

Modulation of the Affinity and Selectivity of RGS Protein Interaction with G_{α} Subunits by a Conserved Asparagine/Serine Residue[†]

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ABSTRACT: The crystal structure of the complex between a G protein α subunit ($G_{i\alpha 1}$) and its GTPase-activating protein (RGS4) demonstrated that RGS4 acts predominantly by stabilization of the transition state for GTP hydrolysis [Tesmer, J. J., et al. (1997) *Cell* 89, 251]. However, attention was called to a conserved Asn residue (Asn¹²⁸) that could play a catalytic role by interacting, directly or indirectly, with the hydrolytic water molecule. We have analyzed the effects of several disparate substitutions for Asn¹²⁸ on the GAP activity of RGS4 toward four G_{α} substrates (G_o , G_i , G_q , and G_z) using two assay formats. The results substantiate the importance of this residue but indicate that it is largely involved in substrate binding and that its function may vary with different G_{α} targets. Various mutations decreased the apparent affinity of RGS4 for substrate G_{α} proteins by several orders of magnitude, but had variable and modest effects on maximal rates of GTP hydrolysis when tested with different G_{α} subunits. One mutation, N128F, that differentially decreased the GAP activity toward $G_{\alpha i}$ compared with that toward $G_{\alpha q}$ could be partially suppressed by mutation of the nearby residue in $G_{\alpha i}$ to that found in $G_{\alpha q}$ (K180P). Detection of GAP activities of the mutants was enhanced in sensitivity up to 100-fold by assay at steady state in proteoliposomes that contain heterotrimeric G protein and receptor.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are transducers in many cellular transmembrane signaling systems. In brief, liganded cell surface receptors catalyze exchange of GDP for GTP on G protein α subunits, which activates G_{α} and drives its dissociation from a stable dimer of $\beta\gamma$ subunits. Both G_{α} -GTP and $G_{\beta\gamma}$ modulate the activities of downstream effector proteins. Hydrolysis of GTP bound to G_{α} terminates signaling and allows rebinding of $G_{\beta\gamma}$. Members of a recently discovered family of RGS proteins act as GTPase-activating proteins (GAPs)¹ toward G protein α subunits, accelerating the rate of inactivation of the signaling complex (1, 2). More than 20 mammalian RGS proteins have been described, generally by reference to a conserved domain of about 115 amino acid residues known as the RGS box.

The crystal structure of RGS4 bound to $G_{i\alpha 1}$ -GDP- AlF_4^- demonstrates that the active core of the RGS box forms a four-helix bundle (3). This bundle stabilizes the transition state for hydrolysis of G_{α} -bound GTP by interacting with the three flexible switch regions of G_{α} , regions whose conformation is dependent on the identity of the bound

guanine nucleotide (GDP or GTP) (4). Although no residue from RGS4 contacts the GTP substrate directly, Asn¹²⁸ appears to play a particularly critical role via interactions with the hydrolytic water molecule itself and/or with Gln²⁰⁴ and Glu²⁰⁷ of $G_{i\alpha 1}$. Gln²⁰⁴ polarizes and orients the hydrolytic water molecule in the transition state, while Glu²⁰⁷ is a strongly conserved feature in the RGS-G protein interaction footprint. Lys¹⁸⁰ from $G_{i\alpha 1}$ is also involved in a van der Waals interaction with Asn¹²⁸ of RGS4. Asn¹²⁸ is strongly conserved among most RGS proteins. However, several RGS proteins that act as high-affinity, $G_{z\alpha}$ -selective GAPs have naturally replaced Asn¹²⁸ with a Ser residue (5–7), indicating that the requirement for this Asn residue is not absolute.

Two groups have examined the effects of mutation of Asn¹²⁸ but came to different conclusions. Srinivasa et al. (8) suggested that the side chain interactions mentioned above (including Asn¹²⁸) are important for the ability of RGS4 to bind and stabilize the transition state of the G_{α} subunit. They found that RGS4 N128A could not detectably bind $G_{12\alpha}$ -GDP- AlF_4^- and that consequently the N128A mutant retained only 4% of the GAP activity of the wild-type protein (8). Natochin et al. (9) confirmed the low GAP activity and G_{α} binding of the cognate Ala mutant in RGS-r, but found that several other mutations of the Asn residue retained substantial GAP activity. They concluded that this residue is involved both in substrate binding and more directly in the hydrolytic event. We have now explored the role of Asn¹²⁸ in more detail by studying the activities of multiple Asn¹²⁸ RGS4 mutants with several different G_{α} -GTP substrates. Our data indicate that the function of Asn128 depends on both the identity of the G_{α} substrate and its environment.

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¹ Abbreviations: GAP, GTPase-activating protein; GTP γ S, guanosine 5'-O-thiotriphosphate; C₁₂E₁₀, dodecyl poly(ethylene oxide) ($n \sim 10$).

Interactions between RGS protein and G_{α} are most sensitively probed during steady-state GTP hydrolysis in proteoliposomes that contain receptor and heterotrimeric G protein.

EXPERIMENTAL PROCEDURES

Materials. $[\gamma\text{-}^{35}\text{S}]\text{GTP}\gamma\text{S}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ were purchased from NEN. $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was purified as described previously (10). Sources of other materials have been described previously (10–12).

