

GGAPs, a New Family of Bifunctional GTP-Binding and GTPase-Activating Proteins

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G proteins are molecular switches that control a wide variety of physiological functions, including neurotransmission, transcriptional activation, cell migration, cell growth, and proliferation. The ability of GTPases to participate in signaling events is determined by the ratio of GTP-bound to GDP-bound forms in the cell. All known GTPases exist in an inactive (GDP-bound) and an active (GTP-bound) conformation, which are catalyzed by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs), respectively. In this study, we identified and characterized a new family of bifunctional GTP-binding and GTPase-activating proteins, named GGAP. GGAPs contain an N-terminal Ras homology domain, called the G domain, followed by a pleckstrin homology (PH) domain, a C-terminal GAP domain, and a tandem ankyrin (ANK) repeat domain. Expression analysis indicates that this new family of proteins has distinct cell localization, tissue distribution, and even message sizes. GTPase assays demonstrate that GGAPs have high GTPase activity through direct intramolecular interaction of the N-terminal G domain and the C-terminal GAP domain. In the absence of the GAP domain, the N-terminal G domain has very low activity, suggesting a new model of GGAP protein regulation via intramolecular interaction like the multidomain protein kinases. Overexpression of GGAPs leads to changes in cell morphology and activation of gene transcription.

A variety of external stimuli, including growth factors, neurotransmitters, hormones, phospholipids, photons, odorants, and taste ligands, can activate the GTP-binding proteins and their signaling pathways in the cell, leading to the regulation of a variety of cellular functions (3, 9, 17, 22, 28, 39). Therefore, the G-protein-coupled receptors and signal transduction pathways represent important specific targets for a variety of therapeutic approaches, ranging from the control of blood pressure, allergic response, kidney function, and hormonal disorders to neurological diseases and cancers (14).

There are two major families of GTPases; one is the Ras superfamily of small G proteins, such as Ras, Rho, and Arf, and the other is the family of heterotrimeric G proteins consisting of G α , G β , and G γ subunits (6, 19, 21, 42, 48). The ability of GTPases to participate in signaling events is determined by the ratio of GTP-bound to GDP-bound forms in the cell. All known GTPases exist in an inactive (GDP-bound) and an active (GTP-bound) conformation, which are catalyzed by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs), respectively (1, 4, 18, 27, 36, 44, 51). In the GDP-bound conformation, the G proteins are inactive. Activation occurs when GDP is released and GTP is bound. In the active GTP-bound conformation, GTPase interact with a variety of effector proteins to regulate their cellular effects or func-

tions. Their activity is time-limited by their intrinsic GTPase activity, which is stimulated by GTPase-activating proteins (GAPs).

The superfamily of small GTPases are monomeric guanine nucleotide-binding proteins with molecular masses of 20 to 25 kDa. They play major roles in the regulation of growth, morphogenesis, cell motility, axonal guidance, cytokinesis, and trafficking through the Golgi, nucleus, and endosomes (3, 16). The first small GTPase to be discovered was Ras, and there are now many members of the Ras superfamily of GTPases that are grouped into five subfamilies, Ras, Rho, ADP-ribosylation factors (ARFs), Rab, and Ran (16). The Ras subfamily is a key regulator of cell growth and proliferation. Ras is found in mutated oncogenic forms in a large number of human cancers. Activation of the Ras signaling pathways has been found in response to diverse extracellular stimuli, such as peptide growth factors, cytokines, and hormones, leading to the activation of the Raf/MEK/ERK cascade of protein kinases and the stimulation of a number of transcription factors involved in cell growth and proliferation. The second subfamily of the small GTPases is the Rho subfamily, which contains seven distinct proteins (Rho, Rac, Cdc42, RhoD, RhoG, RhoE, and TC10) (34). Activated Rho GTPases interact with cellular effector proteins to mediate a wide variety of cellular responses, including the reorganization of actin cytoskeleton, changes in gene transcription, cell cycle progression, and adhesion (3, 43, 47). The ARFs were initially identified and purified because of their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit (38). Many proteins have been

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GGAP1	MNYQ-----QQLA-----NSAAIRAEIQRFESVHPNIIYSIYELLERVEEP	40
GGAP2	MHAQ-----RQFVV---AAVRAEVRREHVAKQALNRLRKLAEERVDPP	39
MRIP-1	MNFQAGGGQSPQQQQLAGGPPQQFALSNSAAIRAEIQRFESVHPNIIYIYDLIERIEDL	60
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GGAP1	VLQNIQIREHVIAIEDAFVNSQEWLRSRVPPELKVIGVGNLSSGKSALVHRYLGTGYVQEE	100
GGAP2	ELQDSIQASLDSIREAVINSQEWLRSRIPPELRLGVLGDARSFKSLIHRFLTGSYQVLE	99
MRIP-1	ALQNIQIREHVISIEDSFVNSQEWLRSRVPPELKVIGVGNLSSGKSALVHRYLGTGYVQEE	120
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GGAP1	SPEGGRFKKEIVVDGQSYLLLRDEGGPPEAQFAMWVDVAVFVFSLEDEISFQTVYHYYS	160
GGAP2	KTESEQYKKEMLVDGQTHLVLIREEAGAPDAKFSGWADAVIFVFSLEDENSFQAVSRLHG	159
MRIP-1	SPEGGRFKKEIVVDGQSYLLLRDEGGPPELQFAAWVDVAVFVFSLEDEISFQTVYNYFL	180
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GGAP1	RMANYRNTSEIPLV--LVGTQDAISSANPRVIDDARARKLSNDLKRCTYYETCATYGLNV	218
GGAP2	QLSSLRGEGRGLALALVGTQDRISASSPRVVDARARALCADMKRCSYYETCATYGLNV	219
MRIP-1	RLCSFRNASEVMV--LVGTQDAISSANPRVIDDSRARKLSTDLKRCTYYETCATYGLNV	238
	* * * * *	
GGAP1	ERVFDVAQKIVATRKKQQLSIGPCKSLPNSPSHSSVCSAQVSAVHISQTSNGGGS-LSD	277
GGAP2	DRVFQEVAKVVTLRKQQQL-LACKSLPSSPSHS--AATP-VAG-QASNGGHT--SD	271
MRIP-1	ERVFDVAQKVVALRKKQQLAIGPCKSLPNSPSHSAVSAASIPAVHINQATNGGGSASFSD	298
	* * * * *	
GGAP1	YSSVSPSTPSISQKELRIDVPPTANTPTP--VRKQSKRRSNLFTSRKGSDDPKKKGLES	335
GGAP2	YSSSLPSSFNVGHRELRAEAAVAGLSTPGSLHRAAKRRTSLFANRRGSDS--EKRLSDS	329
MRIP-1	YSSVSPSTPSISQRELRIETIAASSTPTP--IRKQSKRRSNIFTSRKGADLDREKKAABC	356
	* * * * *	
GGAP1	RADSIGSGRAIPIKQGMILLKRSGLSNKEWKKKYVTLCDNGVLTYPHPSLHDYMQNVHGKE	395
GGAP2	RGETTGSGRAIPIKQSFLLKRSGLSNKEWKKKYVTLSSNGFLLYHPSINDYIHSHTGKE	389
MRIP-1	KVDSIGSGRAIPIKQGILLKRSGLSNKEWKKKYVTLCDNGLLTYHPSLHDYMQNIHGKE	416
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GGAP1	IDLLRRTTVKVPGRPPRATSACAPISSPKTNGLSKMS-----	433
GGAP2	MDLLRRTTVKVPGRPPRAISAFGP--SASINGLVKDMSTVQMGEGLEATTFMPSPSPSPS	447
MRIP-1	IDLLRRTTVKVPGRKRLPRATPATAPGTSPRANGLSVERSNTQLGGGTGAPHSASSASLHSE	476
	* * * * *	
GGAP1	-SLHISPNSDT--GLGDSVCS-SPSIS-S-----TT-----	462
GGAP2	SLQDMSTVQMG--GLEATTFMPSPSPSLQPPDQTSKHLKLPDRNLARALSTDCPT	505
MRIP-1	RPLSSSAWAGPRPEGLHQRSVSSADQWS----EATTS--LPPGMQHPASGPAEVL	528
	* * * * *	
GGAP1	---SPKLD-PPPSPHANRKHRRKSTSNFKADGLSGTAEQEENFEFIIVSLTGQTHW	517
GGAP2	SGDLSPLSREPPSPMV--KKQRKKTTPSKTEG-S-AGQAEENFEFLIVSSTGQTHW	561
MRIP-1	S---SPKLD-PPPSPHSNRKHRRKSTGTPRPDGPSSATEEAESFEFVVVSLTGQTHW	584
	* * * * *	
GGAP1	FEATYEERDANVQAIQSILASLQSCSSKNKSRLLTSQSEAMALQSIRNMRGNSHCVDC	577
GGAP2	FEAASFEERDANVQAIQSILASLQCCSSKVKLRDTSQSEAVAIQAIRNAKGNISFCVDC	621
MRIP-1	FEASTAEERELVWQSVQAQILASLQGCERSAKDKTRLGNQNAALAVQAVRTRVGRSICD	644
	* * * * *	
GGAP1	ETQNFNWSLNLGALMCIECSGIHRNLGTHLSRVRSLLDDWPPELKVMSIENELANS	637
GGAP2	GAPNPTWASLNLGALMCIECSGIHRNLGTHLSRVRSLLDDWPPELTLVLTFAIGNDTANR	681
MRIP-1	DAFNPDWASLNLGALMCIECSGIHRNLGAHLRVRSLLDDWPPELLAVMTAMGNALANS	704
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GGAP1	VWEESSQGRTPKPSVDSTREEKERWIRAKYEQKLFAPLPCTELSLGQHLLRATADEDLRT	697
GGAP2	VWESDTRGAKPSRDSREERESWIRAKYEQLLFLAPLSTSEELPGRQLWAAVQAQDVAT	741
MRIP-1	VWEGALGGYSKPGPDACREKERWIRAKYEQKLFAPLPSDVPFLGQQLLRAVVEDDLRL	764
	* * * * *	
GGAP1	AILLAHGSRDEVNETCGEGDGRTHLACRKGNVVLAQLLIWYGVDVTARDAHNTALA	757
GGAP2	VLLLAHARHGPLDTSVEDPQLRSPHLAAELAHVVITQLLLWYGADVAARDAQVRTALF	801
MRIP-1	LVMLLAHGSKEVNETYGDGGRTHLSSAMANVFTQLLIWYGVDVRSRDARGLTPIA	824
	* * * * *	
GGAP1	YARQASSQECIDVLLQYGCPPERFVIMATPNLSRRN-NNRNNSSGRVPTII	807
GGAP2	YARQAGSHLCADILLQHACPGEGGSAATTPSAATTPSITATPSPRRSSAASVGRADAPVALV	864
MRIP-1	YARRAGSQECADILIQHGCPEGGGLAPTNPREPANGTNPSEALHRSFSL	875

