Human RGS6 Gene Structure, Complex Alternative Splicing, and Role of N Terminus and G Protein γ-Subunit-like (GGL) Domain in Subcellular Localization of RGS6 Splice Variants*

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RGS proteins are defined by the presence of a semi-conserved RGS domain that confers the GTPase-activating activity of these proteins toward certain Gα subunits. RGS6 is a member of a subfamily of RGS proteins distinguished by the presence of DEP and GGL domains, the latter a Gβ/γ-interacting domain. Here we report identification of 36 distinct transcripts of human RGS6 that arise by unusually complex processing of the RGS6 gene, which spans 630 kilobase pairs of genomic DNA in human chromosome 14 and is interrupted by 19 introns. These transcripts arise by use of two alternative transcription sites and complex alternative splicing mechanisms and encode proteins with long or short N-terminal domains, complete or incomplete GGL domains, 7 distinct C-terminal domains and a common internal domain where the RGS domain is found. The role of structural diversity in the N-terminal and GGL domains of RGS6 splice variants in their interaction with Gβγ and subcellular localization and of Gβγ on RGS6 protein localization was examined in COS-7 cells expressing various RGS6 splice variant proteins. RGS6 splice variants with complete GGL domains interacted with Gβγ, irrespective of the type of N-terminal domain, while those lacking a complete GGL domain did not. RGS6 protein variants displayed subcellular distribution patterns ranging from an exclusive cytoplasmic to exclusive nuclear/nucleolar localization, and co-expression of Gβγ promoted nuclear localization of RGS6 proteins. Analysis of our results show that the long N-terminal and GGL domain sequences of RGS6 proteins function as cytoplasmic retention sequences to prevent their nuclear/nucleolar accumulation. These findings provide the first evidence for Gβγ-independent functions of the GGL domain and for a role of Gβγ in RGS protein localization. This study reveals extraordinary complexity in processing of the human RGS6 gene and provides new insights into how structural diversity in the RGS6 protein family is involved in their localization and likely function(s) in cells.

The abbreviations used are: RGS, regulators of G protein signaling; bp, base pair(s); DEP, disheveled, Egl-10, pleckstrin homology; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; PPTC, fluorescein isothiocyanate; Gα, α-subunit of G protein; Gβ, β-subunit of protein; GAP, GTPase-activating protein; Gγ, G protein γ-subunit; G protein, guanine nucleotide-binding protein; GGL, G protein γ-subunit-like domain; NES, nuclear export sequence; NLS, nuclear localization sequence; NOLs, nuclear localization sequence; RACE, rapid amplification of cDNA ends; RGD, RGS domain; PI, propidium iodide.

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Indeed, the R4 subfamily proteins RGS4 and RGS16 possess NESs that function to transport these proteins from the nucleus to the cytoplasm (13) and the N-terminal domain of RGS3 has been implicated in its recruitment to the plasma membrane (14). RGS proteins in the R7 subfamily possess cysteine string motifs and several other protein domains have been identified that are unique to members of a given RGS protein subfamily (3). Of particular interest to the present study is the R7 subfamily of RGS proteins that includes RGS6 as well as RGS7, RGS9, and RGS11. Each of these RGS proteins has an N-terminal domain that contains a DEP (disheveled, Egl-10 and gleckstrin homology) and a GGL (G-protein gamma subunit-like) domain. Although the function of the DEP domain is unknown, the GGL domain has been shown to represent a binding site for Gβ (17, 18), an atypical Gβ subunit (19). Present evidence suggests that R7 family members may represent physiological binding partners for Gβ (10, 20), rather than Gα as observed for other Gβ proteins. The precise function of this interaction is not yet clear although Gβ binding to these RGS proteins has been implicated in RGS and Gβ protein stability (10, 18, 20), in regulation of RGS protein signaling or GAP activity (21–25) and in localization of Gβ (26).

We undertook studies to clone members of the RGS protein family to further our understanding of the structural diversity and complexity within this family. Recent evidence suggests that this complexity may arise not only from the more than 20 mammalian genes encoding RGS protein members, but also by the existence of multiple forms of a given RGS gene product. We identified two major transcripts for human RGS3 (27) and subsequently demonstrated the existence of twelve alternatively spliced forms of human RGS12 (12). RGS9 has also been found to exist in two splice variant forms (28). Here we report identification of 36 splice variant forms of RGS6 that arise by use of two alternative transcription start sites within the human RGS6 gene and by complex alternative splicing of the two primary RGS6 mRNAs. Identification of these transcripts enabled us to deduce the structure of the human RGS6 gene and the splicing mechanisms that generate these novel RGS6 transcripts. Interestingly, these RGS6 transcripts encode proteins with long or short N-terminal domains, complete or incomplete GGL domains, seven distinct C-terminal domains, and a common internal domain where the RGD is located. The existence of diversity outside of but not within the RGD of the RGS6 protein family raises complex questions in relation to a unifying hypothesis that these proteins have a single function dictated by this domain. We examined the role of structural diversity within the N-terminal and GGL domain of RGS6 splice variants in their interaction with Gβ and subcellular localization patterns and assessed whether Gβ alters the subcellular localization of RGS6 proteins. Our results demonstrate unique subcellular distribution patterns of RGS6 protein variants that can be ascribed to N-terminal and GGL domains of these proteins functioning as cytoplasmic retention sequences. The role of the GGL domain in cytoplasmic retention of RGS6 was observed in cells lacking Gβ5, providing the first evidence for a function of this domain independent of its interaction with Gβ5. Moreover, co-expression studies with Gβ5 and RGS6 proteins provide new evidence that Gβ5 interaction with RGS proteins promotes changes in their subcellular distribution. These results demonstrate extraordinary complexity in processing of the human RGS6 gene and provide new insight into how structural complexity in RGS6 proteins dictates their subcellular localization and possible functions.