Mutagenesis. The following oligonucleotides and the Biorad Mutagene kit were utilized to introduce mutations at position 128 of RGS4, CA AAA GAG GTG NNN CTG GAT TCT TG, where NNN was GCG, AGT, TTG, GTG, or GGT for encoding N128A, N128S, N128F, N128V, or N128G RGS4, respectively. Lys¹⁸⁰ of $G_{i\alpha 1}$ was mutated to Pro using the Stratagene Quick Change kit and the oligonucleotide CTC AGA ACT AGA GTG CCA ACG ACG GGA ATT A. An internally hexahistidine-tagged construct of $G_{i\alpha 1}$ was used as the template for mutagenesis to facilitate protein purification (13).

Protein Purification. Mutant and wild-type hexahistidine-tagged RGS proteins were synthesized in *Escherichia coli* and purified as described previously (12). All of these proteins migrated through a Superdex G75 column (Pharmacia) as monomeric species with identical retention times and were homogeneous as determined by SDS–polyacrylamide gel electrophoresis. Hexahistidine-tagged GAIP was synthesized in Sf9 cells infected with the appropriate recombinant baculovirus and purified using Ni^{2+} –NTA resin (Qiagen). The fractional activities of purified wild-type RGS4, wild-type GAIP, and RGS4 N128S were determined by titration with $G_{\alpha}\text{--GDP--AlF}_4^-$ under conditions where prebound G_{α} blocks GAP activity. The RGS protein (130 nM RGS4, 13 μM RGS4 N128S, and 1 μM GAIP) was first incubated with $G_{\alpha}\text{--GDP}$, 30 μM AlCl_3 , 10 mM MgCl_2 , and 10 mM NaF for 5 min at 30 °C and then placed on ice. Each sample was subsequently diluted 13-fold into GTPase assay buffer that contained $G_{\alpha}\text{--}[\gamma\text{-}^{32}\text{P}]\text{GTP}$, and the rate of hydrolysis of the bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was determined as described below. The concentration of $G_{\alpha}\text{--GDP--AlF}_4^-$ in the initial incubation was varied above and below the concentration of the RGS protein, and the concentrations of both proteins were several times higher than the K_d for the $\text{RGS--}G_{\alpha}\text{--GDP--AlF}_4^-$ complex. Plots of residual GAP activity versus the concentration of $G_{\alpha}\text{--GDP--AlF}_4^-$ in the preincubation yield two straight lines that intersect at the concentration of $G_{\alpha}\text{--GDP--AlF}_4^-$ equal to the concentration of the active RGS protein (Figure 1B) (14). The concentration of the G_{α} used in the blocking reaction was determined using a $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ binding assay (15). The active site titrations revealed that more than 80% of each preparation of these RGS proteins was active. RGS mutants with activities too low to be standardized by this procedure were quantitated by measurement of total protein concentrations (16).

Procedures for the purification of N-terminally hexahistidine-tagged G_{α} (17), internally hexahistidine-tagged $G_{i\alpha 1}$, myristoylated $G_{i\alpha 1}$ and G_{α} (18), wild-type and R183C $G_{q\alpha}$ (10), $G_{\beta 1\gamma 2}$ (10), and m1 and m2 muscarinic cholinergic receptors (19) have been described previously. N-Terminally hexahistidine tagged G_{α} was routinely used as the substrate for GAP assays unless otherwise indicated. Myristoylated

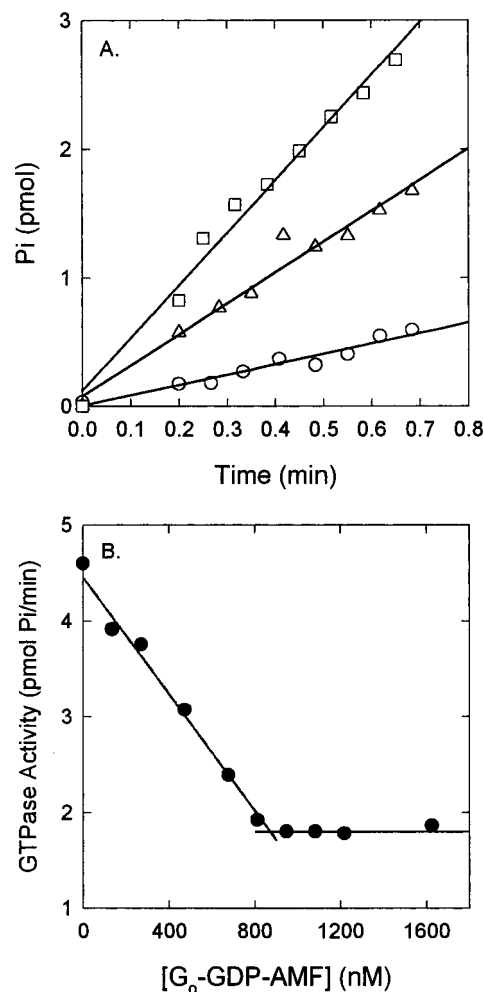
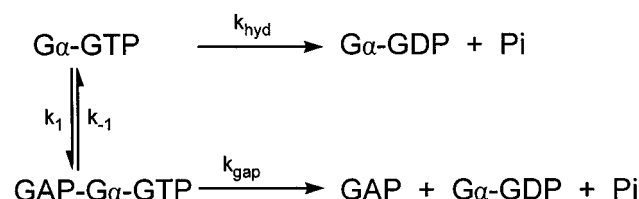


