A Candidate Target for G Protein Action in Brain*

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An effector candidate for G protein action, GRIN1, was identified by screening a cDNA expression library with phosphorylated GTPαS-Gα as a probe. GRIN1 is a novel protein without substantial homology to known protein domains. It is expressed largely in brain and binds specifically to activated Gα1, Gα13, and Gα14 through its carboxyl-terminal region. The protein KIAA0514 (GRIN2) is homologous to GRIN1 at its carboxyl terminus and also binds to activated Gα1. Both GRIN1 and Gα1 are membrane-bound proteins that are enriched in the growth cones of neurites. Coexpression of GRIN1 or GRIN2 with activated Gα1 causes formation of a network of fine processes in Neuro2a cells, suggesting that this pathway may function downstream of Gα1 to control growth of neurites.

It is likely that several targets for the action of G proteinα subunits remain to be identified. Members of the Gα subfamily of these proteins, particularly Gα13 and Gα14, constitute roughly 1% of brain membrane protein, yet the only known effectors for Gα1, the three Gα proteins, and Gα13 are certain isoforms of the enzyme adenyl cyclase (1, 2). We have thus attempted to detect novel effectors that lie downstream of selected G proteinα subunits, initially by utilizing 32P-labeled α subunits activated (essentially irreversibly) with GTPαS to probe cDNA expression libraries. We have taken advantage of the fact that Gα1 can be phosphorylated by protein kinase C at a site near its amino terminus that does not interfere with interaction between Gα1 and adenylyl cyclase (3). We have also appended a site for phosphorylation by cyclic AMP-dependent protein kinase to the carboxyl terminus of Gα1; this region of G proteinα subunits is also not involved in interactions with known effectors. This strategy led to isolation of a novel cDNA, initially designated Z-16, and detection of a homolog, KIAA0514. The protein products of these cDNAs interact selectively with GTPαS- or GDP-AIFα3-bound forms of Gα1 subfamily members in vitro, and they cause extension of neurites in Neuro2a cells when coexpressed with activated forms of Gα1. We thus tentatively refer to these two proteins as (GRIN1) (Z-16) and GRIN2 (KIAA0514) (G protein-regulated inducer of neurite outgrowth).

EXPERIMENTAL PROCEDURES

Preparation of Phosphorylated Gα and Gα—Gα and protein kinase Ca (PKCa) were purified using a recombinant baculovirus-Sf9 cell expression system as described previously (2, 3). The recombinant catalytic subunit of CAMP-dependent protein kinase (protein kinase A) was expressed and purified from Escherichia coli (4). A tag encoding a phosphorylation site for protein kinase A (RRRASLG) followed by six histidine residues was added to the carboxyl terminus of Gα1 by polymerase chain reaction. The protein was then coexpressed with protein N-myristoyl transferase in E. coli and purified as described (5). Other recombinant G proteinα subunits were prepared as described previously (2, 6).

Gα1 (250 μg) was incubated with GTPγS at 30 °C for 60 min in the presence of 5 mM EDTA and 2 mM MgSO4. Activated Gα1 was then phosphorylated with PKCα (4 μg) in buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM MgSO4, 0.125 mM CaCl2, 1 mM DTT, 3 μM 32P-ATP (40,000 cpm/pmol), and 20 μg/ml phosphorylase-α (Sigma) for 30 min at 30 °C. Tagged Gα1 (600 μg) was similarly activated with GTPγS and phosphorylated with the catalytic subunit of protein kinase A (3 μg) at 30 °C for 30 min as described by Baude et al. (4). After phosphorylation, free 32P-ATP was removed by gel filtration (PD-10 column; Amersham Pharmacia Biotech) in Buffer A (20 mM NaHepes (pH 7.4), 100 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 0.05% C12E10 (polyoxyethylene 10-lauryl ether), 1 mM DTT, 10 mM β-glycerophosphate), and the proteins were used as probes for screening cDNA expression libraries.

