RGS18 is a myeloerythroid lineage-specific regulator of G-protein-signalling molecule highly expressed in megakaryocytes

David YOWE*[1], Nadine WEICH*, Mercy PRABHUDAS*, Louis POISSON*, Patrick ERRADA*, Rosanna KAPELLER*, Kan YU†, Laura FARON*, Minhui SHEN*, Jennifer CLEARY*, Thomas M. WILKIE†, Carlos GUTIERREZ-RAMOS* and Martin R. HODGE*

*Millennium Pharmaceuticals, 75 Sidney Street, Cambridge, MA 02139, U.S.A., and †Pharmacology Department, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, U.S.A.

Myelopoiesis and lymphopoiesis are controlled by haematopoietic growth factors, including cytokines, and chemokines that bind to G-protein-coupled receptors (GPCRs). Regulators of G-protein signalling (RGSs) are a protein family that can act as GTPase-activating proteins for $G_{\alpha i}$- and $G_{\alpha q}$-class proteins. We have identified a new member of the R4 subfamily of RGS proteins, RGS18. RGS18 contains clusters of hydrophobic and basic residues, which are characteristic of an amphipathic helix within its first 33 amino acids. RGS18 mRNA was most highly abundant in megakaryocytes, and was also detected specifically in haematopoietic progenitor and myeloerythroid lineage cells. RGS18 mRNA was not detected in cells of the lymphoid lineage. RGS18 was also highly expressed in mouse embryonic 15-day livers, livers being the principal organ for haematopoiesis at this stage of fetal development. RGS1, RGS2 and RGS16, other members of the R4 subfamily, were expressed in distinct progenitor and mature myeloerythroid and lymphoid lineage blood cells. RGS18 was shown to interact specifically with the $G_{\alpha i\text{-}3}$ subunit in membranes from K562 cells. Furthermore, overexpression of RGS18 inhibited mitogen-activated-protein kinase activation in HEK-293/chemokine receptor 2 cells treated with monocyte chemotactic protein-1. In yeast cells, RGS18 overexpression complemented a pheromone-sensitive phenotype caused by mutations in the endogeneous yeast RGS gene, *SST2*. These data demonstrated that RGS18 was expressed most highly in megakaryocytes, and can modulate GPCR pathways in both mammalian and yeast cells *in vitro*. Hence RGS18 might have an important role in the regulation of megakaryocyte differentiation and chemotaxis.

Key words: G-protein-coupled receptors, haematopoiesis, inflammation, megakaryocyte, platelets, regulators of G-protein signalling.

INTRODUCTION

Haematopoiesis is a life-long developmental process that involves the differentiation of haematopoietic stem cells, which are capable of both self-renewal and generation of lineage-specific progenitor cells [1–3]. At least eight major types of mature blood cells are produced from the terminal differentiation of committed lineage-specific progenitor cells [1]. The proliferation and differentiation of cells that give rise to all myeloid and lymphoid lineages are controlled by haematopoietic growth factors [4].

Cytokines are known to have a major role in the regulation of haematopoiesis by promoting the survival, proliferation and differentiation of haematopoietic stem and progenitor cells [2,5]. Several cytokines function primarily within lineage-committed cells, whereas others support cells of a variety of lineages [4]. Furthermore, a specific cytokine can mediate different responses in cells of different lineages, or at different stages of differentiation [4].

Ligands that bind to members of the seven-transmembrane-spanning G-protein-coupled receptor (GPCR) family also have a role in haematopoiesis. For example, chemokines (chemotactic cytokines in the 8–17 kDa range) can have dual effects on haematopoiesis, depending on the maturity of the progenitor cells, whereas others are known to suppress or have no effect on haematopoietic progenitor cell function and proliferation [6]. Intracellular signalling by chemokine receptors is mediated via pertussis-toxin-inhibitable heterotrimeric G-proteins, usually of the G_i subfamily [7].

Inactive heterotrimeric G-proteins are composed of α, β and γ subunits. Upon ligand binding, GPCRs stimulate G_α to release GDP and to bind GTP, resulting in the formation of active G_α-GTP and $G_{\beta\gamma}$, which regulate downstream effectors [8]. Signalling is terminated when the G_α subunit hydrolyses bound GTP to GDP, leading to reformation of inactive $G_{\alpha\beta\gamma}$ [8]. Cells can limit their response to heterotrimeric G-protein signalling by several mechanisms, including agonist removal from the extracellular fluid, receptor desensitization, endocytosis and down-regulation [9,10]. GTPase-activating proteins can also regulate heterotrimeric G-protein pathways by increasing the intrinsic GTPase activity of the G_α subunit [11,12].