FIGURE 1: Effects of RGS proteins on the hydrolysis of $G_{\alpha}\text{--GTP}$. (A) Hydrolysis of 250 nM $G_{\alpha}\text{--}[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was assessed at 4 °C as described in Experimental Procedures either alone (○), with 10 nM RGS4 (□), or with 4 μM RGS4 N128A (Δ). These data are representative of three identical experiments with these proteins. (B) Active site titration of N128S RGS4. Initial rates of hydrolysis of 400 nM $G_{\alpha}\text{--}[\gamma\text{-}^{32}\text{P}]\text{GTP}$ were measured in the presence of 1 μM N128S RGS4 and the concentrations of $G_{\alpha}\text{--GDP--AlF}_4^-$ shown on the abscissa. The data were fit to two straight lines. The concentration of active RGS4 is equal to the concentration of $G_{\alpha}\text{--GDP--AlF}_4^-$ at the intersection of the lines.

G_{α} and $G_{i\alpha 1}$ were used when reconstituted with $G_{\beta\gamma}$ and receptors in phospholipid vesicles. In contrast to previous studies with G_{α} , however, myristoylation of $G_{i\alpha 1}$ or G_{α} had no effect on the GAP activities of the RGS4 proteins used here (data not shown).

Hydrolysis of Bound GTP. Hydrolysis of G_{α} -bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in solution was monitored at 4 °C essentially as described by Berman et al. (12). To prepare $G_{\alpha}\text{--}[\gamma\text{-}^{32}\text{P}]\text{GTP}$, G_{α} was incubated with 30 μM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (700–2000 cpm/pmol), 50 mM NaHepes (pH 8.0), 5 mM EDTA, 3 mM dithiothreitol, and 0.05% $\text{C}_{12}\text{E}_{10}$ for 20 min at 20 °C prior to passage through a gel filtration spin column (G-50 Sephadex, Pharmacia) at 4 °C to remove excess $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $^{32}\text{P}_i$. Assays were initiated by addition of excess unlabeled GTP, MgSO_4 , and RGS protein to $G_{\alpha}\text{--GTP}$ substrate at 4 °C. The final concentrations of components were 50 mM NaHepes (pH 8.0), 5 mM EDTA, 3 mM dithiothreitol, 200 μM GTP, 10 mM MgSO_4 , and 0.05% $\text{C}_{12}\text{E}_{10}$. Reactions were quenched with neutral charcoal (Norit

Scheme 1



A) at time intervals of 3–6 s. At each concentration of G α -GTP, the basal rate of hydrolysis (k_{hyd} , Scheme 1) was determined in the absence of RGS protein and was subtracted from the rates observed in the presence of RGS protein. Values of V_{max} and K_m were determined by fitting the concentration dependence of the corrected initial rates to the Michaelis–Menten equation. Hydrolysis of G α -[γ - ^{32}P]GTP was assessed similarly (except at 8 °C, 7 mM Mg $^{2+}$, and 5 mM GTP), but rate constants were calculated by fitting complete hydrolysis time courses to first-order rate equations (20) (Marquardt–Levenberg algorithm in the SigmaPlot program package). While somewhat less accurate for wild-type G α , this approach gave more reliable data for the slow hydrolysis rates of the K180P G α mutant. To measure GAP activity with G α -GTP as the substrate, the R183C mutant was used to slow hydrolysis and thereby allow loading with [γ - ^{32}P]GTP (21). Assays with R183C G α were performed at 20 °C as described previously (21), with the same order of addition of reactants described above. GAP activity with G α -GTP as the substrate was assayed at 15 °C as described by Wang et al. (11). Quantitative evaluation of GAP assay data has been discussed elsewhere (20).

Steady-State GTPase Assays with Receptor–G Protein Vesicles. Purified m1 or m2 muscarinic cholinergic receptors and heterotrimeric G proteins were reconstituted into phospholipid vesicles (phosphatidylethanolamine/phosphatidylserine/cholesteryl hemisuccinate, 165:98:18) by gel filtration as described previously (10). Each assay (40 μL) contained 10–20 fmol of receptor and 50–70 fmol of G α [as a trimer, with a slight molar excess of G $\beta\gamma$ (10)] as determined in ligand binding assays (15). GTPase assays were performed at 30 °C for G $_i$ and G $_o$ or at 20 °C for G $_q$ exactly as described previously (10). Assays contained either 1 mM carbachol or 10 μM atropine, with or without added RGS proteins. Assays were initiated by addition of vesicles to the reaction mixture. For assays using G $_q$, data are reported only as the increment in activity caused by carbachol; basal activities were negligible (10). For G $_i$ and G $_o$, activity in the absence of agonist was significant, and we report total GTPase activity.

RESULTS

Activities of Mutant and Wild-Type RGS Proteins with G α and R183C G α . RGS proteins act catalytically to accelerate the conversion of G α -GTP to G α -GDP (Scheme 1). Their activity at steady state is most readily described by an analogy to enzyme kinetics, with a maximal turnover number (k_{gap}) and Michaelis constant (K_m) that are obtained by measuring the rate of GTP hydrolysis as a function of substrate concentration (G α -GTP–Mg $^{2+}$) (11, 20). The k_{gap} is the intrinsic rate of hydrolysis of GTP in the RGS–G α -GTP–Mg $^{2+}$ complex. The K_m appears to approximate the

Table 1: Michaelis Parameters for the G α GAP Activity of RGS4 and GAIP^a

	RGS4	GAIP
K_m (μM)	0.6 ± 0.1	0.05 ± 0.015
k_{gap} (min^{-1})	130 ± 10	3.2 ± 0.4
k_{gap}/K_m ($\text{min}^{-1} \text{M}^{-1}$)	$(2.2 \pm 0.4) \times 10^8$	$(6.4 \pm 2.0) \times 10^7$

^a Values were derived from nonlinear least-squares fits to Scheme 1 of the data shown in Figure 2 and one other similar experiment. Error estimates are standard errors of the fit. k_{hyd} for G α under these conditions is 0.10 min^{-1} .