Isolation of Z-16 cDNA—Candidate effector proteins were sought in a Z-16 cDNA library from 16-day mouse embryo cDNA (Novagen) with BL21/DE3 as the host E. coli strain. Approximately 1 × 107 clones were screened using phosphorylated Gα1 as a probe. Phage were plated at a density of 3–5 × 105 per 150-mm plate and incubated at 37 °C for 7 h. The plates were overlaid with Hybond-C filters (Amersham Pharmacia Biotech) that had been saturated with 10 mM isopropl-β-D-thiogalactopyranoside. They were further incubated at 37 °C for 3.5 h to induce expression of proteins encoded by cDNAs. The filters were then rinsed with Buffer A at room temperature and blocked with Buffer A containing 5% dry milk at 4 °C overnight. The filters were probed with 50 nl phosphorylated GTPγS-bound Gα1 in Buffer A with 5% dry milk for 4 h at 4 °C. The filters were washed three times with Buffer A for 5 min at 4 °C, air dried, and exposed to film at −70 °C for 2 days. Secondary and tertiary screens were carried out under identical conditions. Two clones, Z-13 and Z-16, were isolated and cDNA inserts were sequenced. Z-13 had a 0.8-kb insert encoding mouse nucleobindin (7). Z-16 had a 1.8-kb insert encoding 273 amino acid residues of previously undescribed sequence.

To obtain a full-length Z-16 cDNA, a mouse brain agt11 library (generously provided by Dr. Melvin Simon, California Institute of Technology) was screened by plaque hybridization using a fragment from the 5′ end of the initial Z-16 clone as a probe. An additional 0.8 kb of coding sequence was isolated; the reading frame was open throughout. Two rounds of 5′-rapid amplification of cDNA ends reactions were then performed with a mouse brain cDNA library (CLONTECH), using oligonucleotides based on sequences at the 5′ end of the Z-16 cDNA. The final Z-16 cDNA contains an ATG codon that agrees well with Kozak’s translation initiation criteria and an in-frame, upstream stop codon. A related cDNA clone, designated KIAA0514 (8), was identified in public data bases and was generously supplied by Dr. T. Nagase (Kazusa DNA Research Institute).

No insert Analysis—Northern blots of RNA from various tissues or brain regions (CLONTECH) were probed with GRIN1 (Z-16) or GRIN2 (KIAA0514) cDNA fragments labeled with α-[32P]dATP (random primer labeling; Stratagene, Prime-it II). Blots were hybridized with probe (5 × 106 cpn/ml) in ExpressHyb (CLONTECH) at 63 °C for 2 h. The blots were then washed in 2 × SSC and 0.1% SDS for 45 min at room temperature.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AF146569.

The abbreviations used are: G protein, guanine nucleotide binding regulatory protein; GTPαS, guanosine 5′-3-0-thio(triphosphate); C12E10, polyoxyethylene 10-lauryl ether; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).
temperature, followed by a high stringency wash with 0.1x SSC containing 0.1% SDS for 30 min at 50 °C. Then, the blots were finally subjected to phosphorimaging analysis (TR2040S imaging plates and BAS1500 scanner, Fuji Medical System).

Antisera—Two peptides were synthesized based on the amino acid sequence of GRIN1: P1 (550SAAQPQRDTRSIGSLPERMT) and P2 (550EVELMAIQKHERQITML); these peptides also included an additional cysteine residue at their amino termini and a tyrosine residue at their carboxyl termini. The cysteine residue utilized to facilitate cross-linking of peptide to keyhole limpet hemocyanin (Sigma) with m-maleimidobisozol-V-hydroxysuccinimide ester. Antisera were produced in New Zealand White rabbits. Antisera T116 and T114 were generated against peptides P1 and P2, respectively. The specificities of the antisera were confirmed by immunoblotting. An antiserum specific for Gα (U1901) was generated in a rabbit against a synthetic peptide with the amino acid sequence of Gα (550EYGDKRKAAMVMKVCG515) conjugated to keyhole limpet hemocyanin. Other rabbit polyclonal antisera against various Go subunits have been described previously (2, 9–11), as has mouse monoclonal antibody (mAb2A) against Gα (12).

Purification of Recombinant GRIN1 and GRIN2 Proteins—GRIN1 and GRIN2 were subcloned into the pFastBacHTB vector (Life Technologies, Inc.), and recombinant baculoviruses encoding His6-GRIN1 or His6-GRIN2 were generated according to the manufacturer’s protocol. Membranes from SF9 cells infected with baculoviruses encoding His6-GRIN1 or His6-GRIN2 were prepared and extracted with 1% C12E10 resins. His6-GRIN1 and His6-GRIN2 were then purified from these extracts using Ni-NTA chromatography. The proteins were further purified by Mono Q and Superdex 200 column chromatography in 20 mM NaHepes (pH 8.0), 5 mM MgCl2, 2 mM EDTA, 50 mM NaCl, 1 mM DTT, and 0.5% C12E10.

