RGS14 is a novel Rap effector that preferentially regulates the GTPase activity of $G\alpha_o$

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In an attempt to elucidate the physiological function(s) of the Ras-related Rap proteins, we used the yeast two-hybrid system and isolated a cDNA encoding a protein that interacts with both Rap1 and Rap2, but not with Ras; the use of Rap2 mutants showed that this interaction is characteristic of a potential Rap effector. This protein was identified as RGS14, a member of the recently discovered family of RGS ('regulators of G-protein signalling') proteins that stimulate the GTPase activity of the GTP-binding α subunit of heterotrimeric G-proteins ($G\alpha$). Deletion analysis, as well as *in vitro* binding experiments, revealed that RGS14 binds Rap proteins through a domain distinct from that carrying the RGS identity, and that this domain shares sequence identity with the Ras/Rap binding domain of B-Raf and Raf-1 kinases. RGS14 is distinguished from other RGS proteins by its marked preference for $G\alpha_o$ over other $G\alpha$ subunits: RGS14 binds preferentially to $G\alpha_o$ in isolated brain membranes, and also interacts preferentially with $G\alpha_o$ (as compared with

$G\alpha_{i1}$) to stimulate its GTPase activity. In adult mice, RGS14 expression is restricted to spleen and brain. *In situ* hybridization studies showed that it is highly expressed only in certain areas of mouse brain (such as the CA1 and CA2 regions of the hippocampus), and that this pattern closely resembles that of Rap2, but not Rap1, expression. Double *in situ* hybridization experiments revealed that certain cells in the hippocampus express both RGS14 and $G\alpha_o$, as well as both RGS14 and Rap2, showing that the interaction of RGS14 with $G\alpha_o$ and Rap2 is physiologically possible. Taken together, these results suggest that RGS14 could constitute a bridging molecule that allows cross-regulation of signalling pathways downstream from G-protein-coupled receptors involving heterotrimeric proteins of the $G_{i/o}$ family and those involving the Ras-related GTPase Rap2.

Key words: heterotrimeric G-proteins, Ras family, signal transduction, two hybrid.

INTRODUCTION

The first steps of signal transduction originating from many growth factors, hormones and neurotransmitters lead to the activation of G-proteins belonging to two main families: monomeric GTPases of the Ras superfamily [1], and heterotrimeric G-proteins composed of a GTP-binding α subunit and a membrane-associated $\beta\gamma$ dimer that dissociates from the α subunit upon activation [2]. Under physiological conditions, the activation of G-proteins from both families is transient: efficient hydrolysis of the bound GTP ensures termination of the incoming signal. Ras family proteins usually carry a relatively low intrinsic GTPase activity that can be greatly enhanced by GTPase-activating proteins (GAPs) [3]. The intrinsic GTPase activity of $G\alpha$ subunits is 1–2 orders of magnitude greater than that of Ras; nevertheless, proteins of the recently discovered RGS ('regulators of G-protein signalling') family, which now encompasses more than 20 members, act as GAPs for the $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ groups of $G\alpha$ subunits [4].

Many functional connections have been established between signalling pathways involving G-proteins from both the Ras-related and heterotrimeric families. For instance, the activation

of G-protein-coupled receptors involving $G\alpha_i$ or $G\alpha_q$ proteins via lysophosphatidic acid, α -adrenergic agonists or muscarinic agents results in the activation of the ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) cascade via the activation of Ras [5,6]. More recently, p115RhoGEF, a guanine nucleotide exchange factor (GEF) for the Ras-related GTPase Rho, was shown to carry an RGS domain which confers to p115 the ability to specifically enhance the GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$ [7]; in the case of $G\alpha_{13}$, the RGS activity of p115 may be regulated by Rho [8]. Finally, two recent reports showed that there is a direct interaction between $G\alpha$ subunits ($G\alpha_i$ in one report and $G\alpha_o$ in the other) and Rap1GAP, the protein that stimulates the GTPase activity of Ras-related Rap proteins, and that, through different mechanisms in distinct biological systems, this interaction results in activation of the ERK/MAPK cascade [9,10].

Among the family of Ras-related GTPases, the group of Rap proteins (comprising the 60% identical Rap1 and Rap2 proteins) is most closely related to Ras [11]: Rap GTPases share overall approx. 50% amino acid identity with Ras, resulting in a similar three-dimensional structure, and exhibit a conserved effector domain, which is the region through which Ras and Rap proteins

Abbreviations used: DTT, dithiothreitol; ERK, extracellular-signal-regulated kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; Ni-NTA, Ni²⁺-nitrilotriacetate; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate; RalGDS, Ral GDP dissociation stimulator; RID, Rap-interacting domain; RBD, Ras-binding domain; RGL, RalGDS like; RGS, regulators of G-protein signalling; Rlf, RalGDS-like factor; RPIP, Rap-interacting protein.

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interact with the effectors responsible for their biological activities [12–14]. The physiological role of Rap proteins is still poorly understood, in spite of intense efforts to decipher their biological activities. For many years, Rap1 was thought to act as a Ras antagonist, since its overexpression was able to revert to normal the phenotype of Ras-transformed fibroblasts [15]. In addition, Rap1 competes with Ras for interaction with effectors such as Raf-1 [16,17]. Evidence pointing to the ability of Rap1 to function as a Ras signalling suppressor in resting CHO cells has also been presented [18]. However, several reports have shown that Rap1 is activated in intact cells by many incoming signals, including thrombin, epidermal growth factor, insulin and endothelin 1, and have identified GEFs specific for Rap1 that mediate its activation by second messengers such as cAMP, calcium and diacylglycerol [13,19–24]. Apart from the case of PC12 cells, in which activation of Rap1 by cAMP has been reported to activate B-Raf and stimulate the MAPK cascade [25], the nature of downstream pathways triggered by activated Rap1 has not yet been established.

