Cytoplasmic, Nuclear, and Golgi Localization of RGS Proteins

EVIDENCE FOR N-TERMINAL AND RGS DOMAIN SEQUENCES AS INTRACELLULAR TARGETING MOTIFS*

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RGS proteins comprise a family of proteins named for their ability to negatively regulate heterotrimeric G protein signaling. Biochemical studies suggest that members of this protein family act as GTPase-activating proteins for certain Ga subunits, thereby accelerating the turn-off mechanism of Ga and terminating signaling by both Ga and Gβ subunits. In the present study, we used confocal microscopy to examine the intracellular distribution of several RGS proteins in COS-7 cells expressing RGS-green fluorescent protein (GFP) fusion proteins and in cells expressing RGS proteins endogenously. RGS2 and RGS10 accumulated in the nucleus of COS-7 cells transfected with GFP constructs of these proteins. In contrast, RGS4 and RGS16 accumulated in the cytoplasm of COS-7 transfectants. As observed in COS-7 cells, RGS4 exhibited cytoplasmic localization in mouse neuroblastoma cells, and RGS10 exhibited nuclear localization in human glioma cells. Deletion or alanine substitution of an N-terminal leucine repeat motif present in both RGS4 and RGS16, a domain identified as a nuclear export sequence in HIV Rev and other proteins, promoted nuclear localization of these proteins in COS-7 cells. In agreement with this observation, treatment of mouse neuroblastoma cells with leptomycin B to inhibit nuclear protein export by exportin1 resulted in accumulation of RGS4 in the nucleus of these cells. GFP fusions of RGS domains of RGS proteins localized in the nucleus, suggesting that nuclear localization of RGS proteins results from nuclear targeting via RGS domain sequences. RGSZ, which shares with RGS-GAIP a cysteine-rich string in its N-terminal region, localized to the Golgi complex in COS-7 cells. Deletion of the N-terminal domain of RGSZ that includes the cysteine motif promoted nuclear localization of RGSZ. None of the RGS proteins examined were localized at the plasma membrane. These results demonstrate that RGS proteins localize in the nucleus, the cytoplasm, or shuttle between the nucleus and cytoplasm as nucleo-cytoplasmic shuttle proteins. RGS proteins localize differentially within cells as a result of structural differences among these proteins that do not appear to be important determinants for their G protein-regulating activities. These findings suggest involvement of RGS proteins in more complex cellular functions than currently envisioned.

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RGS proteins comprise a family of more than 20 known members that have been implicated in the negative regulation of heterotrimeric G protein1 signaling (1, 2). RGS proteins were discovered as a pheromone desensitization factor (Sat2p, super-sensitive phenotype protein) by genetic studies in yeast and shown to negatively regulate signaling by the yeast homolog of Gαi, Gpa1 (3). Genetic studies in Caenorhabditis elegans identified a homolog of Sat2p involved in negative regulation of signaling by the Goa homolog GOA-1 (4). Subsequent studies have documented the existence of transcripts encoding proteins with RGS domains, a semi-conserved sequence of approximately 120 amino acids found in all RGS proteins, in species ranging from fungus to man (1, 2, 5).

Biochemical studies have supported the genetic evidence that the locus of RGS protein action is at the level of G protein α subunits. RGS proteins bind to Ga subunits in the G1 and Gq families in vitro and dramatically enhance their intrinsic GTPase activity (6, 7). Thus, RGS proteins function as GTPase-activating proteins for certain heterotrimeric Gα subunits. Stimulation of GTP hydrolysis of Ga subunits leads to their conversion from the active Ga-GTP form to the inactive Ga-GDP form and their recombination with Gβγ subunits, effectively terminating signaling by both Ga and Gβγ subunits. The deduced crystal structure of RGS4 bound to G1α1 showed interaction of RGS domain residues with the G protein switch regions, suggesting that the mechanism of GTPase acceleration by RGS proteins may be due primarily to stabilization of the transition state of Gα residues directly involved in catalysis (8).

Additional studies have raised the possibility that RGS proteins may also interact with effectors or receptors to attenuate G protein signaling. These studies reported that recombinant RGS proteins can block PI PLC activation by G1α-GTP-[32P] in vitro (9) and produce receptor-selective attenuation of Gq signaling when added to permeabilized cells (10).