Regulators of G-protein signalling (RGSs) are a recently discovered protein family that can act as GTPase-activating proteins for G_α subunits. At present, 21 mammalian RGS genes are known, which all contain the conserved RGS domain that binds to the G_α subunit and accelerates GTPase hydrolysis [12–14]. Several RGS proteins have been implicated in the function of mature myeloid or lymphoid cells on the basis of their expression pattern, intracellular location and their ability to inhibit *in vitro* chemotaxis, adhesion and/or intracellular signalling [15–21]. Recently, it was shown that mice lacking RGS2 exhibited reduced T cell proliferation and interleukin-2 production, as well as behavioural and neuronal defects [22]. No difference was observed in the differentiation of haematopoietic lineages, including neutrophils, macrophages, erythrocytes and platelets, between wild-type and RGS2-deficient mice [22]. The physiological role of other RGSs in both progenitor and mature

Abbreviations used: CCR, chemokine receptor; CD, cluster of differentiation; C_T, comparative threshold cycle; FAM, 6-carboxyfluorescein; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; RT-QPCR, reverse transcriptase-quantitative PCR; RGS, regulator of G-protein signalling.

[1] To whom correspondence should be addressed (e-mail yowe@mpi.com).

haematopoietic cell proliferation, differentiation and function is largely unknown.

In the present study we describe the isolation and functional characterization of RGS18. RGS18 was shown to be expressed specifically in the myeloerythroid lineage, and was also highly expressed in megakaryocytes. RGS1, RGS2 and RGS16 were also characterized in terms of their expression patterns in progenitor and mature myeloerythroid and lymphoid lineage blood cells. Studies demonstrated that overexpression of RGS18 could inhibit $G_{\alpha i\text{-}1}$ signalling pathways *in vitro*, and specifically interact with $G_{\alpha i}$ subunits. RGS18 may be involved in the regulation of megakaryocyte differentiation and chemotaxis, and in platelet activation.

MATERIALS AND METHODS

Source of tissue and cell samples

Human tissue samples from healthy individuals were obtained from the Millennium Pharmaceuticals Molecular Pathology Department. HEK-293, HL60, Jurkat and K562 cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), and were cultured according to the supplier's recommendations. Purified human adult bone marrow, cord blood and granulocyte-colony-stimulating-factor-mobilized peripheral blood cluster of differentiation (CD)34$^+$ cells were obtained from AllCells (Berkeley, CA, U.S.A.) with purities $> 95\%$. For myeloid cultures, purified granulocyte-colony-stimulating-factor-mobilized peripheral blood human CD34$^+$ cells were cultured at a density of $(1–2) \times 10^5$ cells/ml as follows. Briefly, erythroid progenitors were cultured in X-VIVO-10 (Biowhittaker, Walkersville, MD, U.S.A.) supplemented with 2% (w/v) BSA, 15% (v/v) fetal bovine serum, 20 ng/ml stem-cell factor (R&D Systems, Minneapolis, MN, U.S.A.) and 2–3 units/ml erythropoietin (R&D Systems). CD41$^+$ progenitors were grown in X-VIVO-10 containing 2% (w/v) BSA, 20 ng/ml stem-cell factor and 20 ng/ml thrombopoietin (R&D Systems). Fresh medium was added halfway through the culture period. By day 14, erythroid cultures were generated that were 80–90% glycophorin A$^+$, and after centrifugation consistently exhibited haemoglobin-rich 'red' pellets. In megakaryocyte day-14 cultures, more than 70% of the cells were CD41a$^+$. These cultures were purified by using magnetic cell-sorting technology (Miltenyi Biotec Inc., Auburn, CA, U.S.A.), using antibodies against cell-surface markers conjugated to magnetic micro-beads and passaged through separation columns. Purities of $> 93\%$ were obtained by the method of FACS.

Human peripheral blood mononuclear cells and granulocytes were isolated from whole blood using Ficoll-hypaque density-gradient (1.077 and 1.119 g/ml) centrifugation at 700 ***g*** for 30 min at 4 °C. Peripheral blood mononuclear cells were isolated from the interface between the plasma and the 1.077 g/ml Ficoll layer; granulocytes were isolated from the lower interface with a purity of 90%. Both cell layers were depleted of erythrocytes and washed before subsequent isolation of cell populations. Individual CD3$^+$, CD4$^+$, CD8$^+$ and CD14$^+$ cell populations were isolated using magnetic cell-sorting technology. Human tonsils were macerated, washed with RPMI1640, passed through a 70 μm filter and then centrifuged through a Ficoll density gradient. Cells present at the interface were washed with RPMI 1640 medium, and CD19$^+$ cells were isolated at a purity of $> 90\%$ by magnetic cell-sorting technology.

T helper-1 and -2 cells were differentiated by the method of Palmer and van Seventer [23]. Briefly, human peripheral blood CD4$^+$CD45RA$^+$ cells were cultured in plates containing immobilized anti-CD3 (10 μg/ml), JY cells and exogenous cytokines. For T helper-1 cells, the cultures were grown in the presence of 5 ng/ml interleukin-1β, 5 ng/ml interleukin-6, 20 ng/ml interleukin-12 and 5 μg/ml anti-(interleukin-4) antibody. T helper-2 cells were differentiated by culturing T cells in the presence of 5 ng/ml interleukin-1β, 5 ng/ml interleukin-6, 20 ng/ml interleukin-4 and 5 μg/ml anti-(interleukin-12) antibody. At weekly intervals, the cultures were centrifuged by Ficoll-hypaque density-gradient centrifugation to remove dead accessory cells, and were subsequently cultured with the above cytokines and antibodies. Activated T helper-1 and -2 cells were maintained in this manner for three cycles, and then re-activated using the cytokine combinations described above for the desired period of time. T helper-1 and -2 cultures were characterized by ELISA (Endogen Inc., Woburn, MA, U.S.A. and R&D Systems) for interferon-γ, interleukin-5 and interleukin-13 production.