K_d for G α -GTP–Mg $^{2+}$ binding to the RGS protein, as predicted by the relationships of the individual kinetic constants (20). The available data do not implicate a more complex scheme. Observed values of k_{gap} are generally more than 100-fold greater than k_{hyd} for the same G α , and values of K_m vary from nanomolar to several micromolar (11, 12). RGS proteins do not directly influence nucleotide exchange (12, 22, 23; data not shown for the mutants used here) or bind G α -GDP with high affinity (12, 22, 24, 25).

The GAP activities of Asn 128 mutants of RGS4 toward soluble G α -GTP–Mg $^{2+}$ varied widely. Wild-type RGS4 has a modest K_m of $0.6 \mu\text{M}$ and a k_{gap} of 130 min^{-1} for G α -GTP–Mg $^{2+}$ at 4 °C under our standard assay conditions (Figures 1A and 2A and Table 1). RGS4 thus increases the hydrolytic rate of G α more than 1000-fold. RGS4 N128S retained activity, but the data of Figure 2B do not indicate saturation at the highest concentration of G α -GTP tested. The K_m of RGS4 N128S for G α -GTP–Mg $^{2+}$ is thus clearly elevated (probably about 100-fold), and k_{gap} may also be diminished. GAIP, which naturally has a Ser residue at the position corresponding to Asn 128 , provides a contrast (Figure 2C and Table 1). Although GAIP exhibited a k_{gap} of only 3.2 min^{-1} , its K_m was 50 nM, about 12-fold lower than that of RGS4. RGSZ1, which also has a Ser residue at this position, also binds G α -class substrates with high affinity [K_d for G α -GTP $\gamma\text{S} \sim 5 \text{ nM}$ (11)]. RGS4 proteins with other mutations at position 128 were less active on G α -GTP than was RGS4 N128S. High concentrations of RGS4 N128A (4 μM) were required to observe any effect on the GTPase activity of G α (Figure 1A), and Phe, Val, and Gly mutants (at 4, 8, and 4 μM) had no detectable activity on G α when assayed in solution.

Because a subgroup of RGS proteins that are selective for G $_z$ have a Ser residue at the position homologous to Asn 128 in RGS4 (7), we tested the GAP activities of the RGS4 mutants with G α -GTP as the substrate (Table 2). Although RGS4 N128S was the most active mutant as a G $_z$ GAP, its activity was only 0.1% of that of wild-type RGS4. This decrease in activity primarily reflected an increase in K_m to about 7 μM (data not shown), which is much higher than that of wild-type RGS4 ($\sim 10 \text{ nM}$).² Although we could measure the G $_z$ GAP activities of the other mutants, they displayed as little as 1% of the activity of the Ser mutant (10^{-5} times the value of the wild type). A Ser residue at this position does not therefore independently provide either high activity or selectivity toward G α .

To determine whether effects of mutations at Asn 128 are selective for G α substrates of the G $_i$ or G $_q$ class, we also measured the GAP activities of wild-type and mutant RGS4

² J. Wang, personal communication.