### EXPERIMENTAL PROCEDURES

**Materials**—5′-RACE-ready cDNA, marathon-ready cDNA, Quick-scan cDNA library panel and pEGFP vector were purchased from Clontech. pCR2.1 and pCR3.1 were from Invitrogen. Elongase was from Invitrogen. Antibody to and cDNA encoding mouse Gβ5 was a generous gift of Dr. William Simonds (National Institutes of Health) and Dr. Vladi Slepak (University of Miami), respectively. 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 Facility. Recombinant RGS6 antibodies were generated with a synthetic peptide immunogen corresponding to residues 1–19 of RGS6, by Bio- synthesis Incorporated (Lewissville, TX).

**PCR Amplification of RGS6 cDNAs**—Full-length cDNAs encoding various forms of RGS6 were amplified using a PCR-based strategy we described previously (12). We utilized a 508-base expressed sequence tag (EST) as RGS6 (GenBank™ accession number H09621) as a design primer for use in 5′- and 3′-RACE to amplify overlapping segments of RGS6 cDNAs essentially as we described previously (12). Marathon-ready human brain cDNA (adapter sequences on both cDNA ends) were used as templates for 5′-RACE and 3′-RACE using adapter-specific forward or reverse primers in combination with appropriate RGS6 forward or reverse primers. This cDNA was synthesized from poly(A)- containing mRNA. Resulting PCR products were cloned into pCR2.1, and sequence analysis of multiple clones revealed successful amplification of overlapping 5′- and 3′-cDNA fragments of RGS6 from brain cDNA. Sequence analysis of these clones revealed the existence of multiple splice variant forms of RGS6. cDNAs encoding these splice variant forms of RGS6 were amplified using forward and reverse primers encompassing the translational start and stop sites, respectively. cDNAs were cloned into pCR3.1 and double-stranded sequencing was performed by automated fluorescent dideoxyxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Preparation of EGFP Constructs of RGS6**—Various RGS6 protein cDNAs were PCR-amplified using gene-specific primers incorporating restriction sites to facilitate their cloning into EGFP vector. First, amplified RGS6 protein cDNAs were cloned in the T/A cloning vector pCR2.1 (Invitrogen). Then, restriction enzyme digestion and agarose gel purification of the cloned RGS6 protein cDNAs was performed. RGS6 protein cDNAs were ligated to EGFP vector in-frame with its C-terminal or N-terminal EGFP sequence. Double-stranded sequencing of all cloned RGS6 protein cDNAs was performed by automated fluorescent dideoxyxynucleotide sequencing by the University of Iowa DNA Core Facility.

**Cell Culture and Transfection**—COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and gentamycin (50 µg/ml) (complete DMEM) in a 5% CO2 humidified atmosphere at 37 °C.

COS-7 cells were transiently transfected with vectors containing various RGS6 protein cDNAs and mouse Gβ5 cDNA by electroporation using a BioRad Gene-Pulsar. Typically, COS-7 cells (106/ml) were transfected with 40 µg of plasmid DNA at settings of 0.22 kV and 950 µF. Cells were harvested in complete DMEM and plated in two-chambered slides (Nunc) at a density of 5×104 cells/well. Transfected cells were used in experiments 40 h following transfection.

**Immunofluorescence and Immunohistochemistry**—Cells were rinsed three times with DPBS before fixation for immunofluorescence. For visualization of GFP-tagged RGS6 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 Applied Science) for 20 min at room temperature prior to staining with propidium iodide. For immunocytochemical detection of expressed Gαs in COS-7 cells, cells were fixed and permeabilized with 50% methanol, 50% acetic acid, prior to treatment with RNase A and incubation with anti-Gαs (1:5 × 106/ml) in DPBS containing 5% bovine serum albumin for 1 h at room temperature. Cells were then rinsed three times with DPBS and incubated in FITC-conjugated secondary antibody (1 mg/ml) in DPBS for 20 min at room temperature. RNase A treatment and propidium iodide staining was performed as described above. Cells were then mounted using Vecta Shield mounting solution. Confocal microscopy was performed as described previously (13). Images shown are representative of a minimum of 1000 cells derived from four or more separate transfections.

**Immunohistochemistry** was performed by Molecular Histology (MD) with synthetic peptide immunogen affinity-purified (SulfoLink, Pierce) anti-RGS6L. Briefly, tissue slides were incubated with and without anti-RGS6L, followed by biotinylated goat anti-rabbit antibody and streptavidin-horseradish peroxidase prior to incubation with substrate (DAB). Hematoxylin counterstain (stains nuclei blue) was used.
Identification of human RGS6 cDNAs—We used a PCR-based strategy to first amplify and clone two RGS6 cDNAs (GenBank™ AF073920, AF073921), the first identified RGS6 cDNAs, using sequence information from a 505-bp expressed sequence tag (GenBank™ H09621). The naming of these cDNAs was derived from the conceptual translation of rat brain PCR products of Koelle and Horvitz (5) in which degenerate primers were used to amplify a semiconserved region, now known as the RGS domain, of a family of mammalian proteins sharing sequence identity with EGL-10, the RGS protein in C. elegans. Inspection of the sequence of these two RGS6 cDNAs suggested the possibility of splicing of RGS6 transcripts at their 5′-ends; the larger form encoded a protein with an additional 139 N-terminal amino acids, and the cDNAs had different 5′-untranslated sequences. An apparent difference at the 3′-ends of these two cDNAs was subsequently resolved by identifying a 2-bp sequencing error in the longer form. Thus, the two RGS6 cDNAs appeared to represent long and short N-terminal forms of RGS6 so the short form was named an RGS6 variant to highlight this possibility.

Using 5′- and 3′-RACE and sequence information from these two RGS6 cDNAs, we were able to identify the existence of 36 novel RGS6 transcripts from oligo(dT)-primed cDNA from human brain. Sequence analysis of full or partial RGS6 cDNAs revealed that these variant RGS6 forms, although highly homologous, exhibit differences in their 5′-, 3′-, and internal sequences. Two different 5′-cDNA ends were identified that correspond to proteins with the long or short N-terminal sequences mentioned above. RGS6 cDNAs with each type of 5′-end existed in combination with seven different types of 3′-ends, encoding seven different C-terminal domains of the proteins. In addition, each of the RGS6 transcripts distinguished by different 5′- and 3′-ends existed in two forms that differed in the region encoding the G protein γ-subunit-like (GGL) domain that defines the subfamily of RGS proteins that includes RGS6, RGS7, RGS9, and RGS11. Sequence differences in this region encode RGS6 proteins with complete or incomplete GGL domains. The coding sequences of the 36 different RGS6 cDNAs encode proteins ranging from 284 to 490 amino acids.