Binding of Purified GRIN1 or GRIN2 to G Protein Subunits—Hexa-histidine-tagged GRIN1 or GRIN2 purified from SF9 cells (1.5 μg) was mixed with 5 μg (500 nM) of recombinant Gα or Gβ bound with GDP, GTP-S, or GDP-AlF4, as indicated, in 100 μl of Buffer B (50 mM NaHepes [pH 8.0], 5 mM MgCl2, 10 mM β-mercaptoethanol, 0.1% C12E10) and incubated on ice for 30 min. NaF (10 μM) and AlCl3 (30 μM) were included in the buffer to prepare Go-GDP-AlF4. Ni-NTA resin (Qiagen) was equilibrated with Buffer B and 55 μl was added to the mixture of proteins, followed by further incubation on ice for 5 min. The resin was collected by brief centrifugation, and the supernatant was centrifuged at 11,700 g for 20 min. The pellets were resuspended in Buffer C and collected onto a discontinuous sucrose gradient (10–40%) and centrifuged at 150,000 × g for 2 h. Fractions at the interfaces of the gradient were analyzed by immunoblotting. Plasma membrane is concentrated at the 25–30% sucrose interface. Subcellular fractions of embryonic mouse brain were obtained as described (16, 17).

Immunocytochemistry—To detect endogenous GRIN1, Neuro2A cells were plated on lamin-coated coverslips at 50% confluency and grown for 2 days to generate neurites. For transient transfection, Neuro2A cells were plated on nontreated coverslips at 60–70% confluency and allowed to grow for 1 day. Cells were then transfected with expression plasmids and incubated for 18 h.

Transfected MA104 or Neuro2A cells were rinsed and incubated with Dulbecco’s modified Eagle’s medium without serum for 6 h, fixed with 4% paraformaldehyde in phosphate-buffered saline at 4 °C for 20 min, and washed with Dulbecco’s modified Eagle’s medium containing 5 mM glycine on ice for 5 min. Cells were then permeabilized in buffer (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 2 mM MgCl2, and 0.1% Triton X-100) or cold 100% methanol for 10 min. Cells were then incubated in 5% goat serum in buffer containing 10 mM sodium phosphate (pH 8.0), 150 mM NaCl, and 2 mM MgCl2 for 1 h, followed by incubation at 4 °C overnight with Gα monoclonal antibody (mAb2A, 1:50 dilution), anti-Gα antibody (T116, 1:300 dilution), or GAP43 antibody (Zymed Laboratories Inc., 1:100 dilution). After washing, coverslips were incubated with fluorescent, conjugated secondary antibodies (10 μg/ml; Oregon Green-conjugated goat anti-rabbit IgG or Texas Red-conjugated goat anti-mouse IgG) for 20 min at room temperature. The coverslips were washed twice with 1% Triton X-100 in phosphate-buffered saline, twice with phosphate-buffered saline, and twice with water, and then air-dried for 30 min before mounting with Fluoromount G.

RESULTS

Cloning of Z-16 cDNA—Phosphorylated GDP-S-Gα was utilized to screen a mouse embryo AEX10 expression library. Two clones, designated Z-13 and Z-16, were eventually isolated based on clearly positive signals in several successive screens. The product of the Z-13 cDNA bound GDP-Gα and GTPγS-Gα equally well, whereas the product of the Z-16 cDNA bound GTPγS-Gα selectively (Fig. 1). Sequencing and data base
FIG. 2. A, the nucleotide and deduced amino acid sequence of GRIN1 (Z-16). B, alignment of the amino acid sequences of GRIN1 and GRIN2 (KIAA0514) using NIH Blast 2 (27).