FIG. 1. Sequence alignment of GGAP1, GGAP2, and MRIP1 (GenBank accession numbers: AY033765 for GGAP1, AF384128 for GGAP2, and AF359283 for MRIP1). Identical amino acids are indicated by an asterisk. GGAP1 shares approximately 50 and 70% sequence homology with GGAP2 and MRIP1 at the amino acid level, respectively. A database search identified two cDNAs, KIAA1099 and KIAA0167, that are the same as GGAP1 and GGAP2 (25).

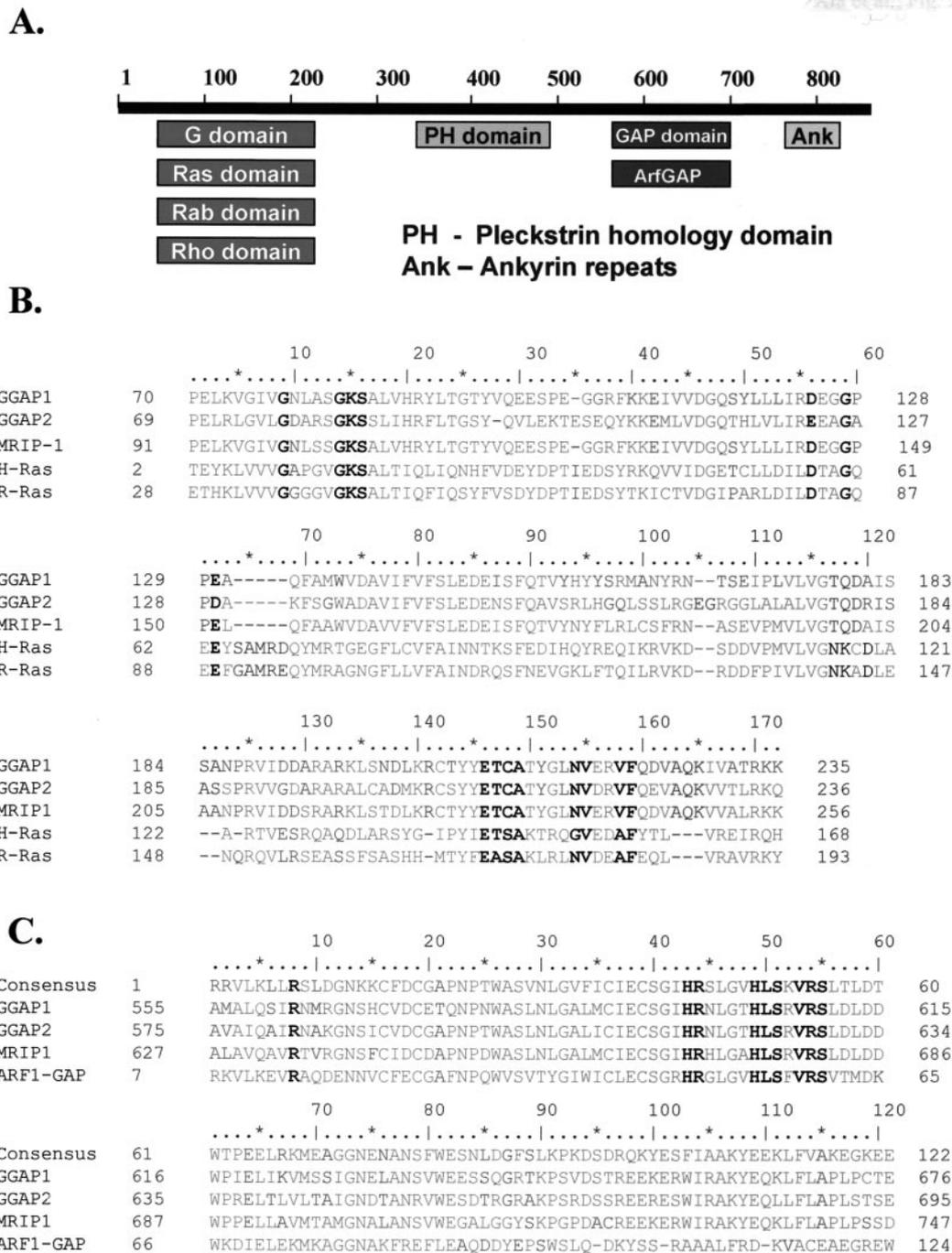


FIG. 2. Domain structure and sequence comparison of GGAP proteins. (A) The three GGAP proteins share the same domain structure with an N-terminal GTPase domain, a PH domain, followed by a C-terminal GAP domain, and an ANK repeat domain. (B) The N-termini of the GGAPs share sequence and motif homology with Ras family of G proteins (12, 32). Residues in boldface type indicate conserved consensus motifs in the proteins. Dark residues are unique insertions in the sequences. (C) Sequence comparison of the C-terminal GAP domain with Arf GAP (20, 36). (D) Sequence homology of ANK domains between GGAP family of proteins and other ANK repeat domain proteins. Residues represent consensus conserved ANK repeat amino acids and nonconserved amino acids. (E) Rooted phylogenetic tree of GGAP family proteins and Ras family of proteins. Nucleotide sequences of the above proteins were obtained from the GenBank. After being multiply aligned using the ClustalW program available at Biology workbench, the obtained multiple alignments were then used to construct rooted phylogenetic tree using ClustalW program and then viewing with DRAWGRAM program (<http://workbench.sdsc.edu>) as described by Li and Gouy (30).

D.