Implicit in the proposed regulatory actions of RGS proteins in cells is their localization at or near the plasma membrane where G proteins, as well as receptors and effectors, are located. Here we examined the cellular localization of RGS proteins expressed as GFP fusion proteins in COS-7 cells and endogenously in other cells. Our results reveal a surprising diversity in subcellular localization and trafficking of RGS proteins. RGS proteins localize in the nucleus, the cytoplasm or shuttle between the nucleus and cytoplasm as nucleo-cytoplasmic shuttle proteins. None of the RGS proteins studied showed localization at the plasma membrane. We identified the molecular determinant for nuclear localization of RGS proteins to the

* The abbreviations used are: G protein, guanine nucleotide-binding protein; DPBS, Dulbecco’s phosphate buffered saline; EGFF, enhanced GFP; GFP, green fluorescent protein; HIV, human immunodeficiency virus; Leb3, inhibitor β a; NES, nuclear export sequence; PI PLC, phosphoinositide phospholipase C; GTP-[γ-32P], guanosine 5’-3-O-(thio)triphosphate; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate.
conserved RGS domain present in all RGS proteins. We also identified sequence elements outside of the RGS domain that result in either nuclear-cytoplasmic transport or cytoplasmic retention of RGS proteins. These results show that RGS proteins localize differentially within cells as a result of structural differences among these proteins that do not appear to be important determinants for their G protein-regulating activities. This study is the first to investigate sequences responsible for and mechanisms underlying localization of RGS proteins within mammalian cells. Our findings suggest involvement of RGS proteins in more complex cellular functions than currently envisioned.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium and serum was provided by the Diabetes Endocrinology Research Center (the University of Iowa). Oligonucleotide primers and other molecular biological reagents were obtained from the University of Iowa DNA Core. Human neuroglioma cells (H4) were from ATCC and mouse neuroblastoma cells (N-18) were a gift from Dr. Murphy (University of Iowa). Antibodies to c-Myc, RGS4, and RGS10 were purchased from Santa Cruz. Leptomycin B was provided graciously by Dr. Barbara Wolf (Novartis, Vienna, Austria). RGS2, RGS4, RGS16, and RGSZ were purchased from Santa Cruz. Leptomycin B was provided graciously by Dr. Barbara Wolf (Novartis, Vienna, Austria).

**Isolation and Cloning of RGS cDNAs**—Full-length cDNAs encoding RGS2, RGS4, RGS16, and RGSZ were PCR-amplified based upon sequences of these cDNAs deposited in GenBankTM. Full-length cDNAs encoding RGS10 were amplified in our laboratory based upon expressed sequence tags found in database of Expressed Sequence Tags (NCBI, National Institutes of Health), assigned as RGS10, using a PCR-based strategy we described previously (11). This strategy utilized both 5’ and 3’ rapid amplification of cDNA ends to obtain overlapping cDNAs encoding RGS10. A full-length cDNA of RGS10 was amplified by PCR using primers to sequences deduced by rapid amplification of cDNA ends. Complete coding sequence of RGS10 (AF045229) was deposited in GenBankTM following cloning and sequencing. The various RGS protein cDNAs (except RGS10; see below) were PCR-amplified using gene-specific primers incorporating restriction sites to facilitate subsequent cloning into EGFP vector (CLONTECH). First, amplified RGS protein cDNAs were cloned in the T/A cloning vector pCR2.1 (Invitrogen). Then, restriction enzyme digestion and agarose gel purification of the cloned RGS protein cDNAs was performed by automated fluorescent dideoxynucleotide sequencing by the University of Iowa DNA Core Facility.

**cDNAs encoding RGS proteins with N-terminal deletions (RGS4Δ1–15, RGS16Δ1–15, and RGSZΔ1–49) were generated by PCR using forward primers that deleted the indicated amino acids (1–15 or 1–49) of each RGS protein and that included a Kozak consensus sequence and ATG start codon for proper translation of the truncated protein. cDNAs encoding alanine mutants of RGS4 (RGS4 ΔL5A/L8/L13A) and RGS16 (L5A/F8A) were prepared by PCR using forward primers incorporating the c-Myc sequence, and the amplified cDNA was cloned in pcR3.1 Uni (Invitrogen). cDNA encoding RGS4 with an N-terminal EGFP sequence was generated by cloning into EGFP vector in frame with its N-terminal EGFP sequence. Double-stranded sequencing of all mutant RGS protein cDNAs was performed by automated fluorescent dideoxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Cell Culture and Transfection**—Human neuroglioma (H4) and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml) (complete Dulbecco’s modified Eagle’s medium). Mouse neuroblastoma (N-18) cells were grown in RPMI medium supplemented with 10% fetal bovine serum (50 μg/ml). All cells were grown in a 5% CO₂ humidified atmosphere at 37°C.

