RGS6 Interacts with SCG10 and Promotes Neuronal Differentiation

ROLE OF THE G GAMMA SUBUNIT-LIKE (GGL) DOMAIN OF RGS6

Received for publication, June 13, 2002, and in revised form, July 18, 2002
Published, JBC Papers in Press, July 24, 2002, DOI 10.1074/jbc.M205908200

Zhengyu Liu, Tapan K. Chatterjee, and Rory A. Fisher

From the Department of Pharmacology, University of Iowa College of Medicine, Iowa City, Iowa 52242

RGS proteins comprise a large family of proteins named for their ability to negatively regulate heterotrimeric G protein signaling. RGS6 is a member of the R7 RGS protein subfamily endowed with DEP (disheveled, Egl-10, pleckstrin) and GGL (G protein gamma subunit-like) domains in addition to the RGS domain present in all RGS proteins. RGS6 exists in multiple splice variant forms with identical RGS domains but possessing complete or incomplete GGL domains and distinct N- and C-terminal domains. Here we report that RGS6 interacts with SCG10, a neuronal growth-associated protein. Using yeast two-hybrid analysis to map protein interaction domains, we identified the GGL domain of RGS6 as the SCG10-interacting region and the stathmin domain of SCG10 as the RGS6-interacting region. Pull-down studies in COS-7 cells expressing SCG10 and RGS6 splice variants revealed that SCG10 co-precipitated RGS6 proteins with complete GGL domains but not those with incomplete GGL domains, and vice versa. Expression of SCG10-interacting forms of RGS6 with SCG10 in PC12 or COS-7 cells resulted in co-localization of both proteins. RGS6 potentiated the ability of SCG10 to disrupt microtubule organization in PC12 and COS-7 cells. Furthermore, expression of SCG10 and RGS6 each enhanced NGF-induced PC12 cell differentiation, and co-expression of SCG10 with RGS6 produced synergistic effects on NGF-induced PC12 differentiation. These effects of RGS6 on microtubules and neuronal differentiation were observed only with RGS6 proteins with complete GGL domains. Mutation of a critical residue required for interaction of RGS proteins with G proteins did not affect the ability of RGS6 to induce neuronal differentiation. These findings identify SCG10 as a binding partner for the GGL domain of RGS6 and provide the first evidence for regulatory effects of an RGS protein on neuronal differentiation. Our results suggest that RGS6 induces neuronal differentiation by a novel mechanism involving interaction of SCG10 with its GGL domain and independent of RGS6 interactions with heterotrimeric G proteins.

RGS proteins comprise a family of more than 20 members named for their ability to negatively regulate heterotrimeric G protein signaling (1, 2). These proteins were defined originally by the presence of a homologous domain of ~120 amino acids called the RGD. RGS proteins, or their RGDs, bind in vitro to Ga subunits of Gα and Gβγ family members and enhance their intrinsic GTPase activity, i.e. accelerating the mechanism that inactivates Ga subunits and terminating intracellular signaling by both Ga and Gβγ subunits. These and other studies suggest that RGS proteins regulate G protein signaling by functioning as GAPs for Ga subunits (3). It must be acknowledged, however, that such a role has been demonstrated in mammals in vivo only for RGS9, whose GAP activity is required for the normal inactivation of transducin during phototransduction in retina (4).

Implicit in the proposed function of RGS proteins is their localization at the plasma membrane where G proteins and their activating receptors are found. Yet, recent studies in our laboratory (5, 6) revealed that several RGS proteins are localized in the nucleus or at intracellular sites other than the plasma membrane, suggesting that some RGS proteins may possess functions other than regulatory actions on G protein signaling. Indeed, we recently reported that a member of the RGS12 family is a nuclear matrix protein and transcriptional repressor (7).

RGS6 belongs to a subfamily of RGS proteins including RGS7, RGS9, and RGS11. Proteins in this family all possess a DEP (disheveled, Egl-10, and pleckstrin homology) and a GGL (G gamma subunit-like) domain (8–10). Recently, we identified 20 splice variant forms of RGS6 and demonstrated differences in their subcellular localization patterns that were related to structural differences in the proteins.2 We found that N-terminal sequences and the GGL domain function as cytoplasmic retention sequences and that splice variants lacking both of these domains localized exclusively in the nucleus. The structural complexity and diverse subcellular localization patterns of RGS6 proteins raises the possibility that RGS6 variants may have functions distinct from or in addition to effects on G protein signaling. RGS6 transcripts are expressed predominantly in brain although the functional role of these proteins remains unknown.

To identify and isolate proteins that interact with RGS6, we employed a yeast two-hybrid screen. We found that SCG10, a neuronal growth-associated protein, interacted strongly with RGS6. SCG10 belongs to the stathmin protein family, all members of which regulate cytoskeleton dynamics by destabilizing microtubules (11). SCG10 expression is high in the developing...
central nervous system and then dramatically decreases in adults (12). Both endogenous SCG10 in PC12 cells and ectopically expressed SCG10 are localized in the Golgi complex (13). Overexpression of SCG10 in PC12 cells enhances neurite outgrowth (14), an indicator of neuronal differentiation.

