Molecular Pharmacology Fast Forward. Published on December 7, 2004 as doi:10.1124/mol.104.003913
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# Functional characterization of Gαo signaling through GRIN1

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Molecular Pharmacology Fast Forward. Published on December 7, 2004 as DOI: 10.1124/mol.104.003913
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**Running title:** Gαo signaling through GRIN1

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Number of text pages: 29

Number of tables: 0

Number of figures: 6

Number of words in

Abstract: 247

Introduction: 459

Discussion: 833

**Abbreviations used** : AMF, 30 μM $AlCl_3$, 5 mM $MgCl_2$, and 10 mM NaF; C12E10, polyoxyethlene 10-lauryl ether; DTT, dithiothreitol; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescence protein; G protein, guanine nucleotide binding regulatory protein; GST, glutathione S-transferase; PAK, p21-activated kinase; PBD, p21-binding domain; RGS, regulator of G protein signaling.

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**Abstract**

GRIN1 (G protein-regulated inducer of neurite outgrowth 1) was initially identified as a binding protein for GTPγS-bound Gαz. GRIN1 is specifically expressed in brain and interacts selectively with activated α subunits of Gi subfamily. GRIN1 colocalizes with Gαo at the growth cone of neuronal cells and promotes neurite extention in Neuro2a cells when co-expressed with constitutively active mutant GαoQ205L. These results suggest that GRIN1 functions as a downstream target for Gαo. However, GRIN1 does not contain domains that are homologous to known signaling motifs. To understand the mechanisms of Gαo-GRIN1 pathway, we analyzed functional domains of GRIN1 that are involved in binding with Gαo or its targeting to the plasma membrane. Using pull down assays with GST-fused GRIN1 deletion mutants, Gαo binding regions were localized to amino acid residues 716-746 and 797-827 of GRIN1. The Gαo binding region of GRIN1 did not demonstrate GTPase accelerating activity for Gαo. GRIN1 localized in the cell periphery in Neuro2a cells, and two cysteine residues at C-terminal region of GRIN1 (Cys818 and Cys819) were shown to be critical for its membrane targeting. Co-expression of GRIN1 with GαoQ205L or GRIN1Δ717-827), which lacks Gαo binding region, promoted microspike formation in Swiss3T3 cells or neurite extention in Neuro2a cells. The dominant-negative mutant of Cdc42 blocked these morphological changes. Co-expression of GRIN1 and GαoQ205L stimulated formation of GTP-bound Cdc42 in Swiss3T3 cells. These results suggest that the binding of activated Gαo to GRIN1 induces activation of Cdc42, which leads to morphological changes in neuronal cells.

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## Introduction

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) transduce a variety of signals from a large number of seven transmembrane type receptors to intracellular effectors (Gilman, 1987; Hepler and Gilman, 1992). Agonist-activated receptors induce dissociation of G protein subunits, generating GTP-bound form of $\alpha$ subunits and free $\beta\gamma$ subunits. Both GTP-$\alpha$ and $\beta\gamma$ can regulate downstream effectors. Hydrolysis of GTP to GDP on $\alpha$ subunit leads to reassociation of $\alpha$ and $\beta\gamma$ to form an inactive heterotrimer. More than thirty RGS proteins have been identified as novel regulators of G protein signaling. RGS proteins associate with GTP-bound G$\alpha$ and, in most cases, accelerate GTPase activity of G$\alpha$ to facilitate the inactivation of G proteins (Berman et al., 1996; Ross and Wilkie, 2000).

Alpha subunits of Gi subfamily are highly expressed in the nervous system. Particularly, G$\alpha$o and G$\alpha$i1 constitute nearly 1 % of membrane protein in brain. However, the only well established effectors of these $\alpha$subunits are certain isoforms of adenylyl cyclase (Taussig et al., 1994; Kozasa and Gilman, 1995). We have recently identified GRIN1 and GRIN2 (G protein-regulated inducer of neurite outgrowth 1 and 2) as novel effector candidates for G$\alpha$o (Chen et al., 1999). GRIN1 was isolated through screening of a mouse embryo cDNA expression library with phosphorylated GTP$\gamma$S-G$\alpha$z as a probe. A homologue of GRIN1, GRIN2 (KIAA0514), was identified by database search. GRIN1 and GRIN2 bind selectively to activated forms of G$\alpha$o, G$\alpha$z and G$\alpha$i. Among different tissues, GRIN1 is specifically

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expressed in the brain. Furthermore, GRIN1 is enriched in the growth cones of neurites similar to Gαo and GAP43 (neuromodulin) (Chen et al., 1999). Co-expression of GRIN1 with the constitutively active mutant of Gαo promoted the extension of neurites in Neuro2a cells. Although GRIN1 does not contain domains that are homologous to other known signaling motifs, these results suggest that the Gαo-GRIN1 pathway may be involved in the regulation of neurite growth.

Similar to heterotrimeric G proteins, Rho family GTPases, Cdc42, Rac and Rho, function as molecular switches by cycling between a GDP-bound, inactive state and a GTP-bound, active state. These GTPases are involved in various cellular processes, such as gene expression, cell cycle progression, cell polarity, or vesicle trafficking (Etienne-Manneville and Hall, 2002; Schmidt and Hall, 2002; Symons and Settleman, 2000). Among them, the most well established role of Rho family GTPases is the reorganization of the actin cytoskeleton, which is crucial for cellular responses such as cell migration, adhesion, phagocytosis, or axonal guidance (Etienne-Manneville and Hall, 2002; Dickson, 2001).

In this study, we characterized functional domains of GRIN1 to understand the molecular mechanisms of Gαo-GRIN1 signaling. We have identified regions in GRIN1 which are involved in Gαo binding or membrane targeting. We also identified a possible link of the Gαo-GRIN1 pathway to Cdc42 activation.

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## Materials and Methods

*Materials* – The anti-GST monoclonal antibody and anti-GFP polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz,CA). Anti-flag (M2) antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and Sigma (St. Louis, MO). The anti-Gαo monoclonal antibody was purchased from Calbiochem (La Jolla, CA). The anti-Rac1 monoclonal antibody was purchased from Transduction Laboratories (BD Biosciences : San Jose, CA). Rhodamine phalloidin and Alexa Fluor 350-F(ab')$_2$ fragment of goat anti-mouse IgG were from Molecular Probes (Eugene, OR). [$\gamma$-$^{32}$P]GTP was purchased from ICN Biomedicals (Costa Mesa, CA). Antibodies against GRIN1 (T116) and Gαo (U1901) were previously described (Chen et al., 1999). VECTASHIELD mounting medium was purchased from Vector Laboratories (Burlingame, CA). Fibronectin and laminin were from Sigma. Plasmids for GST-PAKPBD and Rho family GTPases were kindly provided by Drs. G. Bokoch (Scripps Research Institute, CA) and T. Satoh (Kobe University, Japan), respectively.