RESULTS

Identification of Human RGS6 cDNAs—The 36 different RGS6 cDNAs encode RGS6 proteins with long or short N-terminal domains, seven distinct C-terminal domains, and complete or incomplete GGL domains. RGS6 proteins with the long and short N-terminal domain are named RGS6L and RGS6S, respectively. The identity of the type of C-terminal domain is indicated by α, β, γ, δ, ε, η, and ζ designations with α and β forms existing in two forms, with (α1 and β1) and without (α2 and β2) an 18 amino acid sequence (encoded by exon 18 of the RGS6 gene). Finally, the absence of a complete GGL domain is indicated by (-GGL). Amino acids are indicated by numbers and locations of DEP, GGL, and RGS domains are shown. The C-terminal amino acid sequences of the seven C-terminal variant forms of RGS6 are shown in Table I.

FIG. 1. Structure of human RGS6 proteins encoded by 36 RGS6 cDNAs. The 36 different RGS6 cDNAs encode RGS6 proteins with long or short N-terminal domains, seven distinct C-terminal domains, and complete or incomplete GGL domains. RGS6 proteins with the long and short N-terminal domain are named RGS6L and RGS6S, respectively. The identity of the type of C-terminal domain is indicated by α, β, γ, δ, ε, η, and ζ designations with α and β forms existing in two forms, with (α1 and β1) and without (α2 and β2) an 18 amino acid sequence (encoded by exon 18 of the RGS6 gene). Finally, the absence of a complete GGL domain is indicated by (-GGL). Amino acids are indicated by numbers and locations of DEP, GGL, and RGS domains are shown. The C-terminal amino acid sequences of the seven C-terminal variant forms of RGS6 are shown in Table I.

Co-immunoprecipitation of Gβ5 and RGS6—For co-immunoprecipitation studies, COS-7 cells were co-transfected with RGS6-GFP and mouse Gβ5 cDNA and grown for 48 h in 6-well culture dishes. Cells were harvested by lysis with 1 ml of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 1% Nonidet P-40, 6 mM MgCl₂, 10 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 16,000 × g for 1 min at 4 °C. Resulting supernatants were incubated with anti-GFP antibodies overnight at 4 °C followed by addition of protein A-Sepharose and incubation for an additional 3 h. Immunoprecipitates were collected by centrifugation and washed three times in RIPA buffer, suspended in Laemmli sample buffer and boiled for 3 min. Proteins were subjected to SDS-PAGE and immunoblotting as described previously (12).

Table I. C-terminal amino acid sequences of the seven C-terminal variant forms of RGS6 are shown in

Structure of the Human RGS6 Gene—The finding that the identified RGS6 transcripts encode highly homologous proteins...
that differ only by the presence or absence of sequence cas-
ettes or in their C-terminal sequences suggested these tran-
scripts might arise by alternative splicing mechanisms. Using
BLAST nucleotide sequence analysis of identified RGS6 cDNAs
with human gene data banks we were able to deduce the
complete structure of the human RGS6 gene. The RGS6 gene
spans 629,635 bp of DNA of human chromosome 14 and is
interrupted by 19 introns, and the 20 RGS6 gene exons range
in size from 51 to >323 bp. The intron-exon organization of the
human RGS6 gene in relation to RGS6L1 mRNA is shown in Fig. 2.
Table II shows the sizes of introns and the intron-exon splice
junction sequences in RGS6 transcripts. As shown, introns
vary in size from 155 bp to 387 kb, and all of the splice acceptor
and donor sequences agree with the GT/AG consensus se-
quence (29). The RGS6 gene intron phasing is type 0 (the intron
occurs between codons) for introns 2, 5, 7, 9–11, 15–19; type 1
(the intron interrupts the first and second bases of the codon)
for introns 3, 4, and 6; and type 2 (the intron interrupts the
second and third codon) for introns 8 and 12–14.

Splicing of Human RGS6 Transcripts—We examined the
relationship between exon and intron locations of the RGS6
gene to the structure of the 36 RGS6 cDNA variants we iden-
tified to gain insight into how these unique RGS6 variants
arise. Fig. 3 illustrates the deduced splicing mechanisms in-
volved in generating these 36 RGS6 transcripts. Transcripts
encoding the two N-terminal forms of RGS6 arise by use of
different transcriptional start sites. Exons 1–7 encode the 5’
untranslated region and unique N-terminal domain of RGS6L
proteins while an alternate transcription start site within
intron 7 generates RGS6S transcripts whose 5’-untranslated se-
quence and translation start site are encoded within intron 7
(non-coding exons A, B, C, and D) and exon 8. The translational
start site for RGS6S proteins begins at nucleotide 24 of exon 8,
resulting in no reading frameshift compared with RGS6L pro-
teins. The shared common internal domain found in all RGS6
proteins is encoded by exons 8 to 16 with splicing out of exon 13
in transcripts encoding -GGL forms of these proteins. The
conserved RGS domain present in RGS6 proteins is encoded by
exons 14–16. Complex alternative splicing at the 3’-end of
RGS6 transcripts generates 7 different C termini of RGS6
proteins. The C terminus of α1 forms of RGS6 is generated by
splicing together exons 16, 18, and 20. β1 forms of RGS6 are
formed the same way, except exon 18 is spliced to an alternate
splice site (β splice site) within exon 20 located 298 bp 3’ to the
α splice site (Table II). α2 and β2 forms of RGS6 transcripts
differ from their α1 and β1 counterparts by splicing out of exon
18, resulting in deletion of 18 amino acids encoded by this exon
with no change in reading frame in the encoded proteins. The
unique C terminus present in γ, δ, and ε forms of RGS6 is
encoded by exon 19, these transcripts unique in retention of
this exon. Indeed, alternative splicing within exon 19 generates
transcripts encoding the δ and ε forms, utilizing splice sites
located 9 and 12 bp, respectively, 3’ to the splice site used for γ
RGS6 transcripts (Table II), with splicing out of exon 17 in all

