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Binding of Regulator of G Protein Signaling (RGS) Proteins to Phospholipid Bilayers

CONTRIBUTION OF LOCATION AND/OR ORIENTATION TO GTPase-ACTIVATING PROTEIN ACTIVITY*

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Regulator of G protein signaling (RGS) proteins must bind membranes in an orientation that permits the protein-protein interactions necessary for regulatory activity. RGS4 binds to phospholipid surfaces in a slow, multistep process that leads to maximal GTPase-activating protein (GAP) activity. When RGS4 is added to phospholipid vesicles that contain m2 or m1 muscarinic receptor and G_i, G_z, or G_q, GAP activity increases ~3-fold over 4 h at 30 °C and more slowly at 20 °C. This increase in GAP activity is preceded by several other events that suggest that, after binding, optimal interaction with G protein and receptor requires reorientation of RGS4 on the membrane surface, a conformational change, or both. Binding of RGS4 is initially reversible but becomes irreversible within 5 min. Onset of irreversibility parallels initial quenching of tryptophan fluorescence ($t_{1/2}$ ~ 30 s). Further quenching occurs after binding has become irreversible ($t_{1/2}$ ~ 6 min) but is complete well before maximal GAP activity is attained. These processes all appear to be energetically driven by the amphipathic N-terminal domain of RGS4 and are accelerated by palmitoylation of cysteine residues in this region. The RGS4 N-terminal domain confers similar membrane binding behavior on the RGS domains of either RGS10 or RGSZ1.

The initial events in G protein-mediated signaling take place at the membrane surface. G proteins are peripheral membrane proteins, anchored to the membrane both by intrinsic hydrophobicity and by lipid modifications of their α and γ subunits. G protein-coupled receptors, which activate G proteins by accelerating the binding of GTP, span the membrane bilayer and present one relatively hydrophilic face to the cytoplasmic surface. GTPase-activating proteins (GAPs),[1] which accelerate GTP hydrolysis and consequent deactivation, must also operate in this interfacial environment. G protein GAPs include at least two families of proteins, the phosopholipase C-βs and the RGS proteins (including the RGS-related proteins such as p115 RhoGEF and G protein-coupled receptor kinases) (1). Each group displays different modes of membrane binding. Phospholipase C-β, which is both a G_q-stimulated signaling protein and a G_q GAP, is soluble in aqueous solution but binds to membranes by nonspecific ionic interactions, with contribution from a plekstrin homology domain that can bind to phosphatidylinositol 3,4-bisphosphate (2). The receptor kinases are also soluble and apparently do not bind tightly to phospholipid bilayers but rather are recruited by a combination of palmitoylation and by binding both their receptor substrates and G$\beta\gamma$ subunits (see Ref. 3 for review).

The membrane binding behavior of RGS proteins is more diverse. Although RGS proteins do not contain obvious membrane-spanning domains, many are tightly membrane-associated and behave as hydrophobic molecules. At the extreme, RGSZ1[2] is tightly membrane-bound and can be extracted from natural membranes under non-denaturing conditions only using high concentrations of Triton X-100 at elevated temperatures (4). Members of the RGSZ family (GAIP, RGSZ1, RGSZ2, Ret-RGS) also contain cysteine strings that are probable sites for multiple palmitoylation (5), but RGSZ1 expressed in *Escherichia coli* is hydrophobic and aggregates in the absence of detergent even after exposure to 20 mM dithiothreitol, which removes thioesterified palmitate (6, 7). RGSZ1 tends to form oligomers even on SDS-polyacrylamide gels. At the other extreme, RGS10 is a soluble protein found in the cytoplasm (7, but see Ref. 8). RGS3, RGS4, and RGS16 display intermediate behavior. They are found both in cytoplasm and in membrane fractions, their localization is influenced by reversible palmitoylation at two or more sites, and their N-terminal sequences can form an amphipathic helix that directs binding to anionic lipid surfaces (7, 9–11). In addition to this behavioral heterogeneity, the specific binding sites for individual RGS proteins on membranes (lipids, Gα or G$\beta\gamma$ subunits, receptors, or other proteins) remain unknown in most cases.

The GAP activities of RGS proteins are markedly influenced by their interactions with membranes and, presumably, by the ordering influence that membrane surfaces provide. GAP activity can be readily and accurately measured in detergent solution under conditions where substoichiometric GAP promotes hydrolysis of preformed Gα-GTP complexes (12). However, low GAP activities are more sensitively measured by monitoring stimulation of steady-state GTPase activity in reconstituted phospholipid vesicles that contain trimeric G protein and an appropriate agonist-bound receptor (12). The enhanced GAP activity observed in this system may reflect interaction of the GAP with the lipid bilayer. Removal of the N-terminal region of RGS4 diminishes its activity in the membrane-based assay but not in detergent solution (9, 10, 13).

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[1] The abbreviations used are: GAP, GTPase-activating protein; RGS, regulator of G protein signaling; AChR, muscarinic acetylcholine receptor; PS, phosphatidylserine; PE, phosphatidylethanolamine; CHS, cholesteryl hemisuccinate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

[2] Ret-RGS (31) and RGSZ1 (6) are products of alternatively spliced mRNAs derived from the RGS20 gene (S. A. Barker, J. Wang, D. A. Sierra, and E. M. Ross, submitted for publication). Ret-RGS has an extended N-terminal domain predicted to contain a membrane-spanning segment (31).

RGSZ1, which is significantly more hydrophobic than RGS4, does not incorporate into membranes from solution and is therefore essentially inactive in the membrane-based assay unless it is incorporated into the vesicle bilayer during the reconstitution process (6).

We report here that RGS protein must be able to bind membranes with correct orientation to display optimal GAP activity. In the case of RGS4, binding to the bilayer from solution and reorientation are multiphasic phenomena that become essentially irreversible over a few minutes but that goes to completion over at least 60 min at 30 °C. The low intrinsic GAP activity of RGS10 in the vesicle-based assay can be markedly enhanced by the addition of the N-terminal amphipathic domain from RGS4 while maintaining the Gα selectivity characteristic of RGS10.

