Recruitment of RGS2 and RGS4 to the Plasma Membrane by G Proteins and Receptors Reflects Functional Interactions

ANJU ANNE ROY, KARA E. LEMBERG, and PETER CHIDIAC

Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada

Received October 15, 2002; accepted May 28, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

N-terminally green fluorescent protein (GFP)-tagged regulator of G protein signaling (RGS) 2 and RGS4 fusion proteins expressed in human embryonic kidney 293 cells localized to the nucleus and cytosol, respectively. They were selectively recruited to the plasma membrane by G proteins and correspondingly by receptors that activate those G proteins: GFP-RGS2 when coexpressed with $G\alpha s$, β_2-adrenergic receptor, $G\alpha q$, or AT_{1A} angiotensin II receptor, and GFP-RGS4 when coexpressed with $G\alpha i2$ or M_2 muscarinic receptor. G protein mutants with reduced RGS affinity did not produce this effect, implying that the recruitment involves direct binding to G proteins and is independent of downstream signaling events. Neither agonists nor inverse agonists altered receptor-promoted RGS association with the plasma membrane, and expressing either constitutively activated or poorly activated G protein mutants produced effects similar to those of their wild-type counterparts. Thus, intracellular interactions between these proteins seem to be relatively stable and insensitive to the activation state of the G protein, in contrast to the transient increases in RGS-G protein association known to be caused by G protein activation in solution-based assays. G protein effects on RGS localization were mirrored by RGS effects on G protein function. RGS4 was more potent than RGS2 in promoting steady-state Gi GTPase activity, whereas RGS2 inhibited Gs-dependent increases in intracellular cAMP, suggesting that G protein signaling in cells is regulated by the selective recruitment of RGS proteins to the plasma membrane.

The most prevalent mechanism of intercellular communication in eukaryotic organisms involves the activation of heterotrimeric G proteins by receptors that recognize chemical signals at the cell surface. Receptors turn on G proteins by promoting the binding of the activating nucleotide GTP. G proteins are deactivated by the hydrolysis of GTP to GDP plus inorganic phosphate, a process accelerated by GTPase activating proteins (GAPs), such as the regulator of G protein signaling (RGS) proteins.

RGS-G protein interactions have been detected by a variety of methods, including yeast two-hybrid screening (De Vries et al., 1996) and coimmunoprecipitation (Druey et al., 1998; Tseng and Zhang, 1998). In solution, RGS proteins are able to bind to free GDP-bound $G\alpha$ subunits, and this binding is increased in the presence of fluoride, aluminum, and magnesium ions, which together promote a G protein conformation that occurs immediately before GTP hydrolysis (De Vries et al., 1996). It is unclear, however, to what degree G protein

activation states influence their binding to RGS proteins in vivo (Druey et al., 1998) or how stable RGS protein-G protein association might be over the course of multiple cycles of GTP binding and hydrolysis (Wilkie and Ross, 2000; Ko et al., 2001).

The binding of RGS proteins to G proteins in solution for the most part corresponds to their observed GAP effects, although RGS2 binds to $G\alpha s$-GDP in a fluoroaluminate-sensitive manner (Ko et al., 2001) with no apparent effect on GTP hydrolysis (Ingi et al., 1998). Notwithstanding this lack of GAP activity, RGS2 has been observed to impede Gs signaling, as evidenced by decreases in intracellular cAMP levels (Tseng and Zhang, 1998). Similarly, the affinity of the RGS domain of G protein-coupled receptor kinase 2 (GRK2) for $G\alpha q$-GDP is greatly enhanced by AlF_4^-, and GRK2 inhibits phospholipase $C\beta$ activation by free $G\alpha q$ but has no apparent effect on the GTPase activity of the latter (Carman et al., 1999). The attenuation of G protein signals by RGS proteins in a manner independent of (Hepler et al., 1997) or only partly dependent on GAP activity (Derrien and Druey, 2001) has also been observed in other systems and is thought to reflect the functional inhibition by RGS proteins of G protein-effector coupling. There is also some evidence, however, that

ABBREVIATIONS: GAP, GTPase-activating protein; RGS, regulator of G protein signaling; HEK, human embryonic kidney; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; M_2R, M_2 muscarinic acetylcholine receptor; $AT_{1A}R$, angiotensin II type 1A receptor; β_2AR, β_2-adrenergic receptor.

RGS proteins may directly interact with effector proteins (Dowal et al., 2001; Sinnarajah et al., 2001), suggesting other possible mechanisms.

In whole cells, RGS proteins variously have been found to localize to the plasma membrane, cytosol, nucleus, and Golgi (De Vries et al., 2000). RGS proteins must have access to the inner face of the plasma membrane to interact with receptor-activated G proteins, but the mechanism(s) of and need for plasma membrane association are uncertain. Whereas evidence from studies on *Saccharomyces cerevisiae* suggests that plasma membrane localization is essential for RGS proteins to affect receptor-stimulated G protein signals (Srinivasa et al., 1998), the ability of the *Schizosaccharomyces pombe* protein Rgs1 to decrease pheromone signaling requires an intact RGS domain but does not require visible membrane localization (Pereira and Jones, 2001).

Because RGS proteins can act catalytically (Berman and Gilman, 1998), stable plasma membrane association might not be necessary for intracellular GAP activity. On the other hand, however, there is increasing evidence that RGS proteins can form signaling complexes with other proteins (Sierra et al., 2000), and RGS GAP activity toward G proteins reconstituted with receptors into proteoliposomes is increased by RGS-proteoliposome association (Tu et al., 2001). RGS proteins may attach to the inner surface of the plasma membrane through interactions with membrane phospholipids (Ishii et al., 2002), membrane-bound proteins (Hollinger and Hepler, 2002), or both.