Here we show that the GGL domain of RGS6 is an SCG10-interacting region and the stathmin domain of SCG10 as the RGS6-interacting region. Co-expression of RGS6L with SCG10 resulted in their co-localization and promoted disruption of the microtubule network in COS-7 and PC12 cells. Both RGS6 and SCG10 enhance PC12 cell differentiation induced by NGF treatment and co-expression of SCG10 with RGS6 produced a synergistic effect on NGF-induced PC12 cell differentiation. Effects of RGS6 on microtubules and neuronal differentiation were observed only with RGS6 proteins with complete GGL domains while an RGS6 protein mutant incapable of interacting with G proteins induced neuronal differentiation like wild type RGS6. This work defines a new role of the GGL domain of RGS proteins as a protein interaction domain for SCG10 and new evidence for effects of an RGS protein on neuronal differentiation. Our results suggest that RGS6 induces neuronal differentiation by a novel mechanism involving interaction of SCG10 with its GGL domain and independent of RGS6 interactions with heterotrimeric G proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**pEGFP vector, pGBKTK, pACT2, pretransformed brain MATCHMAKER library, and anti-GFP monoclonal antibody were purchased from CLONTECH. pCR2.1 vector and anti-GFP polyclonal antibody were from Invitrogen and pCMV vector was from Stratagene. Anti-FLAG-conjugated agarose and monoclonal His probe (H3) were from Invitrogen and pCMV vector was from Stratagene. Anti-β-actin antibody was from Sigma. Anti-α-tubulin was from Sigma. Anti-Flag antibody were from Invitrogen and monoclonal His probe (H3) was from Santa Cruz Biotechnology. Anti-α-tubulin was from Sigma. Anti-β-actin antibody was from Sigma. Anti-Flag antibody were from Invitrogen and monoclonal His probe (H3) was from Santa Cruz Biotechnology.

**Preparation of EGFP Constructs of RGS6 and FLAG-tagged Constructs of SCG10—**Various RGS6 protein cDNAs and SCG10 protein cDNA were PCR-amplified using gene-specific primers incorporating restriction sites that facilitate their cloning into EGFP vector or pCMV2B 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 cDNAs was performed. RGS6 protein cDNAs were ligated to pGBKTK vector and SCG10 protein cDNAs were ligated to pACT2 vector. Constructs cloned into the pGBKTK vector were fused to an N-terminal GAL4 DNA binding domain (DB) and constructs cloned into the pACT2 vector were fused to an N-terminal GAL4 DNA activating domain (AC).

pGBKTK-RGS6L was used to screen a human brain cDNA library cloned into the pACT2 vector. The yeast strain AH109 was transformed with pGBKTK-RGS6L and mated with the yeast strain Y187 already transformed with human brain cDNA library. An estimated 10^8 diploid clones were screened. Yeast clones containing interacting proteins were identified by growth on selective medium lacking leucine, tryptophan, histidine, arginine, and adenine and encoded by β-galactosidase.

Full-length RGS6L and different RGS6 truncation mutants fused with GAL4 DB were then co-transformed with full-length SCG10 and SCG10 truncation mutants fused with GAL4 AD into AH109 cells. The activity of the reporter gene β-galactosidase was then assessed by liquid culture assay using ONPG as a substrate.

**Fluorescence and Immunofluorescence—**Cells were rinsed three times with PBS followed by fixation in ice-cold 10% 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. For immunodetection of FLAG, tubulin, or γ-adaptin, 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, incubated with fluorescence group-conjugated secondary antibodies (~1 μg/ml) in DPBS for 1 h at room temperature and washed three times with DPBS. Cells were air-dried and then mounted using Vecta Shield mounting solution. Images shown are representative of a minimum of 400 cells derived from four or more separate transfections. In some cases, the original fluorescence color was changed to a different color (e.g. Fig. 4) where we used the same fluorophore for two antibodies and/or for illustrative purposes.

**Co-immunoprecipitation of RGS6 and SCG10—**For co-immunoprecipitation studies, COS-7 cells were co-transfected with GFP-tagged forms of RGS6 proteins and FLAG-tagged SCG10 and grown for 48 h in 10-cm tissue 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, 60 mM MgCl2, and 10 mM phenylmethylsulfonyl fluoride) plus 1% Nonidet P-40 followed by centrifugation at 16,000 × g for 1 min at 4 °C. Resulting supernatants were incubated with either anti-FLAG-conjugated agarose or with anti-GFP and protein A-agarose overnight at 4 °C. Immune precipitates were collected by centrifugation, washed three times with RIPA buffer, suspended in Laemmli sample buffer, and boiled for 3 min. Proteins were subjected to SDS-PAGE and immunoblotting.