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Fig. 3. Northern and Western analysis. A, Northern analysis of mouse tissue for GRIN1 expression. One μg of poly(A)+ mRNA from mouse heart (lane 1), brain (lane 2), liver (lane 3), spleen (lane 4), lung (lane 5), skeletal muscle (lane 6), kidney (lane 7), or testis (lane 8) was resolved electrophoretically and hybridized with a 32P-labeled GRIN1 probe (1416–1662) (top panel) or a human β-actin probe (bottom panel) as described under “Experimental Procedures.” The migration positions of markers (size in kb) are shown to the left. B, Northern analysis of human brain regions for GRIN1 and GRIN2 expression. One μg of poly (A)+ mRNA from human amygdala (lane 1), caudate nucleus (lane 2), corpus callosum (lane 3), hippocampus (lane 4), whole brain (lane 5), substantia nigra (lane 6), subthalamic nucleus (lane 7), thalamus (lane 8), cerebellum (lane 9), cerebral cortex (lane 10), medulla (lane 11), spinal cord (lane 12), occipital pole (lane 13), frontal lobe (lane 14), temporal lobe (lane 15), or putamen (lane 16) was resolved electrophoretically and hybridized with a 32P-labeled GRIN1 probe (1416–1662) (top panel), a 32P-labeled full-length GRIN2 probe (middle panel), or a 32P-labeled human β-actin probe (bottom panel). C, Western immunoblot analysis of mouse tissues for GRIN1 expression. Homogenate (10 μg of protein) from mouse heart (lane 1), brain (lane 2), liver (lane 3), spleen (lane 4), lung (lane 5), skeletal muscle (lane 6), kidney (lane 7), or placenta (lane 8) was resolved electrophoretically (SDS-PAGE; 9.5% gels). Immunoblots generated with a GRIN1-specific antiserum (T116) and a G_α-specific antiserum (U1901) are shown.

Fig. 4. Interactions of GRIN1 and GRIN2 with G protein α subunits. A, purified recombinant (Sf9 cell-derived) hexahistidine-tagged GRIN1 (left) or hexahistidine-tagged GRIN2 (right) (1.5 μg of each) was mixed with 2 μg of recombinant G_α or G_α bound with GDP, GTPγS, or GDP-AlF_4^−, as indicated. Ni-NTA resin was added to the mixture and processed as described under “Experimental Procedures.” Fractions were resolved by SDS-PAGE, and immunoblots were developed with antibodies reactive with GRIN1 or GRIN2 (top panels, left and right, respectively) or with G_α or G_α, as appropriate. The applied proteins are shown in lane 1 of each panel; lane 2, flow through; lanes 3 and 4, sequential washes with 500 mM NaCl and 10 mM imidazole; lanes 5 and 6, sequential elutions with 200 mM imidazole-HCl. B, immunoprecipitation of G protein α subunits associated with Flag-tagged GRIN1. Flag-tagged GRIN1 was expressed in COS cells and extracted as described under “Experimental Procedures.” Extracts were supplemented with G protein α subunits in various nucleotide-bound states and then subjected to immunoprecipitation as described using an anti-Flag monoclonal antibody. Electrophoretically resolved immunoprecipitates were immunoblotted using antibodies appropriate for the protein listed to the left of each panel. Top two panels: lane 1, GTPγS-bound G_α was mixed with extract from COS cells not expressing Flag-tagged GRIN1; lanes 2–4, GDP-G_α, GDP-AlF_4^−,G_α, and GTPγS-G_α, respectively, were mixed with extracts containing Flag-tagged GRIN1. Lower six panels, lane 1, the indicated G protein α subunit (GTPγS-bound) was mixed with extract from COS cells not expressing Flag-tagged GRIN1. Lanes 2 and 3, the indicated GDP- or GDP-AlF_4^−-bound G protein α subunit, respectively, was mixed with extract from COS cells expressing Flag-tagged GRIN1.

(10.5%), and Ser (13.4%). Data base searches revealed that KIAA0514, a cDNA clone isolated from a human brain cDNA library, and Z-16 encode homologous ~100–150 amino acid residue domains near their carboxyl termini (Fig. 2B). No other matches or homologies were detected, except for a few previously unidentified Z-16 expressed sequence tags (AI427420, W54141, and AI413422). In addition, related genes were not detected by Southern blot analysis of mouse genomic DNA (data not shown). Because of the functional properties of the proteins encoded by the Z-16 and KIAA0514 cDNAs (described below), we refer to these two proteins as GRIN1 and GRIN2, respectively.