**COS-7** cells were transiently transfected with vectors containing various RGS protein cDNAs by electroporation using a Bio-Rad Gene Pulser. Typically, COS-7 cells (10⁷/ml) were transfected with 40 μg of plasmid DNA at settings of 0.22 kV and 950 microfarads. Cells were diluted in complete Dulbecco’s modified Eagle’s medium and plated in two-chambered slides (Nunc) at a density of approximately 10⁶ cells/well. Transfected cells were used in experiments 48 h following transfection.

**Immunofluorescence**—Cells were rinsed three times with DPBS before fixation for immunofluorescence. For immunocytochemical detection of RGS4, RGS10, and c-Myc-RGS4 using antibodies to RGS proteins or to c-Myc, cells were fixed and permeabilized by treatment with 50% methanol/50% acetone for 1 h at 4°C. For visualization of GFP-tagged RGS proteins in COS-7 cells, cells were fixed by treatment with 4% paraformaldehyde for 20 min at room temperature followed by permeabilization with DPBS containing 0.1% Triton X-100 and 0.1% Nonidet P-40 for 10 min at room temperature. After permeabilization, cells were treated with DPBS containing 100 μg/ml RNase A (Roche Molecular Biochemicals) for 20 min at room temperature prior to incubation with antibodies or staining with propidium iodide. For immunodetection of c-Myc, RGS4, and RGS10, RNase A-treated cells were incubated with appropriate antibodies (1 μg/ml) in DPBS containing 5% bovine serum albumin for 1 h at room temperature. Cells then were rinsed three times with DPBS and incubated with FITC-conjugated secondary antibodies (1 μg/ml) in DPBS for 1 h at room temperature. Cells were stained with propidium iodide in DPBS for 20 min at room temperature followed by three washes with DPBS. Cells were air-dried and then mounted using Vecta Shield mounting solution. For staining the Golgi network, paraformaldehyde-fixed and detergent-permeabilized COS-7 cells were washed once with DPBS and then incubated in DPBS containing 10% bovine calf serum for 30 min at room temperature. Cells were then incubated with 0.5 μg/ml 4,6-diamidino-2-phenylindole (Molecular Probes) in DPBS containing 2% bovine calf serum for 1 h at room temperature prior to washing with DPBS, drying, and mounting.

**Confocal microscopy** was performed with a Bio-Rad MRC 1024 confocal microscope equipped with a Krypton/Argon laser at the University of Iowa Central Microscopy Research Facility. EGFP and FITC fluorescence was examined under FITC filter, and propidium iodide fluorescence was examined under Texas Red filter using 60× oil lenses. Images were captured after Kalman averaging. In some experiments, visualization of fluorescence of GFP-tagged RGS proteins was performed in live COS-7 cells plated in 35-mm culture dishes. GFP fluorescence was visualized with a FITC filter while cell structure was simultaneously monitored by phase contrast.

**Fractionation and Immunoblotting**—Subcellular fractionation of COS-7 cells expressing Myc-tagged RGS proteins was performed as described by Brett et al. (12). Briefly, cells were homogenized in a tight-fitting Dounce homogenizer in a hypotonic lysis buffer (5 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol) containing protease inhibitors (1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM leupeptin, 1 μM aprotinin, 1 μM pepstatin, 1 μM bestatin, 1 μM E64). The resulting cell lysate (0.5 ml) was layered onto a solution of 1.1 M sucrose in hypotonic lysis buffer (0.5 ml) and centrifuged at 1500 × g for 10 min at 4°C. The resulting pellet was washed by resuspension in 1 ml of 1.1 M sucrose in hypotonic lysis buffer and recentrifugation at 1500 × g for 5 min at 4°C. The resulting pellet was designated the nuclear fraction. The supernatant from the first 1500 × g centrifugation was subjected to centrifugation at 150,000 × g for 30 min at 4°C. The resulting supernatant and pellet were designated cytosolic and membrane fractions, respectively. Immunoblotting for specific organelle markers using lamin A/C as a nuclear marker, Na⁺/K⁺-ATPase as a plasma membrane marker, and 14-3-3β as a cytosolic marker in COS-7 cells confirmed the identity of these fractions. Immunoblotting was performed essentially as we described previously (13).

