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A novel Rho GTPase-activating-protein interacts with Gem, a member of the Ras superfamily of GTPases

Sandra ARESTA[1], Marie-France DE TAND-HEIM, Florence BÉRANGER[2] and Jean DE GUNZBURG[3]
INSERM U528, Institut Curie – Section de Recherche, 26 rue d'Ulm, 75248 Paris Cedex 05, France

Gem is a Ras-related protein whose expression is induced in several cell types upon activation by extracellular stimuli. With the aim of isolating the cellular partners of Gem that mediate its biological activity we performed a yeast two-hybrid screen and identified a novel protein of 970 amino acids, Gmip, that interacts with Gem through its N-terminal half, and presents a cysteine-rich domain followed by a Rho GTPase-activating protein (RhoGAP) domain in its C-terminal half. The RhoGAP domain of Gmip stimulates *in vitro* the GTPase activity of RhoA, but is inactive towards other Rho family proteins such as Rac1 and Cdc42; it is also specific for RhoA *in vivo*. The same is true for the full-length protein, which is furthermore able to down-regulate RhoA-dependent stress fibres in Ref-52 rat fibroblasts. These findings suggest that the signalling pathways controlled by two proteins of the Ras superfamily, RhoA and Gem, are linked via the action of the RhoGAP protein Gmip (Gem-interacting protein).

Key words: cytoskeleton, *in vivo* activity, RhoA.

INTRODUCTION

Proteins from the Ras superfamily play an important role in the regulation of a variety of cellular processes. They bind GTP or GDP, and cycle in the cell between these two forms. This cycle is regulated by two classes of proteins [1]. Guanine-nucleotide-exchange factors stimulate the exchange of GDP for GTP, hence promoting the appearance of the active GTP-bound form of the protein. In this state, the GTPase interacts with its downstream targets, the effectors. GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity of Ras-related proteins by several orders of magnitude, and act as negative regulators by returning them to their basal, GDP-bound, state. Proteins of the superfamily have been grouped into six different subfamilies, according to their sequences: Ras, Rho, Rab, Arf, Ran and Rad/Gem/Kir (RGK). The principal role of the Ras subfamily is the regulation of cell growth and differentiation [2], Rho proteins are mainly involved in cytoskeleton regulation [3], Ran in nucleo-cytoplasmic transport [4] and Rab and Arf in vesicular transport [5]. The function of RGK proteins remains largely unknown.

The Rho subfamily is composed of 16 mammalian members, the best known being RhoA, Rac1 and Cdc42 [6]. Although their best-characterized function is to control the assembly and organization of the actin cytoskeleton as well as processes that depend on it (cytokinesis, phagocytosis, pinocytosis, cell migration, morphogenesis, axon guidance, etc.) [7], Rho GTPases also regulate a variety of other biochemical pathways such as the serum response factor and nuclear factor κB transcription factors, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways, G1 cell-cycle progression, the assembly of cadherins at cell–cell contacts, cell polarity and cell transformation, activity of the NADPH oxidase complex of phagocytic cells and secretion in mast cells [6].

RGK proteins comprise four members so far: Rad [8], Gem/Kir [9,10], Rem1/Ges and Rem2 [11–13]. They share the general organization of GTP-binding domains, but exhibit many distinctive features compared with other Ras-related GTPases. For instance, they present amino acid substitutions at key positions for GTP/GDP binding and hydrolysis: the conserved Gly in the G1 domain corresponding to position 12 in Ras is replaced by a Gln in Gem/Kir, a Ser in Rem2 and a Pro in Rad, Ges and Rem1, and the entire G3 domain in Ras, $DTAG^{60}$ (Asp-Thr-Ala-Gly, positions 57–60), is replaced by the sequence motif DXWE (Asp-Xaa-Trp-Glu). Additionally, they carry N- and C-terminal extensions compared with Ras, and exhibit an average molecular mass of 35 kDa. Moreover, unlike most Ras family GTPases, RGK proteins do not present canonical C-terminal prenylation motifs (CAAX, CXC or CC); nevertheless, Gem and Rem2 have been shown to localize to the plasma membrane through their C-terminal regions [9,12]. Another distinctive feature of these proteins is that their expression is regulated at the transcriptional level. Rad was found overexpressed in the skeletal muscle of patients with Type II diabetes [8], Gem was first discovered as the product of a mitogen-inducible gene in human T lymphocytes [9], and its mouse orthologue, Kir, was initially described as a protein whose expression is induced by the tyrosine kinase Bcr/Abl in B lymphocytes [10]. Rem, on the other hand, was identified as the product of a gene repressed by lipopolysaccharide treatment in mice [11]. It is not yet known if Rem2 or Ges are also transcriptionally regulated.

Recent progress has been made in understanding the biological function of RGK family members. Gem has been found involved in the regulation of L-type calcium channels [14], and to interact

Abbreviations used: CRD, cysteine-rich domain; DTT, dithiothreitol; GAP, GTPase-activating-protein; Gmip, Gem-interacting protein; GST, glutathione S-tranferase; PAK1, p21-activated kinase 1; PBD, p21-binding domain; PDZ, PSD95/Dlg/ZO-1 homology domain; PKC, protein kinase C; PTPL1, protein tyrosine phosphatase L1; RBD, Rho-binding domain; RGK, Rad/Gem/Kir; ROCK, RhoA-associated kinase.

[1] Present address: Section of Cell and Molecular Biology, Biomedical Sciences Division, Sir Alexander Fleming Building, Imperial College Faculty of Medicine, South Kensington, London SW7 2AZ, U.K.
[2] Present address: IGH du CNRS, UPR1142, 141, rue de la Cardonille, 34396 Montpellier Cedex 5, France.
[3] To whom correspondence should be addressed (e-mail gunzburg@curie.fr).

The nucleotide sequence of human Gmip was deposited in the GenBank Nucleotide Sequence Database under accession no. AF132541.