cDNA cloning and sequencing

The Millennium Pharmaceuticals nucleotide database was searched for expressed sequence tags with similarity to the RGS domain [24]. Expressed sequence tags for human RGS18 and its mouse orthologue were identified, and 5′-rapid amplification of cDNA ends of spleen Marathon-ready cDNA libraries (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) was used to obtain full-length sequences. Several clones were sequenced in both directions using overlapping primers and an automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). DNAStar (DNAStar Inc., Madison, WI, U.S.A.) was used for sequence analysis.

Northern blot analysis

Human multiple-tissue mRNA blots (Clontech Laboratories Inc.) or mouse total-RNA Northern blots were hybridized with PCR products labelled with [α-^{32}P]dCTP using Ready-to-Go DNA labelling beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.). The primers used for human RGS18 PCR probe amplification were 5′-TCCGCTCAACCTACCC-TCCACAGT-3′ (forward) and 5′-TAAGAACCAGATCACTT-CTACACT-3′ (reverse); for mouse RGS18, the primers were 5′-GCGTCCGACAAAGCAGAGTGT-3′ (forward) and 5′-CAAAGCTTCTTCTTACTGACTG-3′ (reverse); and for mouse RGS16, the primers were 5′-ATGTGCCGCACC-CAGCCA-3′ (forward) and 5′-TCAAGTGTGTGAAGGCTC-3′ (reverse). Hybridization was performed overnight at 65 °C with 5×10^6 c.p.m./ml of probe in ExpressHyb (Clontech Laboratories Inc.), following by washing and then exposure to X-ray film (Kodak, Rochester, NY, U.S.A.) according to the manufacturer's instructions. The blots were rehybridized with a β-actin cDNA probe for normalizing the amount of mRNA loaded.

mRNA expression analysis and quantification by reverse transcriptase-quantitative PCR (RT-QPCR)

Real-time RT-QPCR was performed using the Taqman® strategy (PerkinElmer Biosystems, Foster City, CA, U.S.A.). Total RNA was isolated from tissues or cells using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, U.S.A.), treated with DNase I (Ambion Inc., Austin, TX, U.S.A.) for 1 h at 37 °C, phenol-extracted, and then the RNA was precipitated. cDNA was synthesized from 5 μg of total RNA using the Superscript kit and oligo(dT) (Gibco BRL, Rockville, MD, U.S.A.). A no-amplification control containing RNA without reverse transcriptase was prepared to ensure that the DNase I treatment was complete. No-tissue controls containing buffer and enzyme only were included. The expression levels of RGSs and the

Table 1 RT-QPCR probe and primer sequences

Probes were labelled with either FAM or VIC.

Human gene	Probe and primer sequences (5′ → 3′)
RGS1	FAM probe: AGCGCAGAAGGAATGTGCCAGTATGGAT
	Forward: TGGCTGAAGGGAATTAACAGATAGTAT
	Reverse: GGCCAAGCTGTTCACCCA
RGS2	FAM probe: AGCTGGTATCAGAACAGCTTCCCTCACTGTGT
	Forward: CAGGAAGCCAGTAACTGACTAGGAG
	Reverse: CCTATTCCCTTCTTGCGTTCTG
RGS16	FAM probe: TGATTGCATCCTGTTTTCCTAATTCCCAGA
	Forward: TTTGCCTGTTGCTCAGTTGACT
	Reverse: CCCTTCCGTGCCCAGAA
RGS18	FAM probe: TCATTCCTAAGTGAACATGGACGTACCCAGTTA
	Forward: TGACTGAGAATAAGATCCACATTTGAA
	Reverse: CATGTTTCTGTGACCAACAGAAGTACT
β2-Microglobulin	VIC probe: TATGCCTGCCGTGTGAACCACGTG
	Forward: CACCCCCACTGAAAAAGATGA
	Reverse: CTTAACTATCTTGGGCTGTGACAAAG

internal reference β2-microglobulin were measured by multiplex PCR using probes labelled with 6-carboxyfluorescein (FAM) or VIC® (PerkinElmer Biosystems) respectively. The primers and probes (Table 1) were designed using Primer Express software (PerkinElmer Biosystems). The simultaneous measurement of RGS-FAM and β2-microglobulin-VIC permitted normalization of the amount of cDNA added per sample. Duplicate PCRs were performed using the TaqMan® Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System (PerkinElmer Biosystems) using the following thermal cycle routine: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. A comparative threshold cycle (C_T) method (User Bulletin Number 2; PerkinElmer Biosystems) was used to determine gene expression relative to the no-tissue control (calibrator). Hence steady-state mRNA levels were expressed as an *n*-fold difference relative to the calibrator. For each sample, the RGS C_T value was normalized using the formula $\Delta C = C_{TRGS} - C_{T\beta 2\text{-microglobulin}}$. To determine relative expression levels, the following formula was used: $\Delta\Delta C_T = \Delta C_{T(1)sample} - \Delta C_{T(1)calibrator}$, and the value used to graph relative RGS expression was calculated using the expression $2^{-\Delta\Delta C_T}$. RNA samples were not used that exhibited FAM- or VIC-labelled RT-QPCR products with C_T values > 37 or > 24 respectively. This is because inaccurate and unreliable relative expression values might be obtained. All samples demonstrated C_T values $\leqslant 24$ for β2-microglobulin-VIC-labelled RT-QPCR products, indicating the samples contained undegraded cDNA that could be amplified.