**RGS6L Binding to Goα1—**COS-7 cells were transfected with GFP-tagged forms of wild type RGS6L or RGS6L-N401V and grown for 48 h in 10-cm tissue culture dishes. Cells were harvested by lysis with 0.5 ml of ice-cold binding buffer (100 mM NaCl, 20 mM Tris-HCL, pH 8.0, 10 mM MgCl2, and 10 mM phenylmethylsulfonyl fluoride) plus 1% Nonidet P-40 followed by centrifugation at 16,000 × g for 1 min at 4 °C. Resulting supernatants were divided into two parts. One part was incubated with ~10 μg of His-Goα1 protein and 10 mM GDP and the other part was incubated with ~10 μg of His-Goα1 protein and 10 mM GDP plus 10 mM NaF and 30 μM AlCl3. After incubation at room temperature for 20 min, 70 μl of anti-RGS6L-conjugated agarose was added to the reaction. After incubation at room temperature for 30 min, the agarose beads were spun down, washed three times with 1 ml of binding buffer, and suspended in Laemmli sample buffer and boiled for 3 min. Proteins were subjected to SDS-PAGE and immunoblotting.

**Effects of RGS6 on SCG10-mediated PC12 Cell Differentiation—**PC12 cells were transfected with GFP-tagged forms of SCG10, RGS6L, RGS6L-GGL1, RGS6L-N401V or co-transfected with SCG10 and these three different forms of RGS6L as described above. Twenty-four hours later, GFP-positive cells were collected by fluorescence-activated cell sorting (~250,000 cells for each transfection condition). Cells were resuspended in 100,000 cells/dish in medium treated with 50 ng/ml NGF. Cells bearing neurites, defined as processes at least 2 times longer than the cell body, were scored at 24, 36, 48, and 72 h. At these same time points, cells were collected for analysis of 165 kDa neurofilament expression. Cells were collected by centrifugation in DPBS, Laemmli sample buffer was added to cell pellets, and samples were subjected to SDS-PAGE followed by anti-neurofilament immunoblotting. Tubulin was used to normalize protein loading.
RESULTS

Identification of SCG10 as an RGS6-binding Protein—We used the yeast-two-hybrid system to identify human brain proteins that interact with RGS6, whose expression is limited primarily to the brain (10). Full-length RGS6L was fused to the GAL4 DNA binding domain and was used as a bait to screen a human brain library. A clone with strong interaction with RGS6, encoding SCG10, was subsequently recovered by bacterial transformation. SCG10 belongs to the stathmin protein family, a family of proteins characterized by the presence of a stathmin domain of ~120 amino acids. The expression of this exclusively neuronal protein is high in developing neurons and low in mature neurons, and it induces neuronal differentiation (14).

RGS6-SCG10 Minimal Interaction Requirements—To identify the regions of RGS6 and SCG10 required for their interaction, we established several truncation mutants of both proteins and used yeast 2-hybrid β-galactosidase assays to assess the relative strength of interaction of these mutants. Previously, we devised a nomenclature for different RGS6 splice variants based upon the presence of a long (RGS6L) or short (RGS6S) N terminus, the presence or absence (−GGL) of a complete GGL domain, and five different C-terminal tails (α1, α2, β, γ1, γ2) of the encoded RGS6 proteins. RGS6L proteins have a 139 amino acid N-terminal sequence (which includes the DEP domain) not present in the otherwise homologous RGS6S proteins, and RGS6 proteins lacking the C-terminal 25 amino acids of the GGL domain (−GGL) and adjacent 12 amino acids are otherwise homologous to RGS6 proteins containing this 37 amino acid sequence. In the present study, we used RGS6L and RGS6S proteins or their (−GGL) splice variants, which all possessed the α2 C-terminal tail.

Fig. 1 illustrates the RGS6 and SCG10 constructs that were prepared to identify the interaction domains of RGS6 and SCG10. The RGS6 protein constructs were prepared as fusion proteins of GAL4 DNA binding domains, and the SCG10 constructs were prepared as fusion proteins of GAL4 DNA activating domains. The relative strength of interaction of RGS6 protein constructs with SCG10 protein constructs was therefore measured by β-galactosidase activity assays. The RGS6 protein constructs included full-length RGS6L and RGS6S, constructs N-terminally truncated before the GGL domain or the GRS domain and the GGL domain alone. p53 interaction with T antigen served as a positive control, and negative controls included RGS6L interaction with the pACT2 vector alone and SCG10 interaction with the pGBK7 vector alone. Fig. 1 shows that deletion of sequences N-terminal to the GGL domain of RGS6 protein constructs did not prevent or reduce RGS6 interactions with SCG10. However, further deletion of the GGL domain caused a loss of interaction with SCG10. The isolated GGL domain alone exhibited a stronger interaction with SCG10 than did RGS6L, RGS6S, or RGS6 mutants containing a GGL domain. These results show that the GGL domain of RGS6 proteins is necessary and sufficient for interaction with SCG10. The findings also suggest that sequences N-terminal or C-terminal (including the RGD) to the GGL domain are not required for this interaction and possibly may weaken the interaction slightly.