The closely related Rap2 protein has remained even more elusive. In contrast with Rap1, its overexpression does not interfere with signalling through the Ras pathway [26,27], and although the existence of a specific exchange factor activity for Rap2 has been shown (E. Pasheva and J. de Gunzburg, unpublished work), this protein, as well as the signals leading to its activation, have not yet been characterized. Using the yeast two-hybrid method, we have searched for effectors of Rap2 that may mediate its biological effects, and hence shed some light on its physiological role. This has led us to identify a novel protein, RPIP8 (Rap-interacting protein 8), that interacts specifically with Rap2 (exclusive of other Ras family proteins) and is preferentially expressed in neurons and neuroendocrine cells [28]. We also showed that Rap2, similarly to Ras and Rap1, interacts with RalGDS (Ral GDP dissociation stimulator), RGL (RalGDS like) and Rlf (RalGDS-like factor), which are GEFs for the Ras-related protein Ral; however, whereas Ral is activated in response to the activation of Ras, neither Rap1 nor Rap2 leads *in vivo* to the activation of Ral [27,29].

In the present paper we show that screening for Rap effectors by using the yeast two-hybrid method also led to the isolation of a cDNA encoding a protein that interacts with both Rap1 and Rap2, but not with Ras. Sequencing of this cDNA identified the encoded protein as RGS14, a member of the RGS family that had been isolated independently by a systematic search for novel RGS proteins [30,31]. We presently report the detailed characterization of this protein; we show that it stimulates the GTPase activity of α subunits of heterotrimeric proteins from the $G_{i/o}$ families, and interacts preferentially with the α subunit of the heterotrimeric protein G_o . The fact that its expression pattern in mouse brain overlaps with those of G_{α_o} and Rap2, but not that of Rap1, suggests that RGS14 could enable the Ras-related GTPase Rap2 to regulate signalling pathways transduced from G-protein-coupled receptors via the heterotrimeric protein G_o .

MATERIALS AND METHODS

Cloning and deletion analysis of RGS14

A mouse brain cDNA library was screened with pLexA-Rap2A(V12) as bait, as described in [28]. The pGAD-RGS14 plasmid containing the full-length RGS14 cDNA obtained from the screen was used as template to amplify by PCR, using *Pfu* polymerase (Stratagene), the coding sequence from the initiation codon to the stop codon using forward primer 5' AGTAGG-ATTCATGCCAGGGAAGCCCAAGCAC 3' and the reverse

primer 5' AGCCCTCGAGCTATGGTGGAGCCTCCCGAGA 3'. The amplification product was cleaved with *Bam*HI and *Xho*I and cloned into pGAD3S2X, a variant of pGADGH (J. Camonis, personal communication), to yield plasmid pGADcodR, or into pBluescript to yield pBScodR; the absence of PCR-induced mutations was confirmed by sequencing. For expression in mammalian cells, the same insert was cloned into pRK5, in-frame with a Myc epitope tag, to yield pRK5mycRGS14.

The interaction of RGS14 with Ras-family GTPases was assessed in the yeast two-hybrid system by mating [32]. Rap2 effector domain mutants were generated by overlapping PCR in the pVJL10 vector. L40 cells expressing fusions between the DNA binding domain of LexA and Ras-family GTPases were mated with AMR 70 cells expressing fusions of their effectors with the activation domain of GAL4. Interaction was assessed by the ability of diploid conjugants to grow in the absence of histidine and to express β -galactosidase, as in [27].

In order to generate fusion proteins of the activation domain of GAL4 with various regions of RGS14, we took advantage of unique *Bgl*III, *Ecl*XI and *Nco*I restriction sites in the RGS14 cDNA, as depicted in Figure 1. Deletion mutants corresponding to 5' regions, reciprocal 3' regions and the Rap-interacting domain (RID) between the *Ecl*XI and *Nco*I restriction sites were subcloned into appropriate pGAD vectors in-frame with the GAL4 transcription activation domain. The integrity of the constructs was confirmed by sequencing. The interaction of these RGS14 mutants with Ras, Rap1 and Rap2 was assessed using the two-hybrid method by co-transformation of the relevant constructs into *Saccharomyces cerevisiae* strain L40 [28]; both β -galactosidase and his3 reporters gave similar results. Expression of the mutant RGS14 proteins was checked by Western blotting of extracts from the co-transformed yeast with a monoclonal antibody directed against the activation domain of GAL4 (Clontech).

Production and purification of recombinant proteins

The bacterial expression vector for the production of (His)₆-tagged RGS4 in pQE30 (Qiagen) was kindly provided by Professor Tom Wilkie (University of Texas Southwestern Medical Centre, Dallas, TX, U.S.A.). The coding sequences of RGS14 excised from pBScodR with *Bam*HI and *Sal*I were also cloned into pQE30; both vectors were co-transformed along with pREP4 (encoding the lac repressor) into *Escherichia coli* BL21. Sequences corresponding to the RID between residues 300 and 427 were amplified by PCR using forward primer 5' GGGTATACCAT-ATGCGGCCG GGAAGTATTGCTGC 3' and reverse primer 5' AATACTCGAGATTAATCCATGGGCTGCTTC TCTCC-T 3', and cloned into pET15b (Novagen) cut with *Nde*I and *Xho*I. The coding sequences from G_{α_o} and $G_{\alpha_{11}}$ (kindly provided by Dr John Kehrl, NIAID, NIH, Bethesda, MD, U.S.A.) were amplified by PCR and cloned into pET15b as above. Production of proteins was induced with 30–100 μ M isopropyl β -D-thiogalactoside for 16 h at 30 °C in *E. coli* BL21. (His)₆-tagged proteins were purified on Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose beads (Qiagen) following the supplier's instructions. Purified proteins were dialysed against 50 mM Tris buffer, pH 8.0, containing 1 mM EDTA, 2 mM dithiothreitol (DTT) and 10 % glycerol, snap-frozen in liquid nitrogen and stored at –80 °C. Protein concentrations were measured according to Bradford [33] using a commercial kit (Bio-Rad). Recombinant Rap2 was purified as in [34], and purified Ras and Rap1 proteins were generously donated by Dr N. van den Berghe and A. Wittinghofer (Max-Planck Inst. für Molekular Physiologie, Dortmund, Germany).

Figure 1 Determination of the functional domains of RGS14 by deletion analysis

Deletion mutants of RGS14 fused to the activation domain of GAL4 were assessed by the yeast two-hybrid method for their interaction with Ras, Rap1 and Rap2. Expression of the deletion mutants was assessed by Western blotting (Exp). Restriction enzyme sites used to generate the deletion mutants are shown (enz).