| Table I
| Different C-terminal ends and exon 18 variants of RGS6 protein |
|-----------------|-----------------|-----------------|
| Name            | PSE0GR74SLEKPFIRV | C Terminus      |
| α1              | +               | GKL4A4451G1M2Q6S |
| α2              | -               | GKL4A4451G1M2Q6S |
| β1              | -               | LYS4TFLAKR5       |
| β2              | -               | LYS4TFLAKR5       |
| γ               | +               | CLQ4L2F          |
| δ               | +               | L1F              |
| ε               | +               | G                |
| η               | -               | V4WLL            |
| ζ               | -               | KVS4K4VELP       |

three forms as found in α and β splice forms of RGS6. The
unique C terminus of η forms of RGS6 arise from transcripts in
which exon 16 is spliced to exon 17 while that of ζ forms of
RGS6 arise from transcripts in which exon 16 is spliced to an
alternate splice site in exon 18 (Table II). These 3’ splicing
mechanisms thus generate transcripts encoding seven differ-
ent C-terminal tails of RGS6, with two of these forms existing
with or without exon 18-encoded sequences. Thus, use of two
alternate transcription start sites to generate two different
N-terminal domains of RGS6 and alternative splicing to gen-
erate RGS6 proteins with complete or incomplete GGL domains
and nine different C-terminal to the conserved GGL domain
leads to unusual complexity in the RGS6 protein family.

Interaction of RGS6 proteins with Gβ5—The subfamily of
RGS proteins that contain GGL domains are unique in their
ability to interact with the atypical Gβ subunit Gβ5, indeed,
the GGL domain of these RGS proteins mediates interactions
with Gβ5 (17, 18). The precise function of Gβ5 interaction with
members of this RGS protein subfamily is unclear although
recent evidence suggests that such interactions may have a role
in Gβ5 protein stability (10, 18, 20), in regulation of RGS
protein signaling or GAP activity (21–25) and in localization of
Gβ5 (26). The existence of natural splice variants of RGS6
differing by the presence of a complete or incomplete GGL
domain immediately raised the question of whether these pro-
teins differed in their ability to interact with Gβ5. Therefore,
we examined the ability of both long and short forms of RGS6
and their -GGL splice variant forms to interact with Gβ5 in
COS-7 cells by co-immunoprecipitation studies. We focused our
attention on the α2 splice variant forms of RGS6 for these
studies. COS-7 cells were co-transfected with Gβ5 and GFP-
tagged forms of RGS6 proteins, and cell lysates were subjected
to immunoprecipitation with anti-GFP and immunoblotting
with anti-GFP and anti-Gβ5. Fig. 4A shows that Gβ5 co-pre-
cipitated with RGS6α2 but not RGS6α2(-GGL) or GFP alone
and, in a similar fashion, RGS6α2 but not RGS6α2(-GGL)
effectively co-precipitated Gβ5 from cell lysates (Fig. 4B).
These results demonstrate that Gβ5 interacts with both long
and short splice variant forms of RGS6 with complete GGL
domains but does not interact with the corresponding splice
variant forms lacking the approximate C-terminal half of the
GGL domain in these proteins.

Role of N Terminal and GGL Domain in Subcellular Local-
ization of Human RGS6 Proteins—Different splice variant
forms of RGS6 were tagged with GFP to examine their subcel-
ular distribution by confocal microscopy. We focused our ex-
perimental attention on RGS6 proteins possessing a common C
terminus so we could examine the role of N-terminal and GGL
domain variants in subcellular localization. For these ex-
periments, we studied the α2 C-terminal variants because of their
good expression in COS-7 cells and their characterized inter-
action with Gβ5 (Fig. 4). Fig. 5 shows confocal microscope
images of COS-7 cells expressing GFP-tagged forms of
RGS6α2, RGS6α2(-GGL), RGS6α2, and RGS6α2(-GGL).
Green represents GFP fluorescence from expressed RGS6 pro-
teins, red represents fluorescence from propidium iodide stain-
ing of nuclei in these cells and yellow (in the overlay) shows
overlapping red and green fluorescence. RGS6α2 was local-
ized exclusively in the cytoplasm of cells with a fine granular
distribution pattern (Fig. 5A). In contrast, RGS6α2 was vari-
ably localized in the cytoplasm, nucleus, and nucleoli of COS-7
cells. Fig. 5B shows the three major patterns of intracellular
distribution of RGS6α2 that were observed. Approximately
60% of cells exhibited a predominant nuclear pattern of
RGS6α2 (middle panel) while ~30% of cells exhibited a punc-
tate pattern of distribution throughout the cytoplasm and nucleus (left panel). This latter pattern is most similar to that of RGS6L/H9251 and differs by its localization in the nucleus and its punctate appearance. Nucleolar localization of RGS6L/H9251 was observed in 2-5% of transfected cells (right panel). These three distinct patterns of expression of RGS6L/H9251 were reproducibly observed and did not appear to be related to the levels of expression of RGS6L/H9251 in cells. Because RGS6L/H9251 differs from RGS6L/H9251 only by its unique N-terminal domain, these results suggest that the N-terminal domain of RGS6L/H9251 is responsible for its exclusive cytoplasmic localization.

To examine the role of the GGL domain in RGS6 protein localization, we compared the intracellular distribution of RGS6L/H9251 and RGS6L/H9251 to their splice forms lacking a complete GGL domain. Fig. 5A shows that RGS6L/H9251(-GGL) exhibited a cytoplasmic pattern of distribution like that observed for its GGL domain-containing counterpart. However, RGS6L/H9251(-GGL) was localized exclusively in the nucleus and nucleolus.