EXPERIMENTAL PROCEDURES

Materials—All RGS proteins, mutant and wild-type, were purified from *E. coli* as described (6). Gα_z (14), Gα_q (14, 15), G$\beta_1\gamma_2$ (14, 15), m2AchR, and m1AchR (16) were purified from Sf9 cells. Myristoylated Gα_{i1} was purified from *E. coli* (17). Brain PS and liver PE were purchased from Avanti Polar Lipids and were used without further purification. Phosphatidylglycerol, phosphatidic acid, CHS, dioleoylphosphatidylcholine, and L-α -dimyristoylphosphatidylcholine were purchased from Sigma. [γ-^{32}P]GTP was synthesized and purified as described (15).

cDNA Constructs—cDNA constructs used for expression of wild-type RGS proteins (6), for the RGS box domains of RGS10 and RGS4 (18), and for RGS4ΔN57 (7) have been described. cDNAs used for expression of other truncated and chimeric RGS proteins were constructed in pQE60 (Qiagen) by polymerase chain reaction using as templates the cDNAs for human RGSZ1 (6), rat RGS4 (19), and human RGS10 (18). Sequences of the chimeras are as follows: RGS10:4, (MGHHHHHHG)-(RGS10 ^{1}M–S^{26})-(MG)-(RGS4 ^{58}K–A^{205}); RGS4:10, (MGHHHHHHG)-(RGS4 ^{1}M–S^{53})-(G)-(RGS10 ^{26}S–T^{154}); RGSZ1ΔN82, (MGHHHHHHG)-(RGSZ1 ^{83}E–A^{217}); RGS4:Z1, (MGHHHHHHG)-(RGS4 ^{1}M–S^{53})-(GS)-(RGSZ1 ^{83}E–A^{217}); RGSZ1:4, (MGHHHHHHG)-(RGSZ1 ^{1}M–E^{83})-(DMG)-(RGS4 ^{58}K–A^{205}).

Receptor-G Protein Vesicles and Protein-free Liposomes—Heterotrimeric G proteins and m2AChR, with or without added GAPs, were reconstituted in to phospholipid vesicles essentially as described previously (15, 16). Lipids (25 μg; PE:PS:CHS, 55:35:10) were suspended in 25 μl of 20 mM NaHepes (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.2% sodium deoxycholate, 0.02% sodium cholate and sonicated until translucent. The suspension was then mixed with G protein, m2AChR, and GAP (when used) to yield a final volume of 50 μl. This mixture was then chromatographed on a 3 × 150-mm column of Sephacryl AcA34 in the resuspension buffer, but without detergent. The usual fraction volume was 75 μl.

To prepare small, unilamellar vesicles, lipids were dissolved in chloroform (10 mg/ml) at an appropriate molar ratio. Solvent was evaporated under argon to form a thin lipid film, and buffer A (50 mM NaHepes (pH 8.0), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) was added to yield the desired concentration. The lipid dispersion was vortexed and then sonicated under argon at room temperature for 30 min until an almost clear solution was obtained. Liposomes were analyzed by gel filtration under the same conditions used to prepare protein-lipid vesicles.

Fluorescence Measurements—RGS4 or RGS10 (0.5 μM) was diluted in 300 μl of 50 mM NaHepes (pH 7.5) plus 1 mM dithiothreitol in the presence or absence of 100 μM sonicated lipid vesicles at 25 °C. After 10 min of incubation, fluorescence was recorded either at 328 nm or between 305 and 405 nm with excitation at 285 nm in a Hitachi F-2000 fluorescence spectrophotometer.

GAP Assays—GAP activity was assayed in two formats. In "single turnover" assays, Gα is first bound to [γ-^{32}P]GTP, and the rate of hydrolysis of [γ-^{32}P]GTP-Gα is measured in a solution that contains 25 mM NaHepes (pH 7.5), 1 mM EDTA, 6.8 mM $MgCl_2$ (1 mM free Mg^{2+}; see Ref. 6), 1 mM dithiothreitol, 0.1% Triton X-100, 5 mM GTP (12). Activity is calculated as the increase in the rate constant for hydrolysis of the bound [γ-^{32}P]GTP. Single turnover assays, using either 1.5 nM [γ-^{32}P]GTP-Gα_z at 15 °C or 10 nM Gα_{i1} at 8 °C, were performed as described (12). A more sensitive and presumably more physiological assay for GAP activity monitors the enhancement of agonist-stimulated, steady-state GTPase activity in proteoliposomes reconstituted with receptor and heterotrimeric G proteins. Reconstitution of purified m2AChR with either G_z or G_i and of m1AChR with G_q were performed as described (6, 12, 15). RGS proteins were either coreconstituted into the vesicles by mixing with the detergent-dispersed receptor, G protein, and lipid prior to formation of the vesicles or added to the vesicles in detergent-free solution. Added RGS proteins were incubated with the vesicles for 1 h at 30 °C prior to assay unless otherwise indicated. Steady-state assays were carried out at 30 °C for 5–10 min. Data are given as increases in the steady-state GTPase activity. Interpretation of GAP assay data has been discussed elsewhere (12).