**RESULTS**

**Localization of RGS Proteins in Cytoplasm and Nucleus**—The intracellular distribution patterns of various human RGS proteins were examined by transfection of COS-7 cells with GFP-tagged RGS protein constructs. We selected RGS2, RGS4, RGS8, RGS10, and RGS16 for our studies based upon their similar sizes (167–211 amino acids) and extensive characterization in previous studies. These five RGS protein constructs are most homologous in their RGS domains (Fig. 1), a conserved region of approximately 120 amino acids found in all RGS proteins that comprises a domain required for their interaction with and GTPase-activating activity toward Gα subunits (8, 14). Fig. 1 also illustrates the lack of sequence similarity among these proteins in sequences located outside of their RGS domains. Fig. 2 shows confocal microscope images of COS-7 cells
expressing these GFP-tagged RGS proteins. The green color represents GFP fluorescence from expressed RGS proteins, and the red color represents fluorescence from propidium iodide that was used to identify nuclei in these cells. To confirm any apparent nuclear localization of expressed RGS proteins by visualization of GFP fluorescence alone, an overlay image of the GFP and propidium iodide fluorescence is also shown. In these overlay images, the yellow color represents RGS proteins that are localized in the nucleus. As shown, RGS2 and RGS10 are localized predominantly in the nucleus of COS-7 cell transfectants (Fig. 2), whereas RGS4 and RGS16 are localized primarily in the cytoplasm of these cells (Fig. 2). It appears that the intensity of the GFP signal for both RGS4 and RGS16 is high in the perinuclear region, although they are distributed throughout the cytoplasm. No evidence for accumulation of any of these RGS proteins at the plasma membrane was observed.

The observation that these GFP-tagged RGS proteins are expressed in COS-7 cells predominantly as either cytoplasmic or nuclear proteins raised interesting questions concerning the structural basis underlying cellular targeting of these proteins. It seemed unlikely that the presence of a GFP tag on these proteins could be responsible for their cellular localization, i.e. because some RGS proteins were localized in the nucleus and others in the cytoplasm. However, we performed several control experiments to address this possibility. First, COS-7 cells were transfected with GFP alone to assess its cellular distribution. As found in previous studies, GFP was distributed throughout cells with a homogenous pattern of expression (Fig. 3, top panel). We next examined whether the location of the GFP tag or the presence of GFP itself had any influence on RGS protein localization in cells. RGS4 tagged at its N terminus with either GFP (Fig. 3, middle panel) or c-Myc (Fig. 3, bottom panel) showed the same pattern of cytoplasmic localization as observed with RGS4 tagged with GFP on its C terminus (Fig. 2). These results indicate that cytoplasmic localization of RGS4 is not influenced by the presence of a GFP tag. Finally, RGS4, RGS16, RGS2, and RGS10 showed identical patterns of intracellular distribution in live versus fixed COS-7 cells expressing GFP tagged forms of these proteins. Fig. 4A illustrates the predominant cytoplasmic and nuclear localization of RGS4 and RGS2, respectively, in live COS-7 cells expressing these proteins. Similarly, anti-c-Myc immunoblots of cell fractions from COS-7 cells expressing c-Myc-tagged forms of RGS4 and RGS2 demonstrated the predominant cytoplasmic and the exclusive nuclear localization of RGS4 and RGS2, respectively (Fig. 4). Interestingly, RGS4 proteins migrated as two size variants, although it is less clear for RGS2, and the fraction of RGS4 that was present in the nuclear fraction corresponded to more slowly migrating form of the protein. The molecular basis of heterogeneity in apparent size of these RGS proteins and its functional relevance is not clear.

**Cellular Localization of Endogenously Expressed RGS Proteins**—To evaluate whether endogenously expressed RGS proteins show a pattern of cellular localization like that observed in COS-7 cells expressing GFP-tagged RGS proteins, we examined the cellular localization of RGS4 and RGS10 in cells naturally expressing these proteins. RGS4 and RGS10 were selected for study because of the availability of antibodies to these proteins. As shown, RGS4 and RGS10 are localized predominantly in the nucleus of COS-7 cells expressing RGS4 and RGS10, respectively, in live COS-7 cells expressing these proteins. The green color represents GFP fluorescence from expressed RGS proteins, and the red color represents fluorescence from propidium iodide that was used to identify nuclei in these cells. To confirm any apparent nuclear localization of expressed RGS proteins by visualization of GFP fluorescence alone, an overlay image of the GFP and propidium iodide fluorescence is also shown. In these overlay images, the yellow color represents RGS proteins that are localized in the nucleus. As shown, RGS2 and RGS10 are localized predominantly in the nucleus of COS-7 cell transfectants (Fig. 2), whereas RGS4 and RGS16 are localized primarily in the cytoplasm of these cells (Fig. 2). It appears that the intensity of the GFP signal for both RGS4 and RGS16 is high in the perinuclear region, although they are distributed throughout the cytoplasm. No evidence for accumulation of any of these RGS proteins at the plasma membrane was observed.