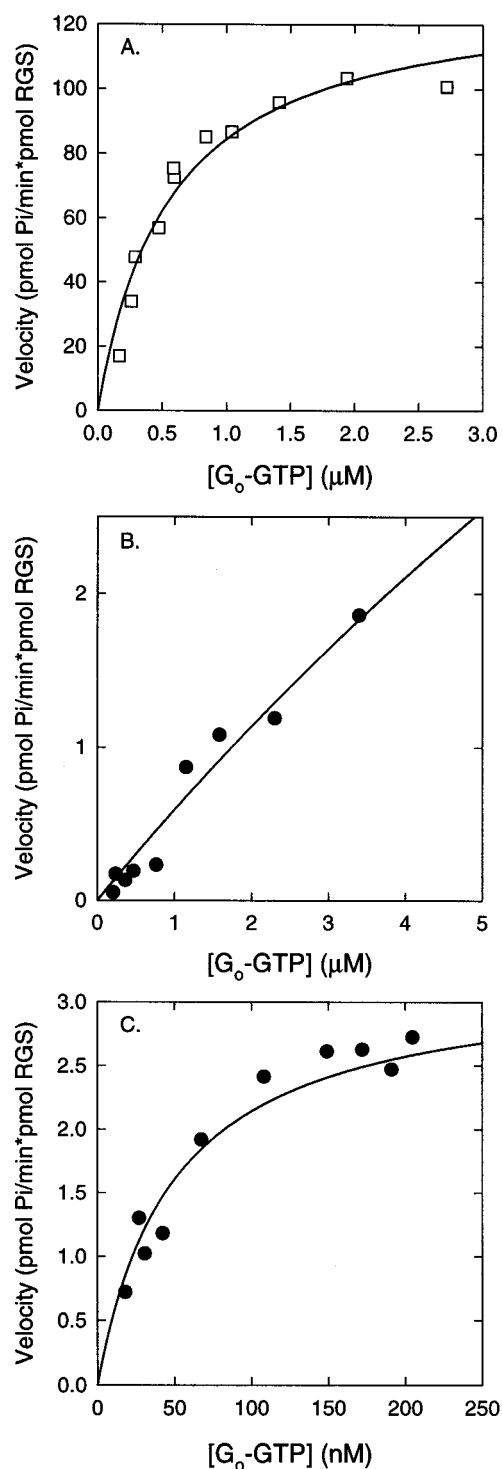


FIGURE 2: RGS-stimulated GTP hydrolysis at increasing G_o-GTP concentrations. Initial rates of hydrolysis of G_o-[γ-³²P]GTP at the concentrations shown were determined in the presence of 10 nM wild-type RGS4 (A), 500 nM RGS4 N128S (B), or 20 nM GAIP (C). These data are representative of two such complete data sets.

proteins with G_{qα} in solution. Because of the slow rate of dissociation of GDP from G_{qα}, we used a mutant (R183C) G_{qα} protein, for which GTPase activity is slower than nucleotide exchange. This permits preparation of the G_{qα}-GTP-Mg²⁺ substrate (21). We could not determine Michaelis parameters for these reactions because of the high values of *K_m*, and therefore report only initial rates of GAP-stimulated GTP hydrolysis corrected for the concentration of the RGS protein (Table 3). The Ser and Phe mutants of

Table 2: G_{zα} GAP Activities of RGS4 Asn¹²⁸ Mutants^a

wild-type RGS4	$(3.7 \pm 0.4) \times 10^7 \text{ min}^{-1} \text{ M}^{-1}$
N128S	$(3.4 \pm 0.6) \times 10^4 \text{ min}^{-1} \text{ M}^{-1}$
N128A	$(7.9 \pm 1.7) \times 10^3 \text{ min}^{-1} \text{ M}^{-1}$
N128F	$(2.5 \pm 0.1) \times 10^3 \text{ min}^{-1} \text{ M}^{-1}$
N128V	$(1.1 \pm 0.2) \times 10^3 \text{ min}^{-1} \text{ M}^{-1}$
N128G	$(4.8 \pm 2.1) \times 10^2 \text{ min}^{-1} \text{ M}^{-1}$

^a Hydrolysis rate constants for G_{zα}-GTP (2 or 3.7 nM) in the presence of appropriate concentrations of each RGS4 protein were determined from complete hydrolysis time courses (20). The increment over the intrinsic rate constant (0.014 min^{-1}) (11) was divided by the concentration of RGS4 protein to yield a second-order catalytic constant. Data shown are means (\pm one-half the range) from two to five determinations at different GAP concentrations. Expressed similarly, the G_z GAP activity of GAIP or RGSZ1 is $\sim 2 \times 10^9 \text{ min}^{-1} \text{ M}^{-1}$ (7).

Table 3: G_{qα} GAP Activities of RGS4 Asn¹²⁸ Mutants and GAIP^a

RGS	<i>k_{ss}</i> (min ⁻¹)	RGS	<i>k_{ss}</i> (min ⁻¹)
wild-type	1.1 ± 0.2	N128A	$(3.0 \pm 0.2) \times 10^{-4}$
N128S	$(3.0 \pm 0.3) \times 10^{-3}$	GAIP	$(4.6 \pm 0.3) \times 10^{-3}$
N128F	$(3.0 \pm 0.3) \times 10^{-3}$		

^a Initial rates of hydrolysis of R183C G_{qα}-GTP ($\sim 30 \text{ pM}$) were measured as described in Experimental Procedures in the presence of appropriate concentrations of wild-type GAIP (1 μM), wild-type RGS4 (20 nM), and the Asn¹²⁸ mutants of RGS4 that are listed (1 μM N128S and N128F and 4 μM N128A). *k_{ss}* represents the RGS-promoted increments in the initial rates of hydrolysis (moles of GTP per minute) divided by the amount of RGS protein. These data are from one of two experiments that yielded similar results. RGS4 N128G and N128V were not assayed.

RGS4 and GAIP were about 300–400-fold less active as G_q GAPs than wild-type RGS4, and RGS4 N128A was substantially worse. Effects of the Asn¹²⁸ mutations on G_q GAP activity were thus significant, as was the case with G_o, although the effects of individual mutations varied. Most notably, RGS4 N128F was much more active than N128A, the reverse of the order determined with G_{oα}. G_q GAP activity was not assayed for N128G and N128V RGS4.

GAP Activities of Mutant and Wild-Type RGS Proteins during Receptor-Stimulated, Steady-State GTP Hydrolysis. The GAP activity of RGS4 is greater and more easily observed in a steady-state GTPase assay using proteoliposomes that contain heterotrimeric G protein and a suitable receptor (21, 26). GAP activity of an RGS protein with a specific G protein substrate may be undetectable in solution-based assays and still detected readily in the vesicle-based assay (21). In such an assay, agonist-bound receptor stimulates nucleotide exchange on G_α. At low concentrations of a GAP, nucleotide exchange is relatively rapid and the observed steady-state rate of production of ³²P_i reflects the rate-limiting hydrolytic step. We therefore assayed the mutant RGS proteins in the receptor-coupled system.