		10	20	30	
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consensus	1	DGNTPLHLAARNGHLEVVKLLLE	----	AGADVNRADK	33
GGAP1	715	DGRTALHLACRKGNVLAQLLIW	----	YGVDTVARD	747
GGAP2	734	QLRSPLHLAAELAHVVITQLLIW	----	YGADVAARDA	766
MRIP1	785	DGRTALHLSSAMANVVFTQLLIW	----	YGVDVRSRDA	818
Bcl-3 ChainA	8	DGDTPLHIAVVQGNLPAVHRLVNlfqq	GGRELDIYNN		44
I-Kappa-B	147	NGRTALHLAVDLQNPDLVSLLLK	----	CGADVNRVTY	179
gi 4335700	712	TGTPALHLATAAGNHRTAMLLLD	----	KGAPATQRDA	744
NF-kB-p105	682	SGRTALHLAVEQENVPLAGCLLLE	----	GDADVSTTY	715
α -latrotoxin	1137	DKDTALHLAVYYKNLQMIKLLIK	----	YGIDVTIRNA	1169
gi 728859	163	EGRTALMQAASKGETGVLPLLE	----	GGADLEARDK	195
gi 3877162	63	DGATPLHYACTHDNVAMAQLLIT	----	FGADPMSADK	95
gi 6176569	734	QLRSPLHLAAELAHVVITQLLIW	----	YGADVAARDA	766

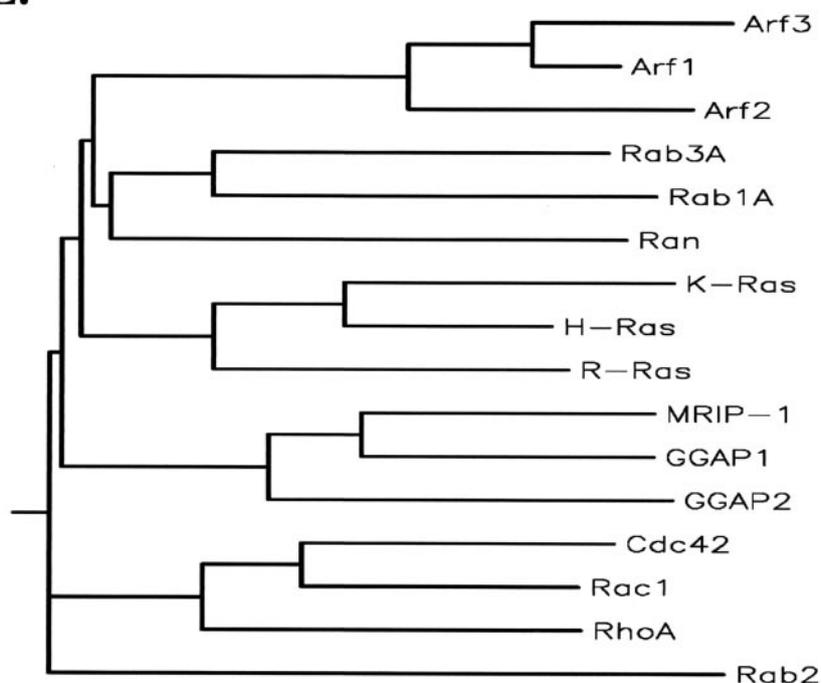
E.

FIG. 2—Continued.

shown to interact with ARF to regulate its state of activation or are involved in its intracellular function, including different vesicular trafficking pathways in all eukaryotic cells, and as activators of specific phospholipase Ds (8, 13, 15, 23, 24, 36, 38, 41, 45).

In this study, we have identified and characterized a new family of bifunctional multidomain proteins that contain an N-terminal Ras homology domain, called G domain, followed by the PH domain, the C-terminal GAP domain and the ankyrin (ANK) repeat domain. These proteins can bind to GTP and exhibit GTPase activity in the native form. GTPase assays demonstrate that the C-terminal GAP domain can stimulate the N-terminal GTPase activity by direct intramolecular interaction between these two domains, suggesting a new mode of activation for this family of proteins. Overexpression of

GGAPs results in changes in cell morphology and activation of gene transcription.

MATERIALS AND METHODS

Cloning of GGAPs. In our effort to clone novel genes involved in cardiovascular development and functions, we identified a fragment of MRIP1 by *Saccharomyces cerevisiae* two-hybrid screening using a human heart cDNA library (Clontech Laboratories). We further identified two new expression tags that belong to the same gene family using database search and analysis. To obtain the full-length cDNAs encoding GGAP1, GGAP2, and MRIP, we screened a cDNA library to obtain clones encoding different regions of the genes, and then we used 5' rapid amplification cDNA ends and reverse transcription-PCR to get other regions of the gene. We constructed the full length of the genes by restriction digestion and ligation. We confirmed the final sequences of the three genes by sequencing.

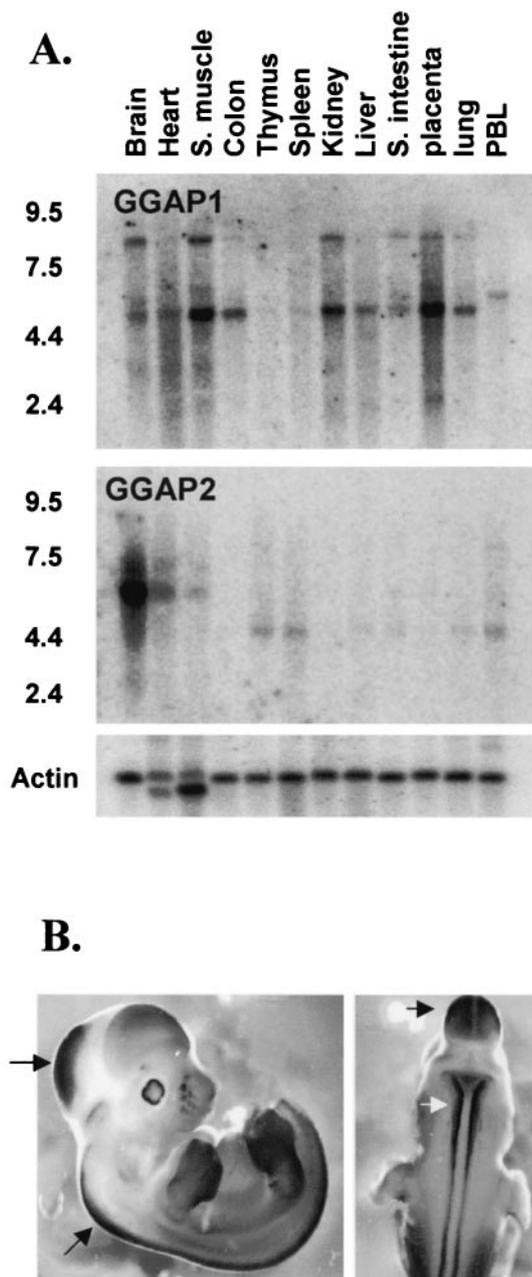


FIG. 3. Expression of GGAP1 and GGAP2 in human tissues and mouse embryo. (A) Human multitissue Northern blot hybridized with a probe derived from N-terminal domains of GGAP1 and GGAP2, respectively. For GGAP1, two message RNAs (~ 5 and 8 kb) were detected in most of the human tissues for GGAP1 while a different splicing form was detected in periphery blood leukocytes (PBL). GGAP2 is highly expressed in brain. Different sizes of transcripts were detected in excitable tissues (brain, heart, and smooth muscle [S. muscle]) compared to immune tissues (thymus, spleen, and PBL). S. intestine, small intestine. (B) Expression of GGAP1 in 12.5-day mouse embryo. Whole-mount in situ hybridization shows GGAP1 is highly expressed in forebrain, middle brain, and neural tubes during embryo development (arrows). Whole-mount in situ hybridization, sectioning and staining of tissue sections were performed as described elsewhere (33).

Cell culture, transfection, and reporter assays. COS-7, HeLa cells or NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum 24 h before transfection. Cells were transfected with Lipofectamine in serum-free Opti-MEM (GIBCO-BRL) as previously described (31, 52). A cytomegalovirus vector pCIS encoding β -galactosidase was used to maintain a constant amount of cDNA and equalize the amount of a particular cDNA in each set of experiments. For transcriptional reporter assays, the PathDetect AP-1 cis reporting system (pAP1-Luc, 7x AP-1 enhancer elements), the PathDetect NF- κ B cis reporting system (pNF- κ B-Luc, 5x NF- κ B enhancer elements), and the pSER-Luc from Stratagene were used in the assays. The pSAP1-Luc and pElk1-Luc were kindly provided by Kuanliang Guan at the University of Michigan Medical School. Luciferase assays were performed as recommended by the manufacturer's instructions (Promega). The data presented are the mean of three individually transfected wells and the experiments are performed at least three times.

Immunoprecipitation, immunoblotting, immunocytochemistry, and fluorescence imaging. Immunoprecipitation of individual proteins was carried out as previously described (53). In brief, cell lysates (1 mg of protein) were incubated with antibodies (1 to 10 μ g) at 4°C for 2 h in a final volume of 1 ml modified RIPA buffer (10 mM sodium phosphate [pH 7], 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, leupeptin [4 μ g/ml], 1 mM phenylmethylsulfonyl fluoride) with constant rocking. After the addition of protein A-agarose beads, reactions were incubated at 4°C overnight. Immune complexes were resolved by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting for interacting proteins.

For fluorescence labeling of the cellular components, cells transfected with GGAPs and control vector (pCMV-Tag2B) were fixed with 4% paraformaldehyde for 20 min, blocked with 10% bovine serum albumin (BSA), and incubated with monoclonal antibody against Flag (M2 monoclonal; Sigma). Actin filaments were labeled by rhodamine-conjugated phalloidin (Molecular Probe). Double-label immunostaining was done with appropriate fluorochrome-conjugated secondary antibodies. Fluorescent images of cells were captured on a charge-coupled device camera mounted on an Olympus inverted research microscope using Ultraview imaging software (Olympus).