*Plasmid Constructs* – Mouse GRIN1 cDNA (Chen et al., 1999) was used for the construction of plasmids for GST or GFP fusion proteins. The corresponding fragments of GRIN1 cDNA were subcloned in frame at the C-terminus of GST in pGEX-KG or at the C-terminus of EGFP in pEGFP-C1 vector. The GRIN1 fragment (797-827) was generated by PCR using following primers: 5'-cggaattcagggcgccgccaag-3' and 5'-ccgctcgagttactccgcagtggggcc-3'. C818A and C819A mutations of GRIN1 were generated using Quikchange

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site-directed mutagenesis (Stratagene : La Jolla, CA) with primers: 5'-gtgcgccgaccgcgggctgcatcgcgagcgggcccc-3' and 5'-ggggcccgctcgcgatgcagcccgcggtcggcgcac-3'. Gαo (wild type or Q205L mutant) was fused at the N-terminus of DsRed protein in pDsRed2-N vector (BD Biosciences Clontech). Quikchange mutagenesis was used to remove a stop codon and add a SmaI site in pCMV-GαoQ205L with primers: 5'-ggctgtggcttgtcccgggctcttgtcctg-3' and 5'-caggacaagagcccgggacaagccacagcc-3'. Then the EcoRI-SmaI fragment of pCMV-GαoQ205L was subcloned into pDsRed2-N to generate pDsRed2N-GαoQ205L. PCR reactions were performed by QuikChange mutagenesis using pDsRed2N-GαoQ205L as a template with primers: 5'-cgttggaggccagcgatctgaac-3' and 5'-gttcagatcgctggcctccaacg-3' to make pDsRed2N-Gαo (wild type). To construct bacterial expression vector for Gαo, a hexahistidine tag, a stop codon, and Hind III site were generated at the C-terminus of rat Gαo cDNA by PCR using pQE6-Gαo as a template with primers: 5'-cggcatgcacgagtctctcatgc-3' and 5'-aagctttcaatggtgatggtgatggtggtacaagccacagcc-3'. The PCR fragment was subcloned into pCR4-TOPO, digested with SphI and HindIII, and subcloned into pQE6.

*Expression and purification of recombinant proteins* – GST-fused GRIN1 mutants, GST-PAKPBD, or Gαo-$His_6$ were expressed in BL21 or JM109 by induction with 100 μM IPTG at 30°C or 37°C for 2-3 hrs, respectively. GST fusion proteins were purified using glutathione Sepharose 4B column (Amersham Biosciences : Piscataway, NJ). Gαo-$His_6$ was purified using Ni-NTA column (Qiagen : Valentin, CA).

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*Gαo binding assay* – The lysates of *E. coli* expressing GST-fused GRIN1 mutants were mixed with purified Gαo in the binding buffer (50 mM Hepes, pH 8.0, 50 mM NaCl, 50 μM GDP, 5 mM $MgCl_2$, 2 mM DTT, 0.1 % $C_{12}E_{10}$, and protease inhibitors) in the presence or absence of $AlF_4^-$ (10 mM NaF and 30 μM $AlCl_3$) and incubated on ice for 1 hr. The resin was washed twice with wash buffer A (50 mM Hepes, pH 8.0, 100 mM NaCl, 50 μM GDP, 5 mM $MgCl_2$, 1 mM DTT, 0.1 % $C_{12}E_{10}$, and protease inhibitors), and once with wash buffer B (50 mM Hepes, pH 8.0, 50 μM GDP, 5 mM $MgCl_2$, 2 mM DTT, 0.1 % $C_{12}E_{10}$, and protease inhibitors). The bound proteins were eluted by boiling the resin in SDS sample buffer and analyzed by SDS-PAGE, followed by immunoblotting using an anti-Gαo antibody, U1901 (Chen et al., 1999).

*Cell culture* – Neuro2a murine neuroblastoma cells or Swiss3T3 fibroblast cells were cultured in DMEM containing 10 % fetal bovine serum with 10 % $CO_2$.

*Fluorescence microscopy* **-** Neuro2a cells on laminin-coated chambered coverslips were transiently transfected with appropriate expression plasmids using LipofectAMINE Plus (Invitrogen : Carlsbad, CA). Two hours after transfection, medium was changed to DMEM with 7.5% fetal bovine serum for 2 hrs and then to DMEM without serum for 24 hrs before capturing fluorescence images.

Swiss3T3 cells plated on fibronectin-coated coverslips were subjected to serum starvation overnight. Then, expression plasmids with total DNA

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concentration of 0.05 mg/ml were microinjected into the nucleus of cells using computer-assisted microinjection system (Eppendorf, and Cell Biology Trading). After 20-22 hrs, cells were rinsed with PBS, fixed in 4 % paraformaldehyde/0.5 % glutaraldehyde/PBS, permeabilized in 0.1 % TritonX-100/PBS, then stained with rhodamine phalloidin. For indirect immunofluorescence staining, cells were fixed and permeabilized as described above and incubated with anti-flag antibody (1:200 dilution in PBS/2 % BSA). Cells were washed in PBS before incubating with secondary antibody Alexa Fluor 350 (anti-mouse IgG, 1:200 dilution in PBS/2 % BSA). Coverslips were mounted with VECTASHIELD mounting medium. Fluorescence images were captured using a LSM510 confocal microscope (ZEISS).

*Cdc42-GTP pull down assay* **-** For Cdc42-GTP pull down assay, Swiss 3T3 cells were transfected with flag-tagged Cdc42 expression plasmid. After 40 hr, they were further transfected with expression plasmids for GαoQ205L and/or GFP-GRIN1. After 6hr, the cells were lysed with lysis buffer (25 mM Hepes (pH 7.5), 1 % Nonidet P-40, 10 mM $MgCl_2$, 100 mM NaCl, 5 % glycerol, 1 mM sodium vanadate, and protease inhibitors). The lysates were incubated for 30 min at 4$^oC$ with GST-PAKPBD (15 μg) in binding buffer (25 mM Hepes (pH 7.5), 0.5 % Nonidet P-40, 30 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) (Benard et al., 1999; Royal et al., 2000). The beads were washed five times with washing buffer (25 mM Hepes (pH 7.5), 0.5 % Nonidet P-40, 30 mM $MgCl_2$, 40 mM NaCl, 1 mM DTT), and boiled in SDS sample buffer. The

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amount of GTP-bound Cdc42 was analyzed by 12.5 % SDS-PAGE, followed by immunoblotting with anti-flag antibody (for Cdc42) or anti-Rac1 antibody.

*GAP assay* –GAP assays of Gαo were performed as described (Nagata et al., 2001; Rochdi et al., 2002).

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## Results

**Gα□ interaction region of GRIN1** – The initial characterization of GRIN1 demonstrated that its carboxy-terminal region (amino acid residues 555-827) contains the Gαo binding domain (Chen et al., 1999). In order to further characterize the Gαo-GRIN1 interaction, several GST-tagged deletion mutants of GRIN1 were constructed. The lysates of *E. coli* expressing these GST-GRIN1 constructs were mixed with purified Gαo in the presence or absence of $AlF_4^-$, a reversible activator of Gα subunits. Their interaction was assessed by GST pull down experiments. As previously shown, in the presence of $AlF_4^-$, the binding of Gαo was detected with full length GRIN1 as well as with GRIN1 (562-827) (Fig. 1A, data not shown). As shown in Fig. 1B, with further deletion, the binding of Gαo was detected in the region of GRIN1 (716-827). In contrast, the mutant without this carboxyl-terminal region, GRIN1 (562-716), did not interact with Gαo. Within 716-827 of GRIN1, Gαo binding was detected in the region of 716-746 or 797-827 but not in the region of 746-771, although the interaction with these short segments was weaker than that with GRIN1 (716-827). These experiments indicate that the both regions of GRIN1 (716-746 and 797-827) are involved in the association with Gαo. RGS protein recognizes the activated form of Gα and accelerates its GTPase activity. Given that GRIN1 specifically recognizes activated Gαo, we tested whether the interaction with GRIN1 stimulates its GTPase activity similar to RGS proteins. However GRIN1 (716-771) showed no effect on the GTPase activity of Gαo (data not shown). Thus, GRIN1 interacts with Gαo without GAP activity.