We investigated the effects of wild type G proteins and receptors on the intracellular localization of GFP-tagged RGS2 and RGS4. Here we show that GFP-RGS fusion proteins are recruited to the plasma membrane by G proteins to which they bind with relatively high affinity. When receptors that activate each G protein were cotransfected in place of the G proteins, corresponding patterns of GFP-RGS relocation were observed. G proteins that interacted weakly with each RGS protein did not appreciably promote membrane association. The most plausible explanation for RGS protein localization to the plasma membrane is that they bind to G proteins there and that binding is enhanced when these G proteins are increased in number or coupled to an appropriate receptor.

Materials and Methods

Plasmid and Baculovirus Constructs. pCDNA1 vectors encoding rGαs WT (Levis and Bourne, 1992), mGαi2 WT, mGαi2-Q205L (Wong et al., 1991), and mGαq WT (Wedegaertner et al., 1993), were purchased from ATCC, deposited by Paul Herzmark and Henry R Bourne. Other plasmids encoding mutant G proteins were kindly provided by John Hepler (Emory University, Atlanta, GA; Gαq-Q209L, Gαq-R183C), Roger Sunahara (University of Michigan, Ann Arbor, MI; Gαi1-R178C), Paul Albert (University of Ottawa, Ottawa, ON, Canada; hGαi2-Q205L-G184S, hGαq-Q209L-G188S) and Catherine Berlot (Geisinger Clinic, Danville, PA; rGαs-α3β5/G226A/A366S), or were supplied by the Guthrie Institute (Sayre, PA; hGαs-Q227L/D295N, hGαi2-Q205L/D273N, hGαq-Q209L/D277N). H6-rRGS4/pQE60 and RGS2.H10 pET 19b were kindly provided by John Hepler. pcDNA3.1mRGS2 was a gift from David Siderovski (University of North Carolina, Chapel Hill, NC) and was constructed as described previously (Ingi et al., 1998). 12CA5 epitope-tagged angiotensin II $_{1A}$ receptors (AT$_{1A}$R) and flag epitope-tagged β$_2$-adrenergic receptors (β$_2$AR) were constructed as described previously (Zhang et al., 1996) and were kindly provided by Stephen Ferguson (Robarts

Research Institute, London, ON, Canada). Adenylyl cyclase VI was generously provided by Ross Feldman (Robarts Research Institute). pcDNA3.1hM$_2$ muscarinic receptor and baculoviruses Gα$_{i2}$, Gβ$_1$, and Gγ$_2$ and encoding N-terminally c-myc–tagged M$_2$ muscarinic receptor (M$_2$R) were gifts from Terry Hebert (Montreal Heart Institute, Montreal, PQ, Canada). The baculovirus β$_2$-adrenergic receptor-Gαs fusion protein construct was a gift from Michel Bouvier (University of Montreal, Montreal, PQ, Canada). pEGFP-C2/3 was purchased from BD Biosciences Clontech. H6-rRGS4/pQE60 was ligated into the pEGFP-C2 vector at *Bam*HI and *Eco*RI restriction sites. pcDNA3.1 mRGS2 was digested with *Hin*dIII and *Apa*I and ligated into pEGFP-C3. Plasmids were verified by DNA sequencing.

Purification of RGS Proteins. N-terminally histidine-tagged RGS proteins were expressed in *Escheria coli* and purified to >95% purity by nickel chromatography followed by size exclusion using a Superdex 75 HR 10/30 column (Amersham Biosciences, Piscataway, NJ), as described previously (Cladman and Chidiac, 2002).

Cell Culture and Transfection. HEK293 and COS-7 cells were transfected in 10-ml dishes using calcium phosphate precipitation. Cells were replated with fresh medium approximately 18 h after transfection and used for assays on the following day. Protein expression was verified by immunoblotting. Sf9 insect cells were cultured in serum-containing medium at 27°C and infected with baculoviruses as indicated below.

Intracellular cAMP Assay. Cells cotransfected with Gαs plus adenylyl cyclase were incubated with 2 μCi/ml [^{3}H]adenine (30 Ci/mmol) for 2 h at 37°C to allow for the accumulation of intracellular [^{3}H]ATP. After cells were rinsed with phosphate-buffered saline and given fresh medium, the assay was started by the addition of 0.5 mM 3-isobutyl-1-methylxanthine at 37°C. After 2.5 min, cells were lysed with 10% trichloroacetic acid, 2 mM cAMP, and 2 mM ATP. [^{3}H]cAMP was recovered by sequential chromatography over Dowex and Alumina (Salomon et al., 1974). For each sample, cellular uptake of [^{3}H]adenosine was estimated from the lysate, and recovered cAMP was calculated as a fraction of total cellular tritium. Cells transfected with Gαs yielded about twice as much cAMP as cells transfected with vector only, and cells transfected with Gαs plus adenylyl cyclase yielded roughly 10 times as much as vector controls.

Assay of GTP Hydrolysis. Sf9 cells were infected with baculoviruses encoding N-terminally c-myc–tagged M$_2$ muscarinic receptor, Gα$_{i2}$, Gβ$_1$, and Gγ$_2$, or a baculovirus encoding a β$_2$-adrenergic receptor-Gsα fusion protein. After 48 h, cellular membranes were prepared as described previously (Cladman and Chidiac, 2002) and stored at −80°C. Membranes were assayed at 30°C for muscarinic receptor-stimulated Gi GTPase activity in 1 μM [γ-^{32}P]GTP, 20 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin, plus 50 mM NaCl and 1.5 mM MgCl$_2$ for 15 min and for β$_2$-adrenergic receptor-stimulated Gs activity in 1 μM [γ-^{32}P]GTP, 20 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM ascorbate plus 50 mM NaCl and 2 mM MgCl$_2$ for 10 min.

Assays were stopped by the addition of 5% Norit in 0.05 M NaH$_2$PO$_4$, pH 3.0; the mixture was centrifuged and ^{32}P$_i$ was recovered from the supernatant. The nonspecific membrane signal was estimated by adding 1 mM unlabeled GTP and GTPase activity attributable to the purified RGS proteins (<2% of specific signal) was estimated in the absence of membranes and subtracted off as appropriate. Agonist-dependent GTPase activities were calculated as described previously (Cladman and Chidiac, 2002).

