GC-GAP, a Rho Family GTPase-activating Protein That Interacts with Signaling Adapters Gab1 and Gab2*

Received for publication, May 2, 2003, and in revised form, June 15, 2003
Published, JBC Papers in Press, June 19, 2003, DOI 10.1074/jbc.M304594200

Chunmei Zhao‡, Hong Ma‡, Ella Bossy-Wetzel‡, Stuart A. Lipton‡, Zhuohua Zhang‡, and Gen-Sheng Feng‡

From The ‡Burnham Institute, La Jolla, California 92037 and the §Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Gab1 and Gab2 are scaffolding proteins acting downstream of cell surface receptors and interact with a variety of cytoplasmic signaling proteins such as Grb2, Shp-2, phosphatidylinositol 3-kinase, Shc, and Crk. To identify new binding partners for GAB proteins and better understand their functions, we performed a yeast two-hybrid screening with hGab2-(120–587) as bait. This work led to identification of a novel GTPase-activating protein (GAP) for Rho family GTPases. The GAP domain shows high similarity to the recently cloned Cdgap and displays activity toward RhoA, Rac1, and Cdc42 in vitro. The protein was named GC-GAP for its ability to interact with GAB proteins and its activity toward Rac and Cdc42. GC-GAP is predominantly expressed in the brain with low levels detected in other tissues. Antibodies directed against GC-GAP recognized a protein of ~200 kDa. Expression of GC-GAP in 293T cells led to a reduction in active Rac1 and Cdc42 levels but not RhoA. Suppression of GC-GAP expression by siRNA inhibited proliferation of C6 astroglia cells. In addition, GC-GAP contains several classic proline-rich motifs, and it interacts with the first SH3 domain of Crk and full-length Nck in vitro. We propose that Gab1 and Gab2 in cooperation with other adapter molecules might regulate the cellular localization of GC-GAP under specific stimuli, acting to regulate precisely Rac and Cdc42 activities. Given that GC-GAP is specifically expressed in the nervous system and that it is localized to the dendritic processes of cultured neurons, GC-GAP may play a role in dendritic morphogenesis and also possibly in neural/glial cell proliferation.

Cells respond to environmental changes and communicate with neighboring cells through different signal transduction pathways. By participating in protein complex assembly, adapter proteins play an active role in transducing signals from cell surface receptors. Grb2-associated binder 1 (Gab1)1 and Gab2 belong to a unique family of adapter proteins, also known as docking or scaffolding proteins. The GAB proteins are characterized by a PH domain at the N terminus of the molecules and multiple consensus tyrosine residues that serve as SH2-domains. Three GAB proteins (Gab1–3) have been identified in mammals, and Daughter of Sevenless (DOS) in Drosophila and suppressor-of-Clr-1 (SOC-1) in Caenorhabditis elegans are apparently their homologues with the same molecular architecture (1–9).

The PH domains of GAB proteins interact with phosphatidylinositol phosphates and bring the host molecules to the plasma membrane. A mutant DOS protein with PH domain deletion is non-functional (10). The PH domain of Gab1 is required for its localization to plasma membrane and cell-cell contacts. It is also required for Gab1 to induce morphological changes in MDCK cells (11).

Both Gab1 and Gab2 interact with the adapter protein Grb2, presumably through their proline-rich motifs and the Grb2 SH3 domain (4, 12, 13). This interaction may serve as a bridge between Gab1 and receptor tyrosine kinases such as c-Met and EGF receptor (14, 15). Grb2/Gab2 interaction brings the complex to another adapter protein Shc and GM-CSF receptor. Gab3 interacts with C-terminal SH3 domain of a Grb2-related adapter protein Mona/Gads (16). It is not clear whether the Grb2 or Gads interactions are essential for the function of GAB proteins, because no mutations specifically disrupting Grb2/Gads interactions are generated for GAB molecules.

All GAB proteins contain multiple docking sites for SH2 domains. Gab1 and Gab2 both can interact with a number of SH2 domain containing proteins, such as Shp-2, p85 subunit of PI3-K, PLC-γ, Crk, and Shc. The function of GAB proteins is highly dependent on the interaction with the protein-tyrosine phosphatase Shp-2 or CSW for DOS in Drosophila (2, 10, 17). DOS has putative binding sites for Shc, PLC-γ, PI3-K, and CSW (8, 9, 18). When single tyrosine residues were mutated to phenylalanine, DOS mutants defective in binding Shc, PLC-γ, and the regulatory subunit of PI3-K can still function during development. Furthermore, a DOS mutant, with essentially all the tyrosine residues outside the PH domain deleted, removed, or mutated except for the two CSW-binding sites (Tyr-801 and Tyr-854), was still able to rescue the loss of endogenous DOS.

PI, phosphatidylinositol; PLC-γ, phospholipase C-γ; RNAi, RNA interference; SH, Src homology; Shp-2, SH2 domain containing protein-tyrosine phosphatase-2; SFV, Semliki Forest virus; RBD, Rho binding domain; oligo, oligonucleotide; HA, hemagglutinin; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; DAPI, 4,6-diamidino-2-phenylindole; GEFs, guanine nucleotide exchange factors; BrdUrd, 5-bromo-2-deoxyuridine; EGF, epidermal growth factor; GFP, green fluorescent protein.

