentrations of purified \( \beta \) or \( \gamma \) shown on the abscissa (○). Hydrolysis of \( \gamma \)-GTP was also measured without GAP at the same concentrations of \( \beta \) or \( \gamma \).
Because the ratio of GAP activity to silver-stained protein is low between the peaks, we suspect that major silver-stained bands in this region are contaminants and that the activity represents distribution of active proteolytic fragments of the 28-kDa GAP. We approached the question of proteolysis during purification by analyzing unfractionated brain membranes by SDS-gel electrophoresis. The principal peak of activity was at about 28 kDa, with tailing to about 20 kDa, but we could also detect small peaks of activity higher in the gel. These larger forms are not observed in purified preparation; peptides of $M_r > 30,000$ in the phenyl-Sepharose fractions have no GAP activity.

**Mechanism, Selectivity, and Regulation of $G_\alpha$ GAP—** Purified cerebral $G_\alpha$ GAP is highly specific in its action on $G_\alpha$. It displayed only slight activity with either $G_{\alpha i1}$-GTP or $G_{\alpha i1}$-GTP as substrates under conditions where hydrolysis of $G_{\alpha i1}$-GTP was accelerated over 30-fold (Table IV). According to competitive inhibition, however, the affinity of the GAP for $G_{\alpha i1}$ is only about 3-fold greater than that for $G_{\alpha o}$ and about 10-fold higher than for $G_{\alpha z}$. Evidently, $G_\alpha$ GAP can bind these other $G$ proteins with high affinity but cannot efficiently promote their deactivation. This unusual pattern of selectivity suggests that other members of the $G$ family may inhibit $G_\alpha$ in cells, where they are much more abundant than is $G_{\alpha o}$. Given the selectivity of the purified GAP for $G_{\alpha i1}$, it was initially surprising that there is significant activity in cells and tissues that express little if any $G_{\alpha o}$ (Table I). It is likely that this activity is that of GAPs for other $G$ family members but which act on $G_{\alpha i1}$ with low efficiency (47, 48).

Cerebral $G_{\alpha z}$ GAP behaves generally as an integral membrane protein, although it was unusually refractory to solubilization by non-denaturing detergents. This behavior is reminiscent of caveolar proteins (42–44). However, caveolae are reported to be solubilized by octyl glucoside ($G_{\alpha}$ GAP was not) and, in one experiment, $G_{\alpha}$ GAP activity did not co-fractionate with caveolin in lysates of MA104 cells. We suspect that $G_{\alpha}$ GAP is a markedly hydrophobic protein because of its resistance to solubilization and its tendency to aggregate. This conclusion is supported by its functional co-reconstitution with m2 muscarinic receptors and trimeric $G_{\alpha}$ into phospholipid vesicles (Fig. 9), in contrast to its inactivity when added to preformed receptor-$G_{\alpha}$ vesicles. We have been unable to perform the standard tests for monomeric solubility of purified $G_{\alpha}$ GAP because removing detergent by dilution or chromatography before assay led to loss of GAP activity. This was true even though the assay was performed in the presence of Triton X-100. Some of this behavior is similar to difficulties encountered in solubilizing adenyl cyclase, a $G$ protein-regulated effector that is a much larger, multi-span membrane protein. There is inadequate information to compare this aspect of $G_{\alpha}$ GAP with RGS proteins, although RGS4 and GAIP are both sensitive to ionic strength (15).

The enzymologic mechanism of $G_{\alpha}$ GAP action is apparently straightforward. $G_{\alpha_{i1}}$-GTP is essentially stable over the usual assay interval. The GAP binds the GTP-bound form of $G_{\alpha_{i1}}$ with nanomolar affinity (Figs. 5 and 7), and the GAP-$G_{\alpha_{i1}}$-GTP complex then hydrolyzes GTP fairly quickly ($t_{1/2} \approx 15 s$ at $15^\circ C$; $t_{1/2} \approx 2 s$ at $30^\circ C$). The GAP binds $G_{\alpha_{i1}}$-GDP weakly if at all, such that the complex rapidly dissociates after hydrolysis. This mechanism allows $G_{\alpha}$ GAP to act catalytically; i.e., one GAP molecule can cycle among multiple molecules of $G_{\alpha_{i1}}$-GTP. A corollary to this behavior is that the rate-limiting step in the GAP-mediated GTPase reaction is hydrolysis of the GAP-$G_{\alpha_{i1}}$-GTP complex. This conclusion is supported by the finding that the maximum reaction rate at saturating and super-stoichiometric concentrations of GAP (Fig. 5B) is the same as the