Analysis of Data. GTPase and intracellular cAMP data are expressed as mean value ± S.D. Mean values of intracellular cAMP recovered from cells transfected with adenylyl cyclase VI −/+RGS2/+RGS4 from six independent experiments were compared via repeated-measures analysis of variance followed by Student-Newman-Keuls multiple comparisons test.

Confocal Microscopy. GFP-tagged RGS proteins were visualized in live HEK293 and COS-7 cells. Microscopy was performed using a Zeiss LSM 410 confocal microscope equipped with a Krypton/Argon laser. EGFP fluorescence was examined under fluorescein isothiocyanate filter using a 63× oil immersion (Figs. 1 and 3) or 40× water immersion lens (Fig. 2). Images for real-time movement of RGS proteins were recorded as 5-s scans every 10 s for a duration of 10 min. For each experimental condition, fluorescence distribution patterns similar to the image shown were observed in the majority (60–90%) of cells inspected.

Results

Intracellular Localization of GFP-RGS2 and GFP-RGS4 Is Affected by G Proteins. In the absence of exogenous G protein or receptor, we found GFP-tagged RGS2 to localize predominantly to the nucleus, whereas GFP-tagged RGS4 localized to the cytosol in HEK293 cells (Fig. 1). To investigate the effects of G proteins on RGS intracellular distribution, we coexpressed these GFP fusion constructs together with wild-type Gαi2, Gαq, and Gαs. The coexpression of G proteins affected the intracellular localization of GFP-RGS2 and GFP-RGS4. In the presence of either Gαq or Gαs, GFP-RGS2 localized to the plasma membrane, whereas

Gαi2 coexpression resulted in the plasma membrane recruitment of GFP-RGS4 (Fig. 1).

Previous studies have shown that constitutively activated "QL" forms of Gαq (Heximer et al., 2001) and Gαi (Druey et al., 1998) cause RGS2 and RGS4, respectively, to localize to the plasma membrane. Similarly, we found that two different constitutively activated forms of each of the three G proteins tested (Gαi2-Q205L, Gαi1-R178C, Gαq-Q209L, Gαq-R183C, Gαs-Q227L, Gαs-R201C) yielded RGS localization patterns like those of their wild-type counterparts (data not shown). The present results with wild-type G proteins indicate that constitutive activity is not required for promoting RGS protein association with the plasma membrane.

In addition to wild-type and constitutively activated G proteins, we also tested several mutant forms expected to show reduced interactions with RGS proteins. We found that poorly activated "dominant-negative" mutants, including Gαs(α3β5/G226A/A366S) (Berlot, 2002), Gαs-Q227L/D295N, Gαi2Q205L/D273N, and Gαq-Q209L/D277N (Yu and Simon, 1998) produced effects similar to those of their wild-type counterparts on intracellular RGS-GFP localization (Fig. 2). In contrast, constitutively activated G proteins containing an additional point substitution (Lan et al., 1998) that substantially reduces RGS protein affinity (Gαi2-Q205L/G184S, Gαq-Q209L/G188S) (H. Mao, Q. Zhao, A. Goto, M. Daigle, M. H. Ghahremani, P. R. Albert and P. Chidiac, submitted) did not promote GFP-RGS association with the plasma membrane (Fig. 2), consistent with the findings of others (Sterne-
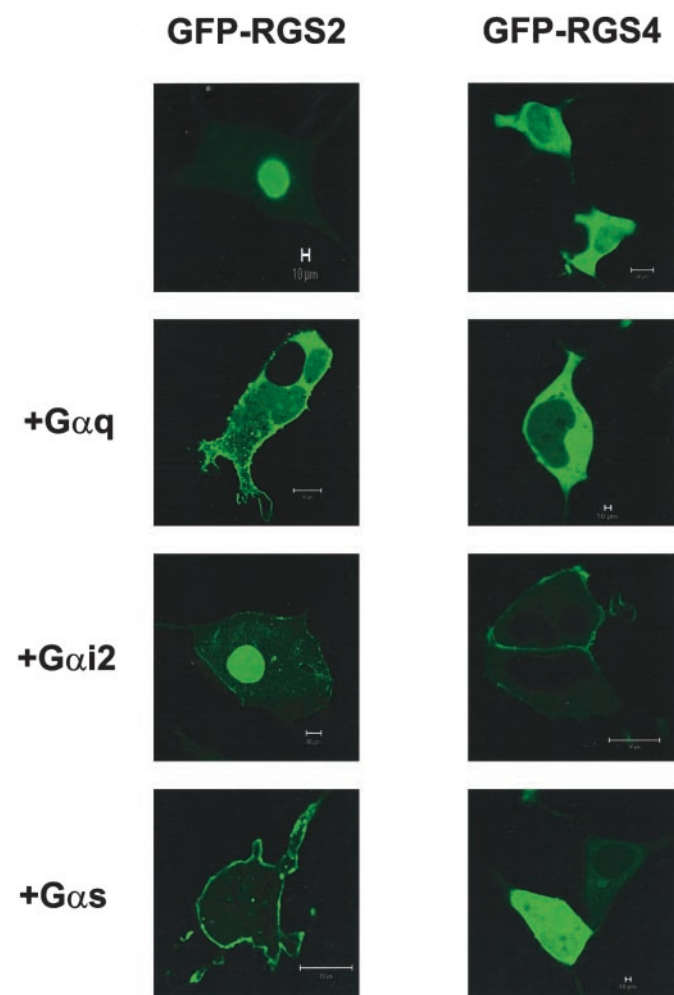
Fig. 1. Effects of G protein expression on the intracellular localization of GFP-RGS2 and GFP-RGS4. HEK293 cells were transiently transfected with GFP-RGS2 or GFP-RGS4 either alone or in the presence of Gαs, Gαi₂, or Gαq as indicated under *Materials and Methods*. Confocal microscopic images shown are representative of at least 100 living cells.