A similar approach was used to identify the region of SCG10 required for its interaction with RGS6. Deletion of the N-terminal 38 amino acid targeting sequence did not affect the ability of SCG10 to interact with an RGS6 construct N-terminally truncated before the GGL domain. Thus, the stathmin domain, conserved among members of the stathmin protein family, represents the structural domain required for interaction with RGS6 proteins.

RGS6 Interaction with SCG10 in COS-7 Cells and Role of the GGL Domain—It seemed essential to determine whether the interactions between RGS6 and SCG10 observed in yeast occurred also in mammalian cells and whether this interaction was mediated by the GGL domain of RGS6 proteins. The existence of naturally existing splice variant forms of RGS6 provided a unique opportunity to study the specificity of interaction of RGS6 proteins with SCG10 and to derive insights into the structural requirements for this interaction. Thus, we performed co-immunoprecipitation assays in COS-7 cells transfected with FLAG-tagged SCG10 and GFP-tagged forms of RGS6L, RGS6S, and their splice forms possessing incomplete GGL domains (designated −GGL). We examined co-immunoprecipitation of SCG10 by RGS6 proteins, and vice versa, by subjecting cell lysates to immunoprecipitation with anti-FLAG or anti-GFP antibodies followed by immunoblotting with anti-GFP or anti-FLAG. Immunoblotting was also performed with the antibody used for immunoprecipitation to show the efficacy of the precipitation reaction. Fig. 2 shows the results of these
studies with the upper and lower parts of the figure showing immunoprecipitation of SCG10 (anti-FLAG IP) and RGS6 proteins (anti-GFP IP), respectively. Fig. 2 shows that SCG10 co-precipitated RGS6L and RGS6S but not their splice forms lacking complete GGL domains. Similarly, RGS6L and RGS6S but not their splice forms lacking complete GGL domains efficiently co-precipitated SCG10. These differences were not due to differences in the level of expression of RGS6 protein variants or SCG10 or in the efficiency of their precipitation. These results provide the first evidence for interaction of SCG10 with RGS6 proteins in mammalian cells and demonstrate that this interaction requires a complete GGL domain. These results confirm our yeast two-hybrid results identifying the GGL domain as the region of RGS6 that is required for interaction with SCG10 and localize this region to the C-terminal 25 amino acids of the GGL domain that are missing in the −GGL splice forms. While an additional 12 amino acids C-terminal to the GGL domain are spliced out in −GGL forms of RGS6, this region does not appear required for RGS6−SCG10 interactions (Fig. 1). These results also show that SCG10 interaction with RGS6 proteins is not mediated by the RGD present in all of these RGS6 splice forms or by N-terminal sequences present on RGS6L proteins. The differential interaction of SCG10 with RGS6 proteins with complete or incomplete GGL domains suggests that RGS6 splice variants may have different functional activities based upon their ability to interact with SCG10.

Co-localization of RGS6 and SCG10—Endogenous SCG10 in PC12 cells and ectopically expressed SCG10 in COS-7 cells are localized in the Golgi complex (13). In contrast, we recently showed that RGS6L proteins, with complete or incomplete GGL domains, localize exclusively in the cytoplasm when expressed in COS-7 cells.2 In view of our evidence demonstrating high affinity interactions between RGS6 proteins and SCG10 and the dependence of this interaction on a complete GGL domain, we performed experiments to see whether we could detect any evidence of co-localization of SCG10-interacting forms of RGS6L. Therefore, we examined the subcellular localization patterns of SCG10 and RGS6L or RGS6L(−GGL) during their individual and combined expression in COS-7 and PC12 cells. Cells were transfected with FLAG−SCG10 alone or with GFP−tagged RGS6L or RGS6L(−GGL) and their subcellular localization patterns were assessed by indirect immunofluorescence and direct measurement of GFP fluorescence, respectively. We also assessed the Golgi localization of SCG10 by evaluating its co-localization with the Golgi marker γ-adaptin, also detected by indirect immunofluorescence. We found essentially identical results in PC12 and COS-7 cells. Fig. 3 shows the results of our studies in PC12 cells. When expressed alone, SCG10 was localized in the Golgi complex, as shown by co-localization (yellow) in the overlay images of anti-FLAG (green) and anti-γ-adaptin (red) immunofluorescence (upper panels). RGS6L exhibited a predominant cytoplasmic expression pattern when expressed alone in both PC12 and COS-7 cells (Fig. 4), as did RGS6L(−GGL).6 Co-expression of RGS6L and SCG10 in PC12 cells resulted in co-localization of these proteins in the perinuclear region (lower panels). Thus, co-expression of SCG10 and RGS6L resulted in their co-localization, and we saw no loss of the distinct Golgi-localized SCG10 observed in cells expressing SCG10 alone. In contrast, RGS6L(−GGL), which does not interact with SCG10 (Fig. 2), failed to co-localize with SCG10 or perturb its distinct Golgi localization. These results show that RGS6L and SCG10 exhibit co-localization during their overexpression in mammalian cells, in keeping with our evidence for direct interactions between these proteins, and that their interaction disrupts their individual subcellular localization.