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sion of RGS4 in N18 mouse neuroblastoma cells and of RGS10 in H4 human neuroglioma cells by indirect immunocytochemistry using primary antibodies to each RGS protein and FITC-conjugated secondary antibody. Immunodetectable RGS4 in N18 cells was present throughout the cytoplasm and perinuclear region in most cells (Fig. 5). Occasionally, we observed nuclear-localized RGS4 in N18 cells that appeared indistinct from the majority of cells expressing RGS4 in the cytoplasm. In contrast, RGS10 immunoreactivity was found predominantly in the nucleoplasm of H4 cells with some immunoreactivity also apparent at the nuclear rim and perinuclear region (Fig. 5). Thus, the patterns of distribution of RGS4 and RGS10 in cells endogenously expressing these proteins are unique and consistent with those observed in COS-7 cells expressing GFP fusions of these proteins. These findings indicate that RGS4 is predominantly a cytoplasmic protein, whereas RGS10 is expressed in the nucleus and perinuclear regions of cells. Based upon the predominant localization of RGS proteins in the cytoplasm or nucleus (Fig. 2), we classified RGS4 and RGS16 as cytoplasmic RGS proteins and RGS2 and RGS10 as nuclear RGS proteins.

The structural basis underlying the predominant cytoplasmic or nuclear localization of these five RGS proteins is unknown. In view of the extensive homology among these proteins within their RGS domains (Fig. 1), it seemed unlikely that nuclear versus cytoplasmic targeting of these proteins resulted from differences within this domain. It seemed more likely that less-conserved sequences located outside the RGS domain of these proteins played a role in their unique patterns of cellular localization. Therefore, we examined these “extra RGS domain” sequences carefully for possible similarities and differences among these two groups of RGS proteins. No obvious similarities in size or sequence outside of the RGS domains of nuclear RGS proteins were apparent. However, RGS4 and RGS16 shared considerable homology in sequences N-terminal to their RGS domains, particularly within their first 30 amino acids (Fig. 2).

Role of N Terminus of RGS4 and RGS16 in Cytoplasmic Localization—To evaluate the possible contribution of N-terminal sequences of RGS4 and RGS16 to their cytoplasmic targeting in cells, we prepared N-terminal deletion mutants of these two proteins and examined their cellular expression as GFP fusions in COS-7 cells. Fig. 6 shows the confocal microscopic findings of COS-7 cells expressing RGS4 and RGS16 lacking the first 15 amino acids, i.e., RGS4D1–15 and RGS16D1–15. Deletion of the N-terminal 15 amino acids of RGS4 and RGS16 resulted in accumulation of both proteins in the nucleus of COS-7 cells, in contrast to the cytoplasmic localization of the native proteins (Fig. 2). These results suggest that this conserved N-terminal region of these two RGS proteins comprises or contains sequence elements that are responsible for the cytoplasmic localization of these proteins. These findings also are consistent with the hypothesis that a default pathway directs these and possibly other RGS proteins to the nucleus unless sequence elements are present that promote cytoplasmic retention of the proteins. Therefore, we hypothe-
Nuclear Export Sequences in RGS4 and RGS16—Inspection of the conserved N-terminal regions of RGS4 and RGS16 revealed the presence of a leucine repeat motif found in both HIV Rev and IκBα (Fig. 7). The second of the three leucines in this repeat is replaced conservatively by phenylalanine in RGS16. HIV Rev and IκBα are nucleo-cytoplasmic shuttle proteins that shuttle in or out of the cell nucleus. Nuclear export of both of these proteins is critically dependent on the presence of these three strategically spaced amino acids. Alanine substitution of these leucine residues in both HIV Rev and IκBα inhibits their nuclear export, resulting in their retention in the nucleus. Thus, this leucine repeat motif comprises a NES of both HIV Rev and IκBα. The presence of a similar motif in a region of RGS4 and RGS16 required for their cytoplasmic targeting in cells (Fig. 7) suggests that this motif may function as a NES for these two RGS proteins. To address this possibility, we examined the effects of alanine substitution of the leucine triplet in RGS4 and of a leucine and phenylalanine in RGS16 on localization of these two RGS proteins expressed as GFP fusion proteins in COS-7 cells. Fig. 8 shows that alanine substitution of the leucine repeat motif in the N-terminal domain of RGS4 and RGS16 resulted in nuclear localization of these proteins, as observed with HIV Rev and IκBα. The dramatic conversion of RGS4 and RGS16 from cytoplasmic to nuclear proteins by alanine substitution of the leucine repeat motif demonstrates that these key residues are critical for cytoplasmic accumulation of these RGS proteins. The observed accumulation of these alanine mutants of RGS4 and RGS16 in the cell nucleus indicates the existence of a default pathway that directs these proteins into the nucleus. These findings are clearly consistent with the hypothesis that RGS4 and RGS16 are nucleo-cytoplasmic shuttle proteins possessing both nuclear import sequences and the leucine repeat motif that functions as an NES, as observed for HIV Rev and IκBα. However, it is also possible that the leucine repeat motif prevents the normal nuclear import of these proteins and that its removal allows that process to occur.