TABLE I
GAP activities measured in detergent solution and in phospholipid vesicles

The GAP activity of each RGS protein was measured in detergent solution ("micelle") in a single-turnover assay according to the increase in the rate of hydrolysis of Gα-[γ-^{32}P]GTP ("Experimental Procedures"). Assays were performed at 15 °C for Gα_z-GTP (1.5 nM) or 8 °C for Gα_{i1}-GTP (10 nM). GAP activity is expressed as an increase in the hydrolytic rate constant (min^{-1}) per pmol of RGS protein. GAP activity was also measured according to the stimulation of steady-state GTPase activity of phospholipid vesicles that contained m2AChR and trimeric G protein ("vesicle"). RGS proteins were added to the vesicles just before initiation of the assay. Steady-state GAP activity, measured at 30 °C, is expressed as the increase in the GTPase turnover number (min^{-1}) per pmol of RGS protein. Turnover numbers for m2-G_z vesicles were 0.11 and 1.3 in the absence and presence, respectively, of 10 nM RGS4 vesicles and 1.0 and 8.7 for m2-G_i vesicles in the absence or presence, respectively, of 5 nM RGS4. Concentrations of GAIP and RGSZ1 were varied between 2 and 16 μM to obtain quantifiable stimulation. Vesicles used in these assays contained 1 nM G_z and 0.26 nM m2AChR or 1 nM G_i and 0.25 nM m2AChR. Note that GAP activities for G_i and G_z cannot be compared directly because the intrinsic GTPase activities of the two proteins (and their interactions with receptor) vary markedly. Interpretation of these assays has been discussed elsewhere (12).

	Medium	GAP activity			
		RGS4	RGS10	GAIP	RGSZ1
		min^{-1}			
G_z	Micelle	1.28	0.2	4.8	7.7
	Vesicle	2.0	0.004	<0.02	0.005
G_i	Micelle	3.3	1.4	0.46	0.15
	Vesicle	26	0.1	0.04	0.005

RESULTS

Several experimental findings initially suggested that the GAP activity of RGS proteins depends on the extent and mode with which they bind to phospholipid membranes. First, when RGSZ1 was assayed for its ability to increase agonist-stimulated, steady-state GTPase activity in receptor-G protein proteoliposomes, it was essentially inactive when added to the vesicles in buffer; it was ~200-fold more active when inserted into the nascent bilayer along with G protein and receptor during formation of the vesicles (Ref. 6 and Table I). GAIP, a close homolog of RGSZ1, behaved similarly (data not shown). In contrast, RGS4 and phospholipase C-β, a non-RGS GAP, are active when added from solution (6, 15). A third pattern was displayed by RGS10. It was relatively ineffective in the vesicle-based assay either when added from solution or when coreconstituted with the other proteins and lipids, even though its GAP activity with soluble Gα_i-GTP substrate is not much different from that of the other two RGS proteins (Fig. 1, Table I).

One explanation for the low activity of RGS10 in the vesicle-based assay is that it neither incorporates into nor binds tightly to phospholipid vesicles. To test this idea, stable incorporation of RGS proteins was monitored during preparation of receptor-G protein vesicles by gel filtration. When RGS4 and RGS10 were mixed with receptor, G protein, and phospholipids prior to reconstitution, only about 5% of the RGS10 eluted with the vesicle peak (Fig. 1). In contrast, about 80% of RGS4 coeluted with the vesicle fraction. RGSZ1 and GAIP also coeluted with

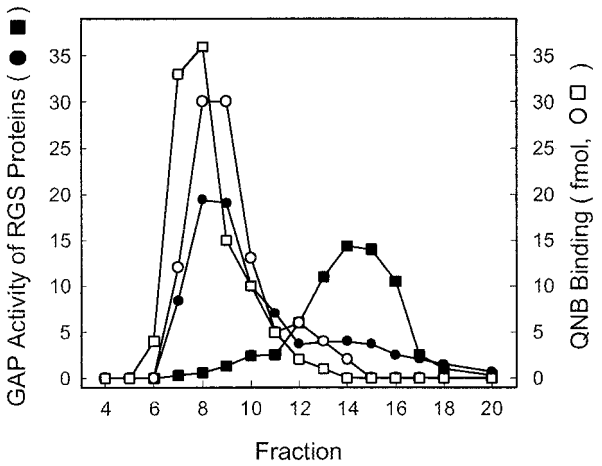


FIG. 1. **Coreconstitution of RGS4 or RGS10 with m2AChR and G_i.** G_i (40 pmol) and m2AChR (8 pmol) and either RGS4 (100 pmol; ■, ●) or RGS10 (280 pmol; ○, ■) were mixed with lipids and subjected to the standard reconstitution protocol ("Experimental Procedures"). A similar batch of vesicles was prepared without any RGS protein. Fractions were assayed either for m2AChR (□, ○) or for G_z GAP activity in a solution-phase single turnover assay (■, ●). The pooled vesicle peak (fractions 7–9, 250 μl) contained 28% of loaded RGS4 (110 nM; underestimated because anionic lipid inhibits solution-phase GAP activity) and 4% of loaded RGS10 (48 nM) according to solution-phase G_z GAP assay. The concentrations of G_i, determined by [^{35}S]GTPγS binding, were as follows: RGS4 vesicles, 11.4 nM; RGS10 vesicles, 13.2 nM; GAP-free vesicles, 12.0 nM. The concentrations of m2AChR, determined by [^{3}H]quinuclidinylbenzilate (QNB) binding (16), were as follows: RGS4 vesicles, 2.5 nM; RGS10 vesicles, 2.9 nM; GAP-free vesicles, 2.8 nM. Carbachol-stimulated GTPase activities of the vesicles (fmol of GTP hydrolyzed/min/5 μl of vesicles) were as follows: RGS4, 1370; RGS10, 190; no GAP, 60.

the vesicles in such experiments (Ref. 6 and data not shown), although their tendency to aggregate in the absence of lipid precludes interpretation of their behavior on gel filtration.

Coreconstitution experiments do not indicate whether RGS4 binds to phospholipids or to the receptor and G protein in the vesicles. As shown in Fig. 2*A*, RGS4 spontaneously binds to protein-free phospholipid vesicles when added after their formation (see also Ref. 9). Association is stable over the time course of gel filtration, suggesting that the affinity of RGS4 for phospholipids is high. In contrast, RGS10 showed no evidence of stable binding to preformed phospholipid vesicles (Fig. 2). These data support the idea that the major determinant of the low GAP activity of RGS10 in vesicle-based assays is its inability to bind to the vesicle surface. This idea is supported by our previous observation that palmitoylation of RGS10 at Cys^{66} markedly increases its GAP activity in vesicle-based assays (7) and allows it to bind to phospholipid vesicles with about the same efficiency as seen for RGS4 (data not shown). The importance of membrane binding is emphasized by the fact that palmitoylation of RGS10 at Cys^{66} increases net GAP activity in the vesicle-based assay even though it inhibits the intrinsic GAP activity of the RGS10 protein (7).