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grants RO1HL66208 and RO1GM53660 (to G. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: The Salk Institute, 10010 N. Torrey Pines Rd., La Jolla, CA 92037.

‡ To whom the correspondence should be addressed: The Burnham Institute, 10011 N. Torrey Pines Rd., La Jolla, CA 92037.

§ The abbreviations used are: Gab, Grb2-associated binder; CAS, Crk-associated substrate; CSW, Corkscrew; DOS, Daughter of Sevenless; GAP, GTPase-activating protein; IRS, insulin receptor substrate; Erk, extracellular signal-regulated kinase; MDCK, Madin-Darby canine kidney; MAP, mitogen-activated protein; PH, plekstrin homology;
A Link between GAB Proteins and Rho GTPases

function, albeit at reduced efficiency. On the other hand, when either Tyr-801 or Tyr-854 was mutated to Phe, DOS protein no longer functions, indicating interaction between DOS and CSW is critical for DOS function (10). Similarly, both Gab1 and Gab2 have tandem tyrosine motifs (YIDL, YVYV for Gab1, and YLAL, YVQV for Gab2) at the C-terminal tail for Shp-2 binding. Both tyrosine residues are critical for Gab2 function in the MAP kinase pathway. When Gab1 and Gab2 were overexpressed in cells, they could promote Erk activation. However, when the Shp-2-binding sites were mutated, they lost their ability to enhance Erk activity (13, 17, 19, 20).

The GAB proteins and PI3-K are mutually important for each other. There are three consensus tyrosine motifs (YIV/ MxV) (21) for p85 SH2 domains in both Gab1 and Gab2. When those tyrosine residues were mutated, Gab1 and Gab2 failed to interact with p85. As a result, they no longer associate with PI3-K activities (22–24). The Gab1Δp85 mutant was able to induce apoptosis in PC12 cells, whereas expression of wild-type Gab1 protected cells from apoptosis (23, 25, 26). Furthermore, bone marrow-derived mast cells from Gab2−/− mice were defective in PI3-K and Akt activation following FceRI activation (27). On the other hand, LY294002 treatment of MDCK cells led to the identification of a novel GTPase-activating protein c-Jun N-terminal kinase activation (30). The significance of receptors. Crk/Gab1 interaction may play a role in Crk-induced MAP kinase pathway. When Gab1 and Gab2 were overexpressed in cells, the association with the receptor tyrosine kinase c-MET (3). Recently, Gab1 was shown to associate with Erk2, and the Met binding domain may be responsible for the interaction (31). In another study, Gab2 was reported to form a stable complex with PKB (Akt) (32). Furthermore, both proteins were shown to be substrates of their associated kinases (31, 32). It is suggested that Gab2 phosphorylation by PKB leads to a feedback inhibition on ErbB receptor signaling (32).

The closely related adapter protein IRS1 is also phosphorylated on Ser/Thr residues, and the Ser/Thr phosphorylation may inhibit tyrosine phosphorylation of IRS-1 by the insulin receptor, therefore down-regulating insulin receptor signaling. Both Erk and the PI3-K/Akt pathways have been indicated as responsible for IRS1 phosphorylation on Ser/Thr residues (33, 34). Gab1 and Gab2 are both highly enriched in Ser/Thr residues. The interactions of GAB proteins with Ser/Thr kinases suggest that they may also be subjected to regulation by Ser/Thr phosphorylation and dephosphorylation.

Although many proteins were identified to bind GAB proteins, the function of these molecules is not clear. Most of these protein/protein interactions are dependent on Tyr(P) residues and originally identified by the presence of consensus SH2-binding motifs within GAB molecules. In order to identify protein/protein interactions independent of Tyr phosphorylation of GAB proteins, a Gab2 fragment (amino acids 120–587 of human Gab2 protein) was used in a yeast two-hybrid screen. This led to the identification of a novel GTPase-activating protein (GAP) for Rho family GTPases, GAB-associated Cdc42/Rac GAP (GC-GAP).

MATERIALS AND METHODS

Yeast Two-hybrid Screen—The cDNA fragment encoding amino acids 120–587 of human Gab2 was inserted into vector pGDBu-C2 in-frame with the DNA binding domain of Gal4. The bait plasmid was transformed into the host cell PJ69-4A. PJ69-4A bearing pGBD-C2/hGab2-(120–587) was transformed with Mouse 17-Day Embryo MATCHMAKER cDNA Library (Clontech ML4006AB). A total of 1.8 × 108 independent colonies were screened for Gab2 interaction. Colonies positive for Ade were further plated on SC-Ura-Leu-His + 2 mM 3-amino-1,2,4-triazole and analyzed for β-galactosidase activity. Yeast DNA from confirmed positive colonies were prepared (Zymoprep Yeast Plasmid Mini preparation Kit, ZYMO Research) and rescued in bacteria strain RR1 (35) under Leu selection. The plasmid DNA was then introduced back into yeast to test for bait dependence. All bait-dependent clones were sequenced and analyzed with the NCBI BLAST program.