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**Membrane targeting region of GRIN1** – We next investigated the domain of GRIN1 involved in its subcellular localization. Neuro2a cells were transiently transfected with GRIN1 constructs which were fused with GFP at their amino terminus, and their expression was analyzed by fluorescence microscopy (Fig. 2). Full length GRIN1 localized mainly at the cell periphery. GRIN1 (716-827) and GRIN1 (766-827) also demonstrated a membrane distribution pattern. In contrast, GRIN1Δ (717-827) and GRIN1 (716-771), constructs lacking the carboxyl-terminal region, showed cytosolic distribution. These results indicate that the carboxyl-terminal region of GRIN1 (772-827) is involved in its membrane targeting.

Since the amino acid sequence of GRIN1 does not contain any apparent transmembrane region, the membrane distribution of GRIN1 suggests the possibility of lipid modification of GRIN1. A variety of signaling proteins undergo palmitoylation at cysteine residues. It is known that palmitoylation affects the activity of the protein in addition to its subcellular distribution (Osterhout et al., 2003; Tu et al., 1999; Wedegaertner and Bourne, 1994). Within the membrane targeting region of GRIN1 (772-827), potential palmitoylation sites were found at Cys818 and Cys819. These cysteine residues were conserved in both human and mouse GRIN1, suggesting the functional importance of these residues. We thus generated a GRIN1 mutant GRIN1CCAA in which both cysteine residues were mutated to alanine. The GRIN1CCAA mutant demonstrated a cytosolic pattern of distribution (Fig. 2). The results indicate that Cys818 and Cys819 residues are critical to target GRIN1 to plasma membrane.

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**Effect of the Gαo-GRIN1 pathway on cell morphology** – When co-expressed with GαoQ205L, GRIN1 induced neurite formation in Neuro2a cells or fine process formation in MA104 cells (Chen et al. 1999). In order to further characterize this morphological change induced by Gαo-GRIN1, a GRIN1 mutant lacking the Gαo binding region, GFP-GRIN1Δ(717-827), was expressed in Neuro2a cells with or without GαoQ205L. As shown in Fig. 3A, expression of GRIN1Δ(717-827) in Neuro2a cells promoted neurite extensiton similar to cells co-expressing wild type GRIN1. Furthermore, this effect of GRIN1Δ(717-827) did not require co-expression of GαoQ205L. These results suggest that Gαo binding domain may have inhibitory effect on GRIN1 activity and the deletion of Gαo binding domain of GRIN1 may change its conformation to a constitutively active form.

In Fig. 3B, we examined the neurite promoting activity of GRIN1CCAA mutant. GRIN1CCAA demonstrated membrane distribution when co-expressed with GαoQL but not with Gαo wild type, suggesting the activation dependent interaction with Gαo. It also promoteed neurite extension in the presence of activated Gαo. These results suggest that although Cys818 and Cys819 are required for the membrane distribution of GRIN1, they are not critically involved in the interaction with Gαo or the neurite promoting activity for GRIN1.

We also examined the specificity of GRIN1-induced morphological changes using activated mutants of several different Gα subunits. In this experiment, Swiss3T3 cells were used to eliminate the morphological changes through endogenous GRIN1. As shown in Fig. 4, Swiss3T3 cells co-

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expressing GRIN1 and Gαi1Q204L, formed microspikes similar to cells expressing GαoQ205L and GRIN1. However, no such morphological changes were detected in cells co-expressing GRIN1 and GαqR183C or GαsQ227L. The results are consistent with the results from *in vitro* experiment showing that GRIN1 specifically interact with α subunits of Gi subfamily. The results also support that these morphological changes are induced by the interaction of GRIN1 with Gαi/o.

**Involvement of Rho family GTPases in the Gαo-GRIN1 signaling pathway.** It is well established that Rho family GTPases, Rho, Rac, and Cdc42 are involved in morphological changes of cells through regulation of the actin cytoskeleton. We thus investigated whether the morphological changes induced by the Gαo-GRIN1 pathway involves these Rho family GTPases. Swiss3T3 cells were microinjected with expression plasmids encoding dominant negative mutant of Rho GTPases, Cdc42N17, Rac1N17, or RhoAN19, along with GαoQ205L-DsRed and GFP-GRIN1. As shown in Fig. 5A, co-expression of dominant negative Cdc42 blocked microspike formation in Swiss3T3 cells, while dominant negative Rac1 or RhoA had no pronounced effect on Gαo-GRIN1 induced morphological changes. We also conducted similar experiments using Neuro2a cells (Fig. 5B). Both dominant negative Cdc42 and dominant negative Rac1 inhibited neurite extention in Neuro2a cells expressing GαoQ205L and GRIN1 whereas dominant negative RhoA showed no effect. These results suggest that Gαo-GRIN1 signaling may involve activation of Cdc42 in its downstream to induce microspike formation.

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**Co-expression of GαoQ205L and GRIN1 activates Cdc42** – Finally, we examined whether Gαo-GRIN1 pathway activates Cdc42 in cells. The amount of GTP-Cdc42 in cells was quantified by pull down assays using GST-PAK-PBD as described in Materials and Methods. As shown in Fig. 6, co-expression of GαoQ205L and GRIN1 in Swiss3T3 cells increased GTP-Cdc42 about two-fold. The expression of GRIN1Δ(717-827) induced similar levels of increase of GTP-Cdc42. This suggests again that GRIN1Δ(717-827) is constitutively active and independent of Gαo. These results agree well with the morphological data in Fig. 5 and further supports the model that Gαo-GRIN1 signaling pathway activates Cdc42 to induce morphological changes in cells.

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## Discussion

In this study, we have characterized the mechanism of the Gαo-GRIN1 signaling pathway. We identified the Gαo binding domain of GRIN1 and also the amino acid residues critical for membrane targeting of GRIN1. We also presented evidence suggesting the possible link of Gαo-GRIN1 pathway to the activation of Cdc42, which is likely to be responsible for the induction of neurite growth by Gαo-GRIN1.

Using deletion mutants of GRIN1, the Gαo binding domain of GRIN1 was mapped to its carboxyl-terminal regions (716-746 and 797-827). Gαo interacts with both of these regions and the presence of either region in GRIN1 could show activation dependent association with Gαo. Amino acid sequences of these binding regions are highly conserved in GRIN2, suggesting that GRIN2 will likely interact with Gαo through the corresponding regions. The Gαo binding domain did not show apparent homology with any known Gα interacting motifs, and GRIN1 did not demonstrate GAP activity for Gαo. Thus, these regions are considered to be novel Gα interacting motifs. Further characterization and identification of the critical residues of GRIN1 for interaction with Gαo will be pursued.

The inhibitory effect of GRIN1 on the GAP activity of RGS4 for Gαo indicates the competition of the interaction of RGS4 and GRIN1 on Gαo. However, the results do not exclude the possibility that other RGS proteins might associate Gαo simultaneously with GRIN1, and regulate the Gαo-

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GRIN1 signaling pathway. For example, in retinal phototransduction system, Gαt has been shown to interact simultaneously with its effector, PDEγ, and its GAP, RGS9-1 (Slep et al., 2001). The presence of RGS9-1 was critical for the temporal resolution of Gαt-mediated visual signal transduction (Skiba et al., 2001). In contrast to RGS4, which is an effective GAP for both Gαo and Gαi, members of R7 subfamily of RGS protein, such as RGS6 and RGS7, show specific GAP activity for Gαo but not for Gαi (Posner et al., 1999). Furthermore, they are expressed only in the brain similar to GRIN1 (Witherow and Slepak, 2003). It will be important to further characterize the involvement of RGS proteins, such as R7 subfamily members, in the Gαo-GRIN1 pathway in brain.