Northern and Western Analysis of GRIN1—Northern blot analysis of GRIN1 was performed using several regions of the cDNA as probes. Messenger RNA for GRIN1 (about 4 kb) was detected in brain but not in heart, liver, spleen, lung, skeletal muscle, kidney, or testis (Fig. 3A). GRIN1 mRNA is widely distributed in the central nervous system; the highest concentration was detected in the spinal cord (Fig. 3B). Messenger RNA for GRIN2, which is about 8 kb, is also specifically expressed in brain. In contrast to GRIN1, expression of GRIN2 was only detected in cerebellum (Fig. 3B).

Two antibodies specific for GRIN1 (designated T114 and T116) were prepared using GRIN1 peptides as immunogens. In lysates of COS cells transfected with GRIN1 cDNA, both anti-

searches revealed that Z-13 encoded the protein nucleobindin. Interactions of G_α with nucleobindin have been detected previously (18). Nucleobindin is described as a secreted protein that interacts with DNA (7). We did not pursue nucleobindin further because of these characteristics and its failure to recognize GTPγS-bound G_α selectively. The protein product of the Z-16 cDNA also bound to phosphorylated protein kinase A-tagged, GTPγS-bound G_α.

Sequencing of the 1800-base pair insert designated Z-16 revealed that it had an open reading frame encoding 273 amino acid residues fused to the T7 gene 10 product, as anticipated. The sequence was incomplete at the 5′-end. An apparently full-length cDNA was obtained by screening a mouse brain cDNA library and by 5′-rapid amplification of cDNA ends, using a 5′-stretch of randomly primed brain cDNA library (Fig. 2A). The deduced amino acid sequence specifies a protein containing 827 residues, with Mr = 84,700. Nearly 45% of the residues have small side chains: Ala (11%), Gly (9.8%), Pro
bodies detected an immunoreactive band with an apparent molecular weight of 110,000. Consistent with the results of Northern analysis, this 110-kDa band was also detected in mouse brain homogenates but not in homogenates of other tissues (Fig. 3C). The appearance of GRIN1 as a doublet band in brain lysate may reflect proteolysis during sample preparation, or it may indicate the existence of splice variants of the protein. This immunoreactive band was not detected if antisera were first incubated with peptides used as immunogens or with expressed GRIN1 protein (data not shown). We thus conclude that this band represents GRIN1 protein and that the protein migrates anomalously during SDS-polyacrylamide gel electrophoresis. Among several cell lines tested, endogenous GRIN1 was detected in mouse neuroblastoma Neuro2a cells (see Fig. 5B) and rat pheochromocytoma PC12 cells as a doublet similar to its appearance in brain lysate (data not shown).

The peptide used to generate antibody T114 shares 13 amino acid residues (of 18) with GRIN2. This antibody, but not T116, recognized the GRIN2 product expressed in COS or Sf9 cells (data not shown). GRIN2 (461 amino acid residues) has a calculated molecular weight of 47,600 but migrates with an apparent molecular weight of 65,000 during SDS-PAGE.

Interaction of GRIN1 and GRIN2 with Various G Protein α Subunits—Baculoviruses encoding full-length GRIN1 or GRIN2 (with hexahistidine tags at the amino terminus) were generated, and recombinant GRIN1 or GRIN2 protein was purified from Sf9 cells using Ni-NTA, Mono-Q, and Superdex 200 column chromatography. Purified GRIN1 or GRIN2 was mixed with 500 nM Gα or Gα bound with either GDP, GDP-AlF4−, or GTPγS, and interactions were analyzed by co-elution from Ni-NTA columns (Fig. 4A). Both GRIN1 and GRIN2 interacted with the GDPγS- or GDP-AlF4−-bound form of Gα and Gα; interactions with the GDP-bound forms of these two proteins were not detected.

Interactions between GRIN1 and various Gα proteins were also examined using full-length Flag-tagged GRIN1 expressed in COS cells. Detergent extracts containing GRIN1 were then mixed with G protein α subunits, and complexes were precipitated with an anti-Flag monoclonal antibody (Fig. 4B). Specific interactions were detected between GRIN1 and members of the Gα subfamily of Gα subunits, including Gα, Gα, and Gα. A weak interaction was detected with G12α, but none was observed with Gα or Gα. Again, GRIN1 interacted preferentially with the GTPγS or the GDP-AlF4−-bound forms of the Gα proteins compared with the GDP-bound proteins.