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with a new member of the kinesin family [15]. Gem also regulates the morphology of various cells, including neuronal cells [16], through cross-talk with Rho GTPases, namely with the pathway of RhoA-associated kinase (ROCK; one of its effectors) [17].

Here we report the cloning and characterization of a new Gem partner. This novel protein, Gmip (Gem-interacting protein), contains a RhoGAP domain. It interacts *in vivo* with Gem, and increases the intrinsic GTPase activity of RhoA *in vitro* and *in vivo*, therefore providing a new link between signalling pathways controlled by the RhoA and Gem GTPases.

MATERIALS AND METHODS

cDNA library construction

Jurkat cells were grown at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin sulphate, 0.25 μg/ml amphotericin B and 10% (v/v) inactivated fetal calf serum (Roche Biochemicals) until reaching a density of 4×10^5 cells/ml. At this point the cultures were stimulated by addition of 81.1 nM PMA and 67 μM ionomycin for 4 h. mRNAs were extracted using the Fast Track Kit (Invitrogen) and cDNAs prepared with the Copy Kit (Invitrogen), that provides an oligo(dT) primer for the reverse transcription reaction containing a *Not*I restriction site. They were ligated to *Eco*RI adapters (New England Biolabs) and digested with *Not*I. After size selection in a 1% (w/v) agarose gel, inserts larger than 750 bp were extracted by digestion with Gelzyme (Invitrogen), and ligated to the pGADGE vector, creating fusion proteins with the GAL4 transcription-activation domain.

Two-hybrid assays

The bait for the two-hybrid screen was obtained by cloning the complete coding sequence of *gem* into the pGBT10 vector, thus creating a fusion with the GAL4 DNA-binding domain. In order to screen for Gem-interacting proteins, the cDNA library and the bait were co-transformed into the HF7c *Saccharomyces cerevisiae* strain. Yeast cultures and two-hybrid procedures were carried out according to standard methods [18]. Library plasmids from transformed yeast colonies were recovered using *Escherichia coli* HB101 as a recipient strain and selected on M9 minimal medium. cDNA inserts from clones interacting specifically with Gem were sequenced on an ABI Prism 310 automated sequencer (PerkinElmer), and homologies with known sequences in GenBank were searched for using the BLAST algorithm.

The pGAD-GmipA construct was obtained by cloning the most 5′ 1261 nucleotides of the clone obtained from the two-hybrid screen into the pGADGE vector. The pGAD-GmipGAP construct was obtained by PCR amplification and cloning of a cDNA fragment corresponding to amino acids 475–786 of Gmip between the *Eco*RI and *Sal*I sites of the pGADGE vector.

Northern blots

A human 12-lane multiple-tissue Northern blot (Clontech) was hybridized with a *gmip* probe (nt 2562–2993) obtained by PCR amplification with specific primers. To assess equal loading of the different lanes, the membrane was stripped and re-hybridized with a probe for β-actin.

Cloning of the full-length *gmip* sequence

The missing 3′ end of *gmip* cDNA was obtained by screening a cDNA library, prepared from HL60 cells differentiated for 55 h in dibutyryl-cAMP and ligated into the pCDM8 vector (Invitrogen; cDNA library kindly provided by Dr F. Boulay, Centre d'Etudes Nuckéaires de Grenoble, Grenoble, France), with the cDNA insert from the two-hybrid clone as a probe. After three rounds of colony purification, the cDNAs that remained positive were sequenced on an ABI Prism 310 automated sequencer.

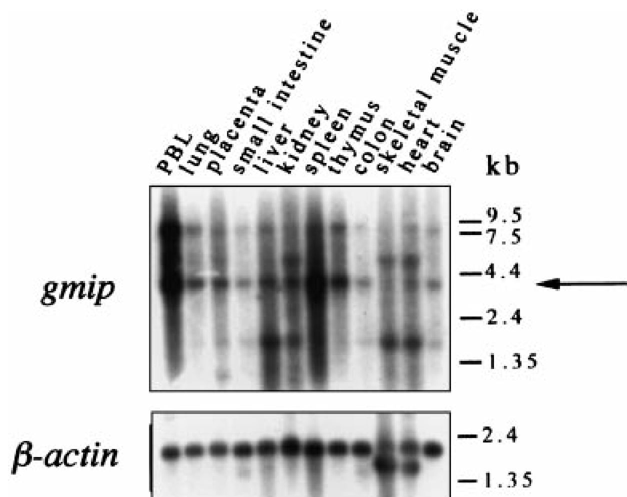

Figure 1 Tissue distribution of Gmip mRNA

A 12-lane multiple-tissue Northern blot (Clontech) was hybridized with a specific probe for *gmip*. Each lane contains 2 μg of poly(A)$^+$ RNA from the indicated tissues. The blot was exposed to film for 4 days. To assess equal loading of the lanes the membrane was stripped and re-hybridized with a β-actin probe. PBL, peripheral blood leucocytes.

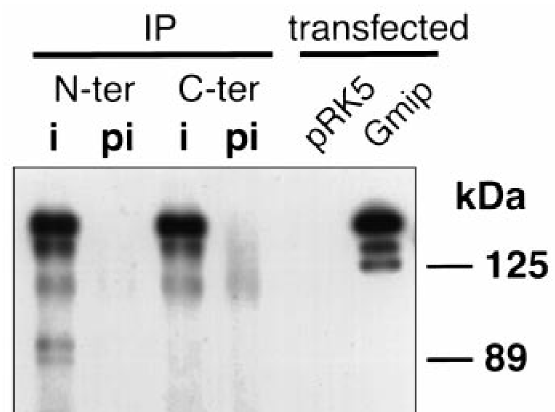

Figure 2 Assessment of the molecular mass of endogenous Gmip

Detergent extracts from Jurkat cells were immunoprecipitated (IP) with rabbit preimmune (pi) or immune (i) sera raised against the N-terminal region (N-ter) and the C-terminal region (C-ter) of Gmip. They were analysed by SDS/PAGE together with proteins ectopically expressed in HeLa cells transfected with pRK5 (empty vector) or pRK5-Gmip as described in the Materials and methods section. The migration of Gmip was visualized after Western blotting with the sera raised against the N-terminal region of Gmip. Western blotting using sera raised against the C-terminal region gave the same result (not shown).