Northern blotting and whole-mount in situ hybridization. To study the expression patterns of GGAPs in different human tissues, an RNA filter comprising poly(A)-selected RNAs of multiple human tissues (Clontech, Inc.) was hybridized with specific 32 P-labeled cDNAs as described previously (53). In brief, human GGAP1 and GGAP2 probes (1 to 900) were radiolabeled with [α - 32 P] CTP by nick translation using random primers. Probes ($\sim 4 \times 10^7$ cpm/ μ g) were hybridized with the RNA filter and analyzed according to manufacturer's protocol.

Whole-mount in situ hybridization, sectioning, and staining of tissue sections were performed as described elsewhere (33).

Guanine nucleotide binding and dissociation assays. Assays of guanine nucleotide binding to GGAPs were performed as described previously (55). Briefly, an equal amount (1 μ g protein) of GST beads and GST-GGAP1NT, GST-GGAP1CT, GST-GGAP2NT and GST-GGAP2CT were incubated with [α - 32 P] GTP (1 μ M), respectively, in the absence or presence of excess unlabeled GTP, after extensive washing, bound radioactivity was counted in a scintillation counter.

The dissociation rates of guanine nucleotide from GGAPs were measured as described previously (56). A 2 μ M concentration of [3 H]GDP or [35 S]GTP γ S (6,000 cpm/ μ mol; Perkin-Elmer) was incubated with the purified GST-G domains (2 μ g) of GGAP1 and GGAP2 at 25°C for 60 min in 160 μ l of buffer containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 10 mM MgCl₂. The dissociation reaction were initiated by adding 2 mM unlabeled GDP or GTP γ S to the incubation mixtures; at the indicated time intervals, aliquots of 20 μ l were withdrawn from the reaction mixture, and the remaining G-protein-bound radionucleotides were quantitated by scintillation counting.

Assay of GTPase activity. In vitro GTPase assays were performed according to Vitale with modification (49). Briefly, purified His-GTPase of GGAP1 and GGAP2 (0.5 μ g each) were washed thoroughly with loading buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 10 mM DTT) and were incubated with [α - 32 P] GTP (0.1 μ M) for 30 min at 25°C in 50 μ l of the loading buffer. The resin was rinsed twice with ice-cold loading buffer followed by resuspension in the reaction buffer (20 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 10 mM DTT) with or without the C-terminal GAP domain (1 μ g each) of GGAP1 or GGAP2. The GTP hydrolysis was conducted at room temperature. Samples were taken at the indicated times and immediately solubilized in the elution buffer (0.2% SDS, 5 mM EDTA, 5 mM GTP, 5 mM GDP) by heating at 65°C for 2 min. The eluted

GTP and GDP were separated by thin layer chromatography on polyethylenimine-cellulose plates as described previously (5).

To further investigate the GAP-stimulated GTPase activities, C-terminal GAP stimulated GTPase activities were measured as described previously by nitrocellulose filter-binding method (57). Briefly, 1.2 μ g of purified N-terminal G domains of GGAP1 and GGAP2 were preloaded, respectively, with [γ - 32 P]GTP (10 μ Ci; 6,000 Ci/mmol) in 40 μ l of buffer containing 50 mM HEPES (pH 7.6), 100 mM NaCl, 0.5 mM EDTA, and BSA (0.2 mg/ml) for 10 min at room temperature before adding MgCl₂ to a final concentration of 5 mM. The [γ - 32 P]GTP-loaded G domains were mixed with 200 μ l of reaction assay buffer containing 50 mM HEPES (pH 7.6), 100 mM NaCl, 5 mM MgCl₂, and BSA (0.2 mg/ml) in the presence or absence of 1.2 μ g of GAP domain. At different time points, the reactions were terminated by filtering aliquots (25 μ l) of the reaction mixture through nitrocellulose filters. After washing with ice-cold buffer containing 50 mM HEPES (pH 7.6), 100 mM NaCl, and 10 mM MgCl₂, the radioactivity retained on the filters was then subjected to quantitation by scintillation counting.

RESULTS

Identification and structural domains of the GAP-containing GTPases. In identification of signaling proteins involved in cardiovascular diseases and tumorigenesis, we cloned and identified two new GTP-binding and GTPase activating proteins, named GGAP1 and GGAP2. Together with the MRIP1 identified in yeast two-hybrid screening in muscle cells, these three proteins consist of a new family of bifunctional GTP-binding and GTPase-activating proteins with multidomain structures. cDNA library screening and 5' rapid amplification cDNA ends were used to clone the full-length cDNAs of the three family members. As shown in Fig. 1, GGAP1 and GGAP2 share approximately 50% sequence homology while GGAP1 and MRIP1 share 70% sequence homology at the amino acid level. The complete protein sequences of this family encode an N-terminal Ras-related G domain, a PH domain, followed by a C-terminal GAP domain and an ANK repeat domain (Fig. 2A). In the N-terminal region of the GGAP proteins, we observe 60% homology to the Ras family of GTPases (Fig. 2B) (7, 12, 32, 42). To further understand the similarity between the Ras superfamily of proteins and the GGAP proteins, we performed a rooted phylogenetic tree analysis using ClustalW program and DRAWGRAM program (30). As shown in Fig. 2E, GGAP family of proteins (GGAP1, GGAP2, and MRIP1) share more sequence homology with the Ras and Rho subfamily of proteins compared to other subfamily of proteins (Fig. 2E). The N-terminal G domain of GGAPs also shares sequence homology with the C-terminal GTPase domain of the newly identified nuclear GTPase, Pike (55). In the C-terminal portion of GGAP proteins, there is GAP domain with sequence homology to ARF GAP protein where the conserved arginine residues are found in the sequences (Fig. 2C) (20, 36). These conserved arginine residues have a structural role and do not point to the active site of ARF while one subunit of the coatamer complex is likely to provide the catalytic arginine (20). Following the C terminus of the GAP domain, tandem ANK repeats are found in this family of proteins (Fig. 2D), suggesting potential protein-protein interactions are involved in this region.

Tissue-specific expression of human GGAP1 and GGAP2.

To examine the tissue distribution of the GGAP proteins, we performed both Northern blot analysis using various human tissues and whole-mount *in situ* hybridization in mouse embryos. As shown in Fig. 3A, GGAP1 mRNA was found in most

of the tissues although the level of expression is varied, with enriched expression in skeletal muscle, brain, placenta, and kidney. Most tissues contain two messages at 5 kb and 8.5 kb, while the peripheral blood leukocytes (PBL) have only one message with the size of 5.5 kb (Fig. 3A, top panel). The expression of GGAP2 is highly enriched in the brain (Fig. 3A, lower panel) with approximately 20-fold higher message level compared to the heart, skeletal muscle, and immune tissues. In addition, the size of the mRNA for GGAP2 is different between excitable tissues (brain, heart, and skeletal muscle) and nonexcitable immune tissues (thymus, spleen, small intestine, and leukocytes) (Fig. 3A, lower panel). Northern blot analysis of human brain regions reveals that a prominent band was found in cerebellum, cerebral cortex, occipital pole, frontal lobe, temporal lobe, and putamen for GGAP1 and GGAP2, while low level of expression was detected in medulla and spinal cord for GGAP2 (data not shown).

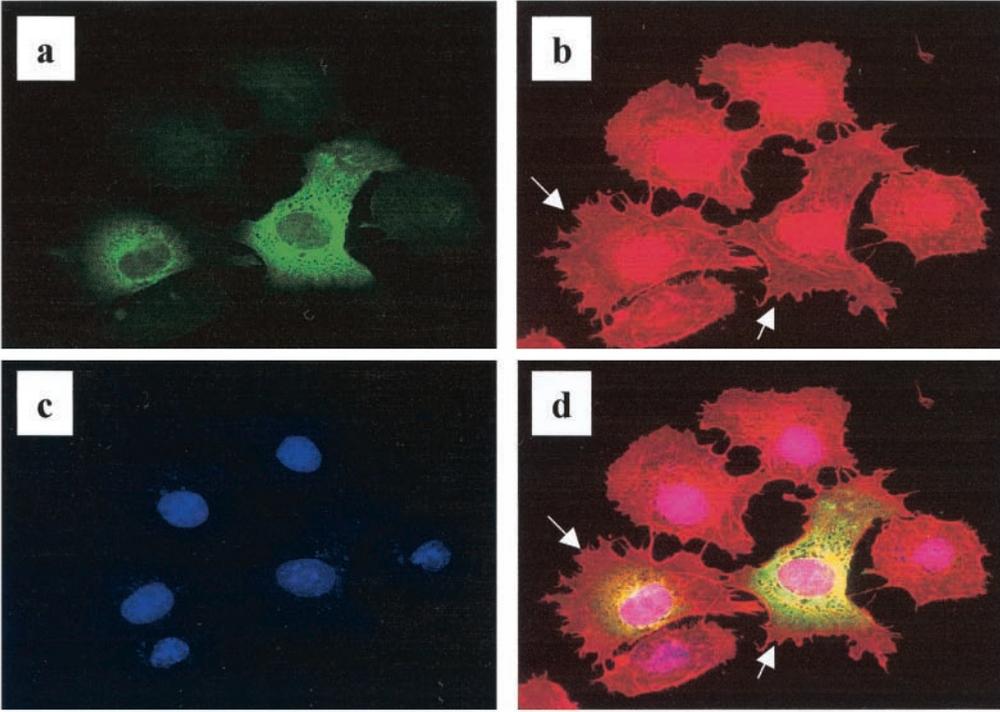
The expression of GGAP1 during mouse embryogenesis was analyzed using whole-mount *in situ* hybridization and specific GGAP1 probe. At 12.5 days postcoitum, GGAP1 expression was restricted to the neural tube, forebrain and midbrain of mouse (Fig. 3B). GGAP1 expression was also detected in the whiskers that contain sensory neurons (Fig. 3B).