We demonstrated that carboxyl-terminal region of GRIN1 was involved in its membrane targeting. In particular, cysteine 818 and 819 were identified as critical residues for membrane localization. Mutation of these cysteine residues abolished the membrane distribution pattern of GRIN1. However, they are not required for the interaction with Gαo or the neurite promoting activity of GRIN1. Similar to other signaling proteins, it is possible that these cysteine residues are modified by palmitoylation. In addition to the regulation of subcellular distribution, palmitoylation also participates in the regulation of biochemical function of various proteins (Osterhout et al., 2003; Tu et al., 1999; Wedegaertner and Bourne, 1994). It will be important to confirm whether GRIN1 is palmitoylated at these residues and, if it is palmitoylated, whether this modification regulates Gαo-GRIN1-mediated signaling.

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Co-expression of GαoQ205L and full length GRIN1 stimulates microspike formation in Swiss3T3 cells, and promotes neurite extention in Neuro2a cells. Similar morphological changes were observed under conditions of GRIN1Δ (717-827) overexpression, which lacks the Gαo binding region. Co-expression of GαoQ205L was not required for the morphological changes induced by GRIN1Δ(717-827). These results suggest that deletion of the Gαo binding domain may change the conformation of GRIN1 into a constitutively active form. It is possible that the Gαo binding domain acts to inhibit GRIN1 function and that the binding of Gαo to GRIN1 releases the autoinhibitory effect of the Gαo binding domain.

We also presented evidence suggesting the possible link of the Gαo-GRIN1 pathway to the activation of Cdc42. Overexpression of dominant negative Cdc42 blocked microspike formation induced by GαoQ205L and GRIN1 in Swiss3T3 cells. Similarly, overexpression of dominant negative Cdc42 inhibited the neurite growth in Neuro2a cells. Furthermore, GST-pull down assays to detect GTP-bound Cdc42 in cells showed that GRIN1 activated Cdc42 synergistically when co-expressed with GαoQ205L. Consistent with the morphological studies, GRIN1Δ (717-827) stimulated Cdc42 independent of GαoQ205L. Thus, the binding of activated Gαo to the carboxyl-terminal region of GRIN1 is likely to induce activation of Cdc42, which will then lead to these morphological changes.

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Rho family GTPases, Rho, Rac, and Cdc42, are well known to play critical roles in the regulation of synaptogenesis, growthcone guidance, or neurite outgrowth (Threadgill et al.,1997, Ziv and Smith, 1996, Jay, 2000). Gαo is most abundantly expressed in the brain and expression of a constitutively active mutant of Gαo promoted neurite extension in PC12 cells (Strittmatter et al., 1994). The molecular mechanisms for these Go-mediated effects has remained poorly characterized. As indicated in this study, one possibility is that Gαo regulates neurite outgrowth through GRIN1 by controlling the activity of Cdc42. Further analysis of the function of endogenous GRIN1 in Gαo-mediated Cdc42 activation and neurite growth, and the characterization of the mechanism of regulation of Cdc42, such as the identification of a GEF for Cdc42 down stream of GRIN1, will be critically important to further understand the physiological role of Gαo-GRIN1 signaling in neuronal cells.

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**Acknowledgements**

We would like to thank Drs. X. Du and N. Yokoyama for helpful suggestions, and Drs. G. Bokoch and T. Satoh for the gift of plasmids for GST-PAKPBD, and Flag-tagged Rho GTPases, respectively. We would also like to thank Dr. S. Nakamura at National Institute of Neuroscience in Japan, for his encouragement and helpful discussions.

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Molecular Pharmacology Fast Forward. Published on December 7, 2004 as DOI: 10.1124/mol.104.003913
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## Footnotes

This work was supported by National Institutes of Health grants GM61454 and NS/GM 41441, and by American Heart Association grant (to T.K.). T.K. is an Established Investigator of the American Heart Association.

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Molecular Pharmacology Fast Forward. Published on December 7, 2004 as DOI: 10.1124/mol.104.003913
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## Figure Legends

**Figure 1. Interaction of GST-GRIN1 deletion mutants with Gαo.** A. GST fused GRIN1 constructs are schematically represented. A homologous region between GRIN1 and GRIN2, the membrane targeting domain, and Gαo binding domains are indicated. The results of Gαo binding assays are shown on the right. B. The binding of Gαo with GST-GRIN1 deletion mutants were assessed by GST pull down methods. Recombinant Gαo was incubated with bacterial lysates expressing indicated GST-GRIN1 mutant and glutathione Sepharose beads in the absence or presence of AMF ($Mg^{2+}/AlF_4^-$). Proteins bound to the beads were eluted and resolved by SDS-PAGE, followed by immunoblotting with anti-Gαo antibody or anti-GST antibody.

**Figure 2. Subcellular localization of GRIN1 mutants in Neuro2a cells.** Neuro2a cells were transiently transfected with expression plasmids for GFP, GFP-GRIN1, or GFP-GRIN1 mutants as indicated. After 24 hours, cells were observed by fluorescence confocal microscopy. Scale bar, 10 μm. Magnification , 1575 x.

**Figure 3. Effects of co-expression of GαoQ205L and GRIN1 or GRIN1Δ (717-827) on morphology of Neuro2a cells. A**. Expression plasmids for GFP, GFP-GRIN1, or GFP-GRIN1Δ(717-827) were transiently co-transfected with either empty vector or pCMV5-GαoQ205L into Neuro2a cells. After 20-22 hrs, the cells were fixed and stained for filamentous actin with rhodamine-phalloidin. The cells were observed by fluorescence confocal microscopy. Scale bars, 10μm. Magnification, 1260 x. The lower graph shows the

percentage of cells with neurite growth of more than one cell body length. Results are mean +/- S.E. from 3-5 experiments (n=103 -142). **B.** Expression plasmid for GFP-GRIN1 or GFP-GRIN1CCAA was transiently co-transfected with DsRed, Gαo-DsRed, or GαoQ205L-DsRed in Neuro2a cells. After 20-22 hrs, the cells were fixed and observed by fluorescence confocal microscopy. Scale bars, 10 μm. Magnification, 1260 x. The lower graph shows the percentage of cells with neurite growth of more than one cell body length. Results are mean +/- S.E. from 3 independent experiments (n=123-188).

**Figure 4. Effects of co-expression of constitutively active Gα subunit and GRIN1 in Swiss3T3 cells.** Expression plasmids encoding the indicated constitutively active mutant of Gα subunit and GFP-GRIN1 were microinjected into the nucleus of Swiss3T3 cells. After 20-22 hours, the cells were fixed and stained with rhodamine-phalloidin and observed by fluorescence confocal microscopy. The top panels show higher magnifications of actin staining images. Scale bar, 10 μm. Magnification of upper panels or lower panels is 3780 x or 1260 x, respectively. The lower graph shows the percentage of microinjected cells with more than ten microspikes per cell. Results are mean +/- S.E. from 3-4 independent experiments (n=56-88).

