Differentially Regulated Expression of Endogenous RGS4 and RGS7*

Regulators of G protein signaling (RGS proteins) constitute a family of newly appreciated components of G protein-mediated signal transduction. With few exceptions, most information available on mammalian RGS proteins was gained by transfection/overexpression or in vitro experiments, with relatively little known about the endogenous counterparts. Transfection studies, typically of tagged RGS proteins, have been conducted to overcome the low natural abundance of endogenous RGS proteins. Because transfection studies can lead to imprecise or erroneous conclusions, we have developed antibodies of high specificity and sensitivity to focus study on endogenous proteins. Expression of both RGS4 and RGS7 was detected in rat brain tissue and cultured PC12 and AtT-20 cells. Endogenous RGS4 presented as a single 27-28-kDa protein. By contrast, cultured cells transfected with a plasmid encoding RGS4 expressed two observable forms of the protein, apparently due to utilization of distinct sites of initiation of protein synthesis. Subcellular localization of endogenous RGS4 revealed predominant association with membrane fractions, rather than in cytosolic fractions, where most heterologously expressed RGS4 has been found. Endogenous levels of RGS7 exceeded RGS4 by 30-40-fold, and studies of cultured cells revealed regulatory differences between the two proteins. We observed that RGS4 mRNA and protein were concomitantly augmented with increased cell density and decreased by exposure of PC12M cells to nerve growth factor, whereas RGS7 was unaffected. Endogenous RGS7 was relatively stable, whereas proteolysis of endogenous RGS4 was a strong determinant of its lower level expression and short half-life. Although we searched without finding evidence for regulation of RGS4 proteolysis, the possibility remains that alterations in the degradation of this protein could provide a means to promptly alter patterns of signal transduction.

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G proteins transduce signals across the plasma membrane by sequential interactions with cell surface receptors and appropriate second messenger-producing effectors (e.g. enzymes and ion channels). These interactions are modulated by nucleation of conformational changes in the α subunits of heterotrimeric G proteins (Gα).1 A ligand-bound receptor catalyzes the exchange of GDP for GTP on its cognate Gα and the dissociation of Gα from the complex of G protein β and γ subunits (Gβγ). These dissociated subunits are competent to modulate the activity of effectors. The duration of G protein-mediated responses are dependent on the intrinsic GTPase rate of Gα and on extrinsic factors, such as regulators of G protein signaling (RGS proteins).

RGS proteins serve to regulate G protein signaling by functioning as GTPase-activating proteins (GAPs). GAP activity can sharpen the termination of a signal upon removal of a stimulus, attenuate a signal either as a feedback inhibitor or in response to a second input, promote regulatory association of other proteins, or redirect signaling within a G protein signaling network (reviewed in Ref. 1). RGS proteins are related by a conserved RGS domain that is composed of ~130 amino acid residues. The RGS domain alone is capable of binding Gα and accelerating GTP hydrolysis, although other domains contribute to affinity and/or selectivity for G protein targets (2, 3). Mammalian RGS proteins, of which ~20 are now known, can be grouped into five subfamilies based on sequence similarity (R4, R7, RI2, RA, and RZ) (4). Although several members of the RGS family are relatively simple ~25-kDa proteins that contain short amino and carboxyl sequences flanking the characteristic RGS domain (such as RGS4), others include more substantial modules that impart other functions. The R7 subfamily is characterized by possession of so-called DEP (disheveled, EGL-10, pleckstrin) and GGL (G protein gamma subunit-like) domains. While not well established, the DEP domain may play a role in directing Gα subunit specificity for the RGS domain (2). The GGL domain apparently specifies an obligate interaction of an RGS protein with the Gαq subunit (5). RGS4 has the capacity to accelerate in vitro GTPase activity of Gαq subfamily (including Gαq, Gα12, and Gα13) and Gαq subfamily members but not Gαi or Gα12 subfamily members. By contrast, GGL-containing RGS proteins exhibit specificity for Gαi and Gαq (7, 8).

Much of the currently available information on mammalian RGS proteins was gained by transfection/overexpression or in vitro experiments, with little known about the endogenous

The abbreviations used are: Gα, α subunit of heterotrimeric G protein; RGS, regulator of G protein signaling; Gβγ, βγ subunits of heterotrimeric G protein; GAP, Gα-interacting protein (also known as RGS19); GAP, GTPase-activating protein; NGF, nerve growth factor; siRNA, small interfering RNA.