In contrast to the marked losses of G_{oα} GAP activity displayed by the Asn¹²⁸ mutants in the solution assay, each of the mutants markedly stimulated the steady-state GTPase activity of the G_o heterotrimer that was reconstituted with m2 muscarinic cholinergic receptors (Figure 3). RGS4 N128S actually produced maximum activity that was higher than that of the wild-type protein, even though it did not saturate. The maximal effects of the other mutant RGS4 proteins were comparable to the maximum attained by wild-type RGS4. In each case, the major effect of the mutations was to decrease potency, about a 20-fold increase in EC₅₀ in the

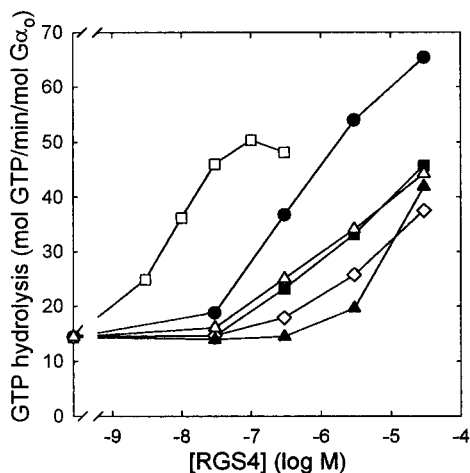


FIGURE 3: GAP activity of RGS4 mutants during steady-state, receptor-stimulated GTP hydrolysis. The carbachol-stimulated, steady-state GTPase activity of m2 muscarinic receptor-G α vesicles was assayed as described in Experimental Procedures in the presence of increasing concentrations of wild-type RGS4 (\square), RGS4 N128S (\bullet), RGS4 N128A (Δ), RGS4 N128V (\blacktriangle), RGS4 N128G (\blacksquare), or RGS4 N128F (\diamond). Similar data were obtained in two other experiments.

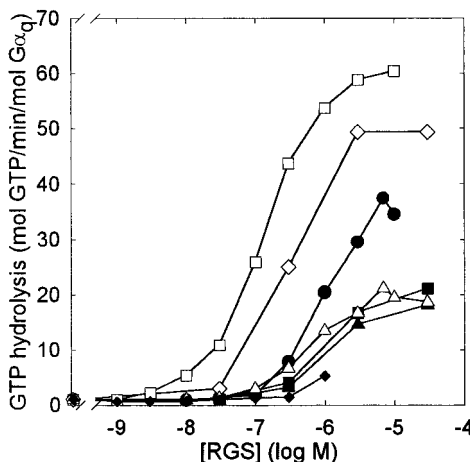


FIGURE 4: G α GAP activity of RGS4 mutants during steady-state, receptor-stimulated GTP hydrolysis. The carbachol-stimulated, steady-state GTPase activity of m1 muscarinic receptor-G α vesicles was assayed as described in Experimental Procedures in the presence of increasing concentrations of wild-type RGS4 (\square), RGS4 N128S (\bullet), RGS4 N128A (Δ), RGS4 N128V (\blacktriangle), RGS4 N128G (\blacksquare), RGS4 N128F (\diamond), or wild-type GAIP (\blacklozenge). Data are representative of four other similar complete experiments.

case of N128S and as much as 2000-fold for N128G and N128F. For each mutant, however, the rank order of activity was qualitatively similar in the two assay formats.

Reconstitution of heterotrimeric G α with m1 muscarinic receptors and phospholipids also amplified the assayable G α GAP activities of the RGS4 mutants and allowed more detailed study of their activities (Figure 4). In this assay, RGS4 N128F was only slightly less potent as a G α GAP than was wild-type RGS4 and displayed almost the same maximal activity. The high activity of the N128F mutant with G α contrasts with its poor activity with G α and confirms its activity in the assay with soluble R183C G α . The other Asn¹²⁸ mutants all were active in the vesicle-based assay, although several saturated at somewhat lower maximal activities and displayed values of EC₅₀ well above that of wild-type RGS4. RGS4 N128S and the other mutants had

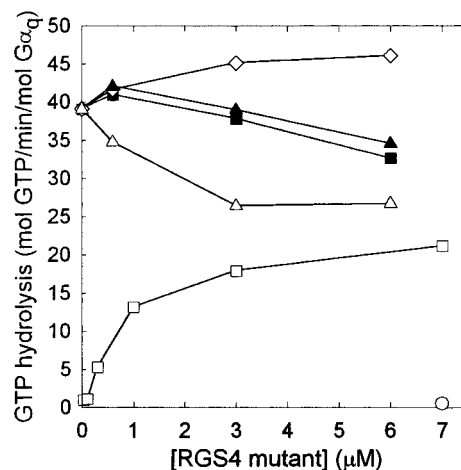


FIGURE 5: Competition between Asn¹²⁸ mutant and wild-type RGS4. The carbachol-stimulated, steady-state GTPase activity of m1 muscarinic receptor-G α vesicles was measured as described in Experimental Procedures. Assays included 300 nM wild-type RGS4 and varying concentrations of RGS4 N128A (Δ), RGS4 N128G (\blacksquare), RGS4 N128V (\blacktriangle), or RGS4 N128F (\diamond). The GAP activity of RGS4 N128A in the absence of the wild-type protein was also assayed (\square). The GTPase activity of the vesicles in the absence of a GAP is shown at the lower right (\circ). Similar data were obtained in two other experiments.

maximal activities that were roughly 30–60% of the wild-type value. Wild-type GAIP was only slightly active as a G α GAP in the vesicle system (Figure 4).