Subcellular localization of GGAP1 and GGAP2. Subcellular localization reveals GGAPs are expressed in different cellular compartments (Fig. 4). In transfected COS-7 cells, Flag-tagged GGAP1 staining occurs in the cytosol, possibly in the internal membrane systems, such as the endoplasmic reticulum (ER) and the Golgi apparatus (Fig. 4A, panels a and d), suggesting a potential role of GGAP1 in protein biosynthesis and modification. On the other hand, the Flag-tagged GGAP2 was stained in both cytosol and nucleus (Fig. 4B, arrows), indicating possible function of the GGAP2 protein in both cytosol and nucleus in the cell. Furthermore, cells overexpressing GGAP1 are more flat and have much more lamellipodia than control cells (Fig. 4A, arrows in panels b and d). In contrast, cell overexpressing GGAP2 are smaller and more rounded than control cells (Fig. 4B, panels b and d). Actin organization in GGAP2-expressing cells is also different from control cells (Fig. 4B, arrows in panels b and d). Together, these data suggest that GGAP1 and GGAP2 may be involved in cell morphology change and intracellular actin cytoskeleton reorganization.

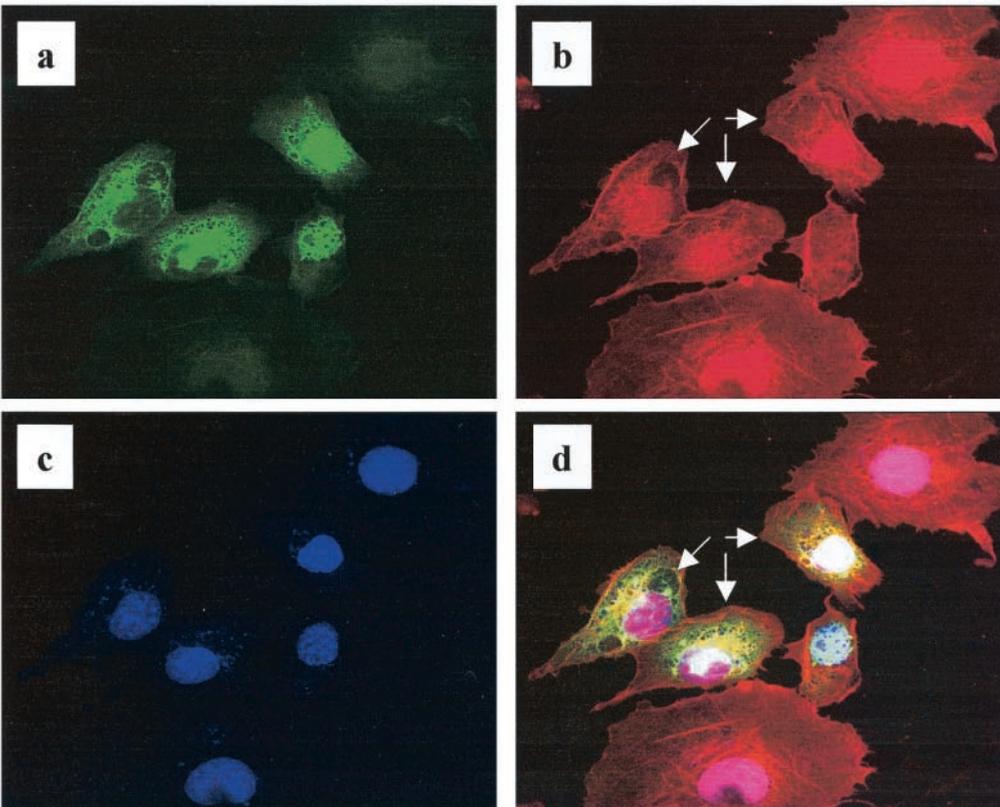
GGAP1 and GGAP2 are a new family of GTP-binding proteins. GGAP proteins contain motifs that have been found to be important for guanine nucleotide-binding and GTPase activity in a variety of cellular proteins (7). Besides the phosphate-binding motif (GXXXXGKS) and the Mg²⁺-binding motif (DXXG), GGAP proteins contains two additional motifs, ETCA and NVXXVF, similar to the ETSA and NVXXAF motifs found only in the Ras-like GTPases (Fig. 2B). The presence of these motifs suggest that the GGAP protein family is likely to exhibit guanine nucleotide-binding and GTP-hydrolyzing activity. To demonstrate the binding of GTP to the GGAPs, we have directly examined the binding of [32 P]GTP to the N-terminal regions of GGAP1 and GGAP2, respectively. As shown in Fig. 5A, the N-terminal G domain of the GGAP proteins binds to [32 P]GTP, while the C-terminal segment of the proteins fail to display GTP-binding.

To further determine the GTPase activity of GGAP pro-

A.



B.



teins, we transiently transfected the cDNAs encoding the Flag-tagged proteins into COS-7 cells. Flag-tagged GGAPs were purified by immunoprecipitation using monoclonal anti-Flag M2 antibody and were assayed for GTPase activity. As shown in Fig. 5B, full-length GGAP1 and GGAP2 immunopurified from the cells possess GTPase activity, hydrolyzing bound GTP into GDP (Fig. 5B), suggesting the GGAP proteins are a new family of proteins that can hydrolyze GTP. Estimation of the release of $^{32}\text{P}_i$ from $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ by the GGAP proteins, the turnover number of GGAPs for GTPase activity is similar to GTP-Ras protein (0.01 min^{-1}).

We further examined the dissociation of $[\text{H}^3]\text{GDP}$ from GGAP proteins. GST-G domain fusion proteins encoding the G domains of GGAP1 and GGAP2 were first loaded with $2 \mu\text{M}$ $[\text{H}^3]\text{GDP}$, and the dissociation of the bound nucleotide was measured in the presence of 2 mM of nonlabeled GDP. The radiolabeled $[\text{H}^3]\text{GDP}$ was rapidly released from GST-G domain fusion proteins in high concentration of unlabeled GDP, indicating that the G domains of GGAPs can quickly exchange GDP (Fig. 5C).

Activation of the N-terminal G domain by the C-terminal GAP domain of GGAPs. GTP-binding proteins cycle between inactive GDP-bound and active GTP-bound states, and therefore, the rate of GTP hydrolysis is regulated in part by the balance of the GTP- and GDP-bound states of the G proteins. Since GGAP proteins contain both the GTPase domain and the GAP domain in one molecule, we examined whether the C-terminal GAP domain could regulate the activity of the N-terminal G domain via direct intramolecular interaction. His-tagged fusion proteins corresponding to the N-terminal G domain and the C-terminal GAP domain were expressed and purified from bacteria. The purified N-terminal G domains of GGAP1 and GGAP2 proteins were assayed for GTPase activity. As shown in Fig. 6A, the N-terminal G domains from GGAP1 and GGAP2 have very low intrinsic GTPase activity in the absence of the C-terminal GAP domains, respectively. Addition of the GAP domain from the same protein (GGAP1 or GGAP2) significantly increased the GTPase activity of the G domains of the GGAP proteins (Fig. 6A). To obtain a quantitative increase of GTPase activity in the presence of GAP domain, we measured the radioactivity of GTP and GDP after thin layer chromatography, and the percentages of GTP/[GTP+GDP] are shown in the bottom of Fig. 6A. Therefore, addition of the GAP domain increased the enzymatic activity of the G domain 7- to 10-fold compared to the control assays.