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counterparts (particularly for the RGS4 subfamily). Because such studies can lead to imprecise or erroneous conclusions, caused by problems such as mislocalization and/or loss of substrate specificity, we have focused study on endogenous proteins. A case in point is the apparent difference in selectivity of RGS2 (an R4 family member) for Gαi and Gαq, when different transfection systems have been utilized (9, 10). Although the methodological design is relatively consistent, in brain, the protein only recently has been reported for this tissue (11). Ubiquitylation and proteasomal degradation may maintain the protein only recently has been reported for this tissue (11).

EXPERIMENTAL PROCEDURES

Reagents—Sources of reagents are indicated in parentheses: MG132 (Calbiochem), lactacystin B (Calbiochem), nerve growth factor (NGF; Promega), and cycloheximide (Sigma). DNA–cDNA for bacterial expression of untagged RGS4 short (initiation site Met-19) was produced by PCR using pQE600-H6-RGS4 (long) (13) as a template. The upstream primer included an appended BspHI restriction site (underlined): 5′-AGATCGTGATGAAAACATCGGCTGGGA-3′. The downstream primer was annealed to a site 3′ of the RGS4 termination site within the pQE bacterial expression plasmid (5′-TCA-ACAGGAGTCCAAGCTCAGC-3′). The PCR product was digested with BspHI and BamHI and subcloned into a compatible NcoI and BamHI-digested pQE60 vector (Qiagen). The newly formed pQE60RGS4 short vector also served as the source for subcloning RGS4 short into the mammalian expression vector, pCMV5 (14, 15), following EcoRI and BspHII digestion.

Proteins—Recombinant RGS4 was produced by transformation of Escherichia coli strain, BL21(DE3), with pQE600RGS4 or pQE600-RGS4 short. The transformed bacteria were allowed to grow at 37°C until A600 of ~1.0, and expression was induced by 100 μM isopropyl-β-D-1-thiogalactopyranoside (Roche Applied Science) for 4 h. Cells were harvested, flash-frozen in liquid nitrogen, and stored at −80°C until lysis, as described (13). Nontagged RGS4 protein (short or full-length) expressed to significantly higher levels than histidine-tagged RGS4 and formed the predominant protein band in the supernatant fraction from high speed centrifugation of lysates (that were resolved by SDS-PAGE and visualized by Coomassie Blue stain). A sample of lysate containing RGS4 short and SDS-PAGE sample buffer served as a control migration standard. Full-length, nontagged RGS4 was purified from the supernatant fraction by successive Mono Q and phenyl-Sepharose columns (Amersham Biosciences) essentially as described for histidine-tagged RGS4 (13).

Antibodies—Untagged recombinant RGS4 (full-length, >95% purity) from E. coli was injected intradermally into a New Zealand White rabbit for production of an antisera designated U1079. 150 μg of protein was divided among multiple sites on the back for the initial injection and each of the subsequent three boosts over a period of 6 months. Crude antisera was employed for Western immunoblotting. Antibodies to RGS7 (designated U1480) were produced from rabbits injected repeatedly with the peptide M41 (modified at the Biopolymer Core Facility, University of Texas Southwest- ern Medical Center), corresponding to amino acids 458–469 of mouse RGS7. The additional Cys residue (shown in parentheses) was appended for conjugation to the carrier protein, keyhole limpet hemocyanin (16). Specific antibodies were affinity-purified from the crude antisera by repeated absorption with the peptide immobilized on Sepharose (17). A similar strategy was employed to produce antisera R-381 against a synthetic peptide representing the 16 carboxyl-terminal amino acids of human Gαi2 (RGS7): (C)YRALLLLQQGPPSQSSSEE. An antisera to the carboxyl terminus of Gαi2 isoform 1 and 2 has been described (18).

Mammalian Cell Culture, Transfection, Fractionation—PC12M (rat pheochromocytoma), AtT20 (human pheochromocytoma), and COS-M6 (simian kidney) cells were obtained from the laboratories of Drs. Paul C. Sternweis, Elliott M. Ross, and Joseph Goldstein, respectively (all of the University of Texas Southwestern Medical Center). Cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose supplemented with 10% fetal calf serum (Invitrogen) and an atmosphere of 10% CO2.

A stably transduced line of human embryo kidney cells (HEK293) was derived as described (19).