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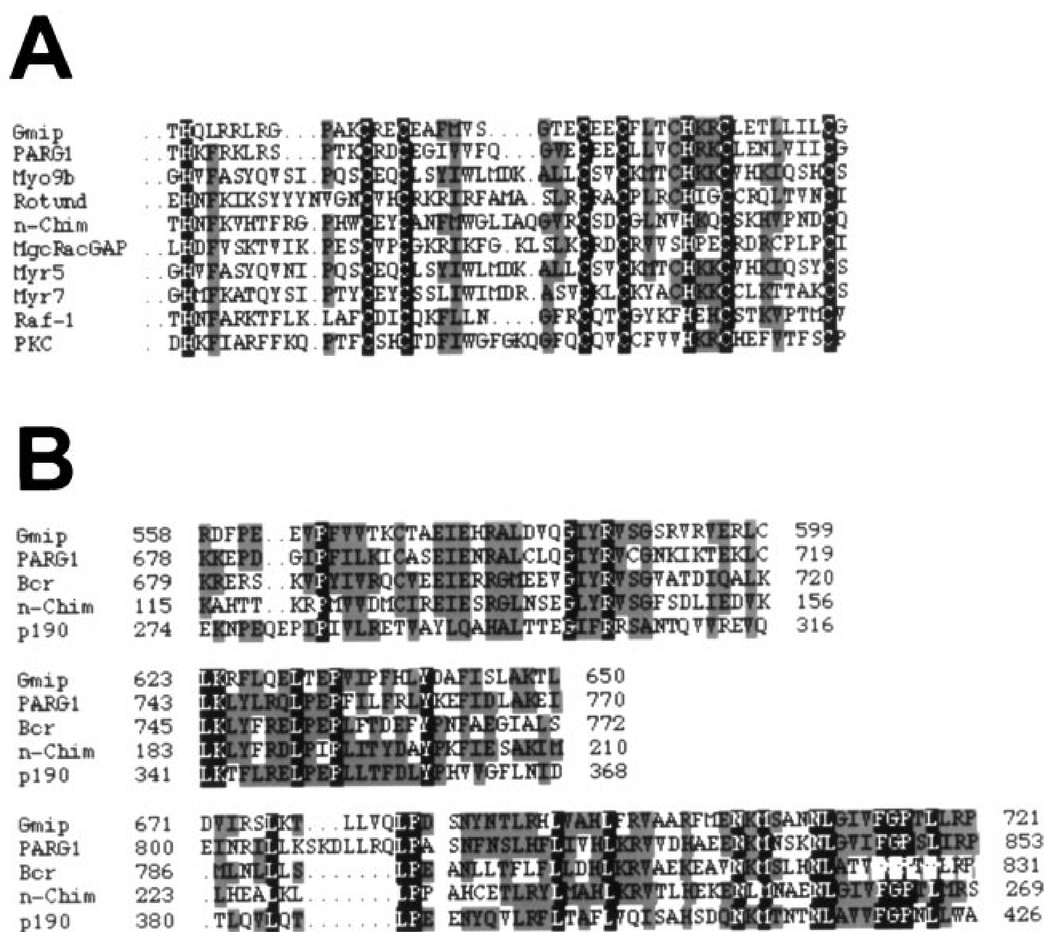

Figure 3 Homology domains present in Gmip

(**A**) Sequence homologies of the CRD. (**B**) Sequence homologies of the RhoGAP domain. N-chim, human n-chimaerin; p190, human p190RhoGAP. Alignments were made using the CLUSTAL W program. Identical amino acids are shaded in black, and conserved amino acids in grey.

Immunoprecipitations

Protein extracts from Jurkat cells were obtained by lysing growing cells in 25 mM Hepes, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA and 1 % (v/v) Triton X-100 for 15 min at 4 °C. Cell lysates were centrifuged at 13 000 ***g*** for 20 min to discard cellular debris, and supplemented with 150 mM NaCl.

Polyclonal antibodies directed against the N- and C-terminal regions of Gmip were produced by injecting rabbits with two different 6 × His-tagged proteins comprising amino acids 1–394 and 394–970 of Gmip, respectively. A 5 μl volume of pre-immune or immune serum was used to immunoprecipitate Gmip from the Jurkat cell lysates. After 3 h incubation at 4 °C, immune complexes were adsorbed on to Protein A–Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, washed four times in 25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.1 % (v/v) Triton X-100, and resolved by SDS/PAGE. The presence of Gmip was detected by Western blotting using the same antisera. The size of endogenous Gmip was compared with that of the protein encoded by the cDNA using an expression construct in the pRK5 vector, starting at the first ATG, introduced by electroporation into HeLa cells.

Co-immunoprecipitations

HeLa cells were electroporated with pRK5myc-Gmip, pRc/CMV-FLAG-Gem (full-length sequences), or both. After 48 h the cells were lysed in 25 mM Hepes, pH 7.5, 100 mM NaCl, 0.1 % (w/v) SDS, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate and protease inhibitors (Roche Biochemicals). Total extracts were centrifuged at 13 000 ***g*** for 20 min at 4 °C to eliminate cellular debris and the supernatant was subjected to immunoprecipitation with the anti-Myc monoclonal antibody 9E10. Immunoprecipitates were resolved by SDS/PAGE and the presence of Gem and Gmip was revealed by Western blotting with anti-FLAG and anti-Myc antibodies respectively.