Subcellular Distribution of GRIN1; GRIN1 Is Found in Growth Cones with Gα—The subcellular distribution of GRIN1 was first examined in transfected COS cells. Exogenously expressed GRIN1 and Gα were largely confined to the particulate fractions, and only small amounts appeared in the cytosol (Fig. 5A). Similarly, endogenous GRIN1 and Gα in Neuro2a cells and brain are predominantly membrane-bound (Fig. 5, B and C). The amino acid sequence of GRIN1 contains no obvious hydrophobic domain sufficient to explain membrane localization. There are also no consensus sequences for covalent modification by myristoylation or prenylation.

Co-localization of GRIN1 and Gα in Neuro2a cells was also demonstrated by immunofluorescence. Neuro2a cells were induced to extend neurites by culture on laminin-coated dishes. Permeabilized cells were then incubated with both rabbit polyclonal anti-GRIN1 antibodies (T116) and a mouse monoclonal antibody specific for Gα (mAb2A). Oregon Green-conjugated goat anti-rabbit IgG or Texas Red-conjugated goat anti-mouse IgG were used as secondary antibodies to detect GRIN1 and Gα.
Fig. 7. Morphological changes of cells transfected with GRIN1 and GRIN2. MA104 cells (A) or nondifferentiated Neuro2a cells (B) were transfected with control vector (a), wild type Goα (b), Goα Q205L (c), GRIN1 (d), GRIN1 and wild type Goα (e), GRIN1 and Goα Q205L (f), GRIN2 (g), GRIN2 and wild type Goα (h), or GRIN2 and Goα Q205L (i). GRIN1 or GRIN2 was stained with Oregon Green, and wild type Goα or Goα Q205L was stained with Texas Red. C, nondifferentiated Neuro2a cells were transfected with GRIN1 and Goα Q205L. After transfection (48 h), cells were further incubated with serum-free medium for 24 h to extend neurites. GRIN1 and Goα Q205L were stained with Oregon Green and Texas Red, respectively. The overlapped images of one transfected cell with long neurites are combined. The calibration bar in each panel corresponds to 10 μm.
G\(_\alpha\), respectively. The specificity of observed fluorescence was confirmed by competition with the immunogenic peptide or GRIN1 protein (data not shown). The pattern of immunofluorescence for GRIN1 and G\(_\alpha\) in differentiated Neuro2a cells is similar, and the two proteins are predominantly found at the plasma membrane (Fig. 5, D–F).

G\(_\alpha\) and GAP43 are enriched in growth cone membranes (19). Growth cone membrane fractions were prepared as described by Pfenniger et al. (16) and Simkowitz et al. (17) and analyzed by immunoblotting using GRIN1 antisera. Fig. 6A shows that GRIN1 is highly enriched in the growth cone membrane fraction (lane 4), as are GAP43 and G\(_\alpha\). By contrast, G\(_\alpha\) and the low-affinity NGF receptor are found in all membrane fractions. Furthermore, immunofluorescence of differentiated Neuro2a cells shows that GRIN1 and GAP43 are concentrated in putative growth cone membranes (Fig. 6, B–D, arrows).

GRIN1 and GRIN2 Cause Morphological Changes in MA104 and Neuro2a Cells—GRIN1 or GRIN2 was cotransfected into MA104 (simian renal epithelium) cells with either wild type G\(_\alpha\) or a constitutively active (GTPase-deficient) mutant of the protein (G\(_\alpha\)Q205L), and the transfected cells were examined by immunofluorescence microscopy. As shown in Fig. 7A, MA104 cells transfected with only wild type G\(_\alpha\), G\(_\alpha\)Q205L, or GRIN1 had a relatively unperturbed morphology. However, cells spread irregularly on coverslips and generated many fine, neurite-like processes if they were cotransfected with GRIN1 and G\(_\alpha\)Q205L. This effect was much less apparent if wild type G\(_\alpha\) was expressed with GRIN1. Expression of GRIN2 alone caused extension of processes in some cells, and co-transfection of G\(_\alpha\) or G\(_\alpha\)Q205L increased both the frequency and extent of these changes. Similar experiments were performed with Neuro2a cells (Fig. 7, B and C). Again, co-transfection of GRIN1 or GRIN2 with G\(_\alpha\)Q205L caused long neurites to appear. In addition, these neurites displayed many hair-like processes. Co-transfection of GRIN1 or GRIN2 with wild type G\(_\alpha\) caused less extensive changes. In contrast to MA104 cells, expression of G\(_\alpha\)Q205L alone induced formation of neurites, albeit with lower efficiency. This effect is perhaps explained by the endogenous content of GRIN1 in Neuro2a cells. These results indicate that G\(_\alpha\) and GRIN1 interact (directly or indirectly) in vivo and that this causes formation and extension of neurite-like processes. Similar effects of G\(_\alpha\)Q205L have been described in PC12 cells (20).