Protein expression and purification of bead-attached fusion protein

The entire open reading frame of human RGS18 was PCR-amplified with *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) using primers which introduced *Bam*HI and *Hind*III restriction sites adjacent to the initiation and termination codons. The PCR product was directionally cloned into the pGEX (Amersham Pharmacia Biotech Inc.) plasmid, and then transformed into DH5α bacterial cells. Expression of glutathione S-transferase human RGS18 protein was induced by adding 1 mM isopropyl β-D-thiogalactoside to cultures with $D_{600} = 0.6$, which were harvested after incubation for 4 h at 37 °C. The cell pellet was resuspended in PBS containing 1 mM dithiothreitol and protease-inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN, U.S.A.), sonicated, and 1 % (v/v) nonylphenoxypolyethoxyethanol was added. After centrifugation, the supernatant was incubated for 1 h at 4 °C with glutathione S-transferase–agarose beads (Amersham Pharmacia Biotech Inc.). The beads were washed with 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % (v/v) nonylphenoxypolyethoxyethanol and 1 mM dithiothreitol. The bead-attached glutathione S-transferase human RGS18 fusion protein was stored at −80 °C.

RSG–G_α-protein binding assay

Cell extracts were prepared by lysing 5×10^7 cells in a solid CO_2/ethanol bath, and then Dounce-homogenized in 10 mM Tris/HCl/1 mM EDTA, pH 7.0. The suspension was passed through a 25-gauge needle several times, and then centrifuged at 3000 ***g*** for 10 min at 4 °C. The post-nuclear supernatant was centrifuged at 100000 ***g*** for 30 min at 4 °C, and the membrane pellet was solubilized in 1 % (w/v) cholate, 1 % (v/v) Triton X-100 and 6 mM $MgCl_2$. Cell membranes (400 μg) were incubated for 30 min at 30 °C with either 30 μM GDP or GDP·AlF_4^- (10 mM NaF/30 μM $AlCl_3$/30 μM GDP) in 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $MgSO_4$, 0.025 % deca(ethylene glycol) monododecyl ether ('$C_{12}E_{10}$'), 10 mM 2-mercaptoethanol and 10 % (v/v) glycerol. After incubation for 30 min on ice, bead-attached glutathione S-transferase RGS proteins were added, and the incubation was allowed to continue for 20 min at 30 °C. The beads were washed twice with 20 mM sodium-Hepes, pH 8.0, 380 mM NaCl, 3 mM dithiothreitol and 6 mM $MgCl_2$, supplemented with either 1 μM GDP or 1 μM GDP/30 μM AlF_4^-. Bound protein was eluted by incubation for 10 min at 100 °C in SDS/polyacrylamide gel buffer, and then analysed by immunoblotting with antibodies specific for individual G_α subunits (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A., and Calbiochem–Novabiochem Corp., La Jolla, CA, U.S.A.).

Yeast pheromone-response assay

The entire open reading frames of human RGS16 or RGS18 were PCR-amplified with *Pfu* polymerase (Stratagene) using primers that introduced *Bam*HI and *Sal*I restriction sites adjacent to the initiation and termination codons. The PCR products were directionally cloned into pYADE4 or pYPGE2 plasmids [25]. Standard yeast media containing synthetic complete medium lacking L-tryptophan were prepared, and yeast genetic manipulations were performed as described previously [26]. Yeast were transformed using standard protocols [27,28]. The MF41 (*MAT*α, *ura3-52*, *trp1*Δ1, *leu2-3,112 lys2-801*, *sst2::URA3*) transformants were subjected to desensitization assays by initially growing this strain to saturation overnight in liquid synthetic complete medium. Each culture was diluted 1:40 into liquid synthetic complete medium, which was added to an equal volume of 1 % (w/v) agar and warmed to 50 °C. The resulting cell suspension was plated on to synthetic complete medium plates, and allowed to solidify. Sterile blank paper quarter-inch disks (Becton Dickinson and Company, Franklin Lanes, NJ, U.S.A.) were spotted with different amounts of synthetic α-factor (Sigma, St. Louis, MO, U.S.A.), and were arrayed on top of the solidified agar plates. The diameter of the halos of growth-arrested cells surrounding the filter disks containing α-factor were photographed and measured after 24 h of growth.

p44/p42 mitogen-activated protein kinase (MAPK) activity assay

HEK-293 cells stably expressing the human chemokine receptor (CCR)2 were maintained in minimum essential medium Eagle's