**Figure 5. Effect of dominant negative mutants of Rho family GTPases on the morphology of cells expressing GRIN1 and GαoQ205L. A**. Expression plasmids encoding for GαoQ205L-DsRed and GFP-GRIN1 were microinjected with the dominant negative mutant of RhoA, Rac1, or Cdc42 into the nucleus of Swiss3T3 cells. After 20 hrs, the expression of flag-tagged

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RhoA, Rac1, or Cdc42 mutant was detected using anti-flag antibody. Scale bars, 10 μm. Magnification, 1260 x. The lower graph shows the percentage of microinjected Swiss3T3 cells coexpressing GRIN1 and GαoQL with more than ten microspikes per cell. Results are mean +/- S.E. from 3-5 independent experiments (n=45-56). **B**. Expression plasmids encoding for GαoQ205L-DsRed and GFP-GRIN1 were transfected with the dominant negative mutant of RhoA, Rac1, or Cdc42 into Neuro2a cells. After 20 hrs, the expression of flag-tagged RhoA, Rac1, or Cdc42 mutant was detected using anti-flag antibody. Scale bars, 10 μm. Magnification, 1260 x. The lower graph shows the percentage of transfected cells with neurite growth of more than one body length is shown. Results are mean +/- S.E. from 3-5 independent experiments (n=127-163).

**Figure 6. Co-expression of GRIN1 and GαoQ205L activates Cdc42.** Expression plasmids encoding the indicated proteins were transiently co-transfected with flag-tagged wild type Cdc42 in Swiss 3T3 cells. The amounts of GTP-bound Cdc42 in cell lysates was measured by the GST-PAKPBD pull down assay as described in Materials and Methods. GTP-bound Cdc42 was detected by immunoblotting using anti-flag antibody (A). The amount of GTP-bound Cdc42 was quantified by densitometry. Cdc42 activity was indicated by the amount of GTP-bound Cdc42 normalized to total flag-Cdc42 in whole cell lysates (B). Expression of Gαo or GRIN1 in cell lysate was detected by immunoblotting with anti-Gαo or anti-GRIN1 antibody. *1*, control; *2*, GαoQ205L; *3*, GRIN1; *4*, GαoQ205L + GRIN1; *5*, GRIN1Δ(717-827); *6*, GαoQ205L + GRIN1Δ(717-827). The values are the mean ± S.E. of four

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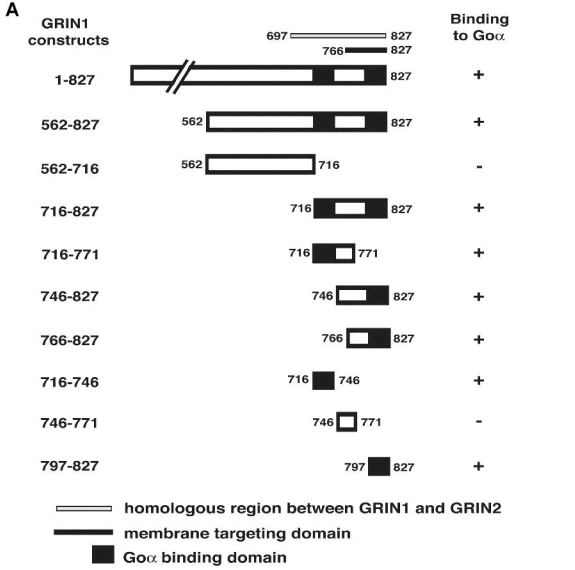
separate experiments. The asterisks indicate the results of the t test analysis.

**, $p<0.01$ , *, $p<0.05$ compared with the control.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GRIN1 constructs
Binding to Goα
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827
766
827
1-827
827
+
562-827
562
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+
562-716
562
716
-
716-827
716
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+
716-771
716
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+
746-827
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+
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766
827
+
716-746
716
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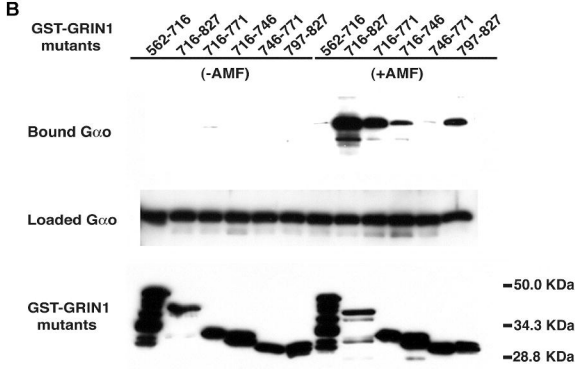
-
797-827
797
827
+
homologous region between GRIN1 and GRIN2
membrane targeting domain
Goα binding domain


B
GST-GRIN1 mutants
562-716
716-827
716-771
716-746
746-771
797-827
562-716
716-827
716-771
716-746
746-771
797-827
(-AMF)
(+AMF)
Bound Gαo
Loaded Gαo
GST-GRIN1 mutants
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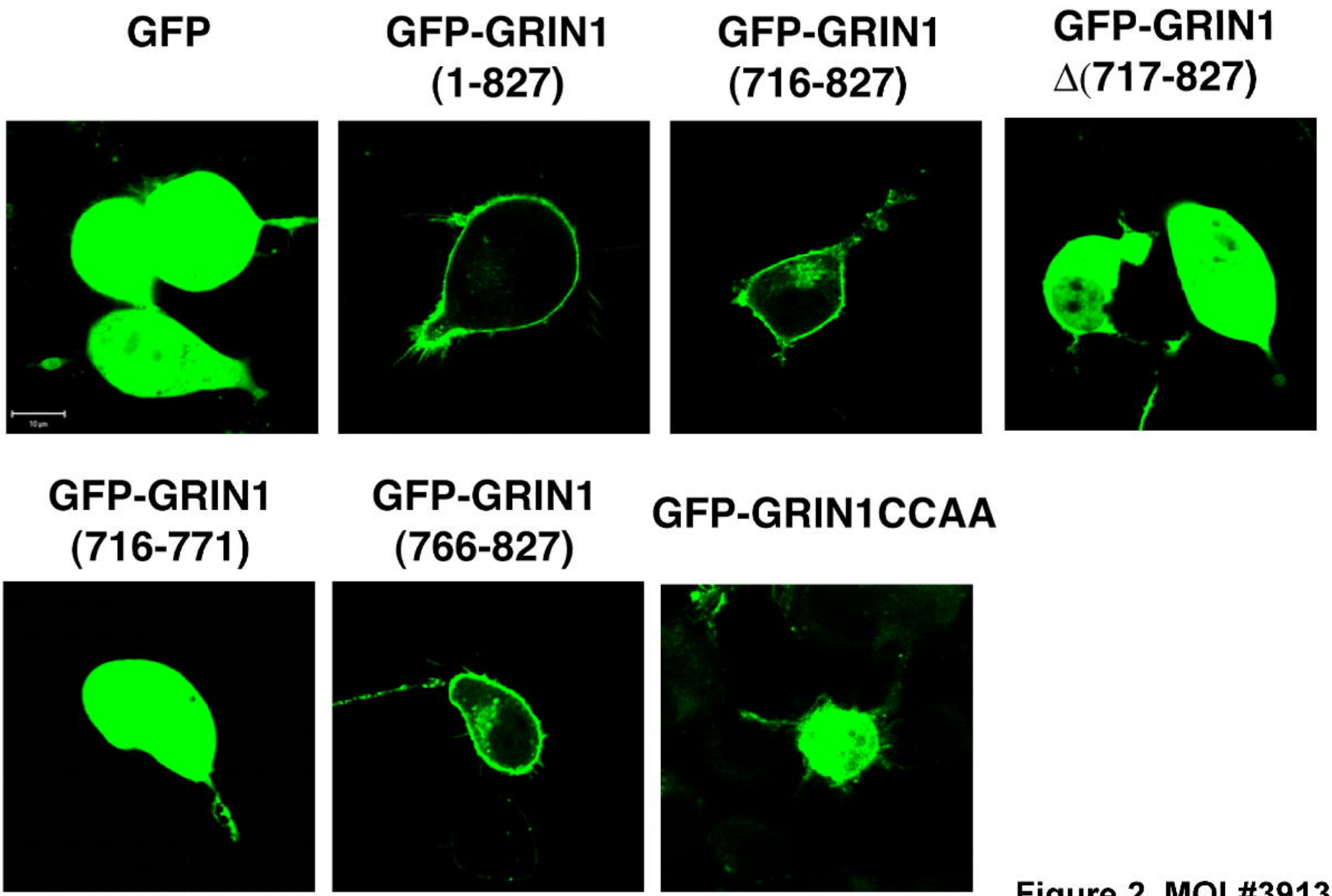
34.3 KDa
28.8 KDa