**DISCUSSION**

We screened a mouse embryonic cDNA expression library to search for proteins capable of interacting with phosphorylated GTP\(_\gamma\)S-G\(_\alpha\). Two clones were isolated, designated Z-13 and Z-16. Z-13 encoded nucelobinidin, the interactions of which with G\(_\alpha\) were detected previously using a yeast two-hybrid screen (18). We did not pursue this lead further because of the apparently similar affinity of GDP-G\(_\alpha\) and GTP\(_\gamma\)S-G\(_\alpha\) for nucelobinidin and the presence of a signal sequence in the protein. By contrast, the protein now designated GRIN1 interacts preferentially with activated forms of G protein subunits in the G\(_\alpha\) subfamily (G\(_\alpha\), G\(_\alpha\), G\(_\alpha\)). Furthermore, GRIN1 is specifically expressed in brain and shares with G\(_\alpha\) substantial enrichment in membranes from neuronal growth cones. We thus hypothesize that GRIN1 may function as a downstream effector for G\(_\alpha\).

A homolog of GRIN1-GRIN2 (KIAA0514), was isolated and sequenced previously as a newly identified brain cDNA by Nagase et al. (8). The regions of GRIN1 and GRIN2 that are similar are at the carboxyl termini of both proteins, and we had shown independently that this is the G\(_\alpha\)-binding domain of GRIN1 (data not shown). Significantly, GRIN2 also interacts preferentially with activated members of the G\(_\alpha\) subfamily of G protein \(\alpha\) subunits. Although GRIN1 is widely distributed throughout the central nervous system, GRIN2 is apparently restricted to the cerebellum.

G\(_\alpha\) is the most abundant G protein \(\alpha\) subunit in mammals. It is expressed predominantly in brain and is enriched in neural growth cones (19). Despite these interesting properties, physiological roles for G\(_\alpha\) have not been identified, other than its interactions with G protein \(\beta\gamma\) subunits and appropriate receptors. The heterotrimeric G protein is responsible for receptor-mediated inhibition of voltage-sensitive N-type or P/Q-type \(\text{Ca}^{2+}\) channels in presynaptic nerve terminals, but this effect appears to be mediated by the G protein \(\beta\gamma\) subunit complex (21). G\(_\alpha\) is a weak inhibitor of some isoforms of adenyl cyclase (1), but the physiological significance of this is difficult to evaluate. G\(_\alpha\) has also been hypothesized to regulate neurite extension. Binding of GTP\(_\gamma\)S to G\(_\alpha\) is stimulated by GAP43 (neuromodulin), an abundant growth cone protein that is important for neural pathfinding (19). The expression of both GAP43 and G\(_\alpha\) starts in brain regions when differentiated neurons begin to extend neurites (22). Furthermore, expression of constitutively activated mutant forms of G\(_\alpha\) stimulates neurite outgrowth in neuronal cell lines (20). The molecular mechanism for this phenomenon has not been defined.

We have shown herein that either GRIN1 or GRIN2 induces extensive outgrowth of neurites from Neuro2a cells when coexpressed with activated forms of G\(_\alpha\). This result implies interaction between proteins in vivo, although not necessarily a direct one. However, the fact that G\(_\alpha\) does interact directly with both GRIN1 and GRIN2 in vitro suggests that these latter proteins may function physiologically as downstream targets for G\(_\alpha\) and/or other members of the G\(_\alpha\) subfamily to regulate neurite outgrowth. The amino acid sequences of GRIN1 and GRIN2 show no significant homology with known kinases, enzymes that generate second messengers, or other identified effectors for G protein action. Although GRIN1 has a proline-rich domain (residues 590–710), we detected no other provocative signatures. Recent evidence suggests that Rho family GTPases are important components of signaling pathways that control axonal growth and guidance (23). Rho itself is involved in collapse of growth cones and retraction of neurites. By contrast, Cdc42 and Rac1 stimulate the formation and advance of growth cones through formation of filopodia and lamellipodia (24). It is thus possible that Cdc42 or Rac1 are downstream components of signaling pathways that include G\(_\alpha\) and GRIN1 or GRIN2. Perhaps relevant is the fact that the G proteins G\(_{12}\) and G\(_{13}\) have recently been shown to control the activity of a guanine nucleotide exchange factor, p115, that activates Rho (25, 26).