Production, purification and characterization of anti-RGS14 antibodies

For the production of antibodies, the RGS14 coding sequence was also inserted into the pMal-c2 vector (New England Biolabs); the resultant MBP-RGS14 fusion protein (where MBP is maltose-binding protein) was purified and injected subcutaneously into rabbits to obtain antibodies. The antibodies were affinity purified on histidine-tagged RGS14 as previously described [35] and detected a single band of 65 kDa on Western blots of cytosol or membranes from brain or spleen, whereas no band was revealed on similar blots from cells or tissues that do not express RGS14 (see Figure 6). The specificity of the antibodies was further established in extracts from HeLa cells transfected with pRK5mycRGS14, as in [27], by showing that the same protein could be immunoprecipitated with anti-Myc antibodies (clone 9E10; Roche) and detected on Western blots with anti-RGS14 antibodies, and vice versa.

Binding of recombinant RGS14 and RGS14-RID to mouse brain membranes

Mouse brains were homogenized at 0 °C in a Teflon/glass Potter-Elvehjem homogenizer in 4 vol. of buffer A (25 mM Tris buffer, pH 7.5, 0.15 M NaCl, 2 mM DTT and 1 mM Pefabloc). After elimination of nuclei and debris by two successive centrifugations at 1000 *g* for 10 min, membranes were separated from cytosol by ultracentrifugation for 1 h at 100000 *g* and then washed once in buffer A by a second ultracentrifugation under the same conditions.

To investigate the binding of RGS14 or of its RID to Rap proteins, membranes resuspended in buffer A, representing 1 mg of total protein, were adjusted to 2 mM EDTA, and loaded for 3 h at 4 °C with 100 μ M GDP or guanosine 5'-[β , γ -imido]-triphosphate (p[NH]ppG) (Roche Biochemicals). $MgCl_2$ was then added to 10 mM, membranes were solubilized with 1% Triton X-100, and soluble material was incubated for 3 h at 4 °C with 10 μ g of purified (His)₆-RGS14 or 30 μ g of (His)₆-RGS-RID bound to Ni-NTA beads in a final volume of 250 μ l. Beads were washed five times with 1 ml of 25 mM Tris buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.15 M NaCl, 2 mM DTT and GDP or p[NH]ppG, and analysed for the presence of Ras, Rap1 and Rap2 proteins by Western blotting using a monoclonal anti-Ras antibody (Transduction Laboratories), or affinity-purified anti-Rap1 or anti-Rap2 antibodies [35,36]. Detection was performed by chemiluminescence using the ECL[®] procedure (Amersham).

In order to investigate the binding of full-length RGS14 to $G\alpha$ subunits, membranes resuspended in buffer A were adjusted to 10 mM $MgSO_4$ and loaded with 20 μ M GDP in the absence or presence of 20 mM NaF and 60 μ M $AlCl_3$ for 30 min at 0 °C. A 100 μ l portion of membrane suspension, representing 1 mg of protein, was incubated with 100 μ l of buffer A containing 10 μ g of purified (His)₆-RGS14 and 10 mM $MgSO_4$ for 1 h at 0 °C. Membranes were solubilized by incubation with 1% sodium cholate for 15 min at 0 °C, and insoluble material was removed by ultracentrifugation as above. Complexes containing (His)₆-RGS14 were recovered on 50 μ l of Ni-NTA-agarose beads and washed five times with 1 ml of 25 mM Tris buffer, pH 8.0, containing 0.3 M NaCl, 2 mM DTT, 10 mM $MgCl_2$, 0.5% Triton X-100, 10 mM imidazole and 10 μ M GDP, in the absence or presence of 10 mM NaF and 30 μ M $AlCl_3$. Complexes were eluted in 70 μ l of the same buffer containing 0.3 M imidazole, and aliquots representing one-seventh of the eluate were analysed for the presence of various $G\alpha$ subunits by Western blotting with the following polyclonal rabbit antibodies: anti- $G\alpha_{11/2}$ and anti- $G\alpha_{13}$ (Calbiochem), anti- $G\alpha_{12}$ and anti- $G\alpha_{13}$ (Santa Cruz), and anti- $G\alpha_o$, anti- $G\alpha_s$ and anti- $G\alpha_q$ (gifts from Dr V. Homburger, CNRS UPR 9023, CCIPE, Montpellier, France).

Expression of RGS14 in mouse organs and HeLa cells

Cytosol and crude washed membranes were prepared from the brain and spleen of an adult mouse, as well as from cultured HeLa cells as indicated above. On average, 45 mg of cytosolic protein and 7.3 mg of membrane protein were recovered from one brain; 34.6 mg and 2.5 mg were recovered respectively from one spleen. Portions of 50 μ g of protein from each extract were analysed for the presence of RGS14 by Western blotting with affinity-purified anti-RGS14 antibodies; detection was performed by chemiluminescence as above, and the relative intensity of bands was quantified using NIH Image software.

Biochemical activities

[³H]GDP binding to, as well as dissociation from, Ras and Rap proteins was assessed by a filter binding assay [34]. Their GTPase activity was measured essentially as previously described, except that [γ -³²P]GTP was used and the amount of [³²P]P_i released was assessed by binding to activated charcoal, as indicated below.

For $G\alpha$ subunits, [³H]GDP binding was measured similarly, except that the assay was performed in the presence of 0.1 mg/ml BSA and 10 mM $MgSO_4$. GTPase activity was measured by a

single-turnover assay essentially as described in [37]. Briefly, 50 pmol of purified active (as assessed by GDP and GTP binding) (His)₆-Gα subunits were loaded with 1 μM [γ -³²P]GTP in a final volume of 600 μl containing 50 mM Tris, pH 8.0, 5 mM EDTA, 1 mM DTT and 0.05% Triton X-100 for 15 min at room temperature, and placed on ice. After adding RGS proteins when indicated, MgSO₄ and GTP were adjusted to final concentrations of 15 mM and 150 μM respectively; 50 μl aliquots were withdrawn at various times thereafter and added to 750 μl of a 5% suspension of active charcoal in 50 mM NaH₂PO₄. After centrifugation, the amount of [³²P]P_i recovered from the supernatant was measured by liquid scintillation counting.

In situ hybridization

Postnatal mice were perfused through the left ventricle with 4% (v/v) paraformaldehyde and post-fixed overnight in the same fixative. Brains were rinsed in PBS after fixation, and cryoprotected in PBS containing 15% (w/v) sucrose for 12 h at 4 °C. After embedding in 15% (w/v) sucrose and 7.5% (w/v) gelatin in PBS, samples were frozen in melting isopentane and sections (20 μm thick) cut on a Microm cryostat were collected on Superfrost plus glass slides (Menzel-Glaser).