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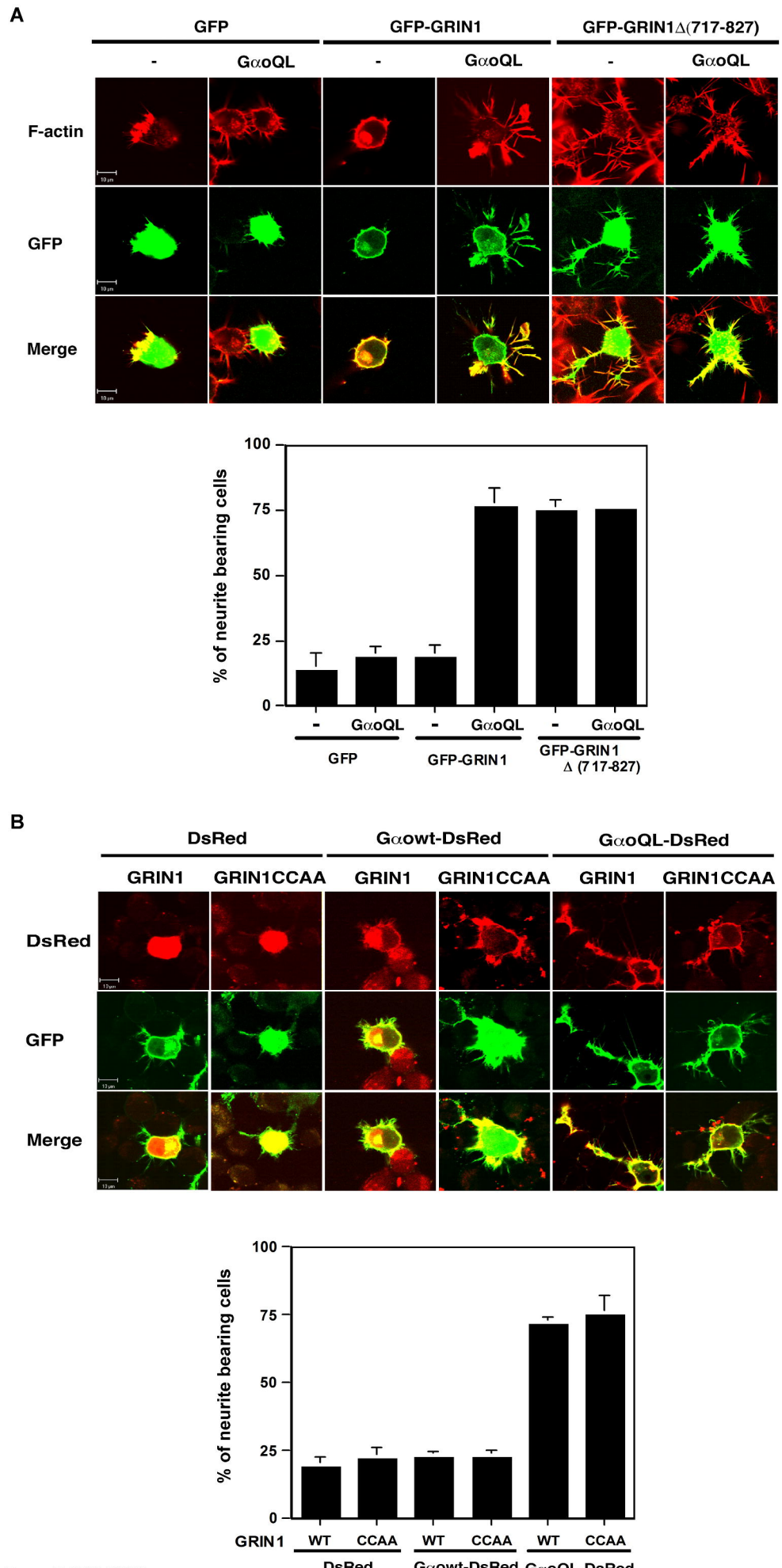


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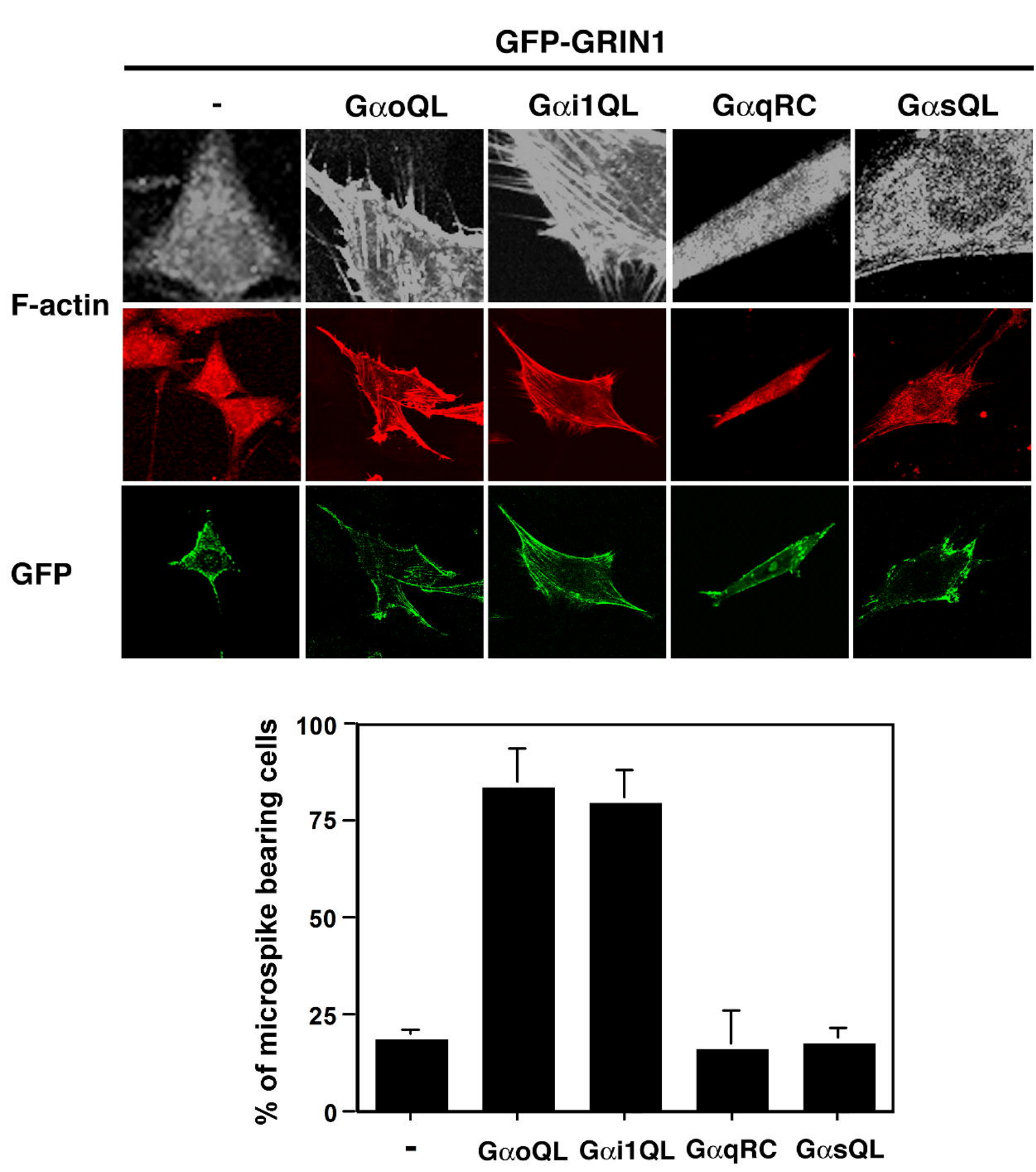