The stimulation of GTPase activity by the GAP domain is time dependent in both GGAP1 (Fig. 6B) and GGAP2 (Fig. 6C). Like RasGAP and ArfGAP proteins (20, 46), the GAP domain of GGAPs contains conserved arginine residues that may be involved in the stability of the protein structure and the

interaction with the active sites of GTPase domains. The exact mechanism of GTPase activation by the GAP domain remains to be determined.

Direct interaction of the N-terminal G domain with the C-terminal GAP domain. Having demonstrated the activation of the N-terminal G domain by the C-terminal GAP domain, we further examined the molecular basis for the activation. We demonstrated that the C-terminal GAP domain could directly interact with the N-terminal G domain in the cell and in vitro. When coexpressed in COS-7 cells, the G domain coimmunoprecipitated with the C-terminal GAP domain, indicating that two domains can directly interact with each other (Fig. 7A), forming an intramolecular complex and modulating the GTPase activity of the proteins. To confirm the coimmunoprecipitation of the two domains, we incubated immobilized His-GAP domains of GGAP1 or GGAP2 with in vitro-translated ^{35}S -labeled N-terminal G domains. Proteins bound to the C-terminal GAP domain were resolved by SDS-polyacrylamide gel electrophoresis, and the ^{35}S -labeled G domain (NT) was found to associate with the GAP domain of the same protein (Fig. 7B). These results suggested that the C-terminal GAP domain could directly interact with the N-terminal G domain and regulate its enzymatic activity. A model of intramolecular activation for the GGAP family of proteins is proposed in Fig. 7C. In this model, intramolecular interaction between the N-terminal G domain and the C-terminal GAP domain activates the GTPase activity of the protein, resulting in an inactive (GDP-binding) conformation of the protein in the cell (Fig. 7C, left panel). Interaction of the C-terminal domains (GAP and ANK repeat domains) with other proteins will change the conformation of the GGAP proteins and disrupt the intramolecular activation of the GTPase by the GAP domain, and therefore, the GGAP proteins exist in the GTP-bound active state (Fig. 7C, right panel). This model of interaction and activation is novel in all known G proteins, but similar to that found in multidomain protein kinases, such as p21-activated protein kinases (PAK) and the guanine nucleotide exchange factor Sos (2, 11, 29).

Regulation of cell signaling pathways by GGAP proteins. Given that GGAP proteins contain an N-terminal Ras-homology G domain, a C-terminal GAP domain, and other protein domains, we further examined the effects of this family of proteins in different cell signaling pathways at the transcriptional level. Cos-7 cells were transfected with pCMV-Tag2B expression plasmids for GGAP1 and GGAP2, along with reporter plasmids in which luciferase expression are driven by promoters for SRE, Elk-1, SAP-1, NF- κB , cJun, and ATF2, respectively. Full length GGAP1 and GGAP2 moderately activate the SRE-luc, Elk-1-Luc, and SAP-1-Luc two to fourfold over vector-only control (Fig. 8). However, when cotransfected

FIG. 4. Intracellular expression and localization of GGAP1 and GGAP2. (A) GGAP1 is expressed in the cytoplasm, possibly in ER and Golgi apparatus in COS-7 cells. Flag-tagged GGAP1 was stained with a specific anti-Flag M2 monoclonal antibody. (a) Expression of GGAP1 in the cytosol, possibly with intracellular membrane structures, such as ER and Golgi apparatus. (b) Actin staining with Texas red-labeled phalloidin. (c) Nuclear staining of COS-7 cells with DAPI (4',6'-diamidino-2-phenylindole). (d) The merger picture of panels a, b, and c, showing expression of GGAP1, actin, and nucleus in the cells. (B) Expression and localization of GGAP2 in cytosol and nucleus in COS-7 cells. (a) GGAP2 expression in both cytosol and nucleus. (b and c) actin and nuclear staining, respectively. (d) Merger picture showing GGAP2 expressed in both cytoplasm and nucleus. Fluorescent images of cells were captured on a charge-coupled device camera mounted on Olympus inverted research microscope using Ultraview imaging software.

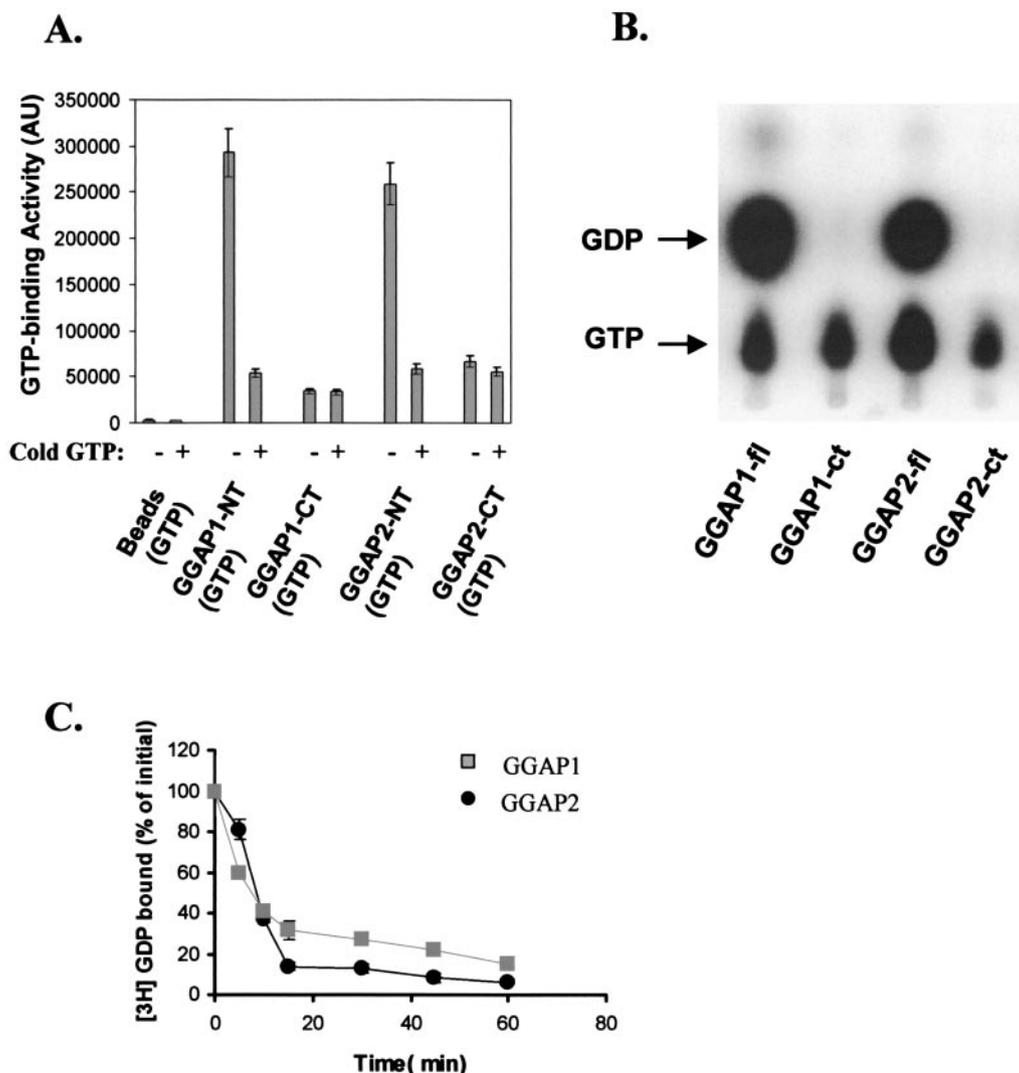


FIG. 5. GTP-binding and GTPase activities of GGAP1 and GGAP2. (A) GGAP1 and GGAP2 bind to $[\alpha\text{-}^{32}\text{P}]\text{GTP}$. Purified His-GGAP1-NT (1 to 292), His-GGAP1-CT (467 to 804), His-GGAP2-NT (1 to 294), His-GGAP2-CT (390 to 826), and Ni^{2+} beads (control) were incubated with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in the absence or presence of excess unlabeled GTP (10 mM). After extensive washing, bound radioactivity was counted in a scintillation counter. Error bars, standard deviations. (B) Enzymatic activity of GGAP1 and GGAP2. Immunopurified Flag-tagged GGAP1 and GGAP2 (0.5 μg) was incubated with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ for 1 h at 30°C. The extent of GTP hydrolysis was assessed by thin layer chromatography. Flag-tagged C-terminal domains of GGAP1 and GGAP2 (0.5 μg) were used as negative control. The eluted GTP and GDP were separated by thin layer chromatography on polyethyleneimine-cellulose plates (J. T. Baker). (C) Dissociation of GDP from GGAPs. Squares present data for GGAP1 and circles represent data for GGAP2. A 2 μM concentration of $[\text{H}^3]\text{GDP}$ was incubated with the purified GST-G domains (2 μg) of GGAP1 and GGAP2 at 25°C for 60 min. The dissociation reaction were initiated by adding 2 mM unlabeled GDP to the incubation mixtures (160 μl), at the indicated time intervals, aliquots of 20 μl were withdrawn from the reaction mixture, the remaining G-protein-bound radionucleotides were quantitated by scintillation counting.

with the Ras protein, the N-terminal G domain of GGAP1 has synergistic activation on the three transcription factors that coupled to ERK signaling pathway (Fig. 8), while GGAP2 has little effect on the transcriptional activation. Together, these data suggest that the N-terminal G domain of GGAP1 has much stronger effects on the activation of signaling pathways in the absence of the GAP domain, consistent with our above observation. Little or no activation was observed with NF- κB , c-Jun, and ATF2 transcriptional reporter genes (data not shown). Thus, the activation of the GGAP1 protein may synergistically act at the serum response element with signals that

activate TCF (ternary complex factors, such as SAP1 and Elk1) transcription factors.