**Table II:** Exon/Intron organization of RGS6 gene

| 5′-Donor site | 3′-Acceptor site | Intron no. | Intron size | Alternate splice sites |
|---------------|------------------|------------|------------|------------------------|
| TGGGATCCGAGACgttagg... | tgcagTGGAGGGGAGACA | 1 | 31469 | |
| ATT GAC CCA GGTgtagg... | ttcagATT GAA GAC ATC | 2 | 387219 | |
| Val Tyr Cys Lys Ile Glu Glu Ile | | | | |
| AGT GTC TCA Ggttagc... | ttcagGT ACT GAC ATT GTC | 3 | 102333 | |
| Ser Val Val Thr G... | ly Thr Asp Ile Val | | | |
| ATT GAG GAC CCA Ggttacct... | ttcagTT GAA GCA ATA CAC | 4 | 3692 | |
| Ile Glu Asp Pro V... | al Glu Ala Ile His | | | |
| TAT CGT TTC CAGgttagc... | ttccagGTC CAG TTC TTT | 5 | 1254 | |
| Tyr Arg Phe Glu Ala Pro Tyr Phe | | | | |
| AAC ACT GAC TAT GGTgtagg... | ctcagCC TAT CTC TGT | 6 | 6074 | |
| Asn Thr Asp Tyr A... | al Glu Tyr Leu Cys | | | |
| GAT TAT GAA GGTgtagg... | ctcagGAA AAC TTA GCA | 7 | 4184 | |
| GAA GCA AAT AAGttagc... | ctccagATT GAC CCG AAA | 8 | 2788 | |
| GLu Glu Glu Val Ly Ile Asp Arg Lys | | | | |
| CAC AGG CCT GGTgtagg... | ctcagCCA GGC TGT GTG | 9 | 1671 | |
| His Arg Pro Val Pro Gly Cys Val | | | | |
| AAC ACT GAC TAT GGTgtagg... | ctcagTT GTC TGC TCG CTC | 10 | 2042 | |
| Lys Val Lys Ser Val Tyr Gly | | | | |
| ATC CGG AAA AAGttagc... | ctcagATA ACA TTT TTT | 11 | 1427 | |
| Ile Arg Lys Glu Ile Thr Phe Leu | | | | |
| AAA GTG GCT GAA GGTgtagc... | tgcagTTA ATT GCC TAC | 12 | 16822 | |
| Lys Val Ala Glu Se... | R Leu Ile Ala Tyr | | | |
| GAC ATA GAG ATG AAGttaga... | ctcagGAAA GAG CCC AGC | 13 | 14891 | |
| Asp Ile Glu Met Se... | R Lys Glu Pro Ser | | | |
| TCA GAA AAC AAGttaga... | ctcagGTT TTC TGG CGT GCT | 14 | 8071 | |
| His Glu Asn Leu Ar... | G Phe Trp Leu Ala | | | |
| GAA GAC GCC CAGgtttg... | ctcagGAG CAC ATC TAC | 15 | 17648 | |
| Glu Asp Ala Glu Glu His Ile Tyr | | | | |
| GCC AAG AAGttagc... | ctcagGTG TGG CTA CTG | 16 | 1242(η) | |
| Ala Lys Lys Lys Ala Trp Leu Leu | | | | |
| GCC AAG AAGttagc... | ttaagCCA GAA AGT GAG | 17 | 3765 | |
| Alas Lys Lys Lys Pro Glu Ser Glu | | | | |
| ACT CGC AGT GGTgtaga... | tgcagGTT CAG CTT | 18 | 155(γ) | Alternate 3′ acceptor site within exon 18 (γ) |
| Thr Arg Ser Val Cys Leu Glu Leu... | tgcagCCT CTC TTC TAG | | | |
| ACT CGC AGT GGTgtaga... | tgcagGTA CTA CAG | 19 | 22073(α) | Alternate 3′ acceptor site within exon 20 (β) |
| Thr Arg Ser Val Gly Lys Ser Leu | | | | |
| ACT CGC AGT GGTgtaga... | tgcagGGA AAG TCG CTT | 19 | 22073(α) | Alternate 3′ acceptor site within exon 19 (δ) |
| Thr Arg Ser Val Gly Lys Ser Leu | | | | |
| ACT CGC AGT GGTgtaga... | tgcagGGA AAG TCG CTT | 19 | 22073(α) | Alternate 3′ acceptor site within exon 19 (ε) |
| Thr Arg Ser Val Gly Lys Ser Leu | | | | |
| ACT CGC AGT GGTgtaga... | tgcagGGA AAG TCG CTT | 19 | 22073(α) | Alternate 3′ acceptor site within exon 20 (β) |
| Thr Arg Ser Val Gly Lys Ser Leu | | | | |

**Fig. 2.** Intron-exon organization of the human RGS6 gene in relation to the coding sequence of human RGS6L/H9251 mRNA. The position of exons (filled) and introns (empty) of the human RGS6 gene are shown at the top. The structure of human RGS6L/H9251 mRNA is shown at the bottom with the locations of introns (vertical lines) indicated by mRNA nucleotide number, with base 1 corresponding to the A of the AUG start codon. kb, kilobases.
Human RGS6 Gene Variants

Fig. 3. Representation of the splicing events occurring in the human RGS6 gene to generate 36 splice variant RGS6 mRNAs. Exons are shown as filled boxes and noncoding sequences as empty boxes. Two primary transcripts encode two 3′-splice forms of RGS6; one is processed to RGS6L transcripts and the other is processed to RGS6S transcripts. Retention or skipping of exon 13 generates transcripts encoding proteins with or without a complete GGL domain, respectively. 3′-splicing generates seven different 3′-ends from each primary RGS6 mRNA. Use of alternate splice sites in exon 20 (Table II) generates α and β forms of RGS6 while γ, δ, and ε forms arise from use of three alternate splice sites within exon 19 (Table II). Splicing of exons 16 to 17 or to an alternate splice site in exon 18 (Table II) generates η and ι forms of RGS6, respectively. RGS6 α and β transcripts each exist in two splice variant forms arising by retention (α1 and β1) or skipping (α2 and β2) of exon 17.

with no perinuclear or cytoplasmic accumulation of the protein (Fig. 5C). In contrast, the GGL domain-containing form of this protein exhibited a punctate distribution throughout the cytoplasm and nucleus in most transfectants while nuclear or nucleolar localization of RGS6S α2 was observed in a small fraction of transfectants and was always accompanied by considerable perinuclear and cytoplasmic protein localization (Fig. 5B). These results show that the GGL domain is required for cytoplasmic localization of RGS6S α2 but does not play a similar role in cytoplasmic targeting of RGS6L α2.