DNA Constructs and Other Reagents—Human HUH7 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 25 mM sodium bicarbonate, penicillin/ streptomycin, and 1% L-glutamine. siRNA was transfected into cells using Hi-PerFect transfection reagent (Qiagen) according to manufacturer’s instructions. The HUH7 cell line was purchased from ATCC (Manassas, VA). GC-GAP was amplified from mouse cDNA by PCR using primer pairs 3-A and 3-B. The GC-GAP cDNA was subcloned into pEGFP-N1 vector (Clontech), yielding GC-GAP/EGFP. The GC-GAP negative control construct was generated by substituting the GC-GAP coding region with a vector backbone. GC-GAP was transiently transfected by lipofectamine. Expression was verified by Western blot analysis using anti-GC-GAP antibodies. The in vivo GC-GAP knockdown was performed using siRNA designed against mouse GC-GAP. The siRNA sequence was 5′-GGUGUACUGCGCACUGCGG-3′.

Northern Blot and in Situ Hybridization—Northern blot was performed as described before (4). Mouse Multiple Tissue Northern (Clontech 7762-1) was used to examine the tissue distribution of GC-GAP. The GC-GAP cDNA probe was labeled with [32P]dCTP (Amersham) according to manufacturer’s instructions. The GC-GAP cDNA fragment was inserted into the pBlueScript II vector (Stratagene) according to manufacturer’s instructions with minor modifications. Antisense GC-GAP-specific primers were used to prime the reaction instead of random primers. The cDNA templates used for probe synthesis encode amino acids 434–761 and 1152–1434 of mouse GC-GAP. In situ hybridization was described before (4).

Cell Culture and Transient Transfection—293T, COS1, NIE115 and C6 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin/streptomycin/glutamine (Invitrogen) and 10% fetal bovine serum (Hyclone). A calcium phosphate transfection system (Invitrogen) and FuGENE 6 (Roche Applied Science) were used to transiently transfect 293T, COS1 and NIE115, respectively, with expression constructs of Myc-tagged wild-type RhoA, Rac1, and Cdc42 and the Nck mammalian expression construct. The Nck mammalian expression construct was from Dr. Lawrence Quilliam. Mammalian expression constructs for Myc-tagged wild-type RhoA, Rac1, and Cdc42 were gifts from Dr. Gary Bokoch. Crk expression construct was obtained from Dr. Kristin Vuori. Anti-GC-GAP antibodies were generated against GST fusion proteins of mouse GC-GAP 803–911 (Bur273) and 1590–1740 (Bur281). A 1.100 dilution of GST pre-cleared Bur273 antiseraum was used for GC-GAP Western blot. Antibody was prepared against the highly conserved portion of GC-GAP (amino acids 585–605, anti-GC-GAP antibody). Antibodies against FLAG (M2), HA (12CA5), and c-Myc (9E10) were from Sigma, Roche Applied Science, and Santa Cruz Biotechnology, respectively. Nck and Crk antibodies were from Transduction Laboratories. Anti-Gab2 was described previously (4).

GAP Activity Assay—GAP activity assays were performed as described before (4). GAB proteins, Gab1 and Gab2, were purified from bacterial strain BL21 (DE3) (37), and the GAP assay was performed as described before (38). Briefly, the GAP reaction was carried out at room temperature with 0.2 pmol of purified GAP, 0.02 pmol of γ[32P]-labeled small GTPase, 25 nm Tris-Cl, 1.5 mg/ml bovine serum albumin, 7.5 mM MgCl2, and 2 mM dithiorthreitol. The reaction was stopped at different time points by 1 ml of stop buffer (25 mM
Fluor 488 goat anti-rabbit (1:100, Molecular Probes). At the last wash two secondary antibodies, Alexa Fluor 594 goat anti-mouse and Alexa 1:100 and anti-MAP2 1:100, Sigma). The cells were then treated with serum, the cells were incubated with primary antibodies (anti-GC-GAP, X-100 for 5 min. After blocking nonspecific binding with phosphate-hyde in phosphate-buffered saline and permeabilized with 0.1% Triton twice with phosphate-buffered saline and fixed with 4% paraformalde against Myc (9E10).

The level of active small GTPases was determined by Western blot RBD and GST-PBD pull-down assays, 48 h after transfection (39, 40). Cdc42. Active RhoA and Rac1/Cdc42 levels were determined by GST-GAP and GST-PBD pull-down assays.

For the affinity-based GAP activity assay, GC-GAP was co-transfected into 293T cells with HA-Gab2 (Fig. 1A). When Gab2 and clone 10, one of the three identical positive clones. To confirm that the polypeptide derived from the cDNA fragment indeed interacts with Gab2, we inserted the fragment into CMV-FLAG II and co-transfected clone 10 into 293T cells with HA-Gab2 (Fig. 1B). HA-Gab2 was pulled down by FLAG monoclonal antibody M2 only when FLAG-C10 was present (Fig. 1B, upper two panels). Likewise, FLAG-C10 was present in the Gab2 antibody complex only when Gab2 was expressed (Fig. 1B, lower panels). These results suggest that there is a specific interaction between Gab2 and clone 10.