Gene silencing of RGS4 and RGS7 in PC12M cells was accomplished by transient transfection of cells (80–95% confluent) with LipofectAMINE 2000 (2.8 μg/ml; Invitrogen) and plasmid (0.33–1 μg/ml) and/or short interfering RNA (siRNA; 100 nt) according to the manufacturer’s instructions. The sequences of the sense strands of the siRNA duplexes used for targeting RGS4 and RGS7 were CCUGCUGUUUC- UCAAGUCuTdTG and GCAGAGGAUAUCGCCAACAcTdTG, respectively. The targeted regions correspond to nucleotides 494–512 and 42–60 of the respective open reading frames. RNA oligonucleotides were synthesized and deprotected at the RNA Oligonucleotide Synthe- sis Facility (Inventions at The Texas Southwestern Medical Center). Cells transfected with siRNA duplexes were harvested 48 h post-transfection.

Cells were usually harvested with 1:2.5-fold concentrated SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% (w/v) SDS, 12.5% glycerol, 0.2% (w/v) bromphenol blue, 25 mM dithiothreitol, and 1.25% (w/v) β-mercaptoethanol). Detergent-solubilized lysates were subjected to ultracentrifugation (200,000 × g, 30 min, 4°C; Beckman 100.3 rotor), and the resultant supernatant fractions were retained for Western blot analyses. Where noted, cells were alternatively fractionated as described (15).

Tissue Preparations—Tissue samples of various regions of the rat brain were prepared from male Sprague-Dawley rats (200–350 g; Charles River). Rats were decapitated, and the brains removed from the skull were chilled for 1 min in phosphate-buffered saline. Coronal 1-mm slabs were obtained with an acrylic brain matrix (Ted Pella, Inc.). Needle punches of dorsolateral striatum, cerebellum, ventrobasal thal- amus (all 12-gauge, or parietal neocortex (14-gauge) were transferred to a Microfuge (Beckman) tube, rapidly frozen on dry ice, and stored at −80°C until use. Dorsal hippocampal samples were obtained identically, except that they were microdissected from the slab. The various tissue sections were routinely solubilized by sonication in buffer containing 1.0% SDS and protease inhibitors (from Sigma, unless otherwise noted): linal bean trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), aprotinin (0.5 μg/ml), and dithiothreitol (15 μg/ml), 1:1-g-lysyl-2-phenethylthylcholorometh ketone (15 μg/ml), (357-aminolo-1-chloro-3- tosylamino-2-heptanone hydrochloride (15 μg/ml), and MG-132 (10 μM; Calbiochem). Samples were boiled immediately for 3 min, aliquots were removed for protein determination, and the remainder of the lysate was rapidly frozen on dry ice and stored at −80°C until further use.

Protein Determination and Western Blots—For samples with SDS-PAGE sample buffer, protein concentration was determined by Amido Black (20) or by the Lowry method (21). Fractionation samples did not contain detergent and were assayed using Bradford reagent (Bio-Rad) (22). Bovine serum albumin served as standard in all assays. Except where noted, equal masses of total protein were processed by Western blot analysis.

cDNA Preparation and PCR—Total RNA was isolated from PC12 cells using Trizol reagent (Invitrogen). The RNA was primed using random hexamers and oligo(dT) and translated into cDNA using Moloney murine leukemia virus reverse transcriptase. RGS4 (sense primer, CAGCAAGAAGGAGCAAAATATG; antisense primer, GCAGCT- GGAAGATTGGTCA) was detected by PCR using 92°C/1 min of denaturation, 54°C/1 min of annealing, and 72°C/3 min of extension for 35 cycles. Each reaction was separated on a 1% agarose gel, and DNA products were detected with ethidium bromide. RGS4 appeared as a 430-base pair single band.

Northern Blots—Northern blots of total RNA (20 μg/lane) from NIH/3T3L1 (3T3L1) cells were produced by electrophoresis in a denaturing formaldehyde gel (23). Sample quality and equal loading of total RNA in each well was confirmed by ethidium bromide staining to visualize 18 and 18 S RNA. The RNA was transferred to nylon membrane (Genescreen; PerkinElmer Life Sciences) and hybridized with a radiolabeled mouse cDNA probe spanning the RGS domain. The in- corporated radioactivity was detected and quantitated using a Phosphoimager (Molecular Dynamics). BglII (RGS1), 150–587 (DS82, ttgtagctggagctgatt-ag); RGS2, 148–620 (C2: gatcctggctctggctcctg); RGS4, 116–549 (DS16: cagcaagaaagaaagact, DS17: gcag-gagctgtgttag); RGS5, 936–1385 (DS22A: gtggagaaggtcaag, DS62A: taaggctggctggagctgattag); RGS8, 99–555 (DS25:cttggacaaac-gacaagcaagct, DS26: gcgagaaggtcaagagaact). Mouse RGS4 R1 (first 1 is the first nucleotide of partial cDNA AF061933, the RGS domain is encoded within n 784–1106: DS310: atagagatggagctgattag, DS311: gagacactcgctctttcttttgg). Mouse Rgs16, 88–540 (DS80, tcagctggactc-gctac, DS81, cagagcttgctgagat). These probes detect unique re-
**RESULTS**