Recently, we showed that certain RGS proteins localize in the cytoplasm or nucleus based upon structural differences in the proteins (13). The RGS domain was identified as the molecular determinant for nuclear localization of these RGS proteins, and N-terminal sequences were identified that functioned as nuclear export sequences in RGS4 and RGS16 and as a cytoplasmic retention sequence in RGSZ. Thus, the requirement of the GGL domain for cytoplasmic localization of RGS6S α2 could reflect its role as a cytoplasmic retention sequence, as a nuclear export sequence or as a sequence that prevents nuclear import of the protein by unknown mechanisms. This latter possibility seems unlikely, in part because of the observed nuclear and nucleolar localization of the GGL domain-containing RGS6S α2 in some cells (Fig. 5B). In addition, sequence analysis of the GGL domain of RGS6 proteins revealed the absence of prototypical nuclear export sequences like those we described in RGS4 and RGS16.

Recently, Zhang et al. (26) reported that endogenous RGS7 and Gβ5 are present in both the cytoplasm and nucleus in mouse brain and PC12 cells. Ectopically expressed Gβ5, like native Gβ5, localized to the cytoplasm and nucleus of PC12 cells, while a Gβ5 mutant impaired in binding to GGL domains of RGS proteins was localized mainly in the cytoplasm when expressed in PC12 cells. Thus, it was suggested that Gβ5 undergoes nuclear translocation in neurons by an RGS protein-dependent mechanism. Indeed, several previous studies have shown that Gβ5 exists in complex with GGL domain-containing RGS proteins in native tissues and in cells expressing both proteins (17, 18, 20, 23, 30, 31). Therefore, it is of particular interest that we observed a different pattern of subcellular localization of RGS6S α2 and RGS6S α2–GGL in COS-7 cells, which do not express Gβ5 endogenously. That is, any difference in subcellular localization of these two proteins is due to the presence or absence of a complete GGL domain but is unrelated to interactions with Gβ5. In view of these observations and the interaction of RGS proteins with Gβ5 (Fig. 4), it seemed essential to determine whether the patterns of localization of Gβ5 and RGS6 proteins containing GGL domains are influenced by the expression of each other.

GFP-tagged RGS6 proteins and Gβ5 were co-transfected into COS-7 cells, and their subcellular distribution was examined by GFP fluorescence and immunocytochemistry (Gβ5). In cells transfected with Gβ5 alone, Gβ5 was expressed homogenously throughout the cytoplasm and nucleus as has been reported previously (26). Fig. 6 illustrates the subcellular patterns of distribution observed during co-expression of Gβ5 with RGS6L α2 and RGS6S α2. As shown, co-expression of Gβ5 with RGS6L α2 or RGS6S α2 promoted nuclear localization of both RGS6 proteins (Fig. 6A) as well as Gβ5 and their resulting co-localization in the nucleus (Fig. 6B). While it is clear that RGS6 expression with Gβ5 promotes nuclear localization of Gβ5, some Gβ5 is still found in the cytoplasm (Fig. 6). However, the effect of Gβ5 co-expression on RGS6 protein localization in the nucleus is particularly striking. RGS6S α2 is localized exclusively in the nucleus when co-expressed with Gβ5 quite in contrast to its patterns of expression when expressed alone (Fig. 5). Moreover, the exclusive cytoplasmic distribution observed during expression of RGS6L α2 alone (Fig. 5) contrasts with the predominant nuclear pattern of expression of this protein when expressed with Gβ5 (Fig. 6). Some RGS6L α2 is still present in the cytoplasm of these Gβ5 expressing cells, again illustrating that the N-terminal domain of this protein is important in its cytoplasmic targeting (i.e. by comparison to RGS6S α2) as noted above.

Expression of RGS6L—The 36 identified RGS6 transcripts are highly homologous and differ only in sequences encoding the long N terminus of RGS6L, the GGL domain, and the extreme C terminus of splice forms. Sequence overlap among transcripts encoding the C-terminal forms of RGS6 makes it problematic to determine the expression pattern of one transcript independent of others. For example, exon 19 splice forms differ by only 9–12 bp and splice forms with exon 18 and/or 20 sequences share overlapping sequence, e.g. coding in some forms and non-coding in others (Table II, Fig. 3). In addition, the sizes of the coding sequences of transcripts for different C-terminal forms of RGS6 are very similar although differences may exist in their 3′-untranslated regions (i.e. which were not determined for each splice form). However, splicing in or out of exon 13 sequences in RGS6 transcripts enabled us to demonstrate expression of transcripts encoding both RGS6L α2 and RGS6L α2–GGL in human brain (Fig. 7A). In addition, we raised an antibody to a synthetic peptide corresponding to residues 1–19 of RGS6L proteins, a sequence present only in the unique N-terminal domain of RGS6L. Fig. 7B shows that this polyclonal anti-RGS6L antibody recognized ectopically expressed RGS6L α2 as well as RGS6L protein(s) in mouse brain lysates. Immunohistochemistry of adult mouse brain demonstrated RGS6L immunoreactivity in several brain regions including the hippocampus and cerebellum (Fig. 7C). We found patterns of both cytoplasmic- and nuclear-localized RGS6L immunoreactivity in brain regions. It is possible that these patterns of localization in native brain could recapitulate the patterns of localization we observed during expression of RGS6L α2 alone (cytoplasmic) or together with Gβ5 (nuclear), as Gβ5 is expressed in cerebellum (32). However, it is unclear which C-terminal forms of RGS6L are present in these tissues and whether RGS6L proteins with different C-terminal domains localize differ-
ently. It is also possible that signaling-mediated changes regulate RGS6L localization in brain, as we provide evidence for nuclear and nucleolar localization of RGS6 proteins in response to activation of stress signaling pathways in the accompanying article (37).