Expression and purification of recombinant proteins

pGEX-Rho, pGEX-Rac and pGEX-Cdc42 were kindly provided by Dr R. Cerione (Cornell University, Ithaca, NY, U.S.A.). pGEX-p50RhoGAP (amino acids 198–439) was obtained from Dr A. Hall (University College London, London, U.K.), and pGEX-Bcr (amino acids 1050–1271) from Dr G. Gacon (Institut Cochin de Génétique Moléculaire, Paris, France). The RhoGAP

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domain of Gmip (amino acids 475–786) was amplified by PCR and cloned between the *Bam*HI and *Xho*I sites of pGEX4T3. Glutathione S-transferase (GST) fusion proteins were expressed in *E. coli* strain BL21, purified on glutathione–Sepharose beads, and eluted from the beads with glutathione. Purified proteins were dialysed against 20 mM Tris, pH 7.5, 1 mM DTT, 50 mM NaCl and 20% (v/v) glycerol. For the GTPases, 5 mM $MgCl_2$ was added throughout the procedure.

RhoGAP assays *in vitro*

GTPases were preloaded with GTP by incubating 0.2 μM of purified protein with 1 μM [γ-^{32}P]GTP for 10 min at 30 °C in 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.6, containing 1 mM DTT, 5 mM sodium pyrophosphate, 100 mM KCl, 1 mM sodium orthovanadate, 100 μg/ml BSA and 2 mM EDTA. After addition of $MgCl_2$ to 5 mM, GTPases were kept on ice. Single-turnover GTP hydrolysis, in the presence or absence of 0.1 μM GAPs, was initiated at 25 °C (15 °C for Cdc42) after addition of non-radioactive GTP to a final concentration of 200 μM. Aliquots were retrieved from the reaction at different time points and added into a tube containing 800 μl of 5% (w/v) activated charcoal (Sigma) in 50 mM NaH_2PO_4. After centrifugation, the amount of $^{32}PO_4^{3-}$ released into the supernatant was measured in a scintillation counter.

RhoGAP assays *in vivo*

To assess RhoGAP activity *in vivo*, the presence of GTP-bound GTPases was detected by specific binding of Rac1-GTP and Cdc42-GTP to the p21-binding domain (PBD) of p21-activated kinase 1 (PAK1; GST-PBD), and that of RhoA-GTP to the Rho-binding domain (RBD) of Rhotekin (GST-RBD) [19]. In brief, HEK-293 cells were plated at a density of 2×10^6 cells/10 cm culture dish, and transfected 12 h later by the calcium phosphate precipitation method with pRK5myc constructs encoding wild-type, dominant negative or activated mutants of Cdc42, Rac1 or RhoA (constructs kindly provided by Dr A. Hall), in the presence or absence of pRK5mycGmip-GAP (obtained by cloning a cDNA fragment corresponding to amino acids 475–786 of Gmip) or pRK5mycGmip (full-length sequence). Cells were lysed as described [19], and lysates were incubated with GST-PBD or GST-RBD conjugated with glutathione–Sepharose beads for 45 min at 4 °C, washed three times with lysis buffer and eluted with SDS sample buffer. Retained Cdc42, Rac1 or RhoA was detected by Western blotting with the anti-Myc monoclonal antibody 9E10. Whole cell lysates were also analysed for the presence of expressed Cdc42, Rac1, RhoA, Gmip-GAP or full-length Gmip for normalization.

Effect of Gmip on actin stress fibres

Ref-52 fibroblasts grown on glass coverslips were transfected with pRK5mycGmip using the LipofectAMINE reagent (Gibco BRL). After 18 h at 37 °C to allow protein expression, cells were fixed with 10% (w/v) paraformaldehyde for 15 min, and stained with anti-Myc monoclonal antibody 9E10, followed by an Alexa-488-conjugated goat anti-mouse secondary antibody, and Alexa-546-labelled phalloidin (Molecular Probes).

RESULTS

cDNA library construction and yeast two-hybrid screening

To identify proteins that would act as effectors or regulators of Gem, we performed a yeast two-hybrid screen with the complete protein sequence of human Gem fused to the DNA-binding domain of GAL4 as bait. The cDNA library was constructed from Jurkat cells stimulated for 4 h with PMA and ionomycin, conditions that had been assessed to induce maximum *gem* expression in this cell line [20]. Approx. 6.6×10^5 clones were screened and 20 clones positive for both *his3* and *lacZ* reporter genes and that interacted specifically with Gem were analysed further. Amongst them, a 2360 bp sequence was isolated that presented an incomplete reading frame, since no stop codon was present either at the 3′ or the 5′ end of the insert. Homology searches in sequence databases showed that the encoded protein contained an incomplete RhoGAP domain at its C-terminus. We named it Gmip, for Gem-interacting protein.

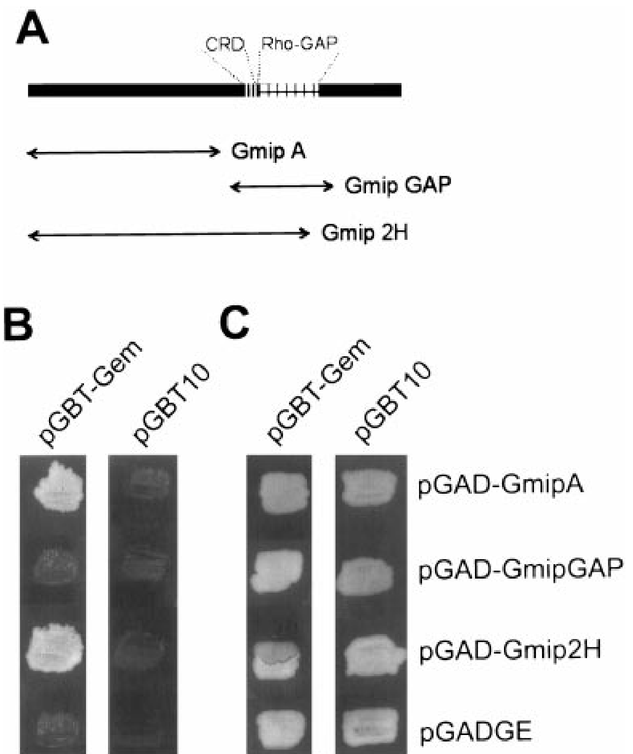

Figure 4 Determination of the Gem-interacting domain

(**A**) Schematic representation of Gmip constructs. Gmip 2H represents the partial cDNA encoding residues 1–721 obtained from the initial two-hybrid screen, Gmip A is residues 1–394, and Gmip GAP is the CRD and RhoGAP domains encompassing residues 475–786. (**B**, **C**) Bait (empty pGBT10, or containing Gem) and target (empty pGADGE, or containing various Gmip fragments) vectors were co-transformed into HF7c *S. cerevisiae*. Colonies were isolated and their ability to grow in the absence (**B**) or presence (**C**) of histidine was assessed.