Although the binding of RGS4 to lipid vesicles appears to be necessary for efficient GAP activity, a second and slower event is apparently also required. When RGS4 is added to receptor-G protein vesicles before a steady-state GTPase assay, some GAP activity is displayed immediately, but prolonged incubation of RGS4 with vesicles before assay further increases GAP activity up to 3-fold (Fig. 3). The rate of activation depends on temperature. Full activation required several hours at 30 °C (Fig. 3) but was not complete even after 10 h at 20 °C (data not shown). The extent of the increase in GAP activity was not altered by temperature, however. Slow activation of GAP activity is not limited to G_i. A similar slow increase in GAP activity of RGS4

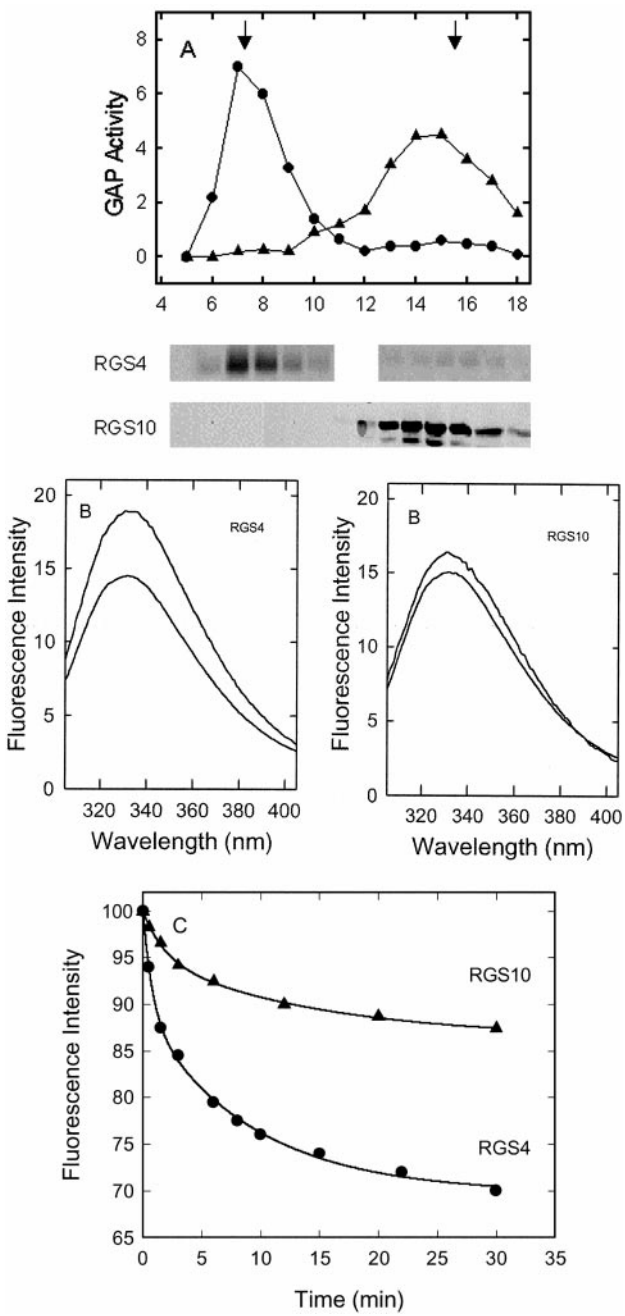


FIG. 2. **Binding of RGS4 and RGS10 to lipid vesicles.** *A*, vesicles (PE:PS:CHS, 55:35:10) were prepared as described under "Experimental Procedures." RGS4 (3 μM; ●) or RGS10 (8 μM; ▲) was mixed with vesicles (0.5 or 1.5 mM total lipid), and the mixture (50 μl) was analyzed by gel filtration chromatography on Sephacryl AcA34 in 20 mM NaHepes (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 1 mM $MgCl_2$. Fractions (75 μl) were analyzed for GAP activity in a solution-based, single turnover assay and for the presence of RGS protein by SDS-gel electrophoresis followed by protein staining. *B*, RGS protein (500 nM) was incubated with (*lower spectra*) or without (*upper spectra*) PS liposomes (100 μM) in 50 mM NaHepes (pH 7.5) and 1 mM dithiothreitol for 10 min at 25 °C. Fluorescence emission spectra were recorded at room temperature with excitation at 285 nm. *C*, RGS4 or RGS10 (0.5 μM) were mixed with a sonicated dispersion of PS (100 μM) in 50 mM NaHepes (pH 7.5), 1 mM dithiothreitol at 25 °C. Tryptophan fluorescence at 328 nm was measured at the times shown. Data were fit to a biexponential decay equation (*solid lines*). For RGS4, the fast component accounted for 37% of the quenching (k_1 = 1.3 min^{-1}; k_2 = 0.11 min^{-1}).

was observed with vesicles that contained G_z. The activation process was not influenced by the presence of agonist. The increased in the GAP activity of RGS4 was accelerated by N-terminal palmitoylation, measured using the C95V mutant

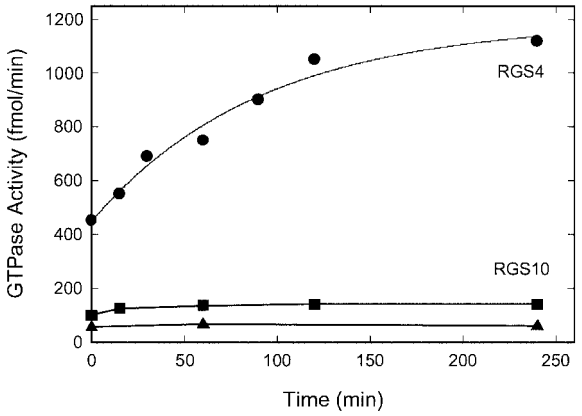


FIG. 3. **Potentiation of GAP activity after vesicle binding.** m2AChR-G_i vesicles (4 μM total lipid) incubated at 30 °C with either RGS4 (●; 20 nM) or RGS10 (■; 4 μM) or without a GAP (▲) in GTPase assay buffer that included 2 mM carbachol but no GTP. At the times shown, 30-μl aliquots were mixed with 30 μl of 4 μM [γ-^{32}P]GTP in complete assay buffer and incubated at 30 °C for 10 min. Separate experiments indicated that the time course of increase in GAP activity is not altered by carbachol.