DISCUSSION

The present study has revealed extraordinary complexity in the processing of the human RGS6 gene to generate 36 distinct forms of RGS6 differing in N- and C-terminal and GGL domain sequences. The possibility of such processing was evident when we first reported the sequence of two RGS6 cDNAs that differed by the presence or absence of a 5'-H11032 sequence encoding the N-terminal domain of one form. However, the identification of 36 RGS6 cDNAs enabled us to determine the structure of the human RGS6 gene and to deduce how these distinct transcripts arise. While it is possible that related transcripts could arise from distinct genes, our analysis shows that all 36 RGS6 mRNAs arise from a single human RGS6 gene by use of two distinct transcription start sites and by complex alternative splicing mechanisms. Although all forms of RGS6 described here retained the hallmark RGS domain characteristic of this protein family, our results show that RGS6 protein variants with structural differences outside of this domain exhibit differences in their subcellular localization patterns and interactions with Gβ5. The observed nuclear or nucleolar localization of certain RGS6 protein variants raises the possibility that their function(s) might be very different from that of other RGS proteins or other RGS6 protein variants that are not localized to these organelles.

Previous studies of RGS6 protein function have been limited to analysis of interaction of one form of RGS6 with Gβ5 and Gα subunits. This form, corresponding to what we have named RGS6Lα2, was amplified by Posner et al. (25) using our RGS6 sequence information and Snow et al. (18) isolated the same RGS6Lα2 cDNA and one with a 3' cDNA end insertion (that corresponds to RGS6Lα1). Snow et al. (18) showed that RGS6Lα2 specifically bound to Gβ5 during co-translation in reticulocyte lysates and that Gβ5 was co-precipitated from COS-7 cells transfected with both cDNAs. Gβ5 did not bind to RGS6Lα in reticulocyte assays when its GGL domain was deleted or mutated. Subsequent studies by Posner et al. (25) demonstrated that RGS6Lα2 complexes purified from Sf9 cells accelerated the GTPase activity of Gαo, but not that of other Gα subunits. These studies suggest the GGL domain of RGS6Lα2 is a binding partner for Gβ5, as observed for other GGL domain-containing RGS proteins (17, 20, 31), and that purified hetero-meric complexes of RGS6Lα and Gβ5 enhance the GTPase activity of Gαo, specifically in vitro. The identification of RGS6 protein splice variants lacking a complete GGL domain and shown here not to interact with Gβ5, obviously raises the possibility of different functional activities of these proteins in vivo. However, it must be acknowledged that the role of RGS6 proteins and of Gβ5 binding to them in cells is not known and will require further cell-based studies.
targeted in the cell. A novel role of the GGL domain and of co-expressed Gβ5 in RGS6 protein localization is suggested from our studies. We limited our studies and the following discussion of the subcellular distribution of RGS6 proteins to variants with a common C-terminal α2 domain. This enabled us to focus on the role of structural differences in the N terminus and GGL domains of RGS6 protein variants and of the GGL domain-binding protein Gβ5 on subcellular targeting of RGS6 proteins. Initially, we found that RGS6Lα2 localized exclusively in the cytoplasm while RGS6Sα2 localized in puncta throughout the cytoplasm and nucleus or, less commonly, localized primarily in the nucleus or nucleoli with reduced cytoplasmic localization. The long N terminus of RGS6L proteins, therefore, is responsible for their exclusive cytoplasmic targeting. A similar role of the GGL domain was suggested by the finding that RGS8Sα2(-GGL) localized exclusively in the nucleus or nucleoli, in contrast to the pattern of localization observed for its GGL domain-containing splice variant counterpart. Although these results show that the GGL domain is required for cytoplasmic targeting of RGS6S proteins, RGS6Lα2(-GGL) had the same subcellular distribution pattern as its GGL domain-containing splice variant. These findings suggest that the N terminus common to RGS6L protein variants and the GGL domain found in both RGS6L and RGS6S proteins function as cytoplasmic retention sequences. The GGL domain of RGS6 proteins may contribute to but is not required for the cytoplasmic localization of RGS6L proteins, i.e. the N terminus of these proteins overcome the need for the GGL domain. This differential requirement of the GGL domain in cytoplasmic targeting of RGS6S and RGS6L proteins was observed in cells not expressing Gβ5, providing new evidence for Gβ5-independent roles of this protein domain.