Sequence analysis of the 2184-bp insert revealed that it displayed over 80% identities in nucleotide sequence to a cDNA clone kiaa0712 in the GenBankTM data base, indicating that clone 10 is the mouse homologue of the human gene kiaa0712. Deduced amino acid sequence of kiaa0712 contains an incomplete GAP domain at the N terminus, highly homologous to the GAP domain of the recently reported CdGAP (68.7% identity). We then performed a 5’-RACE reaction and found that a single nucleotide was missing in the kiaa0712 sequence, which caused a frameshift in the coding sequence. The revised cDNA sequence encoded a protein of 1738 amino acids, which extended 151 amino acid residues from the first Met residue of the original KIAA0712. Several in-frame stop codons were present before the initiation codon AUG. The full-length coding sequence of mouse clone10 was obtained by several 5’- and 3’-RACE reactions. This gene was designated GC-GAP (GAB-associated Cdc42/Rac GTPase-activating protein). The GenBankTM accession numbers for human and mouse GC-GAP are AY194286 and AY194287, respectively. The mouse and human GC-GAP proteins share 86% identity and 88% overall similarity (Fig. 2A). They are identical in the GAP domain. The GAP domain of GC-GAP is closely related to that of CdGAP (Fig. 2B) (41). BLAST search with GC-GAP as query also pulled out a Drosophila protein d-CdGAPr (42). The GAP domain of d-CdGAPr shows over 48% identity to those of GC-GAP and CdGAP (Fig. 2B). In addition to the GAP domain, GC-GAP displays 67% similarity to CdGAP in the region adjacent to the C-terminal end of the GAP domain (Fig. 2C). We designated this region GH domain for GC-GAP homology. Furthermore, GC-GAP contains several proline-rich motifs, indicating putative interactions with SH3 domain or WW domain containing proteins (Fig. 2D) (43). In summary, GC-GAP is a novel Gab2-binding protein. It is a putative Rho family GAP protein and may interact with other proteins through its proline-rich motifs (Fig. 2E).

**RESULTS**

Identification of GC-GAP as a Novel Gab2-binding Protein—In order to identify Gab2-binding proteins, we took a yeast two-hybrid approach using a human Gab2 fragment (amino acids 120–587) as bait. Among the 41 independent positive colonies we obtained from the mouse embryonic day 17 cDNA library, 3 of them contained the same cDNA fragment. The interaction between these positive clones and Gab2DBD-Gab2-(120–587) is dependent on Gab2. Fig. 1A shows the interaction between Gab2 and clone 10, one of the three identical positive clones. To confirm that the polypeptide derived from the cDNA fragment indeed interacts with Gab2, we inserted the fragment into CMV-FLAG II and co-transfected clone 10 into 293T cells with HA-Gab2 (Fig. 1B). HA-Gab2 was pulled down by FLAG monoclonal antibody M2 only when FLAG-C10 was present (Fig. 1B, upper two panels). Likewise, FLAG-C10 was present in the Gab2 antibody complex only when Gab2 was expressed (Fig. 1B, lower panels). These results suggest that there is a specific interaction between Gab2 and clone 10.

Sequence analysis of the 2184-bp insert revealed that it displayed over 80% identities in nucleotide sequence to a cDNA clone kiaa0712 in the GenBankTM data base, indicating that clone 10 is the mouse homologue of the human gene kiaa0712. Deduced amino acid sequence of kiaa0712 contains an incomplete GAP domain at the N terminus, highly homologous to the GAP domain of the recently reported CdGAP (68.7% identity). We then performed a 5’-RACE reaction and found that a single nucleotide was missing in the kiaa0712 sequence, which caused a frameshift in the coding sequence. The revised cDNA sequence encoded a protein of 1738 amino acids, which extended 151 amino acid residues from the first Met residue of the original KIAA0712. Several in-frame stop codons were present before the initiation codon AUG. The full-length coding sequence of mouse clone10 was obtained by several 5’- and 3’-RACE reactions. This gene was designated GC-GAP (GAB-associated Cdc42/Rac GTPase-activating protein). The GenBankTM accession numbers for human and mouse GC-GAP are AY194286 and AY194287, respectively. The mouse and human GC-GAP proteins share 86% identity and 88% overall similarity (Fig. 2A). They are identical in the GAP domain. The GAP domain of GC-GAP is closely related to that of CdGAP (Fig. 2B) (41). BLAST search with GC-GAP as query also pulled out a Drosophila protein d-CdGAPr (42). The GAP domain of d-CdGAPr shows over 48% identity to those of GC-GAP and CdGAP (Fig. 2B). In addition to the GAP domain, GC-GAP displays 67% similarity to CdGAP in the region adjacent to the C-terminal end of the GAP domain (Fig. 2C). We designated this region GH domain for GC-GAP homology. Furthermore, GC-GAP contains several proline-rich motifs, indicating putative interactions with SH3 domain or WW domain containing proteins (Fig. 2D) (43). In summary, GC-GAP is a novel Gab2-binding protein. It is a putative Rho family GAP protein and may interact with other proteins through its proline-rich motifs (Fig. 2E).

**GC-GAP Is Highly Expressed in Brain**—Northern blot analysis was performed to determine the expression pattern of GC-GAP. A band of around 10 kb was detected with GC-GAP cDNA probe. GC-GAP mRNA is most abundant in brain and to a lesser extent in heart, lung, liver, kidney, and testis (Fig. 3A). The closely related CdGAP was highly expressed in heart and lung (41). The weak signal at around 5 kb is likely a cross-reaction or degradation product.