Fig. 2. Effects of G protein mutants on GFP-RGS intracellular localization. HEK293 cells were studied after transient cotransfection with GFP-RGS proteins and mutant Gα proteins as indicated. Confocal microscopic images shown are representative of at least 50 living cells.

Marr et al., 2003). This implies that GFP-RGS2 and GFP-RGS4 bind directly to G proteins in the plasma membrane.

Effects of Receptors on GFP-RGS Intracellular Localization. Receptors activate G proteins and additionally may modulate RGS-G protein interactions. We therefore cotransfected GFP-RGS proteins with plasmids encoding β_2AR, M_2R, and AT_{1A}R. These activate Gs, Gi, and Gq, respectively. As shown in Fig. 3, GFP-RGS2 localized to the plasma membrane in the presence of AT_{1A}R and β_2AR. GFP-RGS4 yielded a different distribution pattern, localizing to the plasma membrane when coexpressed with the M_2 muscarinic receptor. The redistribution of GFP-RGS2 and GFP-RGS4 in response to the presence of these receptors thus corresponds exactly to the effects of their cognate G proteins shown in Fig. 1.

Because receptors promote G protein activation and because RGS proteins tend to bind with higher affinity to activated G proteins in vitro, we anticipated that agonists would promote the recruitment of RGS proteins to the plasma membrane by receptors. In contrast to this expectation, we observed no further changes in GFP-RGS localization upon the addition of the agonists isoproterenol, carbachol, and angiotensin II to HEK293 cells expressing β_2AR, M_2R, and AT_{1A}R, respectively. Such observations suggest that signaling because of spontaneous receptor activity and/or the presence of activators in the culture medium may be sufficient for the localization of RGS proteins to the plasma membrane in HEK293 cells; however, neither the M_2R antagonist atropine nor the β_2AR inverse agonist timolol had any observable effect on intracellular RGS protein distribution.

We also sought to demonstrate agonist effects in these cells

GFP-RGS2   GFP-RGS4

+AT$_{1A}$R

+M$_2$MR

+β_2AR

Fig. 3. Effects of receptor expression on the intracellular localization of GFP-RGS2 and GFP-RGS4. GFP-RGS2 and GFP-RGS4 were studied in live HEK293 cells after transient cotransfection with either the M_2 muscarinic receptor, the angiotensin II$_{1A}$ receptor, or the β_2-adrenergic receptor as indicated. Confocal microscopic images shown are representative of at least 100 living cells.
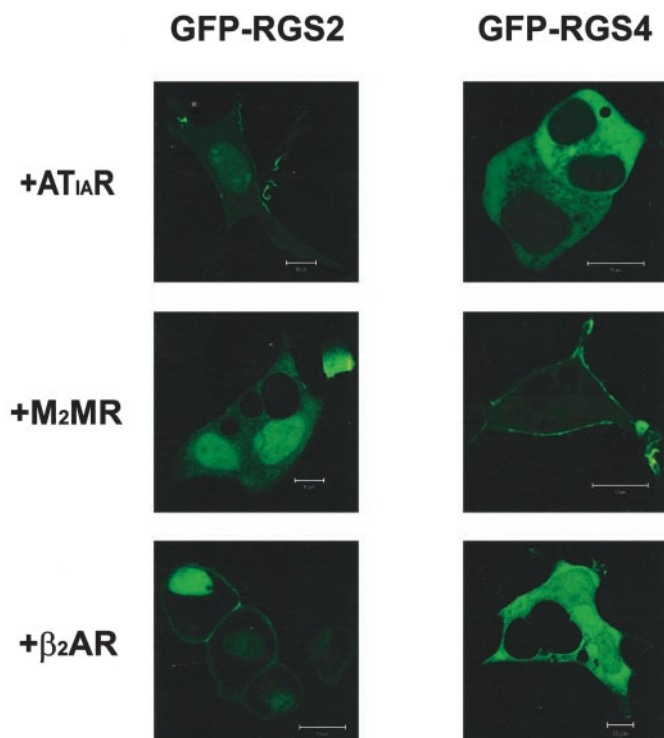
by decreasing the amount of receptor cDNA cotransfected with the GFP-RGS fusion proteins. However, when receptor-encoding plasmids were decreased to levels at which GFP-RGS localization to the plasma membrane did not significantly occur, we still did not detect any agonist-dependent recruitment there (data not shown). In COS-7 cells cotransfected with GFP-RGS2 and the β_2AR, we did observe sporadic evidence of an isoproterenol-dependent decrease in nuclear GFP-RGS2 ($\sim$ 10% of cells tested); however, increased association with the plasma membrane could not be verified (data not shown). The inability of agonists to increase RGS protein association with the plasma membrane implies that the observed effects of receptors are not dependent on their ability to foster guanine nucleotide exchange.

GFP-RGS Plasma Membrane Localization Is Independent of Downstream Signaling Events. The finding that constitutively activated but "non-RGS binding" G proteins Gαi2-Q205L/G184S and Gαq-Q209L/G188S failed to alter intracellular GFP-RGS2 and GFP-RGS4 localization (Fig. 2) suggests that RGS recruitment to the plasma membrane is not caused by events that occur after G protein activation. Consistent with this interpretation, agents that promote events downstream of G protein activation did not alter GFP-RGS localization. Agents tested included the calcium ionophore ionomycin and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (which mimic events downstream of Gq), forskolin (which bypasses Gs to directly activate adenylyl cyclase), as well as the tyrosine phosphatase inhibitor vanadate (data not shown). If downstream processes were in fact responsible for RGS localization to the plasma membrane, these would seem to involve neither adenylyl cyclase nor phospholipase Cβ activation. Furthermore, the G protein- and receptor-specific changes observed herein clearly indicate that what drives RGS2 to the plasma membrane is not the same as what causes RGS4 to go there.