**Heterologously Overexpressed RGS4: Alternative Initiation of Translation**—COS cells transfected with a full-length cDNA for RGS4 expressed a protein that migrated more rapidly on denaturing SDS-PAGE gels than RGS4 protein purified from E. coli (Fig. 1A [lanes 3 and 4] versus the full-length standard (lane 6)). This expression of an apparently shorter than expected form of RGS4 was not cell type-specific, because it was also observed in transfected human embryonic kidney 293 cells and murine Neuro 2A cells (data not shown). We and others (12) noted that the nucleotide sequence surrounding the portion encoding the second methionine at position 19 formed a putative (or alternative) translational start site (26) and thus could explain the production of the short form of RGS4 in transfected mammalian cells. For this reason, RGS4 cDNAs, lacking the portion encoding the first 18 amino acids, were constructed for expression in mammalian cells and E. coli (RGS4 short). The RGS4 short purified from E. coli (Fig. 1A, lane 5) co-migrated with the RGS4 protein expressed in COS cells transfected with the full-length cDNA encoding RGS4 (lanes 1–4). Epitope tags preceding or succeeding full-length RGS4 resulted in the expression of a longer form of the protein in COS cells (lanes 7 and 8). These results suggest that the heterologously overexpressed, untagged RGS4, which we can detect, is predominantly initiated at the methionine at position 19 (of the full-length RGS4).

**Subcellular Localization of RGS4—RGS4 heterologously overexpressed in HEK 293 cells was found predominantly in the soluble fraction. This was the case whether RGS4 was N-terminally tagged (Fig. 1B) or C-terminally tagged, or untagged (not shown). Whereas this observation runs counter to the localization of G protein signaling at the plasma membrane, it is consistent with other reports (27,28). To confirm whether endogenous RGS4 demonstrated a similar subcellular distribution pattern, we required highly sensitive and specific antibodies. Antiserum U1079 was generated against a unique peptide sequence of RGS7, and, as anticipated, the antibodies were specific for this RGS protein (Fig. 2A).**

![Fig. 2. Isoform specificity of RGS antibodies assessed by Western immunoblotting. Blots of 10 ng each of various preparations of purified RGS proteins (indicated by their number designation at the bottom of each panel; 10 ng each) were probed with either the RGS4 antiserum, U1079, at 1:10,000 dilution (A) or 1 μg/ml affinity-purified U1480 antibodies against an RGS7 peptide (B). A: G. Gilman (University of Texas Southwestern Medical Center) provided purified proteins. The migration of molecular weight markers is indicated to the left in A (in kDa).](image-url)
neuroblasts, rat pituitary GH3, rat RBL-2H3, rat C6 glioma, Chinese hamster ovary, or NG108 neuroblastoma/glioma cells; data not shown).

In correlation with the PCR results, Western blots of PC12M and AtT20 cells revealed detectable immunoreactive bands consistent with RGS4 expression. One factor that contributed to our early difficulty in detection of endogenous RGS4 was the dependence of expression on cell density. We discovered that confluent cultures of PC12M (Fig. 3, A and B) or AtT20 cells (not shown) consistently expressed greater levels of RGS4 (per unit of total cell protein) than did cultures harvested at lower cell densities (Fig. 3A). By contrast, the amount of Gαi detected was unaffected by cell density (Fig. 3B). The level of expression of each of these proteins was largely unaffected by coating of the cell cultures with poly-l-lysine or the laminin (Fig. 3, A and B). An additional factor contributing to our early difficulty in detection of endogenous RGS4 was the presence of endogenous RGS7 proteins in these cell lines. The siRNA directed against RGS7 caused partial reduction of endogenous RGS7 protein expression but was without effect on endogenous RGS4. This experiment demonstrated that the siRNA oligonucleotides were RGS-specific and confirmed the identity of the RGS4 band detected by Western blotting with antiserum U1079.