However, co-expression of the GGL domain binding protein Gβ5 with RGS6L promoted the exclusive nuclear and nucleolar targeting of RGS6L. Thus, co-expression of Gβ5 unveiled a cryptic role of the GGL domain of RGS6L proteins in cytoplasmic targeting that is overshadowed by its N-terminal domain in the absence of Gβ5. Our results showed that Gβ5 binding to RGS6L, as well as to RGS6S, is mediated by binding to the GGL domain because no binding was observed in RGS6 splice variants lacking a complete GGL domain. Thus our studies with naturally occurring splice variants of RGS6 confirm the importance of the GGL domain in Gβ5 binding determined by mutational analysis of this domain (18). The equivalent binding of Gβ5 to RGS6L and RGS6S that we observed further suggests that Gβ5 interaction with the GGL domains of these proteins is not hindered or enhanced by the presence of the long N-terminal domain of RGS6L. However the complete conversion of RGS6Lα2 from an exclusively cytoplasmic to exclusively nuclear and nucleolar protein by co-expression of Gβ5 suggests that Gβ5 interaction with RGS6L diminishes or neutralizes the role of both its N terminus and GGL domain in cytoplasmic targeting. It is interesting to speculate that Gβ5 could produce such an effect if its binding to the GGL domain of RGS6L produced both conformational changes in its N terminus that disrupted its role in cytoplasmic targeting and prevented interaction of its GGL domain with protein(s) required for the cytoplasmic targeting function of the GGL domain revealed in our studies. The ability of Gβ5 to enhance the stability of certain GGL-containing RGS proteins might reflect such conformational changes, which will need to be established by structural studies of Gβ5-RGS protein complexes. It is also possible that: 1) Gβ5 possesses NLSs that confer nuclear targeting of the RGS6L-Gβ5 complex, or 2) formation of the RGS6L-Gβ5 complex facilitates its interaction with NLS-containing proteins resulting in a “piggyback” mechanism of nu-
clear/nucleolar transport. Gβ5 does not possess prototypical NLSs although it does exhibit some nuclear localization when expressed alone. However, we did identify, in the accompanying article (37), functional NoLSs that target RGS6 proteins to the nucleus and nucleolus during activation of stress signaling (i.e. NoLSs also represent bona fide NLSs). Deletion mutagenesis identified these motifs in regions corresponding to amino acids 40–121 (DEP domain), 121–182 and the RGD of RGS6L. Indeed, we previously demonstrated that isolated RGS domains possess constitutive NLSs (13). Therefore, it is possible that one or more of these sequences may account for nuclear/nucleolar localization observed with RGS6S and with RGS6L co-expressed with Gβ5. Therefore, we favor the hypothesis that Gβ5 neutralizes the cytoplasmic retention functions of the GGL domain and N terminus of RGS6L to promote its localization to nuclear and nucleolar sites.

Indeed, our results provide the first evidence that Gβ5 interaction with RGS proteins has an effect on RGS protein localization. Co-expression of Gβ5 resulted in the localization of RGS6Lα2 in the nucleus and nucleoli in contrast to the exclusive cytoplasmic localization of RGS6Lα2 in cells not expressing Gβ5. An experiment opposite to ours showed the lack of nuclear localization of a Gβ5 mutant in PC12 cells that was impaired in binding to GGL domains, suggesting RGS-dependent nuclear localization of Gβ5 (26). Indeed, it is possible that RGS6 localization to the nucleus brings along the bound Gβ5 in our studies. The remaining Gβ5 in the cytoplasm of cells in which RGS6Sα2 and RGS6Lα2 were exclusively localized in the nucleus or nucleoli, could reflect saturation of RGS6 GGL domains with Gβ5 due to higher levels of expression of Gβ5 relative to RGS6. It is unknown whether RGS6 is an obligate heteromer with Gβ5 in cells expressing both proteins. It is of note that native RGS6L exhibited both cytoplasmic and nuclear patterns of subcellular localization in mouse cerebellum.

**Fig. 6.** Co-expression of Gβ5 promotes nuclear localization of RGS6L and RGS6S. A, confocal microscopic images of COS-7 cells co-expressing Gβ5 and GFP-tagged RGS6L or RGS6S. Both RGS6 proteins show a predominant nuclear localization in COS-7 cells expressing Gβ5. B, co-localization of Gβ5 with RGS6L (upper panel) or RGS6S (lower panel) in nuclei of COS-7 cells. Green represents GFP fluorescence from expressed RGS6 proteins; red represents propidium iodide (PI)-stained cell nuclei (upper panels); and yellow (in the overlay image) represents overlapping green and red fluorescence. Transfection of COS-7 cells and immunofluorescence measurements were performed as described under "Experimental Procedures."
Evaluation of this finding in light of the present studies in COS-7 cells raises the very interesting possibility that the cytoplasmic and nuclear/nucleolar forms of RGS6 may represent nonmonomeric RGS6L and RGS6L-Gβδ complexes, respectively, as Gβδ is expressed throughout the brain and in cell lines of neuronal origin (32). Alternatively, it is possible that transport of RGS6L-Gβδ complexes to nuclear and nucleolar sites is subject to regulation or that, in the absence of Gβδ, other mechanisms exist for nuclear/nucleolar transport of monomeric RGS6L. Indeed, we now show the existence of this latter possibility in the accompanying article.

The nuclear localization of RGS6 is unique among all RGS proteins studied to date. Studies in our laboratory first showed that some RGS proteins are nuclear proteins (RGS2, RGS10, and RGS12) and that others are nucleo-cytoplasmic shuttle proteins (RGS4 and RGS16) (12, 13). Studies in other laboratories supported these observations and showed that RGS3 and RGS6 are also nuclear RGS proteins (11, 15, 16, 34). Obviously, nuclear RGS proteins would be expected to have functions quite distinct from regulation of cell surface G protein-coupled receptor signaling. Recent findings provide the first insights into possible nuclear roles of RGS proteins. We found that RGS12 is a nuclear matrix protein and possesses cell cycle and transcriptional regulatory effects (35) and a recent study demonstrated that RGS2 promotes adipocyte differentiation (36).

It seems likely that the complex splicing of RGS6 mRNAs to generate 36 distinct forms of RGS6 proteins may be important in not only the localization of RGS6 proteins, but also in their tissue-specific patterns of expression, stability, and cellular functions. We speculate that differences in C-terminal domains of RGS6 protein splice variants, like those in N-terminal and GGL domains, may impact the subcellular localization or function of these proteins. Our results suggest new functions for the GGL domain, that are independent of its interaction with Gβδ, and of the GGL domain-binding protein Gβ5 and the N terminus unique to RGS6L proteins in the subcellular targeting of RGS6 proteins. Thus, splicing of RGS6 transcripts has important implications in generating proteins with different modular functional domains. It seems clear that some forms of RGS6 may be in a position to exert regulatory effects on G protein signaling by virtue of their localization in the cytoplasm or near plasma membrane localized G proteins, while localization of other splice variants or the same (i.e. as a heteromer with Gβδ) RGS6 proteins in the nucleus likely would preclude such effects and/or perhaps position these proteins for specific nuclear or nucleolar functions. Hopefully the present work will facilitate studies to reveal the likely complex roles of the RGS6 protein family.

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