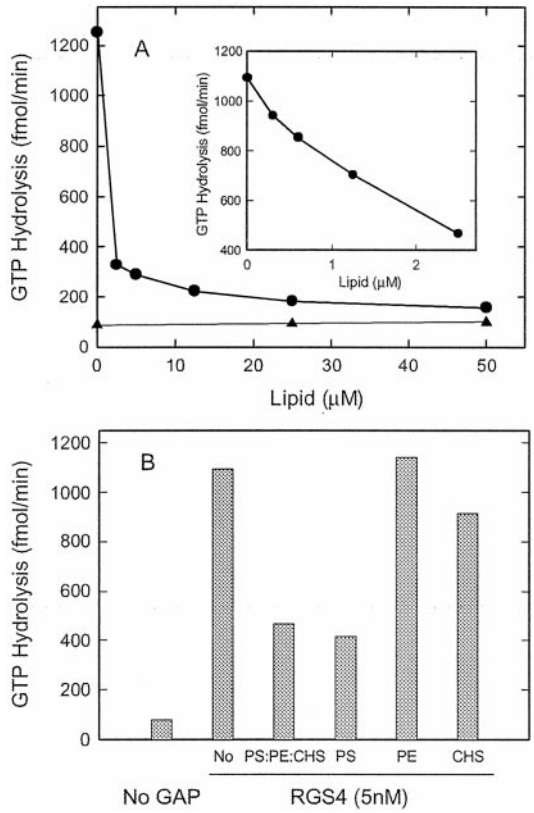


FIG. 4. **Negatively charged liposomes inhibit RGS4 interaction with m2AChR-G_i vesicles.** *A*, m2AChR-G_i vesicles (PE:PS:CHS, 55:35:10; 2 μM final lipid concentration) were mixed with increasing amounts of protein-free liposomes having the same lipid composition. RGS4 (5 nM) was then added, and the mixture was incubated at 30 °C for 60 min. Carbachol-stimulated GTPase activity was assayed as described under "Experimental Procedures." The *inset* shows a separate but similar experiment that covered a lower range of liposome concentrations. *B*, m2AChR-G_i vesicles (2 μM) were mixed with vesicles composed of PS or PE:PS:CHS (55:35:10), both at 2 μM, or similarly prepared dispersions of PE or CHS alone (5 μM each). RGS4 (5 nM) was added, and GTPase activity was measured after 60 min at 30 °C.

to avoid inhibiting GAP activity (see Ref. 7). Palmitoylated C95V RGS4 displayed initially high GAP activity when assayed with m2AChR-G_i vesicles, and activity did not increase with prolonged incubation. RGS10 did not display a time-dependent increase in GAP activity even when added at a high enough concentration to produce an easily measured effect (Fig. 3).

Functional activation of RGS4 upon vesicle binding is preceded by a slow change in either its conformation or orientation, as determined by quenching of its intrinsic tryptophan fluorescence (Fig. 2, *B* and *C*). Phospholipid binding causes ~30% quenching in the fluorescence of RGS4 and a slight blue shift in the emission maximum. This process is biphasic; about 40% of the observed quenching takes place rapidly ($t_{1/2} \sim 30$ s at room temperature), and the remainder occurs over about 30 min (Fig. 2*C*). We do not know the physical basis of the observed quenching, which would not be predicted if the two tryptophan residues in RGS4 were simply inserted into the hydrophobic environment of the membrane. Both tryptophan residues, Trp^{59} and Trp^{92}, are in the conserved RGS GAP domain, and neither faces the exterior of the molecule (23). Trp^{59} is almost completely buried among helices 1, 2, 3 and 9; Trp^{92} lies in the cleft between the two bundles of helices opposite the site of Gα binding (23). It seems most likely therefore that fluorescence quenching observed upon binding to a phospholipid bilayer reflects a conformational change of the RGS4 protein, probably in the environment of Trp^{92}. The fluorescence of RGS10 was also quenched slightly upon exposure to lipid vesicles (~10%; Fig. 2), probably reflecting the same physical phenomenon observed for RGS4, but with weaker binding.

Based on the results above, we used competition for RGS binding between receptor-G protein vesicles and protein-free liposomes to study the lipid binding behavior of RGS4 in greater detail. Mixing RGS4 with receptor-G protein vesicles and empty vesicles causes net inhibition of GAP activity (Fig. 4). Inhibition apparently reflects competition for RGS4 between the two populations of vesicles, because GAP activity is inhibited half-maximally when the concentration of both vesicles is equal (Fig. 4*A*). Maximal inhibition is essentially complete at high concentrations of added liposomes, as is also consistent with competitive binding of RGS4 to available lipid surfaces. Binding of RGS4 to lipid vesicles depends significantly on the presence of anionic lipids (Fig. 4*B*; see also Ref. 9). Although we have not attempted to analyze the selectivity of RGS4 among phospholipids in any detail, individual or mixed neutral lipids such as PE (or phosphatidylcholine; data not shown) do not by themselves bind RGS4. Phosphatidylglycerol and phosphatidic acid are approximately as effective, as is PS. A dispersion of cholesteryl hemisuccinate alone also does not bind RGS4 tightly (Fig. 4*B*).