Once it was clear that endogenous RGS4 could be reliably identified by Western blot, we examined the subcellular localization of endogenous RGS4. PC12M cells were fractionated by differential centrifugation for separation of nuclear (1000 × g; P1) and membrane (200,000 × g; P200) pellets plus cytosolic soluble proteins (S200). Unlike heterologously overexpressed RGS4 (Fig. 1B), endogenous RGS4 was found mostly in the pellet fractions of PC12M cells, including the 200,000 × g pellet, where membranes are expected to be located (Fig. 3E). The presence of RGS4 and Gαi in the 1000 × g (low speed) pellet fractions may, in part, be accounted for by some plasma membrane sheets that became trapped with nuclei and other relatively large subcellular particles. A similar pattern of silencing was observed for endogenous RGS4. The siRNA directed against RGS7 caused partial reduction of endogenous RGS7 protein expression but was without effect on endogenous RGS4. This experiment demonstrated that the siRNA oligonucleotides were RGS-specific and confirmed the identity of the RGS4 band detected by Western blotting with antiserum U1079.

FIG. 3. Detection of endogenous RGS4 in whole cell extracts and subcellular fractions from cultures of PC12M cells. A and B, PC12M cells were cultured at low (subconfluent) or high (confluent) cell density in culture wells that were either untreated (none) or coated with laminin (Lam); or poly-l-lysine (PL). Duplicate samples of 30 μg of cellular proteins were analyzed by Western immunoblotting. Migration of prestained molecular weight markers is indicated at the right in each lane (in kDa). Protein standards (from E. coli) are loaded in the lane designated Std (0.25-ng long RGS4 and 10-ng Gαi). Blots were processed by Western immunoblotting with the RGS4 antisemur (U1079; 1:2000 dilution) (A) or the Gαi antisemur (B087; 1:10,000 dilution) (B). C, relevant portions of the phosphor image from an RNase protection assay of RGS4 and cyclinophillin mRNAs derived from subconfluent and confluent cultures are shown. Migration of standards (right lane) is indicated to the right of the two panels (by nucleotide number). Quantification revealed a 2-fold increase in RGS4 mRNA levels for confluent versus subconfluent cells relative to an internal cyclophilin standard. The data are representative of two separate experiments. Similar results were obtained using β-actin as an internal standard (not shown). D, Western blot analysis (duplicate samples) from an experiment demonstrating siRNA-mediated knockdown of overexpressed green fluorescent protein-tagged RGS4 (top panel), endogenous (Endog) RGS4 (middle panel), or endogenous RGS7 (bottom panel). E, confluent PC12M cells were fractionated by differential centrifugation, and 35 μg of total protein were examined by Western blotting for expression of RGS4 and Gαi. P 1, 1000 × g pellet; P 200, 200,000 × g pellet; S 200, 200,000 × g supernatant fraction.
PC12M cells and frontal cortex from rat. We detected ~3 pg of RGS4 per μg of total protein from PC12M cells and only 1 pg per μg of total protein from frontal cortex. RGS7, on the other hand, was 30-40 fold more abundant: 40 pg/μg PC12M protein and 30 pg/μg frontal cortex protein.

Recently, Muma et al. (11) monitored RGS4 in human brain samples by immunoblotting with an RGS4 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (at 1:5000–10,000 dilution), but no standards for the amount or migration of RGS4 were shown. The Santa Cruz Biotechnology catalog shows migration of a doublet from mouse and rat brain that is >54 kDa, which leads us to question the specificity of the antibody, at least for use with mouse or rat tissues. We calculated the mass of RGS4 to be 23.2 kDa (from sequence data) and estimate size as a singlet of 27–28 kDa in Western blots of PC12 cells using our antisera and molecular weight standards. We tested the antibody from Santa Cruz Biotechnology (1:200 dilution) with known amounts of recombinant human RGS4 standard and found it to be sensitive to 10 ng, whereas our antisera, at 1:2000 dilution, was sensitive to less than 0.01 ng of RGS4 (after a 1-min exposure of chemiluminescent blot to film). Thus, we estimate our antisera to be about 10,000-fold more sensitive than the Santa Cruz Biotechnology preparation that we tested. In total, the available data suggest that either RGS4 is expressed to considerably greater levels in human brain (compared with mouse and rat brain) or that the Santa Cruz Biotechnology antisera is unable to detect bona fide endogenous RGS4.