Patterns of gene transcription were determined by *in situ* hybridization using digoxigenin- or fluorescein-labelled antisense cRNA probes (Roche) transcribed from the coding regions of mouse RGS14, Rap1 and Rap2, and rat Gα_o (which is 97.5% identical to the corresponding mouse sequence), cDNAs cloned into pBluescript KS. Mouse Rap1 and Rap2 cDNAs were generously donated by Dr. Florence Béranger (CRBM, Montpellier, France). *In situ* hybridization of cryostat sections was performed according to [38]. For double *in situ* hybridizations, cRNA probes synthesized with either digoxigenin-UTP or fluorescein-UTP were detected with alkaline phosphatase-conjugated anti-digoxigenin or anti-fluorescein antibodies respectively. The substrate for alkaline phosphatase was either 5-bromo-4-chloroindol-3-yl phosphate (BCIP)/Nitro Blue Tetrazolium chloride (blue) or BCIP/2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (red) (Boehringer). The more abundant of the two transcripts was detected first; the precipitate was then fixed with 4% paraformaldehyde, and alkaline phosphatase was inactivated by incubating the sections for 15 min with 0.1 M glycine/HCl buffer, pH 2.2. The cryosections were blocked, and the second antibody was applied, followed by the second alkaline phosphatase reaction.

RESULTS

Identification of a novel Rap effector

In order to search for effectors of the Rap2 GTPase, we screened a mouse brain cDNA library by the two-hybrid method in *Saccharomyces cerevisiae*, using an activated form of Rap2A [carrying a Gly-12 → Val (G12V) mutation] truncated at residue 168 (this truncation was necessary in order to alleviate the strong transactivation of the *lexA* promoter by the *lexA*-Rap2 fusion protein alone [28]). Such a screen had already led to the identification of two classes of potential effectors of Rap2: RPIP8, a protein preferentially expressed in neurons and neuroendocrine cells that specifically binds Rap2 (but neither Rap1 nor Ras) [28], as well as the three GEFs for the Ras-related GTPase Ral, i.e. RalGDS, RGL and Rlf, that are able to interact equally well with Ras, Rap1 and Rap2 [27]. As shown in Table 1, this two-hybrid screen also led to the isolation of a clone, initially called RPIP1, that interacted with both Rap1 and Rap2,

Table 1 Interaction of RGS14/RPIP1 with Ras-family proteins in the yeast two-hybrid system

L40 yeasts expressing Ras-family GTPases fused to the DNA-binding domain (DBD) of LexA were mated with AMR 70 yeasts expressing effectors fused to the activation domain (AD) of GAL4. The capacity of diploids to grow in the absence of histidine was scored; similar results were obtained by measuring β-galactosidase activity. pGAD RID expressed the RID of RalGDS.

DBD fusions	Growth (+ / -)				
	AD fusions ...	pGAD	pGAD RPIP1	pGAD RID	pGAD RPIP8
pVJL		—	—	—	—
pVJL Ras		—	—	+	—
pVJL Rap1		—	+	+	—
pVJL Rap2		—	+	+	+
pVJL Rap2 G12V		—	+	+	+
pVJL Rap2 S17N		—	—	—	—
pVJL Rap2 T35A		—	—	—	—
pVJL Rap2 D38A		—	—	—	—
pVJL Rap2 D38E		—	—	—	+

but not with Ras. We further characterized this interaction using various alleles of Rap2 in the two-hybrid test; RPIP1 was unable to bind the S17N mutant of Rap2, a form that has substantially lost its affinity for GTP, and is therefore locked in the inactive GDP-bound state [34]. Conversely, RPIP1 bound equally well to the wild-type and the G12V mutant, which has a reduced intrinsic GTPase activity and is therefore likely to remain mainly in the active GTP-bound state in the nucleus of yeast cells. In addition, RPIP1 was unable to bind Rap2 mutants T35A and D38A, which carry invalidating mutations in the effector domain; similar mutations in either Ras or Rap2 impair their interaction with their respective effectors Raf-1 and RPIP8. Furthermore, RPIP1 was unable to interact with Rap2 carrying the more subtle D38E effector domain mutation, which still allows the interaction with RPIP8. Hence RPIP1 exhibits the expected characteristics of a potential Rap effector protein that binds through its effector domain to the activated GTP-bound form of Rap proteins.

RPIP1 is an RGS protein, RGS14

Analysis of the amino acid sequence of RPIP1 (Genbank accession no. U85055) revealed the presence of an RGS domain between residues 63 and 185. This domain, found in more than 20 mammalian, yeast and nematode proteins, confers the ability to stimulate the GTPase activity of the α subunits of heterotrimeric proteins of the G_i, G_q and G_{12/13} families [4,39]. Based on its identity with an RGS domain described in a comprehensive study that used a PCR-based strategy to systematically isolate cDNAs encoding these domains, the RGS-containing RPIP1 protein was named mouse RGS14 [30]. While the present work was in progress, rat RGS14 was also isolated [31], as well as partial human cDNAs encoding the homologous protein. The proteins from all three species are highly conserved, and in particular the mouse and rat proteins exhibit 95.6% identical residues (results not shown). Among the family of RGS proteins, RGS12 is the most closely related to RGS14 (see below and [4,31]); all of the other RGS proteins only exhibited significant identity with RGS14 within their respective RGS domains (results not shown).