Effects of RGS6L and SCG10 Co-expression on Microtubule Organization—SCG10 is a microtubule-destabilizing protein that induces microtubule depolymerization in vitro and during its overexpression in cultured cells (14, 15). Its ability to promote microtubule destabilization during expression in cells is believed to result from its delocalization from the Golgi during high levels of expression. N-terminal truncation of the hydrophobic sequence needed for Golgi localization of SCG10 pro-
with GFP-RGS6L or FLAG-SCG10 alone or together. Upper panels, microtubule organization in cells expressing GFP-RGS6L alone. Images of cells showing GFP-RGS6L (red) and tubulin (green) fluorescence. Yellow represents overlapping green and red fluorescence. Tubulin was detected by indirect immunofluorescence. Middle panels, microtubule organization in cells expressing FLAG-SCG10 alone. Images of cells showing SCG10 (red) and tubulin (green) fluorescence. Both proteins were detected by indirect immunofluorescence. Lower panels, co-expression of RGS6L and FLAG-SCG10 disrupts the microtubule network. Images of cells showing GFP-RGS6L (red), FLAG-SCG10 (blue) and tubulin (green). FLAG-SCG10 and tubulin were detected by indirect immunofluorescence. Transfections and fluorescence measurements were performed as described under “Experimental Procedures.”

**RGS6 Interaction with SCG10**

**FIG. 4.** Effect of RGS6L and SCG10 co-expression on microtubule organization in COS-7 cells and PC12 cells. Cells were transfected with GFP-RGS6L or FLAG-SCG10 alone or together. Upper panels, microtubule organization in cells expressing GFP-RGS6L alone. Images of cells showing GFP-RGS6L (red) and tubulin (green) fluorescence. Yellow represents overlapping green and red fluorescence. Tubulin was detected by indirect immunofluorescence. Middle panels, microtubule organization in cells expressing FLAG-SCG10 alone. Images of cells showing SCG10 (red) and tubulin (green) fluorescence. Both proteins were detected by indirect immunofluorescence. Lower panels, co-expression of RGS6L and FLAG-SCG10 disrupts the microtubule network. Images of cells showing GFP-RGS6L (red), FLAG-SCG10 (blue) and tubulin (green). FLAG-SCG10 and tubulin were detected by indirect immunofluorescence. Transfections and fluorescence measurements were performed as described under “Experimental Procedures.”

**RGS6L Promotes Neuronal Differentiation**—PC12 cells are primary endocrine cells that undergo differentiation into neurons during stimulation with NGF and have been used widely as a model of neuronal differentiation. NGF-induced neuronal differentiation of PC12 cells is characterized by formation of neurites and expression of neuronal markers, including neurofilament protein. Riederer et al. (14) showed that SCG10 expression in PC12 cells enhanced NGF-induced differentiation of these cells. SCG10 is a key regulator of neurite extension through regulation of microtubule instability and is localized in growth cones (14). In view of these observations and the present evidence that RGS6 interacts directly with SCG10 and potentiates its microtubule-stabilizing activity in PC12 cells, it seemed important to assess whether RGS6L possessed any functional ability to modulate neuronal differentiation induced by SCG10. For these studies, we used two measures of neuronal differentiation: formation of neurites and expression of neurofilament protein.

We first examined the influence of SCG10 and RGS6 protein expression on NGF-induced neurite outgrowth of PC12 cells. PC12 cells were transfected with GFP-tagged forms of SCG10, RGS6L, RGS6L(−GGL), or co-transfected with SCG10 and these two splice forms of RGS6L. 24 h following transfection, GFP-positive cells were collected by FACS and cultured in the presence of NGF to induce neuronal differentiation. Untransfected cells were similarly cultured. Neurite outgrowth was assessed by determining the percentage of cells with neurites 24, 36, 48, and 72 h after NGF addition. Fig. 6 summarizes the results of these experiments. In addition to the expected increase in neurite formation observed in cells expressing SCG10, we found that expression of RGS6L, but not RGS6L(−GGL), also increased neurite formation compared with untransfected cells at all time points examined. Of great interest was the finding that co-expression of RGS6L and SCG10 produced more than additive effects on neurite outgrowth at all time points examined. In contrast, neurite outgrowth in cells co-transfected with SCG10 and RGS6L(−GGL) or GFP showed the same neurite outgrowth as cells transfected with SCG10 alone. These results show that RGS6L and SCG10 produce a synergistic effect on neurite outgrowth and demonstrate the requirement for a complete GGL domain in RGS6 for this activity. In view of our evidence for a similar requirement of the GGL domain of RGS6 proteins in their binding to SCG10, these results suggest that the interaction of RGS6 with SCG10 through its GGL domain regulates neural differentiation induced by NGF. Our finding that RGS6L, but not RGS6L(−GGL), alone was capable of inducing neurite formation, may similarly reflect its interaction with endogenous SCG10 in PC12 cells.