Effects of RGS Proteins on Steady-State GTPase Activity and Intracellular cAMP. To investigate functional interactions complementary to the effects of G proteins on GFP-RGS intracellular localization, we examined the effects of purified RGS2 and RGS4 on agonist-driven, steady-state GTPase activities. Using membranes prepared from Sf9 cells expressing M_2 muscarinic receptor plus heterotrimeric Gi, we found that both RGS2 and RGS4 acted as GTPase-activating proteins (Fig. 4). RGS4 was approximately an order of magnitude more potent than RGS2 as a Gi GAP, consistent with the ability of Gi to recruit GFP-RGS4 but not GFP-RGS2 to the plasma membrane.

Because the detection of RGS2 GAP activity on Gi apparently requires the additional presence of an agonist-activated receptor (Ingi et al., 1998), we tested the effects of RGS2 and RGS4 on the steady-state GTPase activity of a β_2AR-Gαs fusion protein in Sf9 insect cell membranes. Although a clear agonist signal was evident with isoproterenol, there was no increase in GTP hydrolysis with either RGS2 or RGS4 (Fig. 5).

Notwithstanding its lack of GAP activity on receptor-activated or free Gαs, we found that RGS2 decreased intracellular cAMP in cells cotransfected with adenylyl cyclase VI plus wild-type Gαs (Fig. 6). Thus, RGS2 is able to impede Gs signaling even though it does not act as a GAP on Gs in either pre-steady-state or receptor-driven, steady-state assays.

Discussion

The present results illustrate previously unseen complexities in the subcellular distribution of RGS proteins. Superficially, the recruitment of RGS2 and RGS4 to the plasma membrane by wild-type G proteins and by receptors that activate those G proteins seems predictable, particularly in light of similar findings with constitutively activated G proteins (Druey et al., 1998; Heximer et al., 2001). Possible explanations for such recruitment include 1) increased RGS affinity for G proteins in the plasma membrane, 2) increased RGS association with the plasma membrane caused by an increase there in the density of RGS protein binding targets,

and 3) changes in RGS proteins and/or their binding targets caused by signaling events downstream of the G protein, for example altered intracellular ion concentrations or changes in the activities of second messenger-dependent kinases. Unlike the first two, the third mechanism can readily be ruled out in the present study, because neither $G\alpha i2$-Q205L/G184S nor $G\alpha q$-Q209L/G188S had any effect on GFP-RGS2 and GFP-RGS4 localization (Fig. 2); moreover, agents that promote downstream events also were without effect. It is worth noting that RGS proteins are not all alike in this regard, because the intracellular localization of RGS3 clearly is influenced by events that occur after G protein activation (Dulin et al., 1999), as is that of RGS10 (Burgon et al., 2001).

Our original expectation was that GFP-RGS2 and GFP-RGS4 would localize to the plasma membrane in response to the binding of GTP to the G proteins with which they interact. Although the present results do not strictly refute this notion, the lack of clearly supportive data suggests that G protein activation is at most a minor contributing factor in the association of RGS2 or RGS4 with the plasma membrane. If G protein activation were a primary determinant, recruitment of RGS proteins should have been evident in response to receptor activation by appropriate agonists, but this could not be demonstrated. Also, we did not observe any major differences between wild-type, constitutively activated, and poorly activated G proteins with respect to their effects on RGS localization, although it is possible that small differences may have been overlooked owing to the limitations of the technique used.
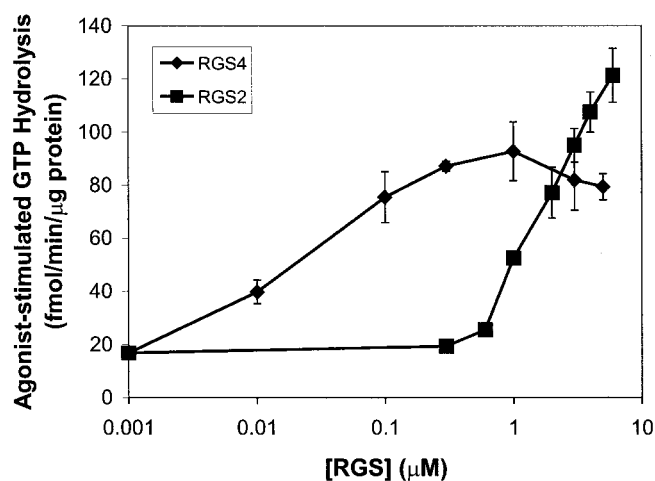
Fig. 4. Effects of purified RGS2 and RGS4 on M_2R-stimulated G protein GTPase activity. Sf9 insect cells were infected with a combination of baculoviruses encoding the M_2 muscarinic receptor, $G\alpha i_2$, $G\beta_1$, and $G\gamma2$ to yield M_2 + Gi–containing membranes. Gi GTPase activity in the presence of 100 μM carbachol was measured at the concentrations of RGS2 (■) and RGS4 (♦) indicated on the abscissa. Background GTP hydrolysis was estimated in the presence of 10 μM atropine and subtracted off to yield the values indicated.
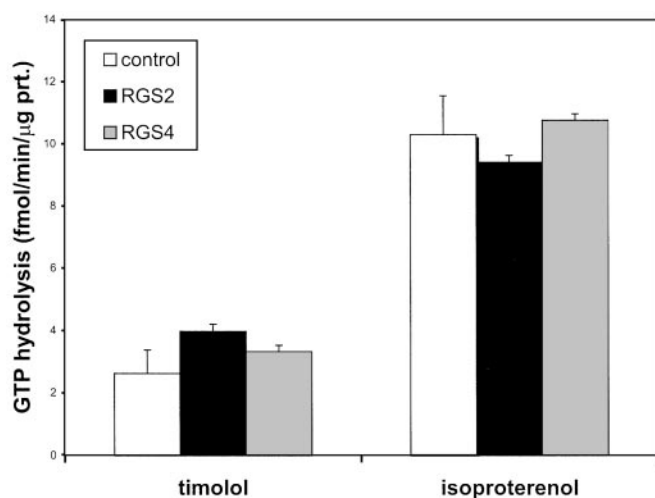
Fig. 5. Effects of purified RGS2 and RGS4 on β_2AR-stimulated Gs GTPase activity. Membranes were prepared from Sf9 insect cells infected with a single baculovirus encoding a β_2-adrenergic receptor-$G\alpha s$ fusion protein, and the hydrolysis of $[\bar{a}\text{-}^{32}P]GTP$ was assessed as described under *Materials and Methods*. Gs GTPase activity was measured in the presence of either 10 μM isoproterenol or 10 μM timolol, either in the absence (□) of RGS protein or in the presence of 4 μM RGS2 (■) or RGS4 (▨).
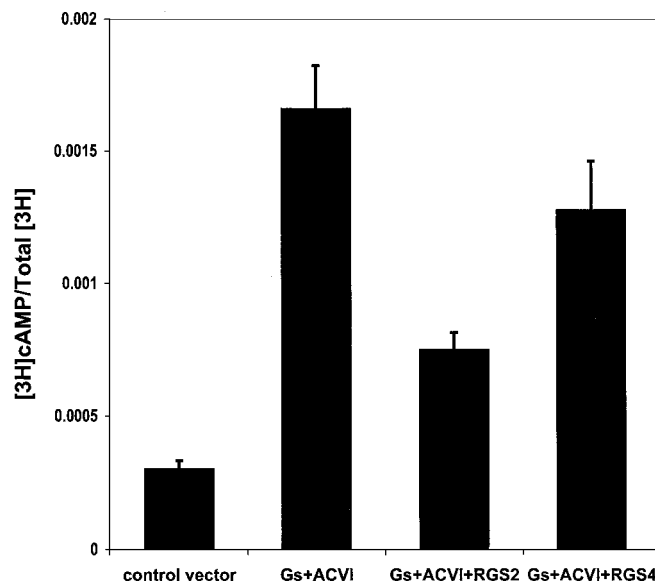
Fig. 6. Effects of RGS proteins on adenylyl cyclase activity. cAMP accumulation was measured in HEK293 cells transfected with control vector only, or $G\alpha s$ plus adenylyl cyclase VI and control vector, RGS2 or RGS4, as indicated. Data are representative of six independent experiments. Compared with cells transfected with $G\alpha s$ and adenylyl cyclase only, RGS2 reduced intracellular cAMP by 51 $\pm$ 14%, whereas RGS4 was associated with a decrease of 28 $\pm$ 14%. Measurements from all six experiments (excluding the control vector only condition) were compared by repeated-measures analysis of variance followed by Student-Newman-Keuls multiple comparisons test, and the signal from RGS2-transfected cells differed significantly ($p < 0.01$) from cells transfected with $G\alpha s$ and adenylyl cyclase only. RGS4-transfected cells differed from those of both of the other two conditions, although these differences were less significant ($p < 0.05$).