Identification of the RID of RGS14

In order to determine which region of RGS14 is involved in its interaction with Rap proteins, we generated constructs encoding

Figure 2 Interaction of RGS14 and its RID with Rap proteins *in vitro*

Mouse brain membranes were loaded with GDP (—) or p[NH]ppG (+), solubilized with 1% Triton X-100, and incubated with the following beads: lanes 1 and 2, Ni-NTA beads; lanes 3 and 4, Ni-NTA beads bound to isolated (His)₆-RID; lanes 5 and 6, Ni-NTA beads bound to full-length (His)₆-RGS14; lanes 7 and 8, GST-RalGDS-RBD (A and B) and GST-Raf-RBD (C) (where GST is glutathione S-transferase) linked to glutathione-Sepharose served as positive controls. Material associated with the beads (lanes 1–8), and 10% input solubilized material (T; lane 9), was analysed by Western blotting for the presence of (A) Rap1, (B) Rap2 and (C) Ras. The data are representative of three independent experiments.

a series of N-terminally and reciprocal C-terminally truncated RGS14 proteins. Their ability to interact with Ras, Rap1 and Rap2 was assessed in the yeast two-hybrid system, and expression of the various RGS14 mutants was controlled in yeast by Western blotting. Prior to this, we had eliminated 5' and 3' non-coding sequences from the RGS14 cDNA to ensure that the observed interactions would only be attributable to the translation product of the RGS14 open reading frame. As shown in Figure 1, deletions of the N-terminal part of RGS14 up to residue 300 still enabled RGS14 to interact with Rap proteins, whereas deletions of the C-terminus lacking sequences further upstream than residue 427 impaired its ability to do so. These mutants therefore define the fragment containing amino acids 300–427 as the minimal region of RGS14 necessary for Rap binding. In order to determine whether this region is sufficient for Rap binding, we fused this fragment with the activation domain of GAL4, and showed that it retained the ability to interact with Rap proteins; this region was further termed the Rap-interacting domain (RID). It is noteworthy that it interacted much better with Rap2 than with Rap1, a difference that was not observed with the full-length RGS14 protein.

We sought to confirm these data, using an independent method,

by investigating the ability of purified (His)₆-tagged RGS14-RID or full-length RGS14 to interact with Ras or Rap proteins extracted from brain membranes. As shown in Figure 2, full-length RGS14 as well as its RID interacted *in vitro* with both Rap1 and Rap2; this interaction was dependent on the active form of the GTPases. It is noteworthy that, as described above with the yeast two-hybrid system, full-length RGS14 seemed to bind Rap1 and Rap2 equally well, whereas its isolated RID exhibited a marked preference for Rap2; such a result might indicate that other domains of the protein are able to modulate the ability of the RID to interact with the active form of Rap proteins. However, neither full-length RGS14 nor its isolated RID exhibited any interaction with Ras. These experiments confirm that RGS14 does indeed constitute a potential effector for Rap proteins, but not for Ras.

A search for proteins exhibiting regions of identity with the RID of RGS14 identified residues 959–1083 of RGS12, as well as the Ras-binding domains (RBDs) of the serine/threonine kinases B-Raf and Raf-1. As shown in Figure 3, the greatest similarities were observed within each functional group: the two RGS proteins were more closely related to each other, as was also the case among the two Raf kinases (exhibiting respectively 39% and 53% identity within the 80-amino-acid region examined), than was RGS12 or RGS14 to either of the kinases. However, there was a significant proportion of identical residues between RGS14 and the Raf kinases, especially within the first 40 amino acids of this stretch (35% with B-Raf and 25% with Raf-1); it is noteworthy that RGS14 is considerably more similar to B-Raf than is RGS12 (14 versus eight out of 40 identical residues). More careful examination of the alignment shows that Arg-336 of RGS14 corresponds to Arg-89 of Raf-1, a residue that is essential for the interaction of Ras and Rap1 with Raf-1 kinase [12,40]; this residue is not conserved in RGS12. Furthermore, none of the other residues involved in the interaction of Raf-1 with Ras or Rap1 (Arg-59, Gln-66, Arg-67, Lys-84, and residues 95–101), as defined by structural, mutational and epitope scanning analysis [12,40–43], are conserved in RGS14. The observations that Rap2 binds Raf-1 with much weaker affinity than do Ras and Rap1, and that RGS14 interacts with both Rap2 and Rap1, and not at all with Ras, probably constitute the biochemical consequences of these structural differences.

Interaction of RGS14 with the α subunits of heterotrimeric G-proteins

In order to characterize the interaction of RGS14 with the α subunits of heterotrimeric G-proteins, we investigated which $G\alpha$

Figure 3 Identity of the RID of RGS14 with Raf kinases

Homologous domains of mouse RGS14 (GenBank accession no. U85055), rat RGS12 (U92280), human Raf-1 (X03484) and human B-Raf (M95712) proteins were aligned using the program Clustal W. Residues identical between RGS proteins and Raf kinases are boxed in black, those identical among Raf kinases are boxed in light grey shading, and those identical between RGS12 and RGS14 are boxed in darker grey shading. Asterisks indicate positions determined to be important for Ras-Raf interactions, according to [12,40–43].

Figure 4 Interaction of recombinant RGS14 with $G\alpha$ subunits in mouse brain membranes

Mouse brain membranes (representing 1 mg of protein) that had been loaded with GDP in the absence (—) or presence (+) of AlF_4^- , were incubated with 10 μ g of purified recombinant (His)₆-RGS14 for 1 h on ice. Following solubilization with 1% sodium cholate, proteins bound to (His)₆-RGS14 were recovered on nickel chelating beads and analysed for the presence of $G\alpha$ subunits by Western blotting. Lane Mb contains 50 μ g of protein from brain membranes. The data are representative of at least three independent experiments.

subunits in membranes isolated from mouse brain (an organ that expresses a wide spectrum of heterotrimeric G-proteins) would interact *in situ* with recombinant purified RGS14 in a pull-down experiment (Figure 4). RGS14 interacted specifically with the high-affinity state of $G\alpha_o$ when membranes had been pre-incubated with GDP in the presence, but not in the absence, of AlF_4^- ; under the same conditions, no interaction was detected with other $G\alpha$ subunits of the G_i family ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$), or with $G\alpha_s$ or $G\alpha_q$.