Competitive Inhibition of GAP Activity by Mutant RGS4. Because several of the RGS4 mutants produced moderately diminished maxima in the vesicle-based G α GAP assay (Figure 4), we tested their ability to bind G α in an abortive complex and thereby competitively block the GAP activity of the wild-type RGS4. As shown in Figure 5, RGS4 N128A inhibited the GAP activity of wild-type RGS4 to a level near that of its own maximum GAP activity. Its IC₅₀ in this assay, $\sim 1.5 \mu\text{M}$, was near its EC₅₀ as a GAP, consistent with the idea that RGS4 N128A can bind G α with only a slight GAP effect, but thereby inhibit competitively the binding of other more active GAPs. There was also a suggestion of competitive inhibition by the Val and Gly mutants, although it was small and we did not try to quantitate its affinity. We found no competitive inhibition of wild-type RGS4 by any of the mutants using the m2 receptor-G α vesicles (not shown), as would be predicted from the observation that all displayed appreciable G α GAP activities (Figure 3).

The K180P Mutation in G α Partially Suppresses the N128F Mutation in RGS4. In the steady-state assay, RGS4 N128F was the most active mutant for G α but the least active for G α (Figures 3 and 4), and it had no detectable G α GAP activity in solution. Models of the structures of the G α -RGS4 N128F and G α -RGS4 N128F complexes based on the known structure of G α -RGS4 (3) predict steric hindrance between Lys¹⁸⁰ in G α and Phe¹²⁸ in RGS4. Asn¹²⁸ in RGS4 also forms a van der Waals contact with Lys¹⁸⁰ that would be lost in the N128F mutant (3). The residue in G α that corresponds to Lys¹⁸⁰ in G α and G α is a proline. We therefore made the K180P mutant of G α and examined its sensitivity to the GAP activity of RGS4 N128F. Although K180P G α hydrolyzes bound GTP about 10-fold more slowly than does wild-type G α , soluble K180P G α was at least 10-fold more sensitive to RGS4 N128F than was the

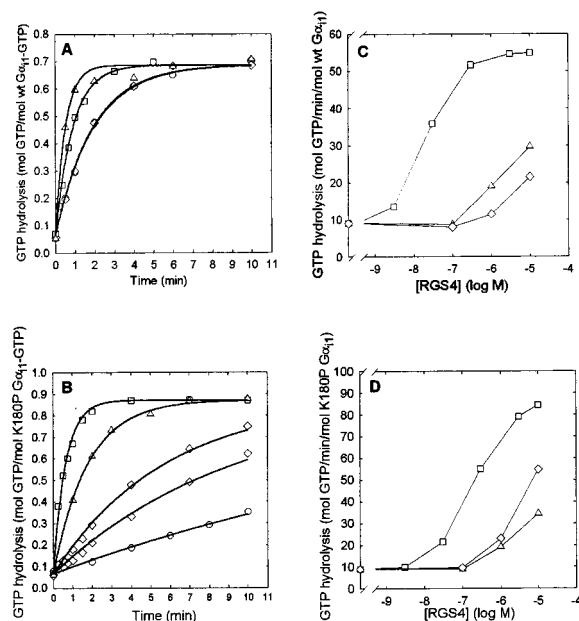


FIGURE 6: K180P G_{αi1} is more sensitive to RGS4 N128F than is wild-type G_{αi1}. (A and B). GAP activity was measured in solution using [γ -³²P]GTP bound to either wild-type G_{αi1} (A) or the K180P mutant (B). Assays contained either no RGS protein (○), 5 nM wild-type RGS4 (□), 1.5 or 3 μM RGS4 N128F (◇), or 3 μM RGS4 N128A (Δ). Solid lines represent fits to first-order rate equations constrained to yield the same total release of ³²P_i. Fitted rate constants (increments over basal) from this and a similar experiment were divided by the concentrations of RGS4 to give the following specific activities in units of min⁻¹ M⁻¹ ($n = 2$ for the wild type and N128A; $n = 3$ for N128F; “±” indicates one-half the range of the determinations). The basal rate for K180P G_{αi1} was 0.045 min⁻¹: $(2.4 \pm 1.2) \times 10^8$ for wild-type G_{αi1} and RGS4, $(0.4 \pm 2.9) \times 10^3$ for wild-type G_{αi1} and N128F, $(6.5 \pm 1.7) \times 10^5$ for wild-type RGS4 and N128A, $(2.3 \pm 0.1) \times 10^7$ for K180P G_{αi1} and RGS4, $(5.0 \pm 0.6) \times 10^4$ for K180P G_{αi1} and N128F, and $(1.7 \pm 0.1) \times 10^5$ for K180P G_{αi1} and N128A. (C and D) Carbamol-stimulated steady-state GTPase activity was measured in vesicles that contained m2 muscarinic receptors and G_{αi1}, either the wild type (C) or K180P (D). Assays contained wild-type RGS4 (□), RGS4 N128F (◇), or RGS4 N128A (Δ).

wild type (Figure 6A,B). This is an underestimate because we were unable to measure any reproducible stimulation of wild-type G_{αi1} by N128F RGS4 (Figure 6A and its legend). In receptor-G_i vesicles, the K180P mutant was less sensitive than wild-type G_{αi1} to wild-type RGS4, but its relative sensitivity to N128F RGS4 was enhanced when compared to its response to either wild-type or N128A RGS4 (Figure 6C,D). The effect of the K180P mutation in G_{αi1} on its sensitivity to RGS4 N128A was much smaller, consistent with the idea that substituting a small side chain for that of Asn¹²⁸ would not cause steric crowding at the binding site.