In retrospect, it may be naive to anticipate RGS recruitment to the plasma membrane in response to G protein activation, regardless of whether one views RGS proteins as catalytically acting GAPs or as participants in multimeric signaling complexes. In solution, RGS proteins form relatively stable associations with G proteins locked into the activated state by fluoroaluminate (Hepler, 1999). However, a single RGS protein molecule can act as a GAP for multiple Gα subunits in pre-steady-state single turnover GTPase assays (Wilkie and Ross, 2000), which implies that the activation state-dependent binding of the two proteins in solution is only transient. Furthermore, RGS4 binds to M$_2$ muscarinic receptor-Gi proteoliposomes in a slow, multistep process that leads to maximal GAP activity (Tu et al., 2001). Because this process is poorly reversible and is insensitive to agonist stimulation (Tu et al., 2001), it follows that G protein activation-dependent RGS association may not be significant in intact cells.

The simplest explanation for the observed effects of heterologously expressed G proteins may be that their increased abundance in the plasma membrane provides more RGS binding sites. Although G protein activation generally increases RGS binding in vitro, it is not an absolute requirement, and appreciable binding to inactive, GDP-bound G proteins has been observed (De Vries et al., 1996; Druey et al., 1998; Ko et al., 2001). Overall, the degree of intracellular RGS protein-G protein binding presumably would depend on their affinity for one another as well as on the concentration of each present, and the effects of G protein activation per se may be of minimal importance.

In the present study, there was little or no apparent association of GFP-RGS2 and GFP-RGS4 with endogenous G proteins in the plasma membrane of HEK293 cells. The recruitment there of RGS proteins by transfected receptors does not seem to reflect G protein activation, because agonists had no effect. One plausible explanation for this ligand-independent phenomenon is that endogenous G proteins in the plasma membrane serve as better RGS targets when coupled to receptors. Whereas G protein-independent effects of receptors on RGS proteins cannot yet be formally ruled out, previous studies have shown that RGS protein-G protein interactions can be promoted by receptors (Ingi et al., 1998; Zeng et al., 1998; Xu et al., 1999). This suggests that GFP-RGS proteins may have greater affinity for receptor-coupled as opposed to free G proteins. Moreover, it seems that different RGS proteins may selectively target different receptor-G protein combinations (Xu et al., 1999; Wang et al., 2002), contrasting with the limited selectivity among RGS proteins found in solution-based assays using only purified Gα and RGS proteins (Berman and Gilman, 1998; Hepler, 1999). In addition, receptors may help to localize and orient RGS proteins to optimize their GAP activities toward Gα at the plasma membrane (Hollinger and Hepler, 2002). Previous work has indicated that the N-terminal domain outside of the RGS box conveys high affinity and receptor selectivity (Zeng et al., 1998), although the corresponding receptor/G protein binding regions have yet to be identified.

In summary, the observed recruitment of GFP-RGS2 and GFP-RGS4 to the plasma membrane in response to heterologous G protein or receptor expression, respectively, seems to reflect increases in the abundance and affinity of target G proteins. In neither case does G protein activation seem to play a major role. Unlike the transient increase in RGS affinity afforded by GTP binding, however, the present results seem to suggest that more long-term associations of G proteins to receptors can significantly affect intracellular RGS-G protein coupling.