**Stability of Endogenous RGS4 and 7**—Both RGS4 and RGS7 have been reported to be susceptible to degradation by the proteasome pathway (12, 35). Accordingly, the Western blot signal for RGS4 was increased significantly when PC12M or AtT20 cells were exposed to a proteasomal inhibitor, MG132 or lactacystin (Fig. 5, A and B). By contrast, these inhibitors had no effect on the endogenous levels of Gαi or Gαq (Fig. 5A). We hypothesized that the expression of RGS4 was limited by a high rate of degradation, and we therefore tested whether inhibition of protein synthesis by cycloheximide would cause RGS4 to diminish more quickly than RGS7 and Gαi. This prediction was supported by the data in Fig. 5C. Only about half of the immunoreactive RGS4 remained detectable after PC12M cells were exposed to cycloheximide for 1 h, whereas the expression of RGS7 and Gαi was apparently stable for at least 7 h (at which time the morphology of the cells had not changed appreciably).

We also examined whether an increase in endogenous RGS4 protein levels, as a result of PC12 cell exposure to the proteasome inhibitor MG132, would correlate with an increase in GAP activity toward Gαi. Of the known mammalian RGS proteins, only RZ and R4 family members have been demonstrated to accelerate the GTPase activity of Gαi. GAP activity in the 200,000 x g pellet fractions was almost 2-fold greater in the membranes from cells exposed to MG132 relative to untreated cells (18 ± 2.3 versus 11 ± 0.47 units/mg, respectively; triplicate determinations). This increase in GAP activity is likely to be related, at least in part, to the increase in the amount of RGS4 present in the membrane fraction from the cells exposed to MG132 (~4-fold measured by densitometry) (Fig. 5D). Additional data supports this inference. No mRNA for RGS2 or RGS22 was identified in PC12M cells by Northern or PCR (not shown). Another member of the RZ family, GAIP (also known as RGS19), could not be detected by Western immunoblotting with an antibody that could detect less than 1 ng of purified GAIP. RGS5 and -16 are additional R4 family members that would be anticipated, based on their N-terminal sequences, to be candidates for proteasomal degradation (12). However, we could not detect RGS16 in PC12M cells by Western immunoblotting with an antibody (that could detect less than 0.1 ng of purified RGS16; data not shown).

**Regulation of Endogenous RGS Proteins**—The relatively rapid turnover of RGS4 (and the accumulation of endogenous RGS4 and GAP activity in cells treated with proteasome inhibitors) prompted us to consider regulation of degradation as a swift means for cells to adjust levels of RGS4 protein. Because RGS4 has the capacity to negatively regulate Gαi- and Gαq-mediated signaling (19), we hypothesized that RGS4 levels would be promptly elevated in response to activation of one or both of these G proteins and thus constitute a mechanism of negative feedback regulation of signaling. We, however, unable to reveal changes in expression of RGS4 protein by acute exposure of cells to G protein activators such as 1 mM carbachol (agonist for Gαq- and Gαq-coupled receptors), 1 μM bradykinin (ligand for a Gαq-coupled receptor), or 20 or 40 μM peptide Mas 07 (a derivative of the Gαi activator, mastoparan, (36), or aluminum fluoride (an activator of G proteins that is effective on some, but not all, varieties of intact cells). Time courses for those reagents (with points ranging from 5 or 15 min to 6 or 8 h) were conducted on confluent and subconfluent cultures, but no changes in RGS4 protein expression were detected. NGF and cAMP signaling pathways promote differentiation in PC12 cells. Pepperell et al. (37) reported that treatment of PC12 cells with forskolin or cAMP analogs decreased RGS4 mRNA by

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2 Y. Tu, personal communication.
Recruitment of RGS4 to the membrane, a translocation that involves a protein in PC12M cells. Cells were cultured for 24 h in the presence of 40 ng/ml NGF. Another addition of half as much NGF was made (to NGF-treated cells only), and the cultures were incubated for another 24 h. Cultures were extracted with SDS-PAGE sample buffer for analysis of protein expression or with Trizol for analysis of mRNA. A, duplicate samples of 45 μg of cellular protein were processed for Western blotting with antibodies as indicated by the proteins and cognate antibodies used for Western blotting. B, duplicate samples of 45 μg of cellular protein were processed for Western blotting with antibodies as indicated by the bands. C, Northern blots were processed with radiolabeled probes for RGS isoforms (as indicated by the numbers at the top of blots). The ticks at the left (for RGS2, -4, and -6) and right (for RGS1, -7, and -16) margins of C indicate the migration of 28 and 18 S ribosomal RNA.