As suggested by the gel filtration experiments (Figs. 1 and 2), binding of RGS4 is at best poorly reversible. If RGS4 is first allowed to bind to receptor-G protein vesicles, subsequent addition of liposomes decreased GAP activity only slightly (Fig. 5, *upper curve*). Whereas binding appeared to be complete by 5 min, stabilization of binding was incomplete at 15 s. Adding liposomes at this time produced almost as much inhibition as did mixing all three components simultaneously. In the converse experiment, prior exposure of RGS4 to liposomes before addition of receptor-G protein vesicles increased inhibition over the same 5-min period (Fig. 5, *lower curve*). In the experiments shown in Fig. 5, mixtures of receptor-G protein vesicles and liposomes were held only 1 min before GAP assay, and there would be little opportunity for exchange of RGS4 among vesicles. To test for possible exchange, we also incubated liposomes for up to 60 min with added vesicles that had been pre-equilibrated with RGS4. The extended incubation did not further inhibit GAP-stimulated GTPase activity (data not shown). These data confirm the idea that once RGS4 binds to an anionic lipid bilayer there is an initially freely reversible interaction that is stabilized over the course of a few minutes. This stabi-

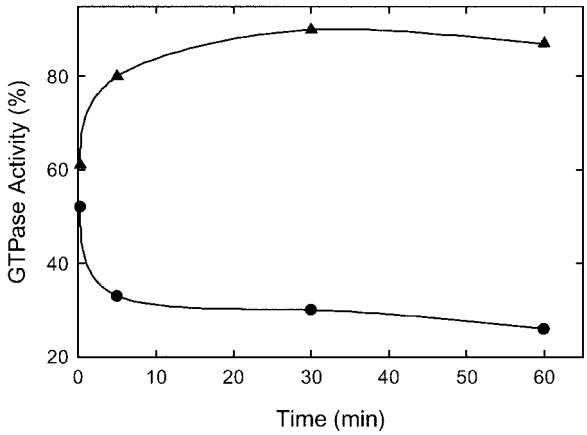
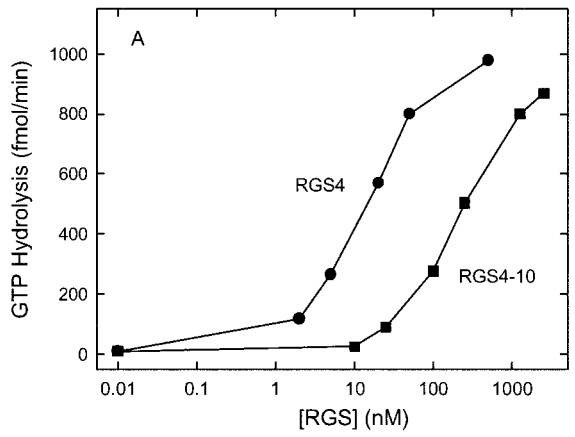


FIG. 5. **Reversibility of RGS4 binding to liposomes or receptor-G protein vesicles.** In one experiment (●), RGS4 was mixed with m2AChR-G_i vesicles (~2 μM lipid) at 30 °C, and protein-free liposomes (2.5 μM lipid) were added at the times shown. After 1 min of further incubation, carbachol-stimulated GTPase activity was assayed over 5 min. Control experiments (data not shown) indicated that prolonged incubation (1–60 min) of liposomes with the vesicles and RGS4 before assay produced no greater inhibition than shown here. In the complimentary experiment (▲), RGS4 was mixed with liposomes at 30 °C, and m2AChR-G_i vesicles were added at the times shown, followed by GTPase assay 1 min later. The initial time points are 15 s, not zero. To correct for the increase in GAP activity as RGS4 is incubated with receptor-G protein vesicles (see Fig. 3), activities are normalized as percentages of activities in similar preparations that were mixed with buffer rather than with liposomes.



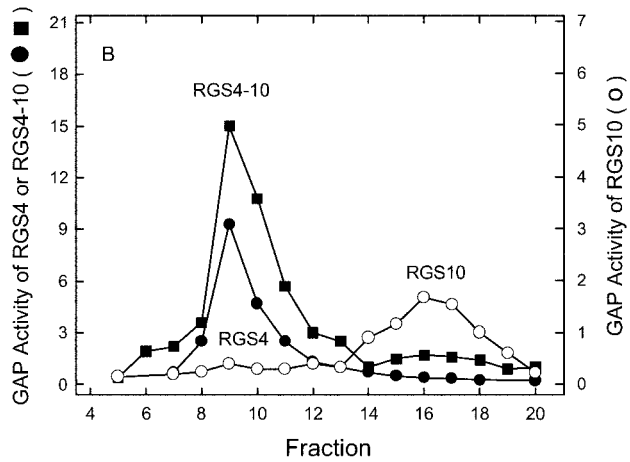


FIG. 6. **The RGS4 N-terminal domain potentiates GAP activity and bilayer binding of RGS10.** *A*, carbachol-stimulated GTPase activity of m1AChR-G_q vesicles was assayed in the presence of increasing concentrations of RGS4 (●) or RGS4:10 (■). Vesicles and RGS protein were pre-incubated for 60 min at 30 °C before assay. For reference, GTPase activity with no added RGS protein was 10 fmol/min, and activity with 16 μM RGS10 was 70 fmol/min. Each assay contained 0.5 nM G_q and 0.085 nM m1AChR. *B*, RGS4, RGS10, or RGS4:10 (1 μM) were incubated with PE:PS:CHS liposomes for 10 min and analyzed by gel filtration on Sephacryl AcA34. Fractions were assayed for G_z GAP activity in a solution-phase, single turnover assay.

lization correlates with the rapid phase of quenching of Trp fluorescence and precedes the conformational or orientational event that increases GAP activity.