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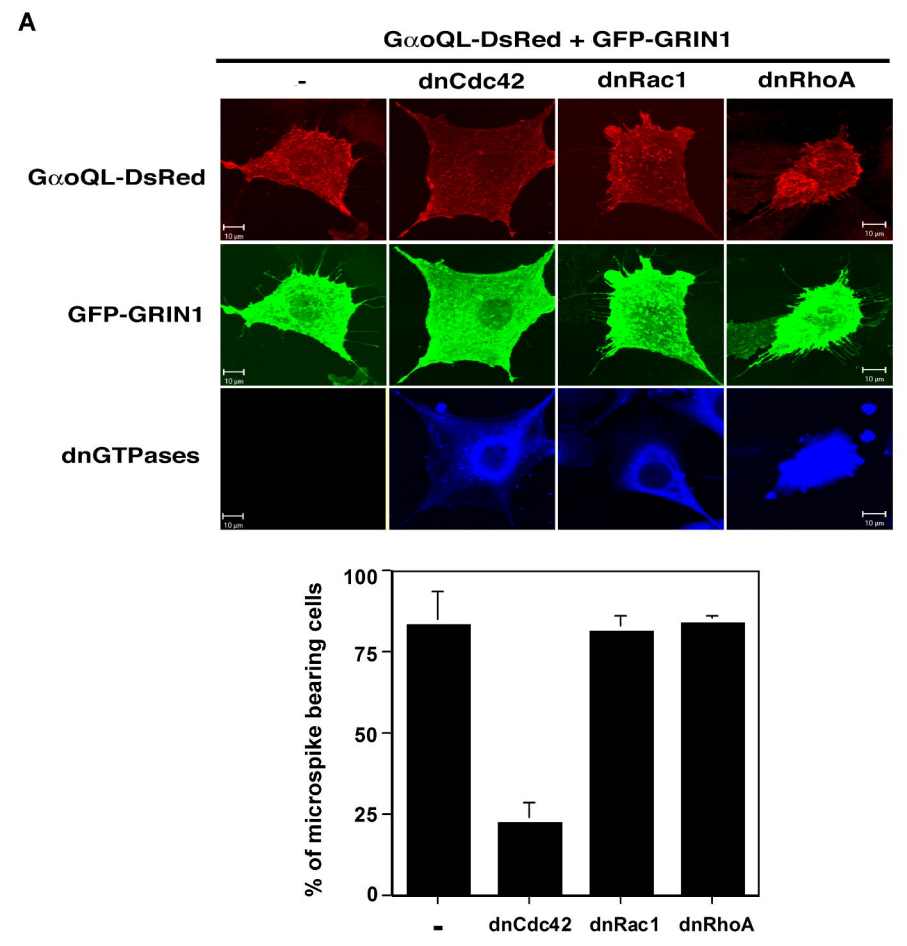