Selectivity of RGS Protein-G Protein Interactions. The effects of G proteins on RGS protein localization essentially mirror the functional effects of the same RGS proteins on G protein-mediated signaling. We showed previously that RGS2 selectively acts as a GAP for Gαq (Ingi et al., 1998). Whereas even micromolar concentrations of RGS2 do not GAP free Gαi (Heximer et al., 1997), such interactions can be observed in the presence of activated M$_2$ muscarinic receptor (Ingi et al., 1998). The relatively low Gi GAP potency of RGS2 compared with RGS4 (Fig. 4) implies a lower affinity of RGS2 for Gi, which in turn may explain why GFP-RGS2 does not localize to the plasma membrane with either M$_2$R or Gαi2, whereas GFP-RGS4 does (Figs. 1 and 3). Consistent with this interpretation, RGS4 seemed to interact with higher affinity than RGS2 for Gi and RGS2 with greater affinity than RGS4 for Gq in cell-based assays (Heximer et al., 1999). A difference in their potencies as GTPase-activating proteins for Gq has not yet been reported.

Although the effects on RGS localization of Gαi, Gαq, and their activating receptors can be rationalized in terms of GAP interactions, this is not possible with Gαs and the β_2AR. So far, only RGS-PX1 has been shown to act as a putative GAP for free Gαs (Zheng et al., 2001), and both RGS2 and RGS4 lack such activity on both free (Ingi et al., 1998) and receptor-activated Gs (Fig. 5).

Although it had no Gs GAP activity, RGS2 did inhibit Gs-dependent increases in intracellular cAMP in this study (Fig. 6) and others (Tseng and Zhang, 1998; Sinnarajah et al., 2001). The simplest explanation for this is that by binding to the G protein, the RGS2 may preclude access to adenylyl cyclase. A potential alternative mechanism is suggested by the findings of Sinnarajah and coworkers (Sinnarajah et al., 2001; Salim et al., 2003), who showed that RGS2 can interact directly with adenylyl cyclase to inhibit cAMP production. RGS2 is known to bind to free Gαs (Ko et al., 2001), but it is unclear whether simultaneous binding to the G protein and effector occurs. In addition, the present results raise the possibility that RGS2 can bind to the β_2AR as well.

Intriguingly, a recent study (Dowal et al., 2001) reported that purified RGS4 bound not only to Gαq but also to the effector phospholipase Cβ1, and the same study showed data indicating the formation of ternary complexes containing RGS4, phospholipase Cβ1, and Gαq-guanosine 5′-O-(3-thio)triphosphate. Analogously, it is possible that RGS2 could bind to and inactivate Gs-adenylyl cyclase complexes. Further complexity is suggested by apparent receptor-RGS interactions (Zeng et al., 1998; Xu et al., 1999); beyond that, there is evidence for RGS4-G$\beta\gamma$ binding (Dowal et al., 2001). Thus, it seems that RGS proteins potentially can bind to all of the major types of proteins that bind to Gα (i.e., receptors, effectors, and G$\beta\gamma$). Whether RGS binding to these proteins occurs sequentially or simultaneously, the impact of these interactions on G protein signaling and the significance of these interactions in vivo are worthwhile issues for further study.

Acknowledgments

References

Berlot CH (2002) A highly effective dominant negative α_s construct containing mutations that affect distinct functions inhibits multiple Gs-coupled receptor signaling pathways. *J Biol Chem* **277:**21080–21085.

Berman DM and Gilman AG (1998) Mammalian RGS proteins: barbarians at the gate. *J Biol Chem* **273:**1269–1272.

Burgon PG, Lee WL, Nixon AB, Peralta EG, and Casey PJ (2001) Phosphorylation and nuclear translocation of a regulator of G protein signaling (RGS10). *J Biol Chem* **276:**32828–32834

Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, Gilman AG, Benovic JL, and Kozasa T (1999) Selective Regulation of $G\alpha_{q/11}$ by an RGS domain in the G protein-coupled receptor kinase, GRK2. *J Biol Chem* **274:** 34483–34492.

Cladman W and Chidiac P (2002) Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G_i. *Mol Pharmacol* **62:**654–659.

De Vries L, Elenko E, Hubler L, Jones TL, and Farquhar MG (1996) GAIP Is membrane-anchored by palmitoylation and interacts with the activated (GTP-bound) form of $G\alpha$ i subunits. *Proc Natl Acad Sci USA* **93:**15203–15208.

De Vries L, Zheng B, Fischer T, Elenko E, and Farquhar MG (2000) The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol* **40:**235–271.

Derrien A and Druey KM (2001) RGS16 function is regulated by epidermal growth factor receptor-mediated tyrosine phosphorylation. *J Biol Chem* **276:**48532–48538.

Dowal L, Elliott J, Popov S, Wilkie TM, and Scarlata S (2001) Determination of the contact energies between a regulator of G protein signaling and G protein subunits and phospholipase Cβ1. *Biochemistry* **40:**414–421.

Druey KM, Sullivan BM, Brown D, Fischer ER, Watson N, Blumer KJ, Gerfen CR, Scheschonka A, and Kehrl JH (1998) Expression of GTPase-deficient $G\alpha_{i2}$ results in translocation of cytoplasmic RGS4 to the plasma membrane. *J Biol Chem* **273:**18405–18410.

Dulin NO, Sorokin A, Reed E, Elliott S, Kehrl JH, and Dunn MJ (1999) RGS3 inhibits G protein-mediated signaling via translocation to the membrane and binding to Galpha11. *Mol Cell Biol* **19:**714–723.

Hepler JR (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* **20:**376–382.

Hepler JR, Berman DM, Gilman AG, and Kozasa T (1997) RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. *Proc Natl Acad Sci USA* **94:**428–432.

Heximer SP, Lim H, Bernard JL, and Blumer KJ (2001) Mechanisms governing subcellular localization and function of human RGS2. *J Biol Chem* **276:**14195–14203.

Heximer SP, Srinivasa SP, Bernstein LS, Bernard JL, Linder ME, Hepler JR, and Blumer KJ (1999) G protein selectivity is a determinant of RGS2 function. *J Biol Chem* **274:**34253–34259.