Isolation of the full-length cDNA sequence: mRNA size and tissue distribution

The partial cDNA, Gmip, described above, was used to screen an oligo(dT)-primed, non-oriented, cDNA library from HL60 cells differentiated for 55 h in dibutyryl-cAMP. Several clones were isolated that contained the 3′ region of the cDNA, with a stop codon 750 nucleotides downstream from the end of the insert obtained from the yeast two-hybrid clone, followed by 490 nucleotides of 3′-untranslated sequence. We assessed the size and tissue distribution of *gmip* mRNA by Northern hybridization of a multiple-tissue blot (Clontech). As shown in Figure 1, a

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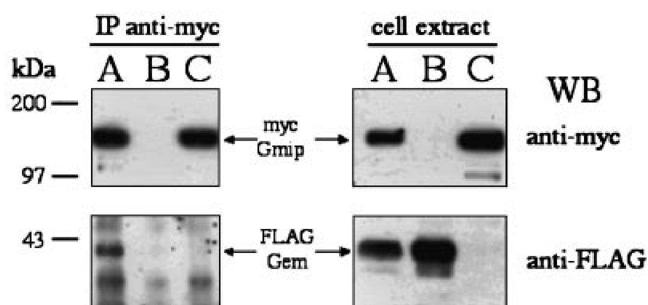

Figure 5 ***In vivo* interaction between Gmip and Gem**

HeLa cells were transfected with either Myc-tagged Gmip (lanes C), FLAG-tagged Gem (lanes B), or both (lanes A), and total cell extracts were immunoprecipitated (IP) with the anti-Myc antibody. Expression of both proteins was assessed by Western blotting (WB) with the anti-Myc or anti-FLAG antibodies in the cell lysates (right-hand panel) or after immunoprecipitation of Myc-tagged Gmip (left-hand panel).

transcript of approx. 4 kb was expressed ubiquitously. Other mRNA species were also detected in many tissues, with different expression patterns. Since there was no stop codon upstream of the first ATG in the cDNA obtained from the yeast two-hybrid clone, it was possible that the complete mRNA could be longer than the 3.6 kb representing the reconstituted sequence from the 5′ end of the two-hybrid clone to the poly(A)$^+$ tail. 5′-Rapid amplification of cDNA ends experiments did not allow us to obtain any additional sequence upstream of the first nucleotide of the two-hybrid clone. In addition, the first ATG presents a favourable translation initiation sequence [21]. The total reconstituted 3.6 kb cDNA that we obtained exhibits an open reading frame of 2910 bp, giving rise to a protein of 970 amino acids. Immunoprecipitation of endogenous Gmip from Jurkat cell extracts allowed the detection of a protein with the same molecular mass as the product of the cDNA expressed in mammalian cells, starting at the first ATG (Figure 2).

A search for functional domains revealed that Gmip contains a cysteine-rich domain (CRD) between residues 505 and 537 (Figure 3A), homologous to those from Raf-1, protein kinase C (PKC) and n-chimaerin. This region is followed by a RhoGAP domain encompassing residues 563 to 754. When compared with other RhoGAP domains, it exhibits the characteristic three blocks of conserved sequences (Figure 3B). The BLAST algorithm was used to search for homologous proteins in international databases. Gmip shares significant identity with two other human proteins that present a similar organization, with a CRD and a RhoGAP domain: the protein tyrosine phosphatase L1 (PTPL1)-associated RhoGAP PARG1 (34% identity) [22] and an uncharacterized cDNA KIAA0223 (33% identity). An open reading frame presenting 30% identity with these proteins