To study the expression of GC-GAP at the protein level, we generated rabbit polyclonal antibodies against mouse GC-GAP (see "Materials and Methods" for details). GC-GAP-specific an-
Fig. 2. Clone 10 encodes C-terminal part of a novel Rho family GAP protein, GC-GAP. A, alignment of mouse (m) and human (h) GC-GAP protein sequences. GCG “Bestfit” program was used to create the alignment. Match display thresholds for the alignment: | = IDENTITY, : = 0.9. The GenBank™ accession numbers for human and mouse GC-GAP sequences are AY194286 and AY194287, respectively. B, GAP domain alignment of mouse GC-GAP, CdGAP, d-CdGAPr, and p50RhoGAP. Identical residues among GC-GAP, CdGAP, and d-CdGAPr are printed in boldface, as well as residues in p50RhoGAP that are identical to the other three GAPs. Residues of similar characteristics are marked +. The three consensus regions in GAP domains are grouped as Blocks 1–3. C, sequence alignment of GH domains from GC-GAP-(215–364) and CdGAP-(214–350). D, potential proline-rich motifs that may mediate GC-GAP interactions with SH3 or WW domain containing proteins. E, schematic structure of GC-GAP, in comparison with CdGAP and d-CdGAPr.
A Link between GAB Proteins and Rho GTPases

Fig. 2—continued
tibodies recognized several bands at around 200 kDa in mouse tissues such as brain and testis (Fig. 3B). The upper band migrated at a similar position as the recombinant GC-GAP protein expressed in 293T cells (data not shown). The lower bands were likely due to cross-reaction of the antibody or degradation products of GC-GAP, because we did not always detect those in protein lysates derived from cell lines (Fig. 4B, input). The result from Western blot analysis was consistent with the Northern blot analysis; both showed that GC-GAP is most abundantly expressed in the brain tissue.

Because GC-GAP was identified from embryonic cDNA library and that it is specifically expressed in the brain, we performed an in situ hybridization to study the regional expression of GC-GAP in the mouse embryo. In the developing forebrain of the E13.75 mouse embryo, GC-GAP RNA was present in cerebral cortex, basal telencephalon, and diencephalons, and its expression pattern overlapped with Gab1 and/or Gab2 in these regions (Fig. 3C). We were then interested in whether GC-GAP has a specific function in neuronal cells. Western blot analysis showed that GC-GAP was indeed expressed in neurons (data not shown). To determine its cellular localization in neurons, GC-GAP antibodies were used to stain cultured neurons from rat embryonic cortices (Fig. 3D). Anti-MAP-2 co-staining was used to reveal neurons. GC-GAP displayed a punctated distribution pattern along the dendritic processes. The localization of GC-GAP to dendritic processes suggests its possible involvement in the regulation of dendritic morphology.
when GC-GAP was identified as a Gab2-binding protein, Gab1 was the only other known mammalian GAB protein. To study whether GC-GAP also interacts with Gab1, we transfected into 293T cells an expression construct of human GC-GAP with a FLAG sequence tagged at its N terminus. Both Gab1 and Gab2 proteins were detected in the GC-GAP complex immunoprecipitated with FLAG antibodies (Fig. 4A). To study the interaction of endogenous GAB and GC-GAP proteins, we used a human neuroblastoma cell line SHSY5Y. Gab1 and Gab2 proteins were purified from the total protein lysate using mouse monoclonal antibodies against Gab1 (Fig. 4B, lower panel). GC-GAP was indeed detected in this GAB protein complex (Fig. 4B). We concluded from these results that GC-GAP interacts with both Gab1 and Gab2.

A Minimal Fragment of GC-GAP Is Required for Gab2 Association—A series of GC-GAP truncations was generated to study the interaction of GC-GAP with Gab2 in yeast cells (Fig. 5A). The shortest fragment retaining the Gab2-binding ability was 1096–1377. Interestingly, all of the truncations showed reduced affinity to Gab2. We then made a deletion mutant (GC-GAPΔ) of the mammalian expression construct of human GC-GAP, which lacks residues 1042–1362 (correspondent to mouse GC-GAP 1046–1365). There was much less Gab2 in GC-GAPΔ protein complex, although GC-GAPΔ was expressed at a higher level than the wild-type GC-GAP. This is consistent with the conclusion from the yeast two-hybrid studies that this region is indeed required for efficient Gab2 interaction. However, this deletion mutant can still weakly interact with Gab2 in 293T cells, suggesting one or more regions outside of this fragment also contribute to GC-GAP/Gab2 interaction (Fig. 5B). Nevertheless, the minimal fragment encompassing 1042–1362 of GC-GAP is the primary region that is responsible for Gab2 interaction, because deletion of this fragment nearly abolished the interaction.