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**REFERENCES**

1. Taussig, R., Tang, W.-J., Hepler, J. R., and Gilman, A. G. (1994) J. Biol. Chem. 269, 6093–6100

2. Kozasa, T., and Gilman, A. G. (1995) J. Biol. Chem. 270, 1734–1741

3. Kozasa, T., and Gilman, A. G. (1996) J. Biol. Chem. 271, 12562–12567

4. Baude, E. J., Dignam, S. S., Olsen, S. R., Reimann, E. M., and Uhler, M. D. (1994) J. Biol. Chem. 269, 2316–2323

5. Mumbay, S. M., and Linder, M. E. (1984) Methods Enzymol. 237, 254–268

6. Lee, E., Linder, M. E., and Gilman, A. G. (1994) Methods Enzymol. 237, 146–164

7. Miura, K., Takan, K., Kurosawa, Y., and Kanai, Y. (1992) Biochem. Biophys. Res. Commun. 187, 375–380

8. Nagase, T., ishikawa, K., Miyajima, N., Tanaka, A., Kotonai, H., Nomura, N., and Ohara, O. (1998) DNA Res. 5, 31–39

9. Mumbay, S. M., and Gilman, A. G. (1993) Methods Enzymol. 215, 213–233

10. Grootes, S., Smrcka, A., Newak, L., Wu, D. G., Simon, M., and Sternweis, P. C. (1991) J. Biol. Chem. 266, 20519–20524

11. Huang, C., Hepler, J. R., Chen, L. T., Gilman, A. G., Anderson, R. G. W., and Mumbay, S. M. (1997) Mol. Biol. Cell 8, 2365–2378
12. Li, X., Mumby, S. M., Greenwood, A., and Jope, R. S. (1995) J. Neurochem. 64, 1107–1117
13. Mumby, S. M., Heuckereth, R. O., Gordon, J. I., and Gilman, A. G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 728–732
14. Muntz, K. H., Gilman, A. G., and Mumby, S. M. (1992) Circulation 86, 1-764
15. Suman, T., Cervato-Franco, G., and Peli, S. (1988) Basic Appl. Histoch. 24, 145–150
16. Pfenniger, K. H., Johnson, E. L., Friedman, L. B., and Somio, S. L. (1983) Cell 35, 573–584
17. Simkowitz, P., Elis, L., and Pfenniger, K. H. (1989) J. Neurosci. 9, 1004–1017
18. Mochizuki, N., Hibi, M., Kanai, Y., and Insel, P. A. (1995) FEBS Lett. 373, 155–158
19. Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J., and Fishman, M. C. (1990) Nature 344, 836–841
20. Strittmatter, S. M., Fishman, M. C., and Zhu, X.-P. (1994) J. Neurosci. 14, 2327–2338
21. Zamponi, G. W., and Snutch, T. P. (1998) Curr. Opin. Neurobiol. 8, 351–356
22. Schmidt, C. J., Zubiaur, M., Valenzuela, D., Neer, E. J., and Drager, U. C. (1994) J. Neurosci. Res. 38, 182–187
23. Luo, L., Jun, L. Y., and Jan, Y. N. (1997) Curr. Opin. Neurobiol. 7, 81–86
24. Kozma, R., Surner, S., Ahmed, S., and Lim, L. (1997) Mol. Cell. Biol. 17, 1201–1211
25. Hart, M. J., Sharma, S., eMasry, N., Qiu, R.-G., McCabe, P., Polakis, P., and Bollag, G. (1996) J. Biol. Chem. 271, 25452–25458
26. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2111
27. Henikoff, S., and Henikoff, J. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10915–10919