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**FIG. 9.** $G_{\alpha}$ GAP amplifies agonist-stimulated steady-state GTPase activity in phospholipid vesicles that contain trimeric $G_{\alpha z}$ and m2 muscarinic cholinergic receptor. M2 muscarinic receptor and trimeric $G_{\alpha z}$ were co-reconstituted into phospholipid vesicles, with (C, □) or without (△, ▲) $G_{\alpha}$ GAP, essentially as described previously (23) and under "Experimental Procedures." A, steady-state GTPase activity was measured in the presence of either 100 $\mu M$ carbachol (○, △) or 5 $\mu M$ atropine (□, ▲). In parallel with the measurement of GTP hydrolysis, the steady-state concentration of $G_{\alpha z}$-[γ-32P]GTP in the assay mixture with carbachol was assayed both at 5 and 15 min. The concentration of $G_{\alpha z}$-[γ-32P]GTP at both times was 460 pm in vesicles with GAP and 640 pm in vesicles without GAP. The concentration of GAP in the assay, when present, was about 30 pm. B, GTP- $S$ binding was measured in the same vesicles. Only a few cell types. By rough comparison with published data (16, 41) and with immunoblotts performed during this study (not shown), $G_{\alpha}$ GAP is about 5–10-fold less abundant than is $G_{\alpha o}$.
maximum specific activity of the GAP at saturating Goα-GTP (Fig. 5A). In apparent contrast, the effect of RGS4 on Goα and Gi1 seems to be limited by substrate binding (12).

The activity of Goα GAP during receptor-stimulated steady-state GTP hydrolysis is evident when it is co-reconstituted in phospholipid vesicles with Goα and m2 muscarinic receptor (Fig. 9). The relative effect of the GAP activity was limited by its concentration and/or by the ratio of its concentration to that of Goα. Goα was in molar excess over GoS and will only accelerate the deactivation limb of the GTPase cycle. The GAP by GoS and GoA favoring the transition state structure of a G analog (45, 46), these authors suggested that RGS4 acts as a GAP by binding to GTP-bound Go than by the same Go bound to GTP-Go4 (47, 48). Because GDP/Go4 binds to Go12 and GoS as a transition state analog (45, 46), these authors suggested that RGS4 acts as a GAP by favoring the transition state structure of a Go over its GTP-bound form. This mechanism would presumably differentiate the GAP activity of RGS proteins from that of effectors, which are activated both by GTPS- and GDP/Go4-bound Go than by the same Go bound to GTP-Go4 (47, 48).

GAP binds the activated form of GoS with about the same affinity when it is bound to GTP (according to Kd), to GTP-Go12, or to GDP/Go4 (according to Ks). In contrast, RGS4, a GAP for the Gi family and Go12, is inhibited much more potently by an GDP/Go4-bound Go than by the same Go bound to GTP-Go4 (47, 48). Because GDP/Go4 binds to Go12 and GoS as a transition state analog (45, 46), these authors suggested that RGS4 acts as a GAP by favoring the transition state structure of a Go over its GTP-bound form. This mechanism would presumably differentiate the GAP activity of G proteins from that of effectors, which are activated both by GTPS- and GDP/Go4-bound G proteins, and would thus suggest that the cerebral Goα GAP is an effector. This distinction may not be generally valid, however, or may perhaps not extend to Goα. In preliminary experiments, we found that RGS4 is potently inhibited by the GTPS-bound form of Goα, a behavior similar to that of the cerebral Goα. The active site and enzymatic properties of Goα differ markedly from those of other Gi family members (16–18), and it is possible that GDP/Go4 is not a transition state analog at the active site of Goα. If true, however, this argument would favor an effector function for the Gi GAP.

Regardless of any yet unknown cellular roles of the Gi GAP, its presence and regulation will influence Goα-modulated signaling. The first mode of regulation so far observed is inhibition of the GAP by Goβγ subunits. Inhibition of GAP activity by Goβγ over a reasonable range of concentrations allows modulation of Goα signaling by other G protein pathways, where activation will release Goα in large excess over Goα. Other controls of GAP activity are also likely, and their understanding should help us understand the cellular pathways uniquely regulated by Goα.

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