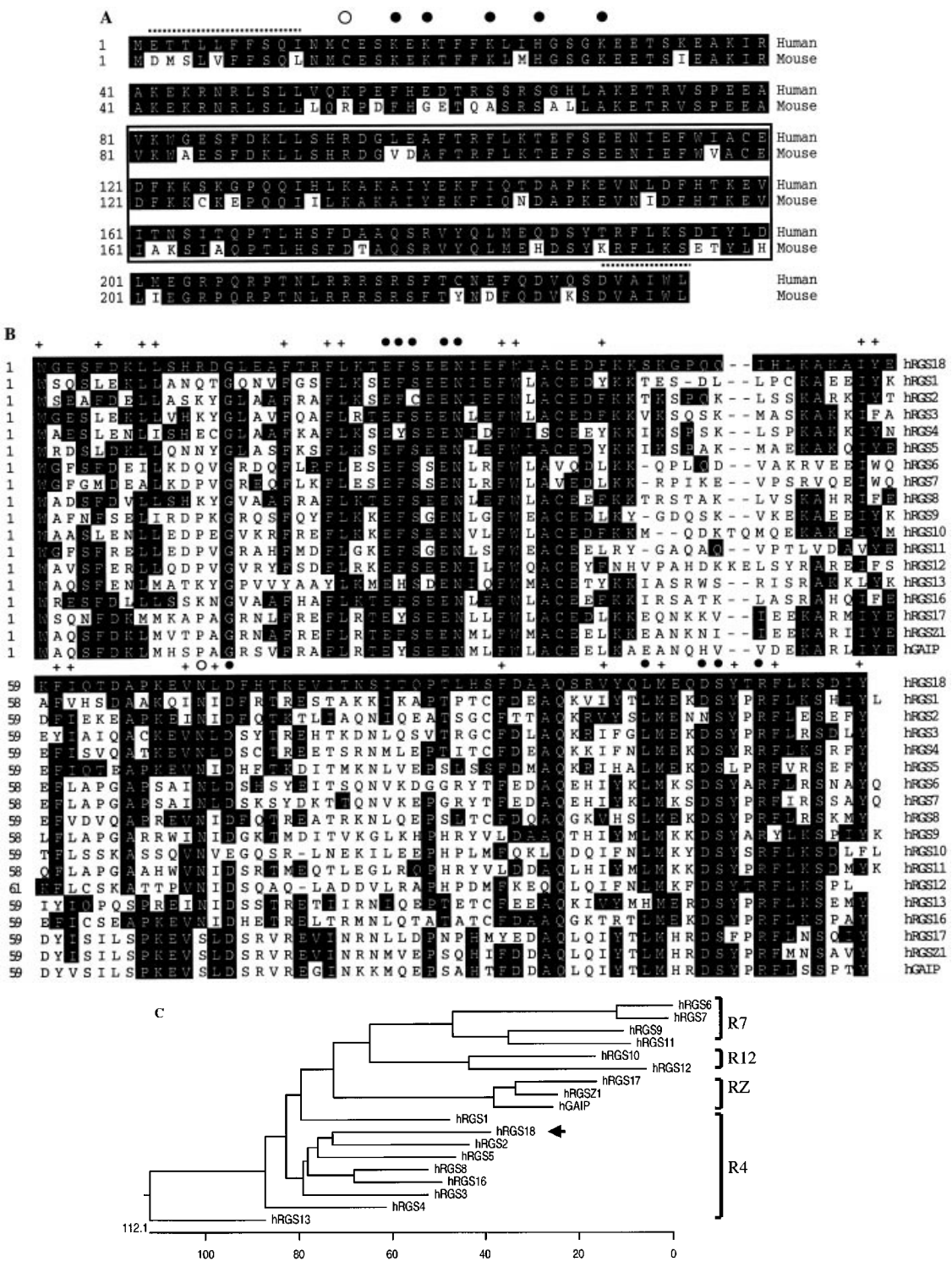

Figure 1 Human and mouse RGS18 amino acid sequences and comparison of RGS domains

(**A**) Alignment of the deduced human and mouse RGS18 amino acid sequences. The RGS domain is indicated by a box and major hydrophobic regions are indicated by dotted lines. A cysteine residue shown to be a secondary palmitoylation site (○) in RGS4 [32] and basic amino acids (●) within the first 33 residues of RGS18 are indicated. The human RGS18 sequence differs at amino acid positions 226 and 227 as compared with the human RGS18 amino acid sequence reported by Park et al. [30]. (**B**) Multiple alignment of human RGS domains using the Clustal method (DNAStar). Residues shown in reversed-out white lettering on a black background indicate amino acids that are conserved with RGS18. RGS4 residues [14] that make direct contact with $G_{\alpha i\text{-}1}$ (●) or form the RGS domain hydrophobic core (+), and the conserved Asp^{128} residue (○) are indicated. (**C**) Cladogram using the Clustal method for illustrating the relationship of RGS18 with the RGS domains of other human RGSs. The nomenclature of Ross and Wilkie [12] was used for describing the RGS subfamilies. Accession numbers for human are as follows: RGS1 (Q08116), RGS2 (P41220), RGS3 (P49796), RGS4 (P49798), RGS5 (AF159570), RGS6 (AF156932), RGS7 (AF090116), RGS8 (P49804), RGS9 (AAC64040), RGS10 (O43665), RGS11 (AF035153), RGS12 (AAC39835), RGS13 (AF030107), RGS16 (AAC39642), RGS17 (AAF08978), GAIP (Gα-interacting protein; GAAC62919), and RGSZ (RGS interacting with Gα-Z subunits; AF074979). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