We then investigated the biochemical activity of RGS14 by assessing its ability to stimulate the GTPase activity of purified

$G\alpha$ subunits *in vitro*, and compared its potency in acting on $G\alpha_o$ and $G\alpha_{i1}$. As depicted in Figure 5(A), the intrinsic GTPase activity of $G\alpha_o$ was strongly stimulated by RGS14; with 83 nM active $G\alpha_o$ in the assay, a concentration as low as 0.7 nM RGS14 caused a significant stimulation of the GTPase activity of $G\alpha_o$, suggesting that, under these conditions, the effect of RGS14 was catalytic. The preferential nature of the RGS14- $G\alpha_o$ interaction to stimulate the GTPase activity of $G\alpha$ subunits was demonstrated in two different ways. First, a 10–20-fold higher concentration of RGS14 was required to induce stimulation of the GTPase activity of $G\alpha_{i1}$ compared with that obtained with $G\alpha_o$ (Figure 5B). Secondly, a 10–20-fold higher concentration of RGS4, as compared with RGS14, was necessary to induce a similar stimulation of the GTPase activity of $G\alpha_o$ (Figure 5C). In contrast, similar concentrations of RGS4 and RGS14 led to comparable stimulation of the GTPase activity of $G\alpha_{i1}$ (Figure 5D). Hence, although RGS14 is able to stimulate the GTPase activity of another $G\alpha$ subunit, such as $G\alpha_{i1}$, its functional interaction with $G\alpha_o$ is at least one order of magnitude more efficient *in vitro*.

Expression of RGS14 in mouse tissues

Northern blot analysis of poly(A)⁺ RNA from adult mouse tissues showed that RGS14 mRNA was strongly expressed in brain and spleen, but barely detectable in or absent from other organs, such as heart, skeletal muscle, lung, kidney, liver and testis (results not shown). Its expression at the protein level was also demonstrated in brain and spleen by Western blotting, with an apparent molecular mass of 65 kDa, slightly higher than the

Figure 5 Comparative effects of RGS14 and RGS4 on the GTPase activities of $G\alpha_o$ and $G\alpha_{i1}$

$G\alpha_o$ or $G\alpha_{i1}$ (83 nM) that had been loaded with [γ -³²P]GTP was incubated in the absence (●) or presence (■, ▲, ▼) of increasing concentrations of RGS14 or RGS4. GTPase activity was measured over a 15 min period by a single-turnover assay, as described in the Materials and methods section. The combination of $G\alpha$ subunit and RGS protein used in each panel was as follows: (A) RGS14 and $G\alpha_o$; (B) RGS14 and $G\alpha_{i1}$; (C) RGS4 and $G\alpha_o$; (D) RGS4 and $G\alpha_{i1}$. Each panel represents the results of at least three independent experiments.

Figure 6 Expression of RGS14 protein in mouse tissues

Whole brain and spleen from an adult mouse, as well as cultured HeLa cells, were homogenized and fractionated into cytosol (c) and membrane (m) fractions as described in the Materials and methods section. Samples of 50 μ g of protein from each fraction were analysed for the presence of RGS14 protein by Western blotting with affinity-purified anti-RGS14 antibodies.

theoretical value of 59 kDa calculated from its amino acid composition (Figure 6); no RGS14 expression was detected in HeLa cells. The respective amounts of RGS14 in cytosol and membranes from brain and spleen were quantified from two

independent experiments (one of which is depicted in Figure 6) in which equal amounts of protein from the two compartments were analysed for the presence of RGS14 by Western blotting, and corrected for the respective contribution of each compartment to the total protein content of the tissue. Although the bulk of the protein was found in the cytosol, a significant amount of RGS14 protein was consistently found associated with membranes: membrane-associated RGS14 represented 15–20 % of total RGS14 in brain, and 4–6 % in spleen.

The distribution of RGS14 transcripts in brain was analysed by *in situ* hybridization. During embryonic development the intensity of the RGS14 signal was weak, but it was detectable at embryonic day 12, mainly in areas lateral to the rhombencephalic floor plate; at embryonic day 17, its expression was more pronounced in the anterior region of the brain, including the telencephalic olfactory nuclei and the hippocampus anlage (results not shown). In the adult, with the exception of the granular layer of the cerebellum, RGS14-expressing cells were mostly restricted to the forebrain (Figure 7A). Cells expressing the highest level of RGS14 mRNA were found in the hippocampus, particularly in the CA2 region (Figure 7D), and to a lesser extent in the CA1 region and the external layer of the dentate gyrus, contrasting with a very weak signal in the CA3 region. RGS14 transcripts were also abundant in the striatum, layer V of the cortex, the olfactory cortex and tubercles, as well as the subthalamic region. Double-labelling experiments with the pan-neuronal marker Hu clearly demonstrated that detected RGS14 transcripts

Figure 7 Localization of RGS14, $G\alpha_o$ and Rap2 transcripts in the brain

Sagittal brain cryo-sections from mice at post-natal day 15 were treated for *in situ* hybridization with RGS14 (A, D), $G\alpha_o$ (B, E) or Rap2 (C, F) digoxigenin- or fluorescein-labelled antisense cRNA probes. (G) Control section, similar to that shown in (D), treated with digoxigenin-labelled RGS14 sense probe. (A)–(C) At a low magnification, note that the patterns of expression of RGS14 and Rap2 mRNAs are very similar, i.e. mostly restricted to the forebrain. In contrast, $G\alpha_o$ -positive cells are more uniformly distributed throughout the brain. (D)–(F) In the hippocampus, the level of detection of RGS14 mRNA is not uniform: it is hardly detectable in the CA3 region, highly expressed in the CA1 region and very highly expressed in the CA2 region. In the dentate gyrus, RGS14-positive cells are mostly seen in the external layer (D). (H) Sagittal sections across the hippocampus were treated for double *in situ* hybridization with digoxigenin-labelled $G\alpha_o$ (purple) and fluorescein-labelled RGS14 (brown–orange) cRNA probes. In the CA2 and CA3 regions, double-labelled neurons appear as dark brown cells. In some neurons of the CA2 region, only RGS14 transcripts were detectable (brown). In contrast, in CA3, only $G\alpha_o$ mRNAs were detected (purple staining). (I) Double staining with digoxigenin-labelled Rap2 (purple) and fluorescein-labelled RGS14 (brown–orange) cRNA probes of the CA2 region shown at higher magnification. The dark brown staining of the CA2 neurons demonstrates the cellular co-expression of the two transcripts. Abbreviations: Cb, cerebellum; Cx, cortex; DG, dentate gyrus; Hi, hippocampus; Ob, olfactory bulb; P, pons; St, striatum. Bar: 1.4 mm in (A)–(C), 150 μ m in (D)–(G), 36 μ m in (H) and 20 μ m in (I).