Endogenous RGS4 and RGS7

FIG. 6. NGF coordinately reduces expression of RGS4 mRNA and protein in PC12M cells. Cultures were exposed to NGF for 24 h in the presence (+) or absence (−) of 40 ng/ml NGF. Another addition of half as much NGF was made (to NGF-treated cells only), and the cultures were incubated for another 24 h. Cultures were extracted with SDS-PAGE sample buffer for analysis of protein expression or with Trizol for analysis of mRNA. A, duplicate samples of 45 μg of cellular protein were processed for Western blotting with antibodies as indicated by the proteins and cognate antibodies used for Western blotting. B, duplicate samples of 45 μg of cellular protein were processed for Western blotting with antibodies as indicated by the bands. C, Northern blots were processed with radiolabeled probes for RGS isoforms (as indicated by the numbers at the top of blots). The ticks at the left (for RGS2, -4, and -6) and right (for RGS1, -7, and -16) margins of C indicate the migration of 28 and 18 S ribosomal RNA.

We did not observe an effect of 10 μM 8-(4-chlorophenyl thio)cAMP, or 1 mM dibutyryl cAMP on the expression of RGS4 protein in PC12M cells (data not shown). Instead, we found that NGF treatment for 48 h decreased RGS4 protein levels by 2–3-fold, with no concomitant change in RGS7 and Goi (Fig. 6A). Northern blot analysis indicated that this decrease in RGS4 protein correlated with a decrease in RGS4 mRNA (Fig. 6, B and C). By contrast, levels of mRNA for RGS6, -7, -8, and -16 were unaffected. Message for RGS1 and -2 was not detected (Fig. 6C). It is possible that the NGF-induced reduction in RGS4 expression would promote Gα inhibition activity and thereby contribute to the process by which this class of G protein participates in NGF-dependent activation of mitogen-activated protein kinase and differentiation of PC12 cells (38).

DISCUSSION

We discovered substantial differences between endogenous and heterologously overexpressed RGS proteins, including start sites utilized for synthesis of protein, subcellular localization, and susceptibility to proteolysis. Davydov and Varshavsky (12) reported that, in addition to full-length RGS4, a shorter more stable form of RGS4, beginning at methionine 19, was produced by in vitro translation. We observed the shorter form exclusively in multiple cell types as a result of transfection with a cDNA that encoded nontagged RGS4. By contrast, however, we found that only the longer form was expressed endogenously in tissue or cultured cells. Thus, we conclude that cells in vivo do not typically utilize the alternative start site at methionine 19 of RGS4.

We found a substantial portion of endogenous RGS4 protein was associated with membrane fractions of PC12M cells. This RGS4 is presumably located strategically for regulation of membrane-bound Go and thereby precludes the necessity for recruitment of RGS4 to the membrane, a translocation that had been concluded from studies of heterologously overex-pressed RGS4 (27, 28). On the other hand, one model of G protein activation suggests that GTP-bound Ga, dissociated from Gβγ, is released from the plasma membrane (39). Perhaps cytosolic proteins, such as the subpopulations of endogenous RGS proteins detected in soluble fractions (Fig. 3E) (6), could serve to inactivate Ga released from the membrane, thus promoting the return of Ga to Gβγ at the plasma membrane.

Highly efficient pools of RGS proteins in multiple subcellular compartments may have prevented some investigators, including us, from finding substantial quantities of activated Go subunits in the cytosol (40).

We found the half-life of endogenous RGS4 to be short, on the order of just 1 h (Fig. 5C). We attribute this brief lifetime to the N-end rule pathway of protein degradation, as elucidated by Davydov and Varshavsky (12) for in vitro produced and transfection-produced RGS4. The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal amino acid. The Cys residue at position 2 of RGS4 is subject to arginylation, which targets the protein for ubiquitylation and degradation by the proteasome. In our mammalian cell transfection experiments, we did not detect full-length, untagged RGS4, presumably because this overexpressed protein was too rapidly degraded. In support of this inference, when an N-terminal Myc tag was added (thus creating a stabilizing amino acid at the N terminus), a protein of the expected size (full length plus tag) was produced (Fig. 1A).