Heximer SP, Watson N, Linder ME, Blumer KJ, and Hepler JR (1997) RGS2/G0S8 is a selective inhibitor of Gqalpha function. *Proc Natl Acad Sci USA* **94:**14389–14393.

Hollinger S and Hepler JR (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacol Rev* **54:**527–559.

Ingi T, Krumins AM, Chidiac P, Brothers GM, Chung S, Snow BE, Barnes CA, Lanahan AA, Siderovski DP, Ross EM, et al. (1998) Dynamic regulation of RGS2 suggests a novel mechanism in G-Protein signaling and neuronal plasticity. *J Neurosci* **18:**7178–7188.

Ishii M, Inanobe A, and Kurachi Y (2002) PIP3 inhibition of RGS protein and its reversal by Ca^{2+}/calmodulin mediate voltage-dependent control of the G protein cycle in a cardiac K+ channel. *Proc Natl Acad Sci USA* **99:**4325–4330.

Ko JK, Choi KH, Kim IS, Jung EK, and Park DH (2001) Inducible RGS2 is a cross-talk regulator for parathyroid hormone signaling in rat osteoblast-like UMR106 cells. *Biochem Biophys Res Commun* **287:**1025–1033.

Lan KL, Sarvazyan NA, Taussig R, Mackenzie RG, DiBello PR, Dohlman HG, and Neubig RR (1998) A point mutation in Gαo and Gαi1 blocks interaction with regulator of G protein signaling proteins. *J Biol Chem* **273:**12794–12797.

Levis MJ and Bourne HR (1992) Activation of the alpha subunit of Gs in intact cells alters its abundance, rate of degradation and membrane avidity. *J Cell Biol* **119:**1297–1307.

Pereira PS and Jones NC (2001) The RGS domain-containing fission yeast protein, Rgs1p, regulates pheromone signalling and is required for mating. *Genes Cells* **6:**789–802.

Salim S, Sinnarajah S, Kehrl JH, and Dessauer CW (2003) Identification of RGS2 and type V adenylyl cyclase interaction sites. *J Biol Chem* **278:**15842–15849.

Salomon Y, Londos C, and Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Anal Biochem* **58:**541–548.

Sierra DA, Popov S, and Wilkie TM (2000) Regulators of G-protein signaling in receptor complexes. *Trends Cardiovasc Med* **10:**263–268.

Sinnarajah S, Dessauer CW, Srikumar D, Chen J, Yuen J, Yilma S, Dennis JC, Morrison EE, Vodyanoy V, and Kehrl JH (2001) RGS2 Regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III. *Nature (Lond)* **409:**1051–1055.

Srinivasa SP, Bernstein LS, Blumer KJ, and Linder ME (1998) Plasma membrane localization is required for RGS4 function in saccharomyces cerevisiae. *Proc Natl Acad Sci USA* **95:**5584–5589.

Sterne-Marr R, Tesmer JJ, Day PW, Stracquatanio RP, Cilente JA, O'Connor KE, Pronin AN, Benovic JL, and Wedegaertner PB (2003) G Protein-coupled receptor kinase 2/Gαq/11 interaction. A novel surface on a regulator of G protein signaling homology domain for binding Gα subunits. *J Biol Chem* **278:**6050–6058.

Tseng CC and Zhang XY (1998) Role of regulator of G protein signaling in desensitization of the glucose-dependent insulinotropic peptide receptor. *Endocrinology* **139:**4470–4475.

Tu Y, Woodson J, and Ross EM (2001) Binding of regulator of G protein signaling (RGS) proteins to phospholipid bilayers. Contribution of location and/or orientation to GTPase-activating protein activity. *J Biol Chem* **276:**20160–20166.

Wang Q, Liu M, Mullah B, Siderovski DP, and Neubig RR (2002) Receptor-selective effects of endogenous RGS3 and RGS5 to regulate mitogen-activated protein kinase activation in rat vascular smooth muscle cells. *J Biol Chem* **277:**24949–24958.

Wedegaertner PB, Chu DH, Wilson PT, Levis MJ, and Bourne HR (1993) Palmitoylation is required for signaling functions and membrane attachment of Gq α and Gs α. *J Biol Chem* **268:**25001–25008.

Wilkie TM and Ross EM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* **69:**795–827.

Wong YH, Federman A, Pace AM, Zachary I, Evans T, Pouyssegur J, and Bourne HR (1991) Mutant alpha subunits of Gi2 inhibit cyclic AMP accumulation. *Nature (Lond)* **351:**63–65.

Xu X, Zeng W, Popov S, Berman DM, Davignon I, Yu K, Yowe D, Offermanns S, Muallem S, and Wilkie TM (1999) RGS proteins determine signaling specificity of Gq-coupled receptors. *J Biol Chem* **274:**3549–3556.

Yu B and Simon MI (1998) Interaction of the xanthine nucleotide binding Goalpha mutant with G protein-coupled receptors. *J Biol Chem* **273:**30183–30188.

Zeng W, Xu X, Popov S, Mukhopadhyay S, Chidiac P, Swistok J, Danho W, Yagaloff KA, Fisher SL, Ross EM, et al. (1998) The N-terminal domain of RGS4 confers receptor-selective inhibition of G protein signaling. *J Biol Chem* **273:**34687–34690.

Zhang J, Ferguson SS, Barak LS, Menard L, and Caron MG (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem* **271:**18302–18305.

Zheng B, Ma YC, Ostrom RS, Lavoie C, Gill GN, Insel PA, Huang XY, and Farquhar MG (2001) RGS-PX1, a GAP for GalphaS and sorting nexin in vesicular trafficking. *Science (Wash DC)* **294:**1939–1942.

Address correspondence to: Peter Chidiac, Ph.D., Department of Physiology and Pharmacology, Medical Sciences Building, University of Western Ontario, London, Ontario N6A 5C1, Canada. E-mail: peter.chidiac@fmd.uwo.ca