Gab2 and GC-GAP Are Co-localized at Cell Membrane—HeLa cells were transfected with HA-Gab2 and GC-GAP, and cells were seeded on fibronectin-coated coverslips for 4 (Fig. 6A) or 24 h (Fig. 6B). These cells were stained with HA antibody 12CA5 and GC-GAP-specific antibodies. Both Gab2 and GC-GAP have a dynamic cellular localization pattern. Gab2 was found mostly at the cytoplasmic membrane and regions close to the membrane. It was also seen in cytosol in some cells. The GC-GAP signal was often detected in the cytosol, but it was concentrated at the membrane, where it was found to co-localize with Gab2. Interestingly, cells with GC-GAP overexpressed were often rounded, whereas the neighboring non-transfected cells spread much better on fibronectin-coated coverslips. The dynamic cellular localization pattern of Gab2 and GC-GAP
suggests that the localization of these proteins may be regulated by cellular activities.

**GC-GAP Stimulates the GTPase Activity of Rho Family Small GTPases**

To examine whether GC-GAP does possess GAP activity toward Rho family small GTPases, we isolated the cDNA fragment encoding the GAP domain of GC-GAP and inserted it into pGEX4T vector. The recombinant GAP domain (GC-GAP200) was purified from bacteria and subjected to an in vitro GAP assay. The endogenous GTPase activities of Rho, Rac, and Cdc42 were considered as negative controls. GC-GAP200 stimulated GTPase activity of Rho, Rac, and Cdc42 (Fig. 7A) but not that of the other family of small GTPases Ras and Rap (data not shown). Similar to p50RhoGAP, GC-GAP200 showed a preference toward Cdc42. The activity pattern is similar to that of Cdc42 (41), consistent with the fact that the two GAP domains are closely related.

To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related. To study the GAP specificity in vivo, we used an affinity assay system to examine the effect of GC-GAP on the level of active RhoA, Rac1, and Cdc42. Rho effector protein Rhotekin has a Rho binding domain (RBD) that specifically interacts with the GTP-bound form of Rho. GST fusion protein of Rho-Rhotekin RBD expressed in bacteria can efficiently and specifically pull-down active RhoA protein in total cell lysate. Likewise, the CRIB domain of Rac/Cdc42 effector p21-activated kinase (PAK) interacts with Cdc42, and these GAP domains are closely related.
A minimal fragment of GC-GAP required for Gab2 association. A, identification of a minimal Gab2 binding region in GC-GAP. A series of GC-GAP truncation mutants was made and tested for interaction with Gab2 in yeast. PJ69-4A was co-transformed with pGBD-Gab2-(120–587) and GC-GAP truncation mutants fused in-frame to a Gal4 DNA activation domain. Colonies were selected first on SC-Ura-Leu for uptake of both plasmids and then grown on SC-Ura-Leu-Ade to test for protein/protein interaction. The interaction was quantified by visual assessment of yeast growth on SC-URA-LEU-ADE. B, deletion of amino acids 1042–1362 significantly reduced hGC/GAP interaction with Gab2 in 293T cells. 293T cells were transfected with Gab2 together with full-length GC-GAP (wt) or hGC-GAP1042–1362 (Δ). The interaction between Gab2 and GC-GAP was determined by FLAG immunoprecipitation (IP) and Gab2 Western blot.

has been used for Rac/Cdc42 activity assays. Although GC-GAP displayed activity toward all three Rho family small GTPases in vitro, only the active Cdc42 and Rac1 levels were significantly reduced in 293T cells when GC-GAP was overexpressed (Fig. 7B), suggesting that GC-GAP is specific for Cdc42 and Rac1 in vivo. We did not detect any changes in the active RhoA level when GC-GAP was co-expressed.

GC-GAP Forms a Complex with the Adapter Proteins Crk, Nck, and p130CAS—In addition to the GAP domain at the N terminus of the molecule, there are several proline-rich motifs throughout the molecule. To test whether these proline-rich motifs can mediate interactions with SH3-containing proteins, we performed a GST pull-down assay with a number of SH3 domains (Fig. 8A). The first SH3 domain of Crk and Nck full-length protein can form a complex with FLAG-GC-GAP expressed in 293T cells; N-SH3 of Grap and Src SH3 domain also interact with GC-GAP, and SH3 domains from Grb2, Abl, Ras, GAP, and PLCγ were not able to bind GC-GAP. To confirm the interaction between Nck and GC-GAP, we co-transfected the cDNA constructs encoding these two proteins into 293T cells and performed immunoprecipitation analysis. Indeed, Nck was present in the GC-GAP complex brought down by the FLAG antibody M2 (Fig. 8B). Similarly, CrkII interacted with GC-GAP in 293T cells (Fig. 8C). These studies confirmed a role of the proline-rich motifs in mediating GC-GAP interactions with SH3 domains. However, the GST fusion proteins used in this study only represent a limited number of SH3 domains; GC-GAP may have other binding partners that are not included in this experiment. These interactions as well as the binding to GAB proteins may specifically regulate the cellular localization of GC-GAP and precisely control the local GTPase activities of Rac and Cdc42.

Sequence analysis of GC-GAP indicates that GC-GAP also contains several tyrosine residues that may be subjected to post-translational modification. To test this hypothesis, GC-GAP was transfected into COS1 cells, and the phosphorylation status of GC-GAP was examined by Western blot with phosphotyrosine (Tyr(P))-specific antibodies. When cells were stimulated with EGF, GC-GAP was phosphorylated on Tyr and interacted with a phosphoprotein with electrophoretic mobility close to the phosphoprotein in the Nck-GC-GAP complex. The size of p130CAS is very likely the reason why the Tyr(P) signal is not present in EGF-treated samples (45). Finally, the size of p130CAS is very close to the phosphoprotein in the Nck-GC-GAP complex. The membrane was then re-blotted with p130CAS-specific antibodies. p130CAS antibodies indeed recognized a specific band at

![Fig. 5. A minimal fragment of GC-GAP required for Gab2 association.](image)
GTPases.