The N-terminal region of members of the RGS4 family (RGS1–4, 16) is important for maintaining both correct subcellular localization (7, 9–11) and cellular interactions with receptors and G proteins (13, 24). This region, usually defined as the first 53–57 amino acid residues for RGS4, contains sites for covalent palmitoylation (7, 11) and behaves as an amphipathic α helix (9). To study the contribution of the N-terminal region of RGS4 to GAP activity in vesicle-based assays, we prepared and assayed several truncated and chimeric RGS proteins with distinct N-terminal domains. As shown in Fig. 6, the N-terminal domain of RGS4 is both necessary and sufficient for most of the interactions with membrane lipids described above for RGS4. Replacement of the N-terminal domain of RGS10 with that of RGS4 increased its potency as a G_q GAP by about 1000-fold in the receptor-coupled, vesicle-based assay relative either to RGS10 itself or the RGS10 box. This effect can be accounted for by the ability of the RGS4:10 chimera to associate stably with receptor-G protein vesicles (Fig. 6*B*). RGS4:Z bound to liposomes, as did RGS4 or RGS4:10, and incubation of these chimeras with the vesicles increased GAP activity, as was seen for RGS4 itself (Fig. 3). RGS10:4 did not bind vesicles, and RGSZ:4 aggregated in aqueous solution such that vesicle binding could not be measured (data not shown). Each case mimicked the behavior of the RGS protein that donated the N terminus.

The experiments shown in Fig. 6 were performed with vesicles that contained G_q and m1AChR, instead of m2AChR and G_i or G_z, to demonstrate the generality of the effect of the N-terminal domain and to confirm a brief report that truncation of the N terminus of RGS4 decreases G_q GAP activity (13). However, similar results were obtained with the m2AChR and either G_i or G_z (Table II). Truncation of the N-terminal domain of RGS4 resulted in loss of GAP activity in the vesicle-based, steady-state assay with either G protein as target. Conversely, activity was significantly restored when the N-terminal region of RGS4 replaced that of RGS10, which does not drive bilayer attachment, or that of RGSZ1, which causes aggregation in aqueous buffer. GAP activities of intact RGS10, the isolated RGS domain of either RGS10 or RGSZ1, or an N-terminally truncated RGS4 were all similarly low. For RGS10, which is already relatively inactive in this assay (Fig. 3, Table I), removal of the short N- and C-terminal domains had little effect. Similarly, replacement of the N-terminal domain of RGS4 with that of RGS10 did not support activity in this assay.

To confirm that manipulation of the N termini of the RGS proteins had not inactivated them in some way, we also assayed the GAP activities of the truncated and chimeric proteins in single turnover assays in detergent solution (Table III). As suggested by the work of Popov *et al.* (18), alteration of the RGS4 N-terminal domain in the constructs shown here had little effect on their activities with $G\alpha_i$-[γ-^{32}P]GTP as substrate. Structural determinants of G_z GAP activity were more complex and indicate that the N-terminal regions of RGS4 contribute to interaction with $G\alpha_z$. Full-length RGS4 displayed

TABLE II
GAP activity of truncated and chimeric RGS proteins in m2AChR-G protein vesicle

RGS proteins were added into proteoliposomes that contained m2AChR and either G_z or G_i and incubated for 60 min at 30 °C. Vesicles were then assayed for GTPase activity in the presence of 1 mM carbachol. Because activities varied dramatically, different concentrations of RGS proteins were used, and relative GAP activity is shown as the GTPase molar turnover number divided by the concentration of RGS protein in the assay (20 nM for RGS4, 100 nM for RGS4:10 and RGS4:Z1, 2 μM for others). Vesicles contained 0.8–1.0 nM Gz and 0.25–0.33 nM m2AChR or 1 nM G_i and 0.29 nM m2AChR. Data shown are averages from two separate experiments performed using a single batch of G_i vesicles and two separate batches of G_z vesicles.

	Steady-state GAP activity	
	m2-G_z	m2-G_i
	min^{-1} μM^{-1}	
RGS4	205	1450
RGS4ΔN57	0.6	4.9
RGS10	0.15	1.3
RGS10Box	0.13	1.4
RGS10:4	0.15	3.9
RGS4:10	30	191
RGSZ1	0.23	1.1
RGSZ1Δ82	0.18	1.0
RGS4:Z1	34	24
RGSZ1:4	0.24	7.4

TABLE III
GAP activity of truncated and chimeric RGS proteins in detergent solution

GAP activity was measured in a single-turnover assay in detergent solution using as substrate either $G\alpha_z$-[γ-^{32}P]GTP (1.5 nM; 15 °C) or $G\alpha_i$-[γ-^{32}P]GTP (7–10 nM; 8 °C). Data are shown as increments in the first-order rate constant for hydrolysis per pmol of RGS protein.

	GAP activity	
	GTP-α_z	GTP-α_i
	min^{-1} $pmol^{-1}$	
RGS4	1.2	3.3
RGS4ΔN57	0.048	2.7
RGS10	0.18	1.4
RGS10Box	0.12	1.7
RGS10:4	0.048	2.5
RGS4:10	3.4	2.9
RGSZ1	7.0	0.21
RGSZ1ΔN82	6.0	0.14
RGS4:Z1	4.8	0.26
RGSZ1:4	0.072	1.8

far more GAP activity toward $G\alpha_z$-GTP than did RGS4ΔN57 (or RGS10), indicating the importance of the N-terminal region in addition to the RGS domain. The RGS4 N-terminal domain thus contributes to interaction with G_z, as is true for G_q (13). Consistent with this positive role, the RGS4 N-terminal domain in the RGS4:10 chimera potentiated the $G\alpha_z$ GAP activity of RGS10, which has slight activity with or without its N-terminal region. The RGS4:10 construct was reproducibly more active than RGS4. Replacement of the N-terminal domain of RGSZ1 with that of RGS4 had relatively little effect. Indeed, RGSZ1ΔN82, which lacks the entire N-terminal region up to the RGS, retained substantial activity in the solution-based assay. Thus, some interaction of RGS proteins with $G\alpha_z$ is contributed by the N-terminal domain as well as by the RGS box. This situation is qualitatively similar to that described for $G\alpha_q$, where removal of the N-terminal region of RGS4 also decreased activity in solution (13).