Although our studies define a role of the GGL domain of RGS6L in its ability to induce neuronal differentiation, likely due to interactions with SCG10, it remained unclear whether RGS6L interactions with heterotrimeric G proteins are required in this process. Therefore, we assessed the effect of mutating a critical Asn in RGS6L (Asn(401)), required for RGS protein interaction with and GAP activity toward Gα subunits (17), on its ability to induce differentiation of PC12 cells. First, we compared the ability of wild type and mutant RGS6L to interact with G proteins by examining their ability to co-precipitate Gα1, in cell lysates by use of anti-RGS6L-conjugated agarose. Fig. 5 shows that Gα1 co-precipitated with RGS6L, and this interaction was specific for the transition state form (GDP + AlF4−) of the G protein. In contrast, Gα1 did not co-precipitate with RGS6L-N401V, confirming the inability of this RGS6L mutant to bind to G proteins. Fig. 6 shows that...
RGS6 Interaction with SCG10

**DISCUSSION**

The present study demonstrates specific interaction between RGS6 and SCG10 and further delineates the structural determinants for this interaction on both proteins. The GGL domain of RGS6 and the stathmin domain of SCG10 are the regions required for their high affinity interaction. RGS6 and SCG10 co-localized in cells expressing both proteins and their co-expression altered the patterns of subcellular localization observed when the proteins were expressed individually. This is the first evidence for RGS6 interaction with a protein other than a G protein α or β subunit. RGS6 co-expression with SCG10 produced a dramatic depletion of the microtubule network, perhaps in part due to its ability to promote delocalization of SCG10 from the Golgi complex. Both RGS6 and SCG10 enhanced PC12 cell differentiation induced by NGF treatment and were present in neurites of differentiated PC12 cells. Moreover, co-expression of SCG10 with RGS6L but not RGS6L(-GGL) produced a synergistic effect on NGF-induced PC12 cell differentiation. Particularly noteworthy is our finding that the ability of RGS6 to promote neuronal differentiation is independent of interactions with heterotrimeric G proteins. This work defines a new role of the GGL domain of RGS proteins as a protein interaction domain for SCG10, the first evidence for an RGS protein on neuronal differentiation and the second example of G protein-independent actions of an RGS protein (7).

RGS6 is a member of the R7 subfamily of RGS proteins that also include RGS7, RGS9, and RGS11. Northern and *in situ* hybridization studies have shown a predominant expression of these proteins in brain, while RGS9 also is expressed in the retina (8, 10, 20, 21). RGS9 is required for normal phototransduction processes in the retina (4), however the precise role of R7 subfamily members in brain has not been established. All members of this family possess a DEP domain and a GGL domain in addition to the semiconserved RGD. Although the role of the DEP domain is not known, the GGL domain has been shown to represent a binding site for an atypical Gβ subunit, Gβα, whose expression is also limited to the brain (22). Complexes of Gβα with RGS6, RGS7, and RGS9 have been isolated from brain or retina (23–25), although the precise function of this interaction is not clear. Levay et al. (9) reported that Gβα interaction with RGS7 decreased the affinity of RGS7 to Gαs without affecting its GAP activity toward Gαs, and that expression of RGS7 and Gβα in cells inhibited M3 muscarinic receptor-induced calcium mobilization mediated by Gαs (24). Complexes of RGS11-Gβα and RGS7-Gβα exhibit GAP activity toward the Gαs subunit *in vitro* (10, 26). A role for Gβα interaction with RGS9 in enhancing the GAP activity of cGMP phosphodiesterase toward transducin has been shown, although part of this effect is independent of the GGL domain of RGS9 (27). Gβα binding to RGS proteins may increase the stability of both proteins by post-transcriptional mechanisms (24). The GGL domain of RGS7 diverges from that of other RGS proteins in that it contains PEST sequences that target proteins for degradation (28). Polycystin binds to the GGL domain of RGS7 and prevents RGS7 degradation (28), however no other RGS proteins have been shown to bind to polycystin. The physiological importance of polycystin-RGS7 interaction is unclear.

Our findings show that SCG10 represents a GGL domain-binding partner for RGS6 and that this interaction has synergistic effects on NGF-induced differentiation of PC12 cells to neurons. This provides the first evidence for effects of an RGS protein on neuronal differentiation and for functional activities of RGS6 distinct from regulatory effects on G protein signaling. This latter conclusion is based upon our finding that RGS6L...
but not RGS6L\((/-GGL)\) enhanced SCG10-mediated microtubule destabilization and differentiation of PC12 cells, although both RGS6 splice forms possess the RGD needed for the GAP activity of these proteins toward \(G_\alpha\) subunits \((29)\). We further demonstrated that a mutation of a critical Asn in RGS6L required for RGS protein binding to and GAP activity toward \(G_\alpha\) subunits, had no effects on its ability to promote neuronal differentiation of PC12 cells. Posner et al. \((26)\) showed that recombinant RGS6L exhibited GAP activity toward \(G_\alpha\) \(\text{in vitro}\). Thus, it is possible that RGS6 may represent a multifunctional RGS protein capable of interactions with different proteins by different protein modules. Perhaps the particularly complex splicing of RGS6 transcripts serves to confer or limit protein interactions and signaling activities of members of the RGS6 protein family.