A) Expression of the Mutant GAP Domain of GC-GAP—protein Nck. The Arg-58 residue is highly conserved among RhoGAP proteins. When the Arg residue is replaced by Ala, the mutant Cdc42GAP protein can still form complex with Cdc42, but the mutation results in 40-fold loss of the catalytic activity (50). Our results suggested that when the mutant GAP was expressed, it might sequester Rho GTPases from endogenous GAP proteins. Because the mutant GAP domain was inefficient to stimulate the GTPase activity of Rho proteins, it may have caused a balance shift of the Rho GTPase activities, leading to the inhibition of NIE115 cell differentiation.

Proliferation of C6 Astroglial Cells Is Inhibited by GC-GAP siRNA—To further investigate the function of GC-GAP in cells, we decided to use siRNA-mediated inhibition of GC-GAP expression. Western blot analysis suggested that GC-GAP is expressed in both purified astroglia and neurons. C6 astroglial cell was chosen for this study because siRNA can be easily delivered with transient transfections. One of the double-stranded RNA oligos designed according to the mouse GC-GAP cDNA sequence completely matched the putative rat GC-GAP mRNA sequence (XM_236020). It was able to block GC-GAP expression (Fig. 10, A and B). The negative control RNAi oligo had at least two mismatches with the putative rat GC-GAP mRNA sequence and had no effect on GC-GAP expression. We did not observe significant cytoskeletal changes when GC-GAP was inhibited in C6 cells. However, we observed a significant decrease in total cell numbers, which could result from decreased cell proliferation, increased cell death, or the combination of both. BrdUrd incorporation assay was then used to determine the proliferation rate of these cells. Interestingly, when C6 cells were treated with GC-GAP siRNA, the proliferation rate was reduced by more than 25% (Fig. 10C, lane RNAi), comparing to cells that were mock-transfected (Fig. 10B, lane M) or cells transfected with nonspecific RNA oligos (Fig. 10B, lane N). In this study, DAPI staining was used to reveal all cell nuclei, and we detected few cells with condensed chromosomes, a characteristic of apoptotic cells, indicating that the reduced cell number with GC-GAP RNAi is mainly caused by a reduction in cell proliferation.

DISCUSSION

To better understand Gab2 mediated signaling events, a yeast two-hybrid screen strategy was used to identify Gab2-binding proteins, and this work led to the identification of a novel Rho family GAP protein, which was designated GC-GAP. The GC-GAP fragment obtained from yeast two-hybrid screening does not show similarity to any known protein domains except for several proline-rich motifs. The polypeptide is highly enriched in Ser residues. The region in Gab2 used as bait has similar characteristics, containing one proline-rich motif and enriched in Ser residues. None of the two proteins contains SH3 domain, WW domain, or any other known domains predicted to bind proline-rich motifs. Both Gab2 and GC-GAP can be phosphorylated on Tyr residues. However, neither of them contains SH2 or phosphotyrosine binding domains that are potential binding modules for Tyr(P) motifs. In short, the interaction between Gab proteins and GC-GAP may represent a novel protein/protein interaction mode. Furthermore, yeast two-hybrid assays with GC-GAP truncations suggest that multiple sites on GC-GAP are involved in Gab2 interaction.

The functions of Gab1 and Gab2 have been extensively studied in the c-MET receptor pathway and hematopoietic systems, respectively. Little is known about their functions in the nervous system, although both genes are expressed in the brain at RNA (1, 2, 4) and protein levels. Previous studies with PC12 pheochromocytoma cells indicated that Gab1 is involved in the regulation of PC12 neurite outgrowth, DNA synthesis, and cell survival (23, 26). Studies with PI3-K inhibitors and a Gab1
mutant suggest that these activities are dependent on PI3-K activity. Because Rho small GTPases are main regulators of actin cytoskeletal changes in response to various extracellular stimuli, it would be interesting to investigate whether Gab1-induced PC12 neurite outgrowth is also dependent on the activation of Rho family small GTPases.

Rho family small GTPases play important roles in a variety of cellular responses, especially the regulation of cytoskeleton assembly. They exist in GTP-bound active form and GDP-bound inactive form. Microinjection studies with fibroblast cells showed that active RhoA induces stress fiber formation; active Rac1 induces lamellipodia and membrane ruffles, and Cdc42 is responsible for formation of microspikes and filopodia (51). They are required in many tissue and cellular responses, such as axonal growth cone guidance, dendritic development, cell polarity, macrophage chemotaxis, mast cell degranulation, MDCK cell spreading, and dissociation, etc. (52–58). Rho family GTPases are regulated by three different proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors. In both Drosophila and human genome, Rho GTPases are outnumbered by their regulators, especially the GEFs and GAPs. There are an estimated 77 or 59 Rho family GAPs encoded in the human genome (59, 60), yet only 18 different Rho GTPases