DISCUSSION

To modulate a membrane-bound G protein signaling pathway, an RGS protein must act at the surface of a cellular membrane, but patterns of membrane attachment vary widely among RGS proteins. Some RGS proteins are hydrophobic and essentially integral membrane proteins (6). For others, such as RGS10, membrane binding has been hard to demonstrate convincingly except when the protein is palmitoylated (7). RGS4 also behaves as a soluble protein when purified but clearly binds to cellular membranes in a process that is dependent on its N-terminal domain and that is apparently driven by direct binding to anionic lipids (Ref. 9 and Fig. 4*B*). Although RGS4 is also palmitoylated, palmitoylation is not required for its binding to natural membranes or phospholipid bilayers (6, 7, 9, 11).

RGS4 binds tightly to receptor-G protein vesicles or to protein-free liposomes such that, after a few minutes, binding is essentially irreversible. Vesicle binding is not appreciably influenced by receptor or G protein, because receptor-G protein vesicles and protein-free liposomes bind RGS4 at similar rates (Fig. 5) and compete equally for limiting RGS4 (Fig. 4). The interaction of RGS4 with membranes consists of several steps that occur after initial contact. Quenching of Trp fluorescence is observed essentially immediately upon mixing RGS4 with liposomes and occurs with a $t_{1/2}$ of about 30 s at room temperature. Additional quenching occurs more slowly and roughly correlates with the loss of reversibility of binding. Onset of irreversibility of binding was measured as the ability of protein-free liposomes to sequester RGS4 and thus inhibit its interaction with subsequently added receptor-G protein vesicles. The coelution of RGS4 with lipid vesicles during gel filtration also supports the idea that binding becomes poorly reversible.

The most interesting of these slow phenomena is the time- and temperature-dependent increase in GAP activity that is observed after initial binding of RGS4 to receptor-G protein vesicles. Increased GAP activity kinetically follows both irreversible binding and the initial quenching of Trp fluorescence (Fig. 2*C*). Activation is apparently dependent on these prior events, because N-terminal palmitoylation increases the rates of onset of all the binding-related phenomena described. Slow activation may result from a change in the orientation of RGS4 with respect to the bilayer or from a change in its conformation. Either effect could be consistent with the slow increase in fluorescence quenching. Enhanced GAP activity might also result from slow association with receptor or G protein. In the latter case, binding could be either to $G\alpha$ or $G\beta\gamma$. $G\beta\gamma$ interacts functionally with RGS proteins (6, 25), and they appear to bind directly to each other (26).[3] We consider this possibility less likely, because the activation process remains even when high concentrations of RGS4 are added to the vesicles. For this same reason, slow activation does not reflect redistribution of RGS4 among vesicles or from an aggregated state to vesicles.

Essentially all of the membrane association behaviors described here for RGS4 are dependent on the contribution to net lipid binding of the N-terminal region. This region binds directly to anionic lipid bilayers, apparently as an amphipathic α helix (9), and contains sites for palmitoylation that further increase hydrophobicity (7, 11). Based on structural similarity and the behavior of RGS16 (10), this lipid binding function of the N-terminal domain is probably conserved through the RGS4 subfamily. This domain appears to function autonomously for lipid binding (9) and functions independently of the RGS domain to which it is attached. The RGS4:10 and RGS4:Z chimeras also bound to lipid vesicles, became irreversibly bound, and underwent slow activation in a manner similar to that of RGS4 (data not shown). The RGS4 N terminus also functioned in the context of the m2AChR and either G_i or G_z or of the m1AChR and G_q. Taken together, these data suggest that the N-terminal region of RGS4 acts primarily to support

[3] Y. Tu, unpublished observation.

adsorption to the bilayer, the first step in positioning a GAP for regulating the receptor-stimulated, steady-state GTPase reaction. The N-terminal domain may also be involved with subsequent reorientation of RGS4, but such an effect cannot readily be distinguished.

Independent of its effect on lipid binding, the RGS4 N-terminal domain also contributes significantly to its GAP activity with $G\alpha_z$ and $G\alpha_q$, although not with $G\alpha_i$. Removal of the N terminus does not interfere with G_i GAP activity (Ref. 18; confirmed in Table III), but N-terminal truncation markedly reduces activity with $G\alpha_z$ (Table III) and $G\alpha_q$ (13). This probably reflects direct interaction between the N terminus and $G\alpha_z$ or $G\alpha_q$, because addition of the RGS4 N-terminal domain to N-terminally truncated RGS10 yielded a chimeric protein, RGS4:10, that displayed about 20-fold more G_z GAP activity than did intact RGS10.

The data presented here point to the complexity of the process whereby RGS proteins must become oriented in their membrane environment to display optimal GAP activity in a receptor-coupled system. They are consistent with previous results that indicated that the amphipathic and cationic RGS4 N terminus and its palmitoylation are important to the energetics of membrane binding (Ref. 9; see also Refs. 27 and 28) but argue that proper orientation or alignment with receptor or G protein subunits or both is a necessary subsequent step. This conclusion agrees with the idea that relatively nonspecific hydrophobic or ionic interactions are frequently the major energetic components of binding of peripheral membrane proteins and that protein-protein binding is needed for specificity (27). The energetic contribution of lipid binding to affinity may in part explain the very high affinities of binding of RGS proteins to multiple partners recently described by Dowal *et al*. (26). We still need to understand how peripheral domains of RGS proteins contribute to these specific interactions, and the ability of the RGS4 core domain to distinguish G_i from G_z or G_q may help us answer the question. The N-terminal regions may be generally used by RGS proteins to determine the mode of membrane attachment, as seems true for the RGS4 and RGSZ families. In addition, though, the presence of diverse N-terminal functional domains in RGS proteins indicates that membrane binding is only one of many roles for this region (1, 29, 30). The present work also leads to the next question of how RGS proteins, which are naturally expressed at levels below those of their G targets, are themselves directed to the specialized sites of receptor-G protein signaling.

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