Nuclear Export of RGS4 in N18 Cells—To distinguish between these two possibilities, we investigated the effects of leptomycin B on the cytoplasmic localization of RGS4 in N18 cells. Nucleo-cytoplasmic shuttle proteins with leucine-rich NES sequences are transported out of the cell nucleus by a protein shuttling mechanism using exportin1 and RanGTP (15–18). Leptomycin B inhibits NES-dependent export of HIV Rev, IκBα, and other NES-containing proteins by binding to exportin1 and preventing assembly of the NES-exportin1-RanGTP ternary complex (16–18). Fig. 9 shows confocal microscopic images of RGS4 in N18 cells treated with vehicle (control) or leptomycin B (5 nM) for 12 h. As shown, leptomycin B treatment of N18 cells caused accumulation of RGS4 in the nucleus where it is not found in control cells. These results confirm that cytoplasmic localization of RGS4 results from its transport from the nucleus to the cytoplasm by interaction with exportin1 via a leucine-rich NES located near its N terminus.

RGS Domain as Nuclear Targeting Domain—An immediate question that arises from these findings is what makes RGS4 go to the nucleus in the first place? Does a default pathway exist that targets RGS4 proteins to the nucleus where they remain unless they have a NES or, alternatively, a cytoplasmic retention sequence? Indeed, the RGS proteins that we have found to be predominantly nuclear proteins (RGS2 and RGS10) do not have leucine-rich NESs like RGS4 and RGS16. We examined whether RGS4, RGS16, or the three nuclear RGS proteins have clusters of basic amino acids or M9-like sequences that have been shown to function as nuclear localization sequences (19). We considered the possibility that sequences responsible for nuclear targeting of RGS proteins, even those that get transported out via NES-exportin1 interactions, are present in sequences that are common to RGS proteins, i.e., their RGS domain sequence. Indeed, RGS2, RGS4, RGS10, and RGS16 possess a consensus nuclear localization sequence (Lys-Lys-Xaa-(Lys/Arg)) within their RGS domains. In addition, RGS4 possesses a sequence within its RGS domain (amino
acids 154–170) that conforms to the consensus bipartite nuclear localization sequence. This sequence is comprised of two adjacent basic amino acids, a spacer of 10 amino acids followed by basic amino acids in three of the next five positions. When we expressed the RGS domains of RGS4 and RGS16 as GFP fusion proteins in COS-7 cells, the RGS domains of both of these cytoplasmic RGS proteins were localized in the cell nucleus (Fig. 10). These results suggest that the nuclear localization of RGS4, RGS16 and other RGS proteins may indeed result from nuclear targeting via their RGS domain sequences.

Cytoplasmic Targeting of RGSZ—RGS GAIP is a membrane-anchored protein located in the trans-Golgi complex (20). Binding of RGS GAIP to membranes in COS cells is associated with its palmitoylation (21). RGS GAIP has a cysteine-rich sequence (8 of 11 contiguous amino acids) in its N-terminal domain thought to represent the site of its palmitoylation (Fig. 11). If RGS GAIP is palmitoylated at this site, the cysteine-rich motif could comprise a cytoplasmic retention sequence. Thus, it is of particular interest that RGSZ has an identical cysteine-rich sequence in its N-terminal domain (Fig. 11) and displays physical properties of a membrane-bound protein (22). Although RGS GAIP has been localized to the trans-Golgi network in cells, the cellular site of localization of RGSZ is not known. RGSZ, unlike RGS4 and RGS16, lacks a leucine-rich NES to promote its cytoplasmic localization. Because RGSZ has an N-terminal cysteine-rich motif implicated in membrane localization of RGS GAIP in the trans-Golgi complex, it was of considerable interest to determine whether RGSZ was retained in the cytoplasmic compartment like RGS GAIP. Fig. 12 (top panels) shows the cellular localization of GFP-tagged RGSZ expressed in COS-7 cells. RGSZ was found predominantly in the cytoplasm with a punctate pattern of distribution consistent with localization in the Golgi complex. Localization of RGSZ in the trans-Golgi was confirmed by showing that RGSZ co-localized with the fluorescence-tagged ceramide analog Bodipy ceramide, which accumulates in the trans-Golgi complex (Fig. 12, middle panels). Also shown in Fig. 12 (bottom panels) is the predominant nuclear localization of an RGSZ mutant (RGSZ Δ1–49) lacking the cysteine-rich motif present in RGS GAIP and sequences N-terminal to this motif. Of note...
Intracellular Localization of RGS Proteins