Figure 8 Localization of RGS14, Rap1 and Rap2 transcripts in the hippocampus, corpus callosum and cerebellum

Sagittal cryo-sections from brains of mice at postnatal day 15 were treated for *in situ* hybridization with digoxigenin-labelled cRNA probes for RGS14 (**A, D**), Rap2 (**B, E**) and Rap1 (**C, F**) transcripts. (**A**)–(**C**) Strong positive signals for RGS14 and Rap2 transcripts are observed in the CA2 region of the hippocampus (**A, B**). In contrast, Rap1 mRNA is hardly detectable in the hippocampus, but is highly expressed in RGS14-negative, Rap2-negative cells in the corpus callosum (CC) (**C**). (**D**)–(**F**) On sagittal sections of the cerebellum, the granular cells are weakly labelled with all three probes. (**F**) Cells with high expression of Rap1 are clearly visible in the deep white matter (WM) and center folia of the cerebellum. Bar: 900 μm in (**A**)–(**C**) and 20 μm in (**D**)–(**F**).

were restricted to neurons (results not shown). We then compared the pattern of expression of RGS14 with that of $G\alpha_o$ (Figure 7B). $G\alpha_o$ mRNA expression appeared to be mostly pan-neuronal, and included RGS14-positive cells; the strongest $G\alpha_o$ signal was also detected in the hippocampus (Figure 7E), but was not distributed similarly to RGS14. Nevertheless, double *in situ* hybridization demonstrated cellular co-expression of $G\alpha_o$ and RGS14 (Figure 7H): in the CA2 region of the hippocampus, where RGS14 expression is strongest, although some cells were only RGS14-positive, many cells co-expressed $G\alpha_o$ and RGS14. It should be noted, however, that in cells co-expressing both transcripts, the intensity of the signal for RGS14 was greater than that for $G\alpha_o$, suggesting that the levels of these two transcripts are not regulated in parallel. Although we cannot exclude the possibility that it could be coincidental, the observed co-expression of RGS14 and $G\alpha_o$ mRNAs at the cellular level strengthens the potential physiological relevance of the preferential interaction of RGS14 and $G\alpha_o$ proteins observed *in vitro*.

Since RGS14 may act as a Rap effector protein, we compared the expression pattern of its mRNA in brain with those of Rap1 and Rap2 transcripts. As shown in Figures 7(C) and 7(F), as well as Figures 8(A), 8(B), 8(D) and 8(E), the pattern of expression of Rap2 was very similar to, although not superimposable with, the pattern of expression of RGS14. It should be noted that the differences observed between the two patterns are more likely to reflect differences in the level of expression of the two transcripts rather than cellular exclusion. In contrast, the patterns of expression of Rap1 and RGS14 did not overlap (Figure 8; compare panels A and D representing RGS14 with panels C and F representing Rap1): Rap1 mRNA was mostly observed in the white matter tracts of the cerebellum (Figure 8F), the fimbria hippocampi (not shown) and the corpus callosum (Figure 8C), suggestive of oligodendroglial expression. Double-labelling experiments performed with RGS14 and Rap2 cRNA probes

(Figure 7I) showed that, at least in the hippocampus, the two transcripts were co-expressed in some cells. Taken together, these observations suggest that the interaction between RGS14 and Rap2, as well as the preferential biochemical interaction of RGS14 with $G\alpha_o$ demonstrated *in vitro*, may actually be physiologically relevant *in vivo*, and indicate a potential regulatory role for the Ras-related GTPase Rap2 in signalling pathways transduced via the heterotrimeric G-protein G_o .

DISCUSSION

Among GTPases of the Ras superfamily, Rap proteins are most closely related to Ras, yet their functional role remains elusive. In the present study, we show that the RGS14 protein, a member of the RGS family, is a potential effector of Rap proteins, and in particular of Rap2, with which it is co-expressed in several areas of the brain; our results suggest that RGS14 may constitute a functional link between signalling pathways involving Rap proteins and heterotrimeric G-proteins of the $G_{i/o}$ family.

Similar to all of the other RGS proteins, RGS14 contains the conserved RGS domain that is responsible for their interaction with the α subunits of heterotrimeric G-proteins, and the ability to stimulate their GTPase activity [4]. Specific to RGS14 is the presence, in a position C-terminal relative to the RGS domain, of an 80-amino-acid stretch that exhibits a significant degree of identity with the RBD of the B-Raf serine/threonine kinase, and to a lesser degree with that of the closely related Raf-1 kinase. The degree of identity of RGS14 with Raf kinases is particularly high in the 40 most N-terminal residues of this domain (14/40 and 10/40 positions identical with B-Raf and Raf-1 respectively); as functionally determined in the present study, this region also represents the N-terminal portion of the domain responsible for the interaction of RGS14 with Rap proteins. It is noteworthy

that Raf kinases not only interact with Ras, but have also been shown to interact with Rap1 [12,16,44] through their RBD; hence the similarity between the RID of RGS14 and the RBDs of Raf kinases is not so surprising, since they share Rap1 as a common partner. Under these circumstances, one would expect that at least some of the residues conserved between RGS14 and Raf kinases would be involved in their interaction with Rap proteins. Unexpectedly, only one of the residues determined by structural analysis [12] to be involved in the interaction between Rap1A and the RBD of Raf-1, or shown by mutational, epitope scanning and functional analysis to play a role in the binding of Ras to the RBD of Raf-1 [40–43], namely Arg-89 of Raf-1, is conserved in RGS14 (Arg-316). This is an indication that the atomic details of the interaction between Ras-family GTPases and RGS14 are probably different from those of their interaction with the RBD of Raf kinases. In effect, distinct mutations in the effector domains of these GTPases result in the loss of their interaction with Raf kinases and RGS14 (J. de Gunzburg, unpublished work). Moreover, the RBD of Raf-1 exhibits an affinity greater by one order of magnitude for Ras than that for Rap1, and an even larger differential is observed between its affinities for Ras and Rap2 [17,27,28]. Conversely, we have shown that RGS14 binds both Rap1 and Rap2 proteins, and that its RID exhibits a marked preference for Rap2; however, it does not interact with Ras, in spite of the identity between the effector regions of Rap1 and Ras. In view of this, it would be interesting to establish the structure of the surface of the interaction between Rap proteins and RGS14, and to compare it with the established structures of the surfaces of interaction between Rap1 and the RBD of Raf-1 [12], as well as between Ras and the RID of the RalGEF Rlf [45], another effector of Ras and Rap proteins [27,46].