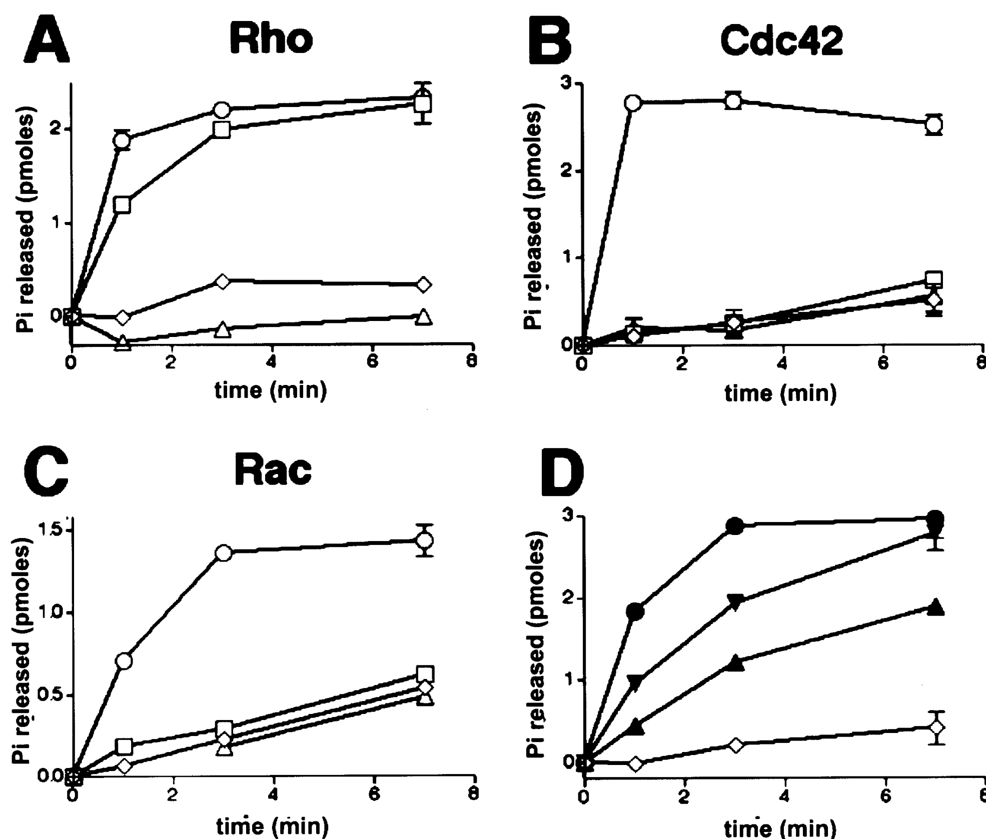

Figure 6 **The RhoGAP domain of Gmip stimulates the GTPase activity of RhoA *in vitro***

(**A**–**C**) Intrinsic GTPase activity (◇), and GTPase activity measured in the presence of 0.1 μM of GST (△), or 0.1 μM GST fused to the RhoGAP domain of Gmip (□), or 0.1 μM GST fused to positive control GAP protein (p50RhoGAP for RhoA and Cdc42, or Bcr for Rac; ○). (**D**) Effect of increasing concentrations of Gmip RhoGAP domain on the GTPase activity of RhoA. ◇, Intrinsic GTPase activity; ▲, 20 nM; ▼, 40 nM; ●, 100 nM RhoGAP domain of Gmip.

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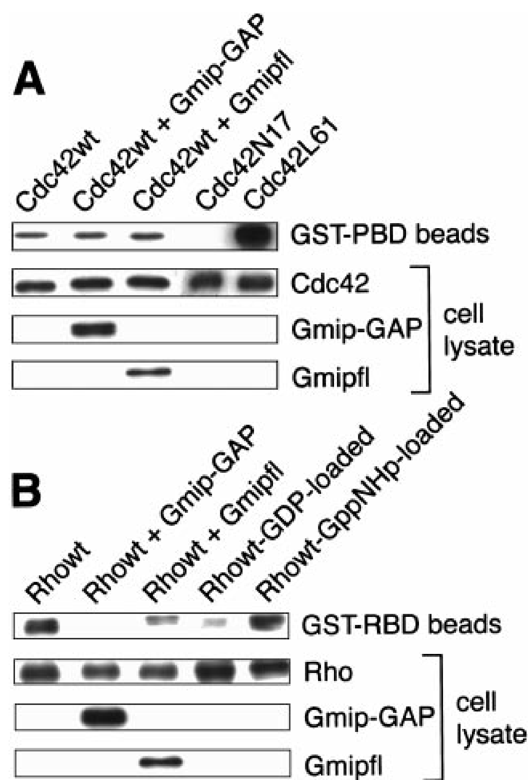

Figure 7 The expression of Gmip decreases the level of GTP-bound RhoA *in vivo*

HEK-293 cells were transfected with wild-type or mutant Myc-tagged GTPases, in the presence or absence of constructs for the expression of Myc-tagged full length Gmip (Gmipfl) or CRD + RhoGAP (Gmip-GAP) domains. Cells were lysed and incubated with GST-PBD or GST-RBD covered beads; proteins retained on the beads were submitted to SDS/PAGE and GTPases were detected by Western blotting with an anti-Myc antibody. A portion of each cell lysate was also submitted to SDS/PAGE and Western blotting with an anti-Myc antibody to visualize the levels of ectopically expressed GTPases, Gimpfl and Gmip-GAP. (**A**) GAP activity of Gmip towards Cdc42. Active Cdc42 levels were detected with GST-PBD-covered beads; their ability to discriminate between the inactive and active forms of Cdc42 was assessed using the N17 and L61 mutants of the GTPase. (**B**) GAP activity of Gmip towards RhoA. Active RhoA levels were detected with GST-RBD-covered beads; their ability to specifically trap the GTP-bound form of RhoA was assessed by adding 10 mM GDP or guanosine 5′-[β,γ-imido]triphosphate (GppNHp) to equal portions of the cell lysate before incubation with the beads. wt, wild type.

is found in *Caenorhabditis elegans* (ZK669.1a), but none was detected in *Drosophila melanogaster*.

Interaction between Gem and Gmip

The domain of Gmip responsible for its interaction with Gem was determined by a deletion analysis using the yeast two-hybrid system. Figure 4 shows that Gem does not interact with the region of Gmip containing the CRD and RhoGAP domains (amino acids 475–786), but binds to the N-terminal part of the protein (amino acids 1–394). This region contains no identifiable functional domains. According to secondary-structure prediction algorithms, it is mainly α-helical, and exhibits a potential coiled-coil between residues 251 and 280. Also, the interaction between Gmip and Gem depends on the GTPase core domain of Gem (amino acids 73–265; results not shown).

We then used an independent approach to check that the full-length Gmip protein was able to interact with Gem, and to this end ectopically expressed both proteins in HeLa cells. The proteins were epitope-tagged with the Myc (Gmip) or FLAG (Gem) epitopes, in order to allow their detection. As shown in Figure 5, following immunoprecipitation of Gmip by the anti-Myc antibody, Gem could be detected in the complex by Western blotting with the anti-FLAG antibody. Hence, Gmip and Gem are able to interact *in vivo*.