Despite a report that RGS7 is also subject to degradation by the proteasome (35), we do not find that this pathway of degradation is a common characteristic of endogenous RGS proteins. Kim et al. (35) reported that heterologously overexpressed RGS7 is subject to degradation by the proteasome because inhibitors of this pathway increased the level of expression of the protein. By contrast, we found that proteasome inhibitors did not affect expression of endogenous RGS7 in PC12M cells; this is consistent with the protein sequence beginning with alanine, which is not a destabilizing amino acid. In addition, our experiments involving inhibition of protein synthesis indicated that endogenous RGS7 was resistant to proteolysis over 7 h (Fig. 5C). We ascribe the stability of endogenous RGS7 in PC12M cells to its obligate association with Gβγ (which may be limiting when RGS7 is overexpressed), as has been demonstrated by Slepak and co-workers (6).

We suggest that the particularly low level of expression of endogenous RGS4 is related to a high rate of degradation relative to synthesis of the protein. The amount of RGS4 detected was about 30-fold lower than RGS7 in frontal cortex. The levels of RGS4 and RGS7 were only 0.0001 and 0.003%, respectively, of total protein in cortex, whereas their substrates, such as Go and Goi, are highly expressed, comprising 1.5% of membrane protein (41). This disparity in the abundance of RGS and G proteins is consistent with RGS proteins acting catalytically in vivo (13). We speculate that localization of RGS proteins within cells of the brain, perhaps in preformed signaling complexes (1), may be a particularly crucial determinant of specifically which molecules of Go and Goi, will be subject to regulation by the relatively small number of RGS proteins.

In our screen of regions of brain and various cell types, we found a positive correlation between the amount of RGS4 mRNA and the amount of protein assayed by Western immunoblotting. For example, we detected RGS4 mRNA and protein in PC12M and AtT20 cells but little or no mRNA and no protein in NG108 or Neuro 2A cells. Whereas we also observed concomitant modulation of expression of RGS4 mRNA and protein by cell density or exposure of cells to NGF, a similar pattern of regulation of mRNA and protein is not necessarily universal. In a separate study, we found that, following acute or chronic
treatment of rats with morphine, the levels of RGS4 mRNA and protein in the locus coeruleus did not change in unison (42). This result points to the importance of monitoring protein (as opposed to just mRNA) in evaluating the impact of modulators on the physiological expression and function of RGS proteins.

Why is the level of RGS4 protein expression dependent on cell density? Reducing the rate of RGS4 degradation and/or increasing its rate of synthesis would increase the steady-state levels of endogenous RGS4. It is unlikely that regulation of the rate of protein degradation makes a major contribution for increased RGS4 protein levels, because treatment of PC12M cells with the proteasome inhibitor, MG132, resulted in increased RGS4 expression regardless of cell density (data for subconfluent cells not shown). Additionally, MG132 treatment of subconfluent cells failed to achieve the level of RGS4 expression found in confluent cells. RNase protection assays suggested that the mechanism of regulation is based, at least in part, on transcriptional control (Fig. 3C). Cell cycle did not appear to be a major factor in transcriptional control, because fluorescence-activated cell cycle analysis did not reveal significant differences between the distributions of cells among phases of the cell cycle. Because RGS4 mRNA and protein levels were coordinately and inversely affected with NGF treatment and higher cell density, the most likely explanation is that elevated RGS4 expression occurs as a result of increased transcriptional activity related to increased cell/cell contacts (vertically in addition to horizontally) that exist at higher cell densities.

In Saccharomyces cerevisiae, the RGS protein, Sst2p, helps overcome cell cycle arrest induced by mating factor (a ligand for a G protein-coupled receptor). Mating factor induces expression of Sst2p via a transcriptional mechanism, and this RGS protein serves as a negative feedback regulator of the mating factor pathway (43). Because we observed a high rate of RGS4 degradation (and increased GAP activity in cells exposed to a proteasome inhibitor), we hypothesized that an appropriate agonist or G protein activator would reduce RGS4 protein degradation in PC12M cells. This could provide the means to increase the level of RGS4 protein for function as a negative feedback regulator, which would be more rapid than a mechanism relying on transcription. To date, however, we were unable to find conditions to regulate proteolysis of RGS4 either by receptor agonist or by direct activation of G proteins. Although our current experience suggests that endogenous RGS4 protein levels in PC12M cells are not dictated by G protein activity, the possibility remains that degradation of RGS4 may be regulated via a mechanism that involves specific receptor(s) or other means that we have yet to address.

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