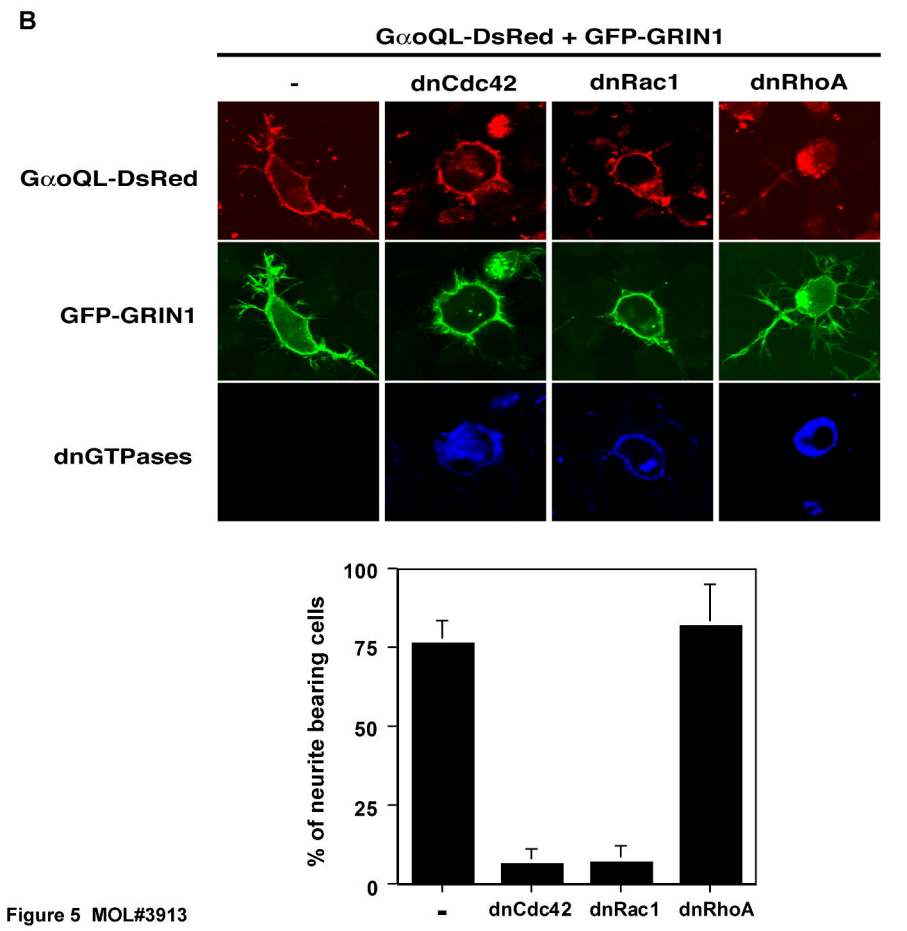


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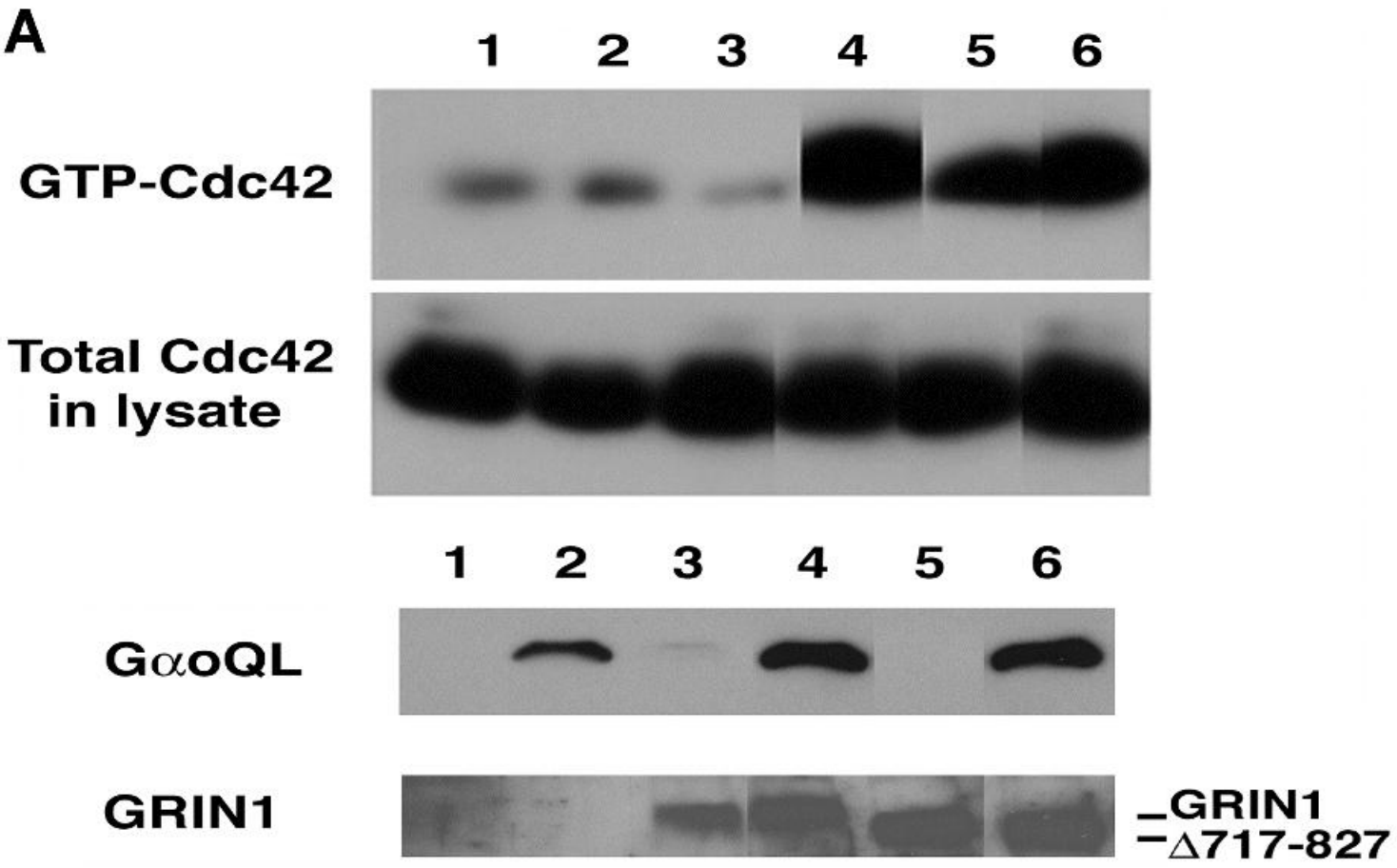
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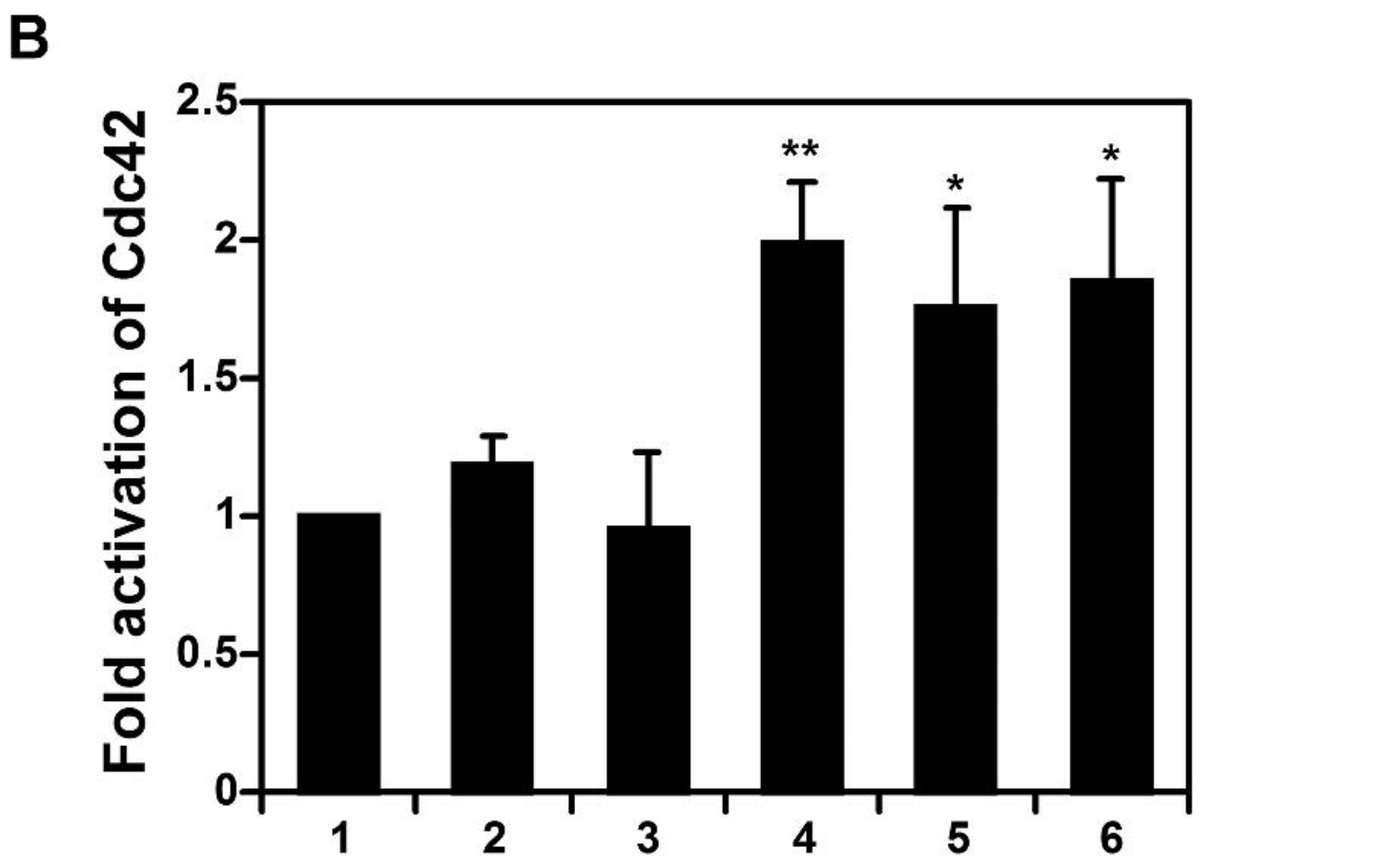
GαoQL
GRIN1
GRIN1
Δ717-827


B
Fold activation of Cdc42
2.5
2
1.5
1
0.5
0
**
*
*
1 2 3 4 5 6