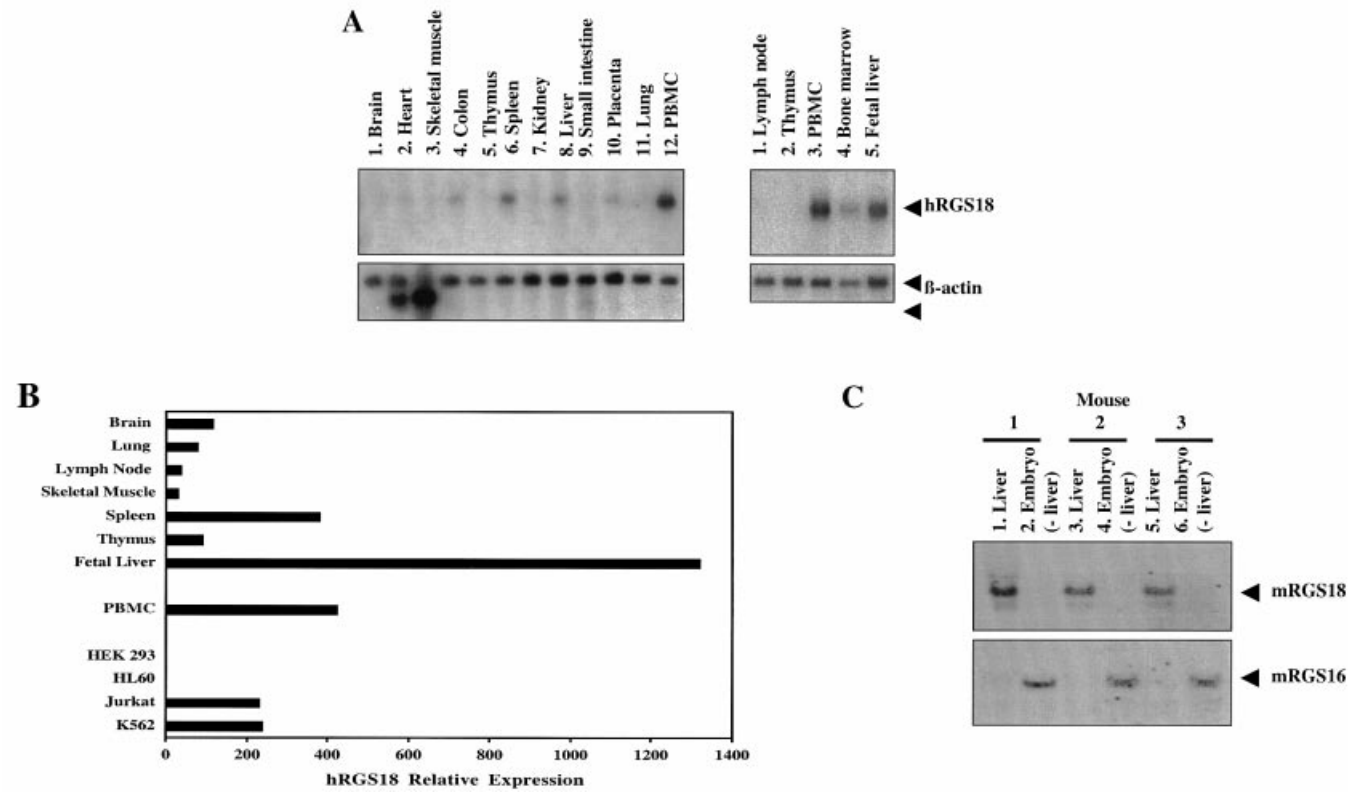

Figure 2 RGS18 is highly expressed in a subset of lymphoid tissues

(**A**) Northern blot of human tissues. The blots were stripped and reprobed with β-actin to normalize for RNA loading. PBMC, peripheral blood mononuclear cells. (**B**) RT-QPCR analysis of human tissue and cultured cell RNA samples. The average expression of duplicate RT-QPCR reactions relative to the no-tissue control are shown. The simultaneous amplification of human RGS18-FAM and β2-microglobulin-VIC-labelled products allowed for normalization of the amount of cDNA added. (**C**) Northern blot analysis of total RNA (10 μg/lane) isolated from three embryonic mouse day-14 livers or the whole embryo after liver removal (—liver). The blots were probed with mouse RGS18, stripped, and then reprobed with mouse RGS16.