While this work was in progress, Nixon et al. \((30)\) reported identification of SCG10 as a binding partner for RGSZ1 using the RGS domain of RGSZ1 as bait. Subsequent GST protein pull-down studies showed that the N terminus of RGSZ1 \((1-\)

**FIG. 6. RGS6L enhances SCG10-mediated PC12 cell neurite outgrowth in response to NGF.** The percentage of cells with neurites in untransfected PC12 cells and PC12 cells transfected with SCG10, RGS6L, RGS6L-N401V, or RGS6L\((/-GGL)\) alone and SCG10 in combination with RGS6L, RGS6L-N401V, RGS6L\((/-GGL)\), or GFP alone was determined following NGF treatment (50 ng/ml) for 24, 36, 48, and 72 h. Neurites were scored as processes greater than two cell diameters in length. Percent neurite-bearing cells were determined as the average of three counts on at least 100 cells. Results shown represent means \(\pm\) S.D. of three separate experiments. Transfections, sorting, and treatment of PC12 cells were performed as described under “Experimental Procedures.”

**FIG. 7. RGS6L enhances SCG10-mediated neurofilament protein expression in PC12 cells in response to NGF.** Untransfected PC12 cells and PC12 cells transfected with SCG10 alone or in combination with RGS6L, RGS6L-N401V, RGS6L\((/-GGL)\), or GFP were treated with NGF (50 ng/ml) for 24, 36, 48, and 72 h. Expression of 165 kDa neurofilament protein was assessed in cells at each time point by immunoblotting, and tubulin immunoblotting was used as a control for sample loading. The results shown are representative of three essentially identical experiments. Transfections, sorting, treatment of PC12 cells, and immunoblotting were performed as described under “Experimental Procedures.”

**FIG. 8. Localization of RGS6L and SCG10 in neurites of NGF-differentiated PC12 cells.** PC12 cells were transfected with GFP-tagged RGS6L or SCG10 or both GFP-RGS6L and FLAG-SCG10 and 24 h following transfection were stimulated with NGF (50 ng/ml) for 45 h to induce differentiation. Confocal microscopic images of the GFP fluorescence (green) and indirect immunofluorescence of FLAG-SCG10 (red) and phase contrast image of cells are shown.
RGS6 Interaction with SCG10

86) and SCG10 (1–34) were required for their interaction. RGSZ1 does not possess a GGL domain, and its site of binding on SCG10 is outside of the stathmin domain and is unique to SCG10. Analysis of in vitro microtubule polymerization/dem polymerization showed that binding of RGSZ1 to SCG10 blocked the ability of SCG10 to induce microtubule disassembly. These results are quite different from those obtained in terms of the structural features required for protein interactions between RGS6 and SCG10 and the effects of this interaction on microtubule dynamics, where we found that RGS6 produced a synergistic effect on SCG10 induced microtubule disruption using intact cells.

The precise mechanism by which RGS6 enhances differentiation of PC12 cells cannot be determined from the present studies. SCG10 is a brain-specific member of the stathmin protein family, all members of which share a common stathmin domain possessing microtubule-depolymerizing activity (11). The present evidence for strong interactions of RGS6 with this domain raises the possibility that RGS6 may affect this stathmin domain function by direct interactions. Indeed, it is believed that SCG10 enhances neurite outgrowth by increasing the dynamic instability of microtubules in the growth cone (14). How Golgi complex-associated SCG10 gets transported to the growth cone and exerts this activity remains an open question. Our finding that co-expression of RGS6 and SCG10 disrupted the subcellular localization of both proteins may be relevant to how these proteins produce synergistic effects on microtubule disruption and NGF-induced PC12 cell differentiation. Certainly, the finding that both proteins are localized in neurites of differentiatied PC12 cells is consistent with a role in neurite outgrowth. Our results do show that RGS6 induces neuronal differentiation by a novel mechanism involving interaction of SCG10 with its GGL domain and independent of RGS6 interactions with heterotrimeric G proteins.

The present results raise intriguing questions concerning the physiological role of RGS6 in brain. Expression of SCG10 is developmentally regulated, with high levels of expression observed in embryonic and postnatal brain (12). Our finding that interaction of RGS6 and SCG10 is mediated via their GGL and stathmin domains, respectively, raises the possibility that RGS6 may interact with other members of the stathmin protein family and that other members of the R7 subfamily of RGS proteins may have similar protein interactions and functional effects on neuronal differentiation. Further studies will be required to evaluate the protein:protein interactions, possible mutual regulation between these two protein families and the role of this interaction in neuronal differentiation and development. It is intriguing that RGS6 transcripts undergo complex alternative splicing to generate at least twenty splice forms of RGS6 with ten of these forms predicted to be incapable of interacting with SCG10 due to splicing within the GGL domain. Hopefully, the present work will facilitate studies to reveal the likely complex functional roles of the RGS6 protein family in brain.