The RhoGAP domain of Gmip is specific for RhoA GTPase *in vitro*

To determine whether Gmip carries a functional GAP activity towards Rho family GTPases, amino acids 475–786 of Gmip encompassing the CRD and RhoGAP domains were expressed in bacteria as a fusion with GST, purified to apparent homogeneity, and assessed for their ability to stimulate the intrinsic GTPase activity of recombinant RhoA, Rac1 and Cdc42. As seen in Figure 6(A), the RhoGAP domain of Gmip considerably stimulated the intrinsic GTPase activity of RhoA, whereas GST alone had no effect. This stimulation by the GAP domain of Gmip was comparable with that obtained with the same concentration of p50RhoGAP, a known regulator of RhoA. In contrast, the GAP domain of Gmip had no such effect on the GTPase activities of recombinant Rac1 and Cdc42 (Figures 6B and 6C), whereas Bcr and p50RhoGAP were respectively able to stimulate significantly the GTPase activities of these proteins in parallel assays. Hence the GAP domain of Gmip behaves *in vitro* as a GAP specific for RhoA. Its GAP activity was dose-dependent and catalytic (Figure 6D), since a concentration of GAP domain as low as 20 nM stimulated 6-fold the GTPase activity of 200 nM RhoA.

Gmip is active towards RhoA *in vivo*

To assess the RhoGAP activity of Gmip *in vivo*, GST fusions of the GTPase-binding domain of PAK1, an effector of Rac1 and Cdc42 (GST-PBD), or that of Rhotekin, an effector of Rho (GST-RBD), were used to pull-down GTP-bound Cdc42, Rac1 or RhoA from cell lysates in a GTP-trapping assay [19]. HEK-293 cells were transfected with Myc-tagged RhoA, Rac1 or Cdc42 alone or together with constructs encoding either the Myc-tagged RhoGAP and CRD domains of Gmip (amino acids 475–786) or the full-length protein. Cell lysates were incubated with GST-PBD or GST-RBD, and the amount of GTP-bound GTPases was assessed by Western blotting with the anti-Myc antibody. The method was indeed specific towards the GTP-bound form of the GTPases (Figure 7). As can be seen in Figure 7(A), neither the RhoGAP domain of Gmip nor the full-length protein exhibited any GAP activity towards Cdc42 *in vivo*, confirming the *in vitro* results. Similar results were obtained with Rac1 (results not shown). However, co-expression of the RhoGAP domain of Gmip in HEK-293 cells dramatically decreased the amount of GTP-bound RhoA (Figure 7B); moreover, full-length Gmip was also able to down-regulate RhoA *in vivo* (Figure 7B). These results extend the biochemical data obtained *in vitro* and demonstrate that Gmip indeed acts as a RhoA-specific GAP *in vivo*.

To characterize further the RhoGAP activity of Gmip, we performed a functional assay for RhoA activity *in vivo*. RhoA is known to control stress fibre formation in fibroblastic cells [23], due to its activation by lysophosphatidic acid, a component of serum. Hence we ectopically expressed Myc-tagged full-length Gmip in Ref-52 fibroblasts growing in media with serum and assessed the presence of actin stress fibres in transfected and non-transfected cells by immunofluorescence. Figure 8 shows that control, non-transfected cells possess many characteristic Rho-dependent stress fibres, while cells expressing Gmip no longer

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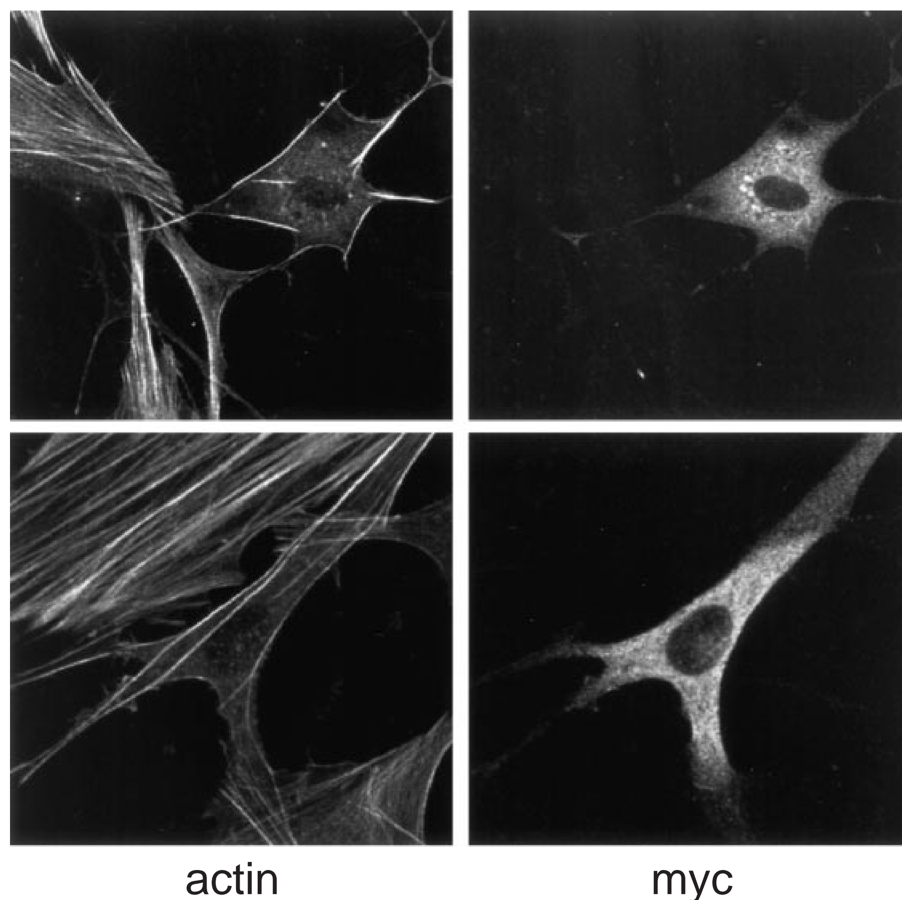

Figure 8 Inhibition of actin stress fibres by Gmip

Ref-52 fibroblasts were transfected with pRK5mycGmip and fixed 18 h after transfection. The cells were kept in serum throughout the experiment to maintain a high level of RhoA activation. The expression of Gmip was detected with anti-Myc antibody (right-hand panels), and actin stress fibres were visualized by staining with Alexa 546-phalloidin (left-hand panels). Cells expressing Gmip no longer presented stress fibres.

exhibit such structures, demonstrating that Gmip is indeed able to inhibit RhoA function *in vivo*.