RGS proteins are named for their ability to negatively regulate signaling by G proteins, located at the plasma membrane and, for some G proteins, in the Golgi complex (23–28). RGS proteins are thought to produce their regulatory effects by physically interacting with Ga subunits, particularly those in the Gq and Gi families, and enhancing their intrinsic GTPase activity by stabilizing the transition state conformation of these proteins for this reaction (7). Numerous studies have provided evidence for interaction of RGS proteins with Ga subunits. Watson et al. (29) demonstrated binding of recombinant RGS1, RGS2, and RGS4 to GDP-αF2-treated Gαi0 in bovine brain membranes, and Berman et al. (7) showed that RGS4 forms a high affinity complex with recombinant GDP-αF2-Gαi0. Co-precipitation of GTPase-deficient forms of Gαi and Gαi with RGS10 (30), of endogenous G12α with RGS3 (31), and of receptor-activated Gαi with RGS2 (28) has been documented in lysates derived from cells over-expressing these RGS proteins. Hepler et al. (9) showed that addition of RGS4 and RGS-GAIP to membrane preparations inhibits receptor-mediated Gαi signaling, and Heximer et al. (32) reported that recombinant RGS2 specifically inhibits Gαi signaling in membrane preparations. Recombinant RGS proteins stimulate the GTPase activity of membrane-bound and recombinant Gαi (6, 7, 9, 29, 32), and mutant RGS proteins defective in binding Ga subunits are inactive in augmenting GTP hydrolysis by Gαi (33).

It also has been proposed that RGS proteins may interact directly with receptors or effectors. Xu et al. (10) showed that recombinant RGS1, RGS4, and RGS16, but not RGS2, produced receptor-selective inhibition of Gαi signaling when added to permeabilized rat pancreatic acinar cells. The differential inhibitory effects of the same or different RGS protein on receptor-mediated Gαi signaling were not due to differences in the type of Gαi activated or the extent of Gαi activation. These observations suggested that RGS proteins interact directly with receptors to modulate G protein-coupled receptor signaling. Zeng et al. (34) reported that the N terminus of RGS4 was responsible for receptor-selective inhibition of Gαs in pancreatic acinar cells dialedyzed with recombinant RGS4 or portions thereof. Interestingly, the N-terminal 33 amino acids of RGS4 produced inhibitory effects on receptor-mediated Gαs signaling that were synergistic with that of the RGS domain of RGS4 and that were not reversed by GTPγS. Thus, the N-terminal domain of RGS4 was suggested to interact with receptors to position the protein between Gαs and the effector PI PLCβ. In such a position, RGS4 could impart receptor-selective inhibition of Gαs activation, act as a Gαs GTPase-activating protein, and act as an effector antagonist toward PI PLCβ. Evidence for this latter concept was provided first by the observation that recombinant RGS4 and RGS-GAIP blocked activation of PI PLC by GTPγS or GTPγS plus Bradykinin in NG-108 membranes (9). These workers also showed that PI PLCβ inhibited binding of Gαi to RGS4, suggesting that RGS4 may compete with Gαi for binding to PI PLC to mediate effector antagonism. RGS4 had no effects on receptor-mediated nucleotide exchange.

Implicit in the proposed regulatory actions of RGS proteins on cell surface receptors, Ga subunits or the effector PI PLC is their localization at or near the plasma membrane. However, our finding that certain RGS proteins (RGS2 and RGS10) are localized predominantly in the nucleus suggests that their interaction with plasma membrane-bound receptors, G proteins, or effectors is unlikely in intact resting cells. This, of course, does not preclude their interaction with such signaling proteins when added as recombinant proteins in vitro or to permeabilized or dialyzed cells as well as under certain cellular situa-
tions that are not as yet defined. Our results show that other RGS proteins (RGS4 and RGS16) are found predominantly in the cytoplasm where it seems more likely that they could interact with signaling proteins at the plasma membrane. We did not observe recruitment of RGS4 or RGS16 to the plasma membrane of COS-7 cells following stimulation of endogenous G_{i}-coupled lysophosphatidic acid receptors in these cells. Druey et al. (35) showed translocation of cytoplasmic RGS4 to the plasma membrane of HEK cells following expression of a GTPase-deficient G_{i}\alpha \_2 (Q207L), although the specificity and physiological relevance of this response is unclear. The mechanism of this recruitment does not involve interaction of RGS4 with mutant G_{i}\alpha because similar recruitment was observed with an RGS4 mutant that does not interact with G_{i}\alpha , and previous studies have shown that RGS4 does not complex with GTPase-deficient G_{i}\alpha \_7 (Dulin et al. (31) reported the presence of RGS3 immunoreactivity in endothelin- or A23187-induced membrane ruffles in HMG/RGS3 cells, although most RGS3 remained in the cytoplasm. Whether RGS3 localization in membrane ruffles is unique to these mesangial cell transfectants and/or important in the regulatory effects of RGS3 in these cells is unknown. However, it is known that expression of RGS4 in several mammalian cells including COS-7 cells attenuates receptor-mediated activation of mitogen-activated protein kinase via G_{i} and G_{q} (13, 23, 24). A key question that remains unanswered is whether these actions of RGS4 and other cytoplasmic RGS proteins require their translocation and physical association with the plasma membrane.