Acknowledgments—We thank Dr. Michael Natochin for helpful suggestions and the expert technical assistance of Dalyz Ochoa and Brian Solway.

REFERENCES
1. Dohlman, H. G., and Thorner, J. (1997) J. Biol. Chem. 272, 3871–3874
2. Burgon, P. G., Lee, W. L., Nixon, A. B., Peralta, E. G., and Casey, P. J. (2001) J. Biol. Chem. 276, 32828–32834
3. Ross, E. M., and Wilkie, T. M. (2000) Annu. Rev. Biochem. 69, 795–827
4. Chen, C. K., Burns, M. E., He, W., Wensel, T. G., Baylor, D. A., and Simon, M. I. (2000) Nature 403, 557–560
5. Grenze, T. K., and Fisher, R. A. (2000) J. Biol. Chem. 275, 24013–24021
6. Chatterjee, T. K., and Fisher, R. A. (2000) J. Biol. Chem. 275, 29660–29671
7. Chatterjee, T. K., and Fisher, R. A. (2002) Mol. Cell. Biol. 22, 4334–4345
8. Snow, B. E., Krumins, A. M., Brothers, G. M., Lee, S. F., Wall, M. A., Chung, S., Mangion, J., Arya, S., Gilman, A. G., and Siderovski, D. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13307–13312
9. Levy, K., Cabrera, J. L., Satgé, D. K., and Slepk, V. Z. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2503–2507
10. Snow, B. E., Betts, L., Mangion, J., Sondek, J., and Siderovski, D. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6489–6494
11. Charbaut, E., Curmi, P. A., Ozon, S., Lachkar, S., Redeker, V., and Sobel, A. (2001) J. Biol. Chem. 276, 16146–16154
12. Stein, R., Mori, N., Matthews, K., Lo, L. C., and Anderson, D. J. (1988) Neuron 1, 463–476
13. Di Paolo, G., Lutjens, R., Peller, V., Stimpson, A. A., Beuchat, M. H., Catsicas, S., and Grenningloh, G. (1997) J. Biol. Chem. 272, 5175–5182
14. Riederer, B. M., Peller, V., Antonsson, B., Di Paolo, G., Stimpson, S. A., Lutjens, R., Catsicas, S., and Grenningloh, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 741–745
15. Gavet, O., Ozon, S., Manceau, V., Lawler, S., Curmi, P., and Sobel, A. (1998) J. Cell Sci. 111, 3333–3344
16. Antonsson, B., Kassel, D. B., Di Paolo, G., Lutjens, R., Riederer, B. M., and Grenningloh, G. (1998) J. Biol. Chem. 273, 8439–8446
17. Posner, B. A., Mukhopadhyay, S., Tesmer, J. J., Gilman, A. G., and Ross, E. M. (1999) Biochemistry 38, 2726–2732
18. Cordes, S. P. (2001) Nat. Rev. Neurosci. 2, 611–623
19. Di Paolo, G., Lutjens, R., Osen-Sand, A., Sobel, A., Catsicas, S., and Grenningloh, G. (1999) J. Biol. Chem. 274, 33339–3344
20. Thomas, E. A., Danielson, P. E., and Sutcliffe, J. G. (1998) J. Neurosci. Res. 52, 118–124
21. Watson, A. J., Katz, A., and Simon, M. I. (1994) J. Biol. Chem. 269, 22150–22156
22. Zhang, J. H., and Simonds, W. F. (2000) J. Neurosci. 20, RC59
23. Witherow, D. S., Wang, Q., Levy, K., Cabrera, J. L., Chen, J., Williams, G. B., and Slepk, V. Z. (2000) J. Biol. Chem. 275, 24872–24880
24. Makino, E. R., Handy, J. W., Li, T., and Arshavsky, V. Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1947–1952
25. Posner, B. A., Gilman, A. G., and Harris, B. A. (1999) J. Biol. Chem. 274, 31087–31093
26. He, W., Lu, L., Zhang, X., El-Hodiri, H. M., Chen, C. K., Slepk, K. C., Simon, M. I., Jamrich, M., and Wensel, T. G. (2000) J. Biol. Chem. 275, 37095–37100
27. Kim, E., Arnold, T., Sellin, L., Benzing, L., Cornell, N., Kocher, O., Tsokas, L., Sukhatme, V. P., and Walz, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6371–6376
28. Popov, S., Yu, K., Konzasa, T., and Wilkie, T. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7216–7220
29. Nixon, A. B., Grenningloh, G., and Casey, P. J. (2002) J. Biol. Chem. 277, 18127–18133