DISCUSSION

GAPs can potentially function via three distinct mechanisms. They may directly contribute to active site chemistry, as is true for GAPs for small monomeric GTP-binding proteins (27–29). Alternatively, they can bind near the active site and reorient specific catalytic residues or, even more indirectly, alter the overall configuration of the GTPase protein to favor faster catalysis. GAPs for heterotrimeric G proteins operate by one or both of the latter two mechanisms

(3), and Asn¹²⁸ in RGS4 might be central to either. The importance of Asn¹²⁸ in RGS4 to the function of RGS proteins is suggested by its almost complete conservation within the family (Ser is the only natural substitution), its location at the G_α-RGS binding interface, and its interaction there with three residues at the G_α active site (Gln²⁰⁴, Glu²⁰⁷, and Lys¹⁸⁰ in G_{αi1}) (3). This importance is reinforced by the finding that its substitution by Ala in RGS4 or by several residues other than Ser in RGS-r markedly reduced GAP activity (8, 9). This led to speculation that Asn¹²⁸ orients these residues within the active site and thus either facilitates catalysis directly or, more generally, forces G_α into its transition-state conformation.

The data presented here suggest that Asn¹²⁸ is primarily a determinant of RGS4 binding to G_α subunits and that its specific interactions with active site residues are less important. Thus, RGS binding primarily stabilizes the normally flexible catalytic switch regions of the G_α subunit to favor interaction with the G_α-GTP transition state (3). This conclusion is supported by data obtained using diverse mutations of Asn¹²⁸ (Ser, Gly, Phe, Ala, and Val), four G_α targets (G_{αo}, G_{αi1}, G_{αq}, and G_{αz}), and two distinct assay formats. Although Asn¹²⁸ may not be an important biological determinant of target selectivity, the rank order of activity among the mutants differed for each G_α: for G_{αo}, WT > GAIP > S > A, G > F, V; for G_{αq}, WT > F > S > A, G, V, GAIP; and for G_{αz}, GAIP > WT > S > A > F, V > G. Lys¹⁸⁰ of G_{αi1} and the corresponding Pro in G_{αq} are also involved in RGS-G_α binding, as indicated by the improved interaction of K180P G_{αi1} with N128F RGS4. The varied sensitivities of the individual G_α-GTP targets to the different RGS4 mutants also suggest that G protein binding is more important than specific reorientation of catalytically important residues, which are generally conserved among the G_α proteins. For m1 receptor-G_q vesicles, several mutations in RGS4 also decreased maximal GTPase rates (Figure 4), and N128A RGS4 apparently lost about 70% of its GAP activity (Figure 5). This decrease in the maximum may reflect a decrease in k_{gap} , but may also point to other functions of RGS proteins in the GTPase cycle that are also altered by mutation of Asn¹²⁸.

This study also points out the sensitivity of the steady-state, reconstitutive assay for GAP function relative to the single-turnover assay using soluble G_α subunits. Although several of the mutants exhibited no detectable GAP activity toward G_{αo} or G_{αq} in solution (at 4–8 μM), all displayed significant activity in the vesicle-based assay. Further, steady-state GAP activity could be quantitated reproducibly with respect to maximum efficacy and affinity over the 3 nM to 30 μM range of RGS concentrations. Results of the two assays agree qualitatively insofar as the rank order of activities of mutant and wild-type proteins were the same in both assays whenever activity in solution could be measured. This was true for both G_{αo} and G_{αq}. One reason for the increased sensitivity of the vesicle-based assay may be the local concentration (or physical orientation) of the RGS protein at the vesicle surface; RGS4 binds relatively tightly to phospholipid vesicles.³ GAPs also accelerate GDP-GTP exchange indirectly by favoring permanent association of the receptor with G protein (10), and RGS4 may thus amplify

³ Y. Tu and E. M. Ross, unpublished.

its net effect on steady-state GTPase activity. Alternatively, RGS4 may interact in an important way with the receptor or G $\beta\gamma$ to enhance its activity (26, 30–32). Regardless, the steady-state assay is operationally more sensitive and intuitively approximates the action of an RGS protein in a cell, and the potencies of RGS4 in the steady-state assay are closer to the very high-affinity interactions described for RGS4 in microinjected cells (26). A weakness of the vesicle-based assay is that the maximal steady-state velocity is, in most cases, limited by the receptor-catalyzed GTP–GDP exchange rate, and simple mechanistic inferences from observed rates are therefore limited.

The effects of the mutant RGS4 proteins on the steady-state GTPase activity of G $_{\alpha}$ (Figure 4) support the possibility of developing useful dominant negative RGS proteins that could bind G $_{\alpha}$ without GAP activity and thus block the GAP activity of other endogenous RGS proteins. Most of the mutants exhibited decreased maximal activities toward G $_{\alpha}$ (Figure 4), and N128A RGS4 competitively inhibited the GAP activity of the wild-type protein (Figure 5). Similar effects were not seen with G $_{\alpha}$, where the only effect of the mutations was to diminish binding. These results thus hold out hope for modifying RGS protein function in cells through the use of mutation, but point out that the appropriately matched RGS–G α pair will need to be used in the development of such approaches.

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