with EGF for 5 min. GC-GAP complex was purified with FLAG antibody M2 and analyzed with specific antibody against TyrP. The membrane was then re-blotted with p130CAS antibodies and FLAG antibody sequentially. Nck and p130CAS expression was examined by Western blot against HA and p130CAS antibodies, respectively. IP, immunoprecipitation.
exist in mammals (61). The abundance of RhoGAPs over Rho GTPases suggests that individual GAPs may have specific functions. Although more than 20 RhoGAP proteins in mammals have been studied, little is known about their regulation. However, several characteristics can be identified from the RhoGAPs studied so far. First, many of the GAPs have specific Rho GTPases as their substrates. For example, p190 RhoGAP is highly specific for RhoA (62). Even though some of the GAPs display activities toward all three major Rho small GTPases (RhoA, Rac1, and Cdc42), the effect on the three GTPases is not identical. Second, many of the RhoGAPs have a distinctive tissue distribution pattern. For instance, CdcGAP is highly expressed in heart and lung (41), and β-chimerin mRNA is detected mostly in the testis at the onset of sexual maturation (63). The third common feature to many of the RhoGAPs is the possession of structural domains or motifs that mediate their interaction with other proteins. Grafs contain an SH3 domain through which they interact with the C-terminal tail of FAK (64). 3BP-1 that contains a proline-rich motif was identified as a binding partner of Abi SH3 domain (65). p190RhoGAP was found as a major p120 RasGAP-binding protein (66). The combination of these three features makes every single RhoGAP distinctive from another. GC-GAP inhibited activities of Rac1 and Cdc42 in 293T cells. We did not detect any effect on RhoA activity. However, GC-GAP significantly induced the GTPase activity of RhoA, Rac1, and Cdc42 in the in vitro GAP assay. We cannot exclude the possibility that our in vivo GAP assay system was not sensitive enough to detect the GAP activity toward RhoA. It is more likely that the specificity of GC-GAP is determined at the level of mRNA and by its subcellular localizations. Both Northern and Western blot analyses showed that GC-GAP is specifically expressed in brain. Recent studies have identified several RhoGAP proteins that are expressed in the nervous system. These proteins are likely involved in the regulation of different functions of Rho GTPases. Nadrin was shown to be involved in calcium-dependent exocytosis (67). SYD-1, a RhoGAP protein in C. elegans, is localized to the presynaptic terminals in mature neurons and required for the specification of axonal identities during differentiation (68). SrGAPs, which are identified by their interaction with the intracellular domain of Robo, are required for neuronal migration regulated by the Robo ligand-Slit (69). These studies confirmed that RhoGAP proteins are specific regulators for Rho GTPases, particularly in brain, where many biological responses are dependent on the dynamic regulation of actin filaments. Our study showed that GC-GAP is localized to the dendritic processes of cultured neurons, indicating that it may be involved in the regulation of dendritic morphogenesis mediated by Rho GTPases. In addition, GC-GAP is also expressed in astrocytes. We showed by GC-GAP RNAi studies that the proliferation of C6 glioblastoma cells was dependent on the expression of GC-GAP protein. This indicates that the controlled cycling of Rho GTPases by GEF and GAP proteins is critical for cell growth. Furthermore, GC-GAP interacts with Gab1, Gab2, Crk, Nck, p130CAS, and possibly c-Src. The interaction between GAB proteins and GC-GAP has been observed both with ectopically expressed proteins in 293T cells and with endogenous proteins in SH-SYSY cells. The interactions between GC-GAP and Nck, Crk, and c-Src still need to be confirmed with endogenous proteins. Although there are few studies about the function of Gab1 and Gab2 in the regulation of Rho GTPases, there is indication that these adapter proteins may be involved in the regulation of Rac activity. A switch from CAS-Crk to Gab1-Crk complex was shown to correlate with c-Met-induced c-Jun N-terminal kinase activation, which requires the activity of Rac1 (70, 71). Detailed studies on the temporal regulations of complex formation involving GAB, Crk, GC-GAP, CAS, and Nck will help us better understand the initial signaling events downstream of cell surface receptors.

While this study was in progress, two groups reported the cloning of the same gene using different approaches. The gene was named Grit and p200RhoGAP, respectively (48, 72). Nakamura et al. (72) reported that Grit interacted with TrkA receptor and co-localized with p130CAS. Okabe et al. (49) reported the identification of RICS while this manuscript was under revision. Together, these studies suggest that this GAP molecule highly expressed in the brain is involved in multiple activities of neuronal/glial cells.

Acknowledgments—We are grateful to Dr. Takahiro Nagase and colleagues at the Kazusa DNA Research Institute for the KIAA0712 cDNA clone. We thank Drs. Lawrence Quilliam, Gary Bokoch, Kristiina Vuori, and Maureen Harrington for kindly providing experimental reagents. We thank Dr. Qiufu Ma for help with in situ hybridization.

REFERENCES
1. Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) Nature 379, 560–564
2. Gu, H., Pratt, J. C., Burakoff, S. J., and Noell, B. G. (1998) Mol. Cell. Biol. 18, 729–740
3. Weidner, K. M., Di Cesare, S., Sacha, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996) Nature 384, 173–176