DISCUSSION

We report here the identification of a novel RhoGAP protein, named Gmip, which is able to down-regulate RhoA both *in vitro* and *in vivo*. Gmip interacts with Gem, a Ras-related GTPase, both in a yeast two-hybrid assay and when ectopically expressed in HeLa cells together with Gem. This interaction provides a way by which Gem could regulate RhoA activity in order to remodel cell morphology, a reported Gem function [15–17].

RhoGAPs constitute a vast family of proteins that have been described in vertebrates, *C. elegans*, *D. melanogaster* and yeast (for a review see [24]), as well as in plants and human pathogens [25,26]. They all share a unique RhoGAP domain characterized by three blocks of conserved amino acids (see Figure 3B), and exhibit a variable degree of specificity towards Rho family proteins *in vitro* and *in vivo* [27]. Like many proteins involved in signal transduction, RhoGAPs are modular, since they contain other domains that might be involved in distinct biochemical activities. Immediately upstream of its RhoGAP domain, several members of this family present a cysteine-rich region homologous to the C1 domain of PKC, and this is also the case for Gmip (Figure 3A). CRDs are able to co-ordinate zinc atoms and bind phorbol esters. Phorbol ester binding to the C1 domain of PKC has been shown to be important for its plasma membrane localization and subsequent activation [28]; moreover, the CRD of n-chimaerin, a known GAP for Rac, allows the modulation of its GAP activity *in vitro* and *in vivo* by binding phorbol esters [29–31]. Although this has not been tested, it seems unlikely that Gmip would bind phorbol esters. Indeed, it has been shown that the CRD of Raf-1, a known Ras effector, does not bind phorbol esters, because of a deletion of four amino acids between the second and the third conserved cysteines as compared with the C1 domain of PKC (see Figure 3A) [32]. Similarly to Raf-1, Gmip also presents a deletion of four amino acids between the second and third consensual cysteines (Figure 3A). However, the co-ordination of zinc atoms remains a possibility that will be assessed in future work.

Gmip's closest homologue is PARG1, a RhoA-specific GAP that has been described as a protein that interacts with the fourth PDZ (PSD95/Dlg/ZO-1 homology domain) of PTPL1, a protein tyrosine phosphatase [22]. We have not addressed the possibility that Gmip would also be able to interact with PTPL1 or other PDZ-domain-containing proteins. However, this seems unlikely, since the interaction with PDZ domains is dependent on the last four C-terminal amino acids of a protein, and those in Gmip

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(-EDHL) do not closely match the consensus established for PDZ-domain binding [33].

Several lines of evidence point to a role for the elevated activity of Rho family proteins in cell transformation and oncogenesis as well in the metastatic process. Indeed, Rho, Rac and Cdc42 were found overexpressed in breast tumours as compared with normal tissue, where these proteins are hardly or not detected [34]. Furthermore, the illegitimate or enhanced expression of Rho guanine-nucleotide-exchange factors is involved in oncogenesis, and in fact several genes coding for these proteins, such as *lbc* and *ost*, have been isolated as oncogenes from naturally occurring human tumours [35]. However, so far there are no reports of genetic alterations leading to cancer of genes encoding RhoGAPs. The genomic sequence of the human *gmip* gene was found on a contig of 286 kb from chromosome 19; the complete cDNA is distributed over 14.13 kb, and encompasses 21 exons (results not shown). By *in situ* hybridization, the human *gmip* gene was localized to chromosome 19p11–12 (results not shown). Two different translocations involving chromosome 19 have been implicated in human cancer [36]: der(19)t(8;19)(q11;p11) is associated with colon adenocarcinoma and (11;19)(q22;p11) with acute myeloid leukaemia. In addition, deletions of the 19p12 region have been associated with acute lymphoblastic leukaemia [36]. Such chromosomal alterations could lead to a reduction or disappearance of the expression of a negative regulator of Rho, the RhoGAP protein Gmip, and therefore create in those tumours an unusually high basal level of Rho activity. Further investigation will be needed to assess the contribution of Gmip to human cancer.

At the cellular level, RhoA plays a major role in regulating the formation of actin stress fibres and focal adhesions, in response to growth factors such as lysophosphatidic acid or bombesin [23]. In neuronal cell lines, Rho is implicated in neurite retraction and cell rounding, through the activation of its effector ROCK [37,38]. Gem is expressed in the developing brain [15] but not in adult brain. Overexpression of Gem in neuronal cell lines induces neurite outgrowth, and blocks cell rounding induced by over-expression of ROCK [16], through a direct interaction between these two proteins [17]. Indeed, this latter report shows that the interaction of Gem with ROCK blocks the kinase's ability to interact with some of its substrates, which would account for Gem's inhibition of the rounding of neuroblastoma cells promoted by ROCK. These data altogether suggest that Gem is involved in neuronal development, and a potential mechanism for its action would be the cross-talk with RhoA signalling. Our work adds a new player, Gmip, to this biological process. It is not known if Gem can regulate Gmip's RhoGAP activity, but the published results lead us to speculate that the interaction with Gem should enhance it. This would decrease the pool of GTP-bound RhoA in the cell, and therefore diminish the amount of active ROCK, leading to the same physiological effect as the direct inhibition of ROCK's activity by Gem, i.e. neurite extension. The same mechanism would also lead Gem to promote the dissassembly of stress fibres and focal adhesions, in accordance with reported experimental data [17]. The precise role of Gmip in co-ordinating the contribution of signals respectively controlled by the RhoA and Gem GTPases will be the focus of future investigation.

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