DISCUSSION

G-protein-coupled signaling pathways mediate a wide array of cellular functions. GTP-binding proteins are regulatory switches whose activity is controlled by cycling between active GTP bound and inactive GDP bound states. However, the GTPase reaction for most G proteins is slow and would not be suitable for most biological signal transduction processes that

require complete inactivation within minutes after GTP loading. Thus, GAPs have been discovered for Ras superfamily of proteins and for heterotrimeric G proteins (called regulators of G protein signaling) (44). In the present study, we have identified and characterized two new GTP-binding and GTPase-activating proteins, GGAP1 and GGAP2. The two new proteins contain an N-terminal G domain, followed by the PH domain, the GAP domain, and the ANK repeat domain in the C-termini (Fig. 2). Based on the structural features, we propose that GGAP1, GGAP2, together with the newly identified MRIP1, constitute a new family of large proteins that contain G domain, GAP domain, and other protein domains. The finding that GGAP family of large proteins contain both the G domain and the GAP domain in one single molecule provides insight into potential new mechanisms for the activation and regulation of this new family of proteins in the cell.

The fact that the new family of GGAP proteins share sequence homology with the Ras family of proteins and synergistic activation of transcription by GGAP1 raise question of whether GGAP1 is important in other Ras signaling pathways and cellular function. Since the multidomain structures of this new family of proteins, it will be interesting to examine whether and how these proteins interact with other key proteins in the Ras signaling pathways and the potential physiological functions of these proteins in cell growth and transformation in our future studies.

The activation of the N-terminal G domain by the C-terminal GAP domain of the same protein via direct protein interaction is an interesting new mechanism for GTP-binding proteins. The use of intramolecular interactions as a mechanism for modulating the activities of proteins has been demonstrated in a number of biological systems, including the Src tyrosine kinases, the PAK, the guanine nucleotide exchange factor Sos, and the ARF-domain protein 1 (ARD1) (2, 11, 29, 37, 49, 50, 53, 54). Among the best examples for this type of intramolecular interaction are the regulation of the enzymatic activities of protein kinases, such as Src and PAK (2, 29, 35, 37, 54). Regulation of Src kinases is involved in the interactions of SH2 and SH3 domains while the regulation of the PAK kinase activity is involved in direct interaction of the N-terminal autoinhibitory domain and C-terminal kinase domain (29, 40). The finding that the C-terminal GAP domain directly interacts with the N-terminal G domain indicates possible intramolecular regulatory mechanisms in this new family of proteins similar to the one reported in protein kinases and in ARD1, a 64-kDa protein with an carboxy-terminal ARF domain and an amino-terminal GAP domain (49, 50). However, the multiple domain structures of this new family of proteins suggest that these proteins are regulated differently compared to the ARD1, possibly by protein-protein interactions via the C-terminal ANK repeat domain and by membrane association via the PH domain in the proteins.

Based on our findings, we propose a simple model for the activation mechanism of this new GTP-binding and GTPase activating proteins as shown in Fig. 7C. In this model, binding of the C-terminal GAP domain with the N-terminal G domain activate the protein's enzymatic activity, and therefore, the GGAP proteins exist in the inactive GDP-bound conforma-

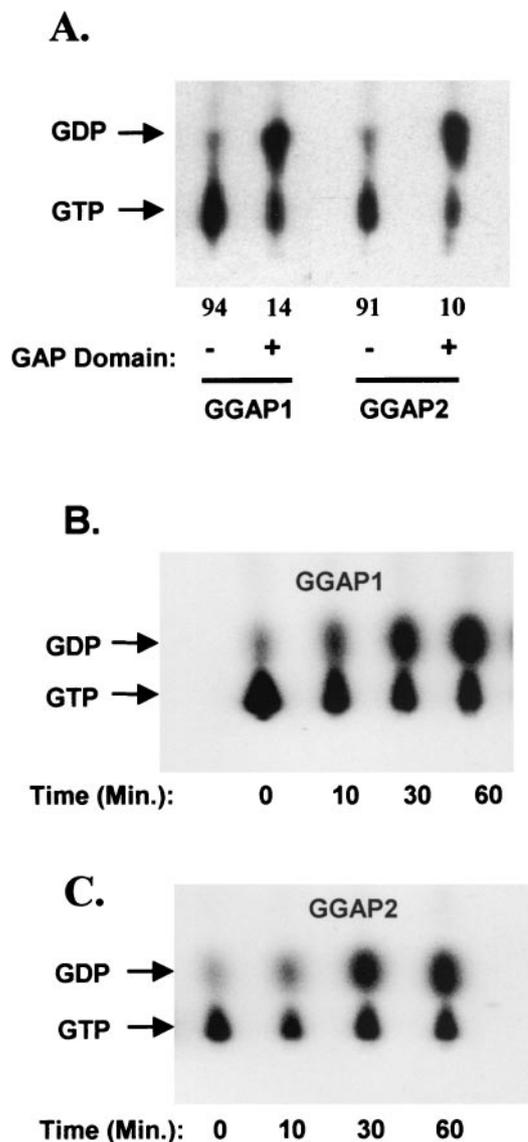


FIG. 6. Activation of G domains of GGAP1 and GGAP2 by the C-terminal GAP domains. (A) GTPase assays of the N-terminal G domain in the absence or presence of the C-terminal GAP domain of GGAP1 and GGAP2, respectively. Purified His-tagged proteins (GGAP1 and GGAP2, 0.5 μ g) were rinsed twice with ice-cold loading buffer followed by resuspension in a 50- μ l reaction buffer (20 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 10 mM DTT). The proteins were incubated with [α -³²P]GTP (0.1 μ M) for 30 min at 30°C with or without the addition of the C-terminal GAP domains (1 μ g each) of GGAP1 and GGAP2, respectively. The extent of GTP hydrolysis was assessed by thin layer chromatography. The radioactivity of GTP and GDP was quantitated, and the percentages of GTP/[GTP+GDP] are shown in the bottom. (B and C) Time-dependent activation of G domains by the GAP domains of GGAP1 and GGAP2, respectively. Purified His-tagged G domains (1 μ g) were assayed for their activities in the presence of the purified GAP domain (1.5 μ g) at indicated time (0, 10, 30, and 60 min). Guanine nucleotides hydrolyzed by the G domains in the presence of GAP domains were separated by thin layer chromatography and detected by autoradiography.