(α modification) media (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 0.5 mg/ml G418. Cells (8×10^5) were plated per well (10 cm^2 surface area) of six-well plates coated with poly(L-lysine) (Sigma) and transiently transfected with 2 μg of pCDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.), pcDNA3.1-human RGS16, pcDNA3.1-human RGS18 or pPCI-β-galactosidase using LIPOFECTAMINE™ 2000 (Gibco BRL). Transfection efficiencies in the range of 80–90% were routinely obtained, as determined from β-galactosidase staining. The RGS constructs were made by *Pfu* polymerase amplification of the human RGS16 or RGS18 open reading frames, and these were then cloned into pcDNA3.1. After transfection, the cells were incubated for 24 h in the medium described above [except that 0.5% (v/v) fetal bovine serum was used], washed in PBS, and then were incubated for an additional 24 h with no fetal bovine serum. HEK-293/CCR2 cells were treated with 5 ng/ml monocyte chemotactic protein-1 (R&D Systems) for 10 min, then PBS washed. Cell lysates were prepared and assayed using the non-radioactive p44/42 MAPK assay kit (New England Biolabs, Beverly, MA, U.S.A.) according to the manufacturer's instructions. Briefly, agarose-bead-immobilized phospho-p44/42 MAPK (Thr^{202} and Tyr^{204}) monoclonal antibody was used to immunoprecipitate active MAPK from cell extracts. This immunoprecipitate was incubated with glutathione S-transferase–Elk-1 (residues 307–428) fusion protein, and the phosphorylation of this protein was measured by immunoblotting with a polyclonal phospho-Elk-1 (Ser^{383}) antibody and subsequent detection of chemiluminescence. For normalization of protein loading, total cell extracts were immunoblotted with a monoclonal antibody against total p42/Erk-2 (Santa Cruz).

RESULTS

RGS18 sequence analysis and comparison with known RGSs

The Millennium Pharmaceuticals nucleotide database was searched for expressed sequence tags derived from lymphoid tissues with similarity to the RGS domain [29]. Expressed sequence tags for a novel human RGS and its mouse orthologue were identified, and 5′-rapid amplification of cDNA ends from spleen libraries were used to obtain full-length sequences. An open reading frame of 235 amino acids was deduced, and the protein was predicted to have a molecular mass of 27.6 kDa (Figure 1A). The mouse RGS clone isolated in this work was found to be identical with the mouse RGS18 sequence published recently by Park et al. [30]. However, the human RGS clone isolated in this study differed at amino acid positions 226 (Asp instead of Met) and 227 (Val instead of Leu) (Figure 1A) in comparison with the human RGS18 amino acid sequence reported by Park et al. [30]. The human RGS18 sequence reported in this work is most likely to be correct because the residues aspartate and valine are also present in mouse RGS18 at positions 226 and 227 respectively (Figure 1A, and Park et al. [30]). The open reading frames of human and mouse RGS18 are 84%