RGS12 is the member of the RGS family that is the closest to RGS14, although it is a much larger protein which carries both N- and C-terminal extensions relative to RGS14 [31]. RGS12 exhibits the most conserved RGS domain relative to RGS14 (results not shown). It also shares some of the residues that are conserved between RGS14 and Raf kinases, except that the residue corresponding to Arg-89 in Raf-1, which is necessary for its interaction with Rap1 and Ras [12,40], is replaced by a histidine. It is noteworthy that the identity between RGS12 and RGS14 in this region extends much further towards the C-terminus of the molecule than the identity with Raf kinases (Figure 3), suggesting that some other protein–protein interaction of RGS12 and RGS14 with an as yet unidentified molecule may also be conserved. Whether these sequence similarities underlie shared functional properties between RGS12 and RGS14 is an attractive hypothesis that deserves careful experimental attention.

Since the biochemical function of RGS proteins is to stimulate the GTPase activity of many α subunits of heterotrimeric G-proteins, we used biochemical techniques to test whether such was also the case for RGS14, and whether this protein exhibited preferences among $G\alpha$ subunits. By investigating the binding of purified recombinant RGS14 protein to $G\alpha$ subunits in isolated brain membranes, we showed that it exhibits a strong preference for $G\alpha_o$ over other $G\alpha$ subunits tested. Such a result is not a mere reflection of the fact that G_o is the most abundant G-protein in brain, since the presence other G-proteins was similarly detected in brain crude membranes. Vast overexposure of the film revealed weak binding to $G\alpha_{13}$, and a barely detectable interaction with $G\alpha_{11}$ and/or $G\alpha_{12}$ (the antibody detects both proteins) (results not shown). This preference of RGS14 for $G\alpha_o$ was confirmed in GTPase stimulation experiments: RGS14 catalytically activates the GTPase activities of $G\alpha_o$ and $G\alpha_{11}$ subunits, but exhibits at least a 10-fold greater efficiency with $G\alpha_o$. In a parallel study, the

group of John Kehrl has similarly found that RGS14 can stimulate the GTPase activity of several $G\alpha_i$ subunits *in vitro*, and that ectopic expression of RGS14 can down-regulate cellular responses to stimuli that are transduced via $G\alpha_i$ subunits; furthermore, they show evidence that RGS14 can interact with another $G\alpha$ protein, $G\alpha_{13}$, and act as an effector antagonist (H. Cho and J. Kehrl, personal communication). While we have detected only a very weak interaction of RGS14 with $G\alpha_{13}$ in intact brain membranes, it is possible that, in cells overexpressing both RGS14 and $G\alpha_{13}$, such an interaction would be more readily measurable, and that in a cell model where both proteins are abundantly expressed such an interaction could be physiologically relevant. Among the many RGS proteins investigated, several exhibit preferences in their interactions with $G\alpha$ subunits. For instance, GAIP interacts specifically with $G\alpha_{13}$ [47], RGS2 with $G\alpha_q$ [48], p115 RhoGEF with $G\alpha_{12}$ and $G\alpha_{13}$ [7], RGSZ1 with $G\alpha_z$ [49], and RGS9 with $G\alpha_i$ [50]. To our knowledge, RGS14 is the first RGS protein demonstrated to exhibit such a marked preference for $G\alpha_o$.

Given the biochemical interactions that we have identified between RGS14 and the α subunits of heterotrimeric G-proteins such as G_o , as well as the Ras-related Rap GTPases, it is tempting to speculate that RGS14 may constitute a link by which members of these two classes of G-proteins could cross-regulate each other's activities. The simplest and most attractive hypothesis would be that Rap proteins directly affect the ability of RGS14 to bind $G\alpha_o$ or to stimulate its GTPase activity. We tested both these possibilities *in vitro* using recombinant purified proteins, and could not demonstrate any effect of Rap1 or Rap2 on either of these two parameters. We also tested whether RGS14, alone or in the presence of purified $G\alpha_o$, was able to modulate the GTP-binding or GTPase activities of purified recombinant Rap proteins, but also failed to detect any effect (results not shown). However, these negative results cannot constitute definite proof. One would have to reconstitute these interactions with prenylated Raps and myristoylated $G\alpha_o$ in liposomes of the appropriate lipid composition, or by ectopic expression in an adequate cellular model, in order to formally demonstrate or exclude these attractive, yet hypothetical, biochemical effects. Such experiments are presently under way.

The biochemical interactions that we have characterized between RGS14 and heterotrimeric G-proteins, as well as Ras-related Rap proteins, are only biologically relevant if they can actually occur *in vivo*. A prerequisite is that the proteins involved must be co-expressed in the same cells, and that at some point in time they should be present in the same, or in communicating, subcellular compartments. The expression of RGS14 is restricted to certain tissues, i.e. brain and spleen (the present work and [31]); brain is precisely the organ where $G\alpha_o$ is predominantly expressed [51], and also contains one of the highest levels of Rap proteins (J. de Gunzburg, unpublished work). We have shown by *in situ* hybridization that the expression of RGS14 mRNA in brain is restricted to neurons and exhibits a highly differential pattern among different regions, with strongest expression in the granular layer of the cerebellum and certain regions of the hippocampus. The expression pattern of $G\alpha_o$ is not dissimilar (Figure 8A, and [52,53]), and at least some cells in the hippocampus were shown to co-express mRNAs for RGS14 and $G\alpha_o$ (Figure 7H). Whereas the expression pattern of Rap1 mRNA is very different from that of RGS14 (Figure 8), the patterns of expression of RGS14 and Rap2 mRNAs are similar and, again, double labelling has shown that some hippocampal cells express both genes. Furthermore, in neurons from primary cultures of rat hippocampus cells, anti-RGS14 antibodies label an intracellular membrane compartment that is compatible with

the previously determined subcellular localizations of $G\alpha_o$ [54] and Rap2 [36] (S. Traver, G. Barbin and J. de Gunzburg, unpublished work). Hence the present study, based on biochemical experiments and the characterization of expression patterns, suggests the occurrence of physiological connections between the functional roles of RGS14, Rap2 and $G\alpha_o$ in signal transduction. We are in the process of establishing adequate cellular models to assess whether and how transduction pathways involving the Ras-related protein Rap2 could, via RGS14, regulate signalling from G-protein-coupled receptors transduced via proteins of the G_i family, and in particular $G\alpha_o$.

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