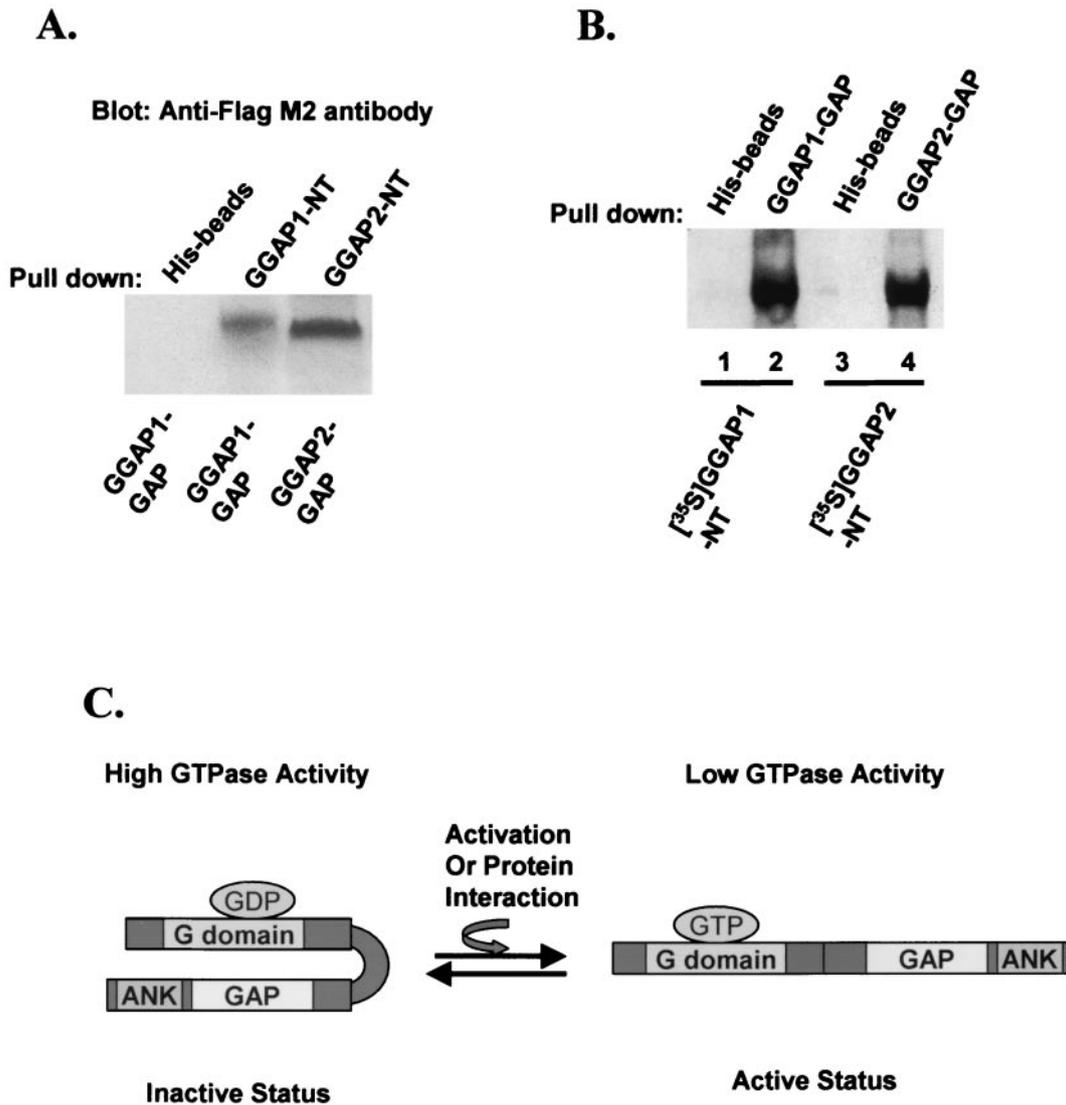


FIG. 7. Direct interaction of the N-terminal GTPase domain with the C-terminal GAP domain of GGAPs. (A) Flag-tagged GAP domains of GGAP1 and GGAP2, respectively, by coimmunoprecipitation. (B) His-tagged GAP domains interact with the ³⁵S-labeled N-terminal GTPase domain of GGAP1 or GGAP2 by protein pull down assay. (C) Model of intramolecular interaction and activation of GGAPs. In the inactive status, the N-terminal GTPase domain interacts with the C-terminal GAP domain, resulting in a protein with high GTPase activity and in the GDP-bound inactive status. Activation or binding of GGAP proteins with other proteins will disrupt the intramolecular interaction of the GTPase domain and the GAP domain. Therefore, the GGAP proteins will have low GTPase activity and exist in the GTP-bound active status.

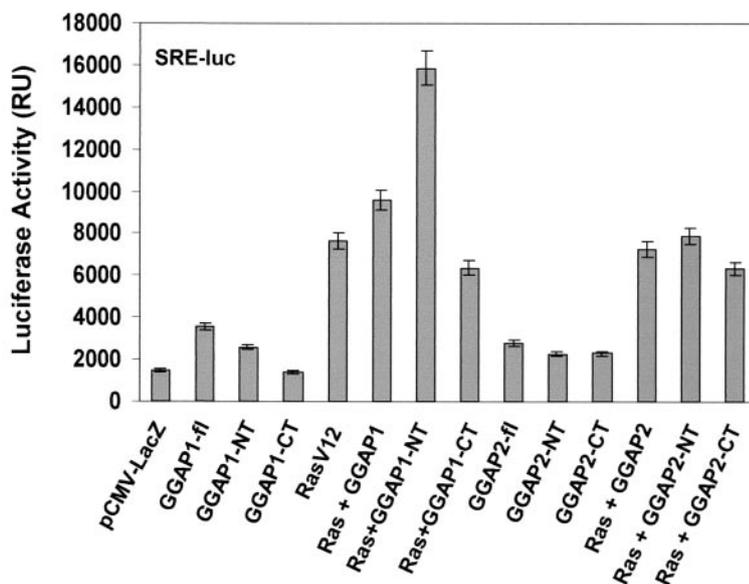
tion. Activation of GGAP proteins or binding of GGAP proteins with other proteins disrupts the intramolecular interaction of the N- and C-terminal domains, thus GGAP proteins have low GTPase activity and exist in the active GTP-bound conformation (Fig. 7C). Although we proposed the potential intramolecular interaction between the N-terminal G domain and the C-terminal GAP domain, we could not rule out the possibility that a trans mechanism exists, where the GAP domain from one GGAP molecule stimulates the enzymatic activity of G domain on another GGAP molecule.

The specific expression patterns and signaling pathways of GGAP proteins indicate potential roles of this family of proteins in a variety of cellular functions. During mouse embryogenesis,

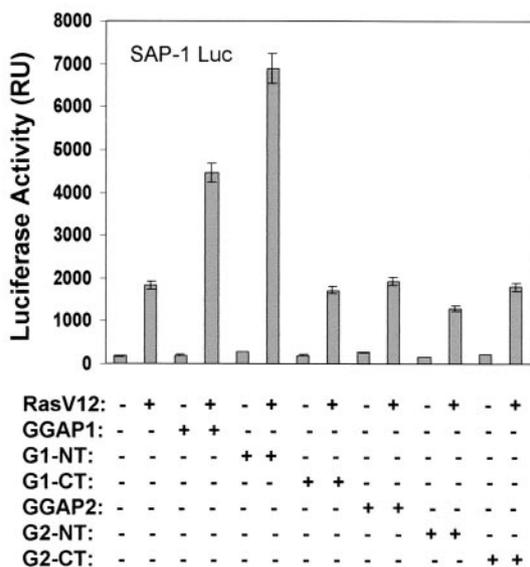
GGAP1 expression was restricted to the neural tube, forebrain, midbrain, and the whiskers that contain sensory neurons (Fig. 3B), suggesting a potential role of this protein in neuronal development and differentiation.

The PH domains of proteins have been shown to bind to phospholipids (10, 26, 58) and therefore might interact with the membrane structures in the cells, determining the localization of the proteins upon activation and inactivation. The ANK repeat domain generally serves as a site for protein-protein interactions. Proteins that interact with the C-terminal ANK repeat might disrupt the intramolecular interaction between the N-terminal G domain and the C-terminal GAP domain, therefore, controlling the GTPase activity and regulating the activation of GGAP proteins. Further experiments will be

A.



B.



C.

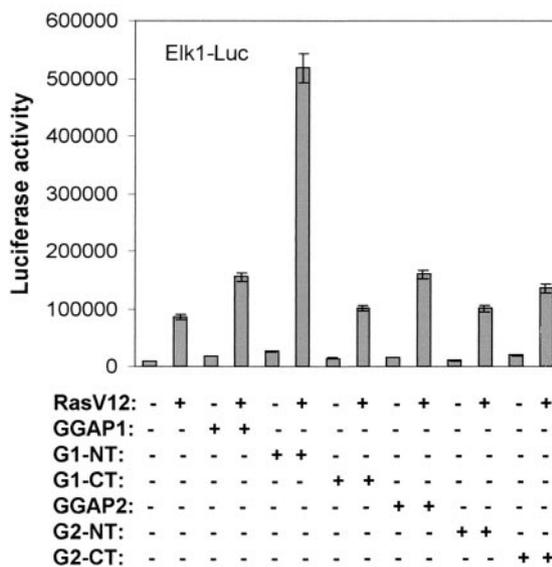


FIG. 8. GGAP1 synergistically activates the Ras-mediated mitogen-activated protein kinase signaling pathway at the serum response element (SRE) with signals that activate TCF (such as SAP1 and Elk1). (A) Activation of the c-fos SRE by GGAP1, GGAP2, and their N- and C-terminal domains, respectively. (B and C) Effects of GGAP1 and GGAP2 on SAP1- and Elk1-mediated transcriptional activation. Cells were transfected with luciferase reporter plasmids controlled by SRE, SAP1, and Elk1, respectively, together with the expression plasmids encoding LacZ (control), GGAP1, GGAP2, and their N- and C-terminal domains as indicated. Data shown are average of three qualitatively similar independent experiments with standard deviations (error bars).

needed to test the mode of regulation for this new family of proteins and their function in the cells.

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