Two recent papers support our evidence that some members of the RGS protein family are localized in the Golgi complex and nucleus. De Vries et al. (20) showed that RGS-GAIP is located on Golgi membranes and not the plasma membrane. Our results show that RGSZ is localized exclusively in the trans-Golgi complex and that this localization is dependent upon sequences in its N-terminal domain, a region that shares with RGS-GAIP 8 cysteines in an 11-amino acid stretch. Because RGSZ shares no homology with RGS-GAIP in this region apart from this cysteine string, we favor the idea that this motif is important in the cytoplasmic retention and targeting of these two RGS proteins to the Golgi complex. In addition, Bowman et al. (36) showed confocal images of L1/L2 lymphoid cells transfected with nuclear GFP fusions of RGS1, RGS2, and RGS3. Although nuclear staining was not shown, the authors suggested that RGS2 was present in a region identified as the nucleus as well as near the plasma membrane. RGS1 was found throughout the cell with equivalent intensity, similar to the pattern of GFP alone, whereas RGS3 seemed to be excluded from the presumed nuclear region. Although these findings are not entirely consistent with our documentation of nuclear localization of RGS2, they support our evidence that some RGS proteins are transported to and remain in the nucleus after synthesis by virtue of their lack of cytoplasmic retention or nuclear export sequences.

The experiments described here do not address whether RGS proteins have functions apart from their ability to regulate membrane-delimited events involved in G protein signaling. It is possible that they have only G protein regulatory functions. In such a case, the separation in space of G proteins at the plasma membrane and RGS proteins at other cellular sites may be of regulatory importance, with these two families of proteins interacting only under certain cellular conditions. Alternatively, it is possible that RGS proteins have functions apart from, or in addition to, their ability to negatively regulate G protein signaling. In view of our evidence for nuclear localization and nucleo-cytoplasmic shuttling of some RGS proteins, it is interesting to speculate that these proteins may possess currently unrecognized functions in nuclear processes. We are unaware of such evidence, although Chuang et al. (37) suggested that RGS4 expression in oocytes caused a larger population of G proteins to be accessible for receptor-mediated GIRK channel activation. Although it has been shown that stimulation of G protein-coupled receptors can induce expression of RGS proteins (23, 28), it is unclear whether RGS protein expression can similarly influence expression of G proteins or other proteins. If interest is our finding that RGS domains of RGS proteins possess, in addition to determinants required for interaction with G\_\alpha proteins, sequences that target these proteins to the cell nucleus. Thus, it seems appropriate to speculate that both conserved and divergent sequences of RGS proteins may encode functions apart from the G protein-binding and -regulating activities of these proteins.

The existence of a large family of RGS proteins immediately prompted questions regarding functional differences among members of this family. Among many possibilities was the notion that sequence differences among these proteins may be involved in determining their specificity(s) toward G proteins. However, most studies have shown that RGS proteins are GTPase-activating proteins for G proteins in the G_{i} and G_{q} family, despite considerable sequence diversity outside of their conserved RGS domains. Thus, the RGS domain may represent the primary determinant for RGS protein interactions with G proteins, a finding supported by the deduced crystal structure of RGS4-G\_\alpha (and in vitro studies (8, 12, 33). Here, we provide evidence that diversity within this protein family has consequences in terms of the localization of RGS proteins within cells. The present study is the first to investigate the sequences responsible for and mechanisms underlying localization of these proteins within mammalian cells. Our results show that RGS proteins localize differentially within cells as a result of structural differences among these proteins that do not appear to be important determinants for their G protein-regulating activities. It seems quite likely that RGS proteins may have different functions within cells as a result of these different localization patterns. Hopefully, the present results will facilitate identification of new activities of the proteins that comprise the RGS protein family.

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