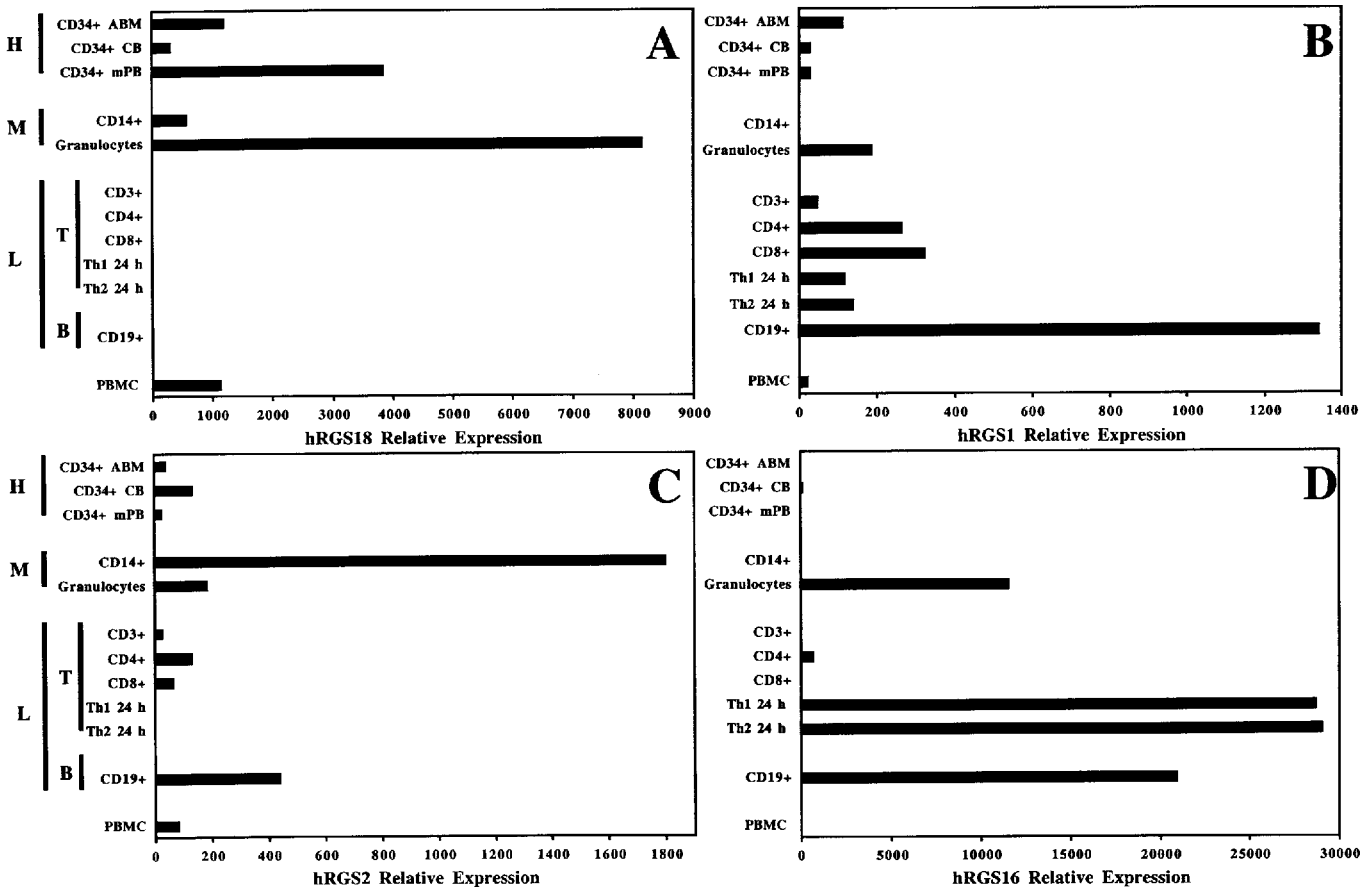

Figure 3 RGS18 is specifically expressed in human CD34$^+$ haematopoietic stem and mature myeloid lineage cells

RT-QPCR analysis was used to determine the average relative mRNA expression levels of (**A**) RGS18, (**B**) RGS1, (**C**) RGS2 and (**D**) RGS16 in human cell samples. The cells were grouped according to being a member of the haematopoietic stem cell (H), myeloid (M) or lymphoid (L) lineages, with subtypes being either B (B) or T (T) cells (lineages/cells are shown on the left-hand side of the Figure, with vertical lines indicating the relevant bars of the charts). The simultaneous amplification of the respective RGS and β2-microglobulin RT-QPCR products allowed for normalization of the amount of cDNA added. All reactions were performed in duplicate and are relative to the no-tissue control. CD34$^+$ cells were isolated from adult bone marrow (ABM), cord blood (CB) or mobilized peripheral blood (mPB). Peripheral blood mononuclear cells (PBMC) and all other cell types shown were isolated from peripheral blood, except for CD19$^+$ and T helper (Th)1 and 2 cells, which originated from tonsil and CD4$^+$CD45RA$^+$ *ex vivo* cultures respectively.

identical at the amino acid sequence level (Figure 1A). This high degree of identity was observed across the entire coding sequences, suggesting they are orthologues in view of the fact that different RGS paralogues exhibit a high degree of identity only within the RGS domain [31].

When the human RGS18 amino acid sequence was used to search protein databases with BLASTP, the top matches were RGS2 and RGS5, with 58 and 55% sequence identity respectively. Importantly, this identity was restricted to the RGS domain of these proteins. The N-terminal sequence of RGS18 was not conserved with other RGSs or genes currently present in protein databases (results not shown). However, RGS18 does contain features within its first 33 amino acids that have been shown in RGS4 [32] to be involved in the interaction of this RGS with the plasma membrane. These features include one of two cysteine residues shown to be palmitoylation sites in RGS4 [32], and clusters of hydrophobic and basic residues that are characteristic of an amphipathic helix (Figure 1A).

The RGS domain was located between amino acids 81–200 (Figure 1A), and exhibited 33–57% identity with other RGSs (Figure 1B). Importantly, 93% of the residues that have been shown in RGS4 [14] to either make direct contact with $G_{\alpha i-1}$ or form the RGS-domain hydrophobic core (Figure 1B) are present in RGS18. The RGS domain of RGS18 also contained the conserved Asn^{128} (Figure 1B), which was postulated in RGS4 to serve a catalytic role by interacting with the G_α side chain of Gln^{204} or the hydrolytic water molecule [14]. Mammalian RGSs have been grouped into five subfamilies (RZ, R4, R7, R12 and RA) on the basis of the sequence identity of their RGS domains [12]. Clustal analysis of known RGSs with human RGS18 showed that this RGS belongs to the R4 subfamily, and is in the same branch as RGS2 (Figure 1C).

Expression pattern of RGS18 in tissue- and cell-culture lines

mRNA from non-lymphoid and lymphoid tissues were screened to determine the expression profile of RGS18. Northern blot analysis detected an RGS18 mRNA transcript of approx. 2.4 kb, which was most abundant in human peripheral blood mononuclear cells, fetal liver, spleen and bone marrow (Figure 2A). Lower levels of RGS18 were detected in adult human liver, heart, colon and placenta (Figure 2A). Interestingly, no expression was detected in the thymus and lymph nodes (Figure 2A). Importantly, this pattern of mRNA expression was also consistent with RT-QPCR analysis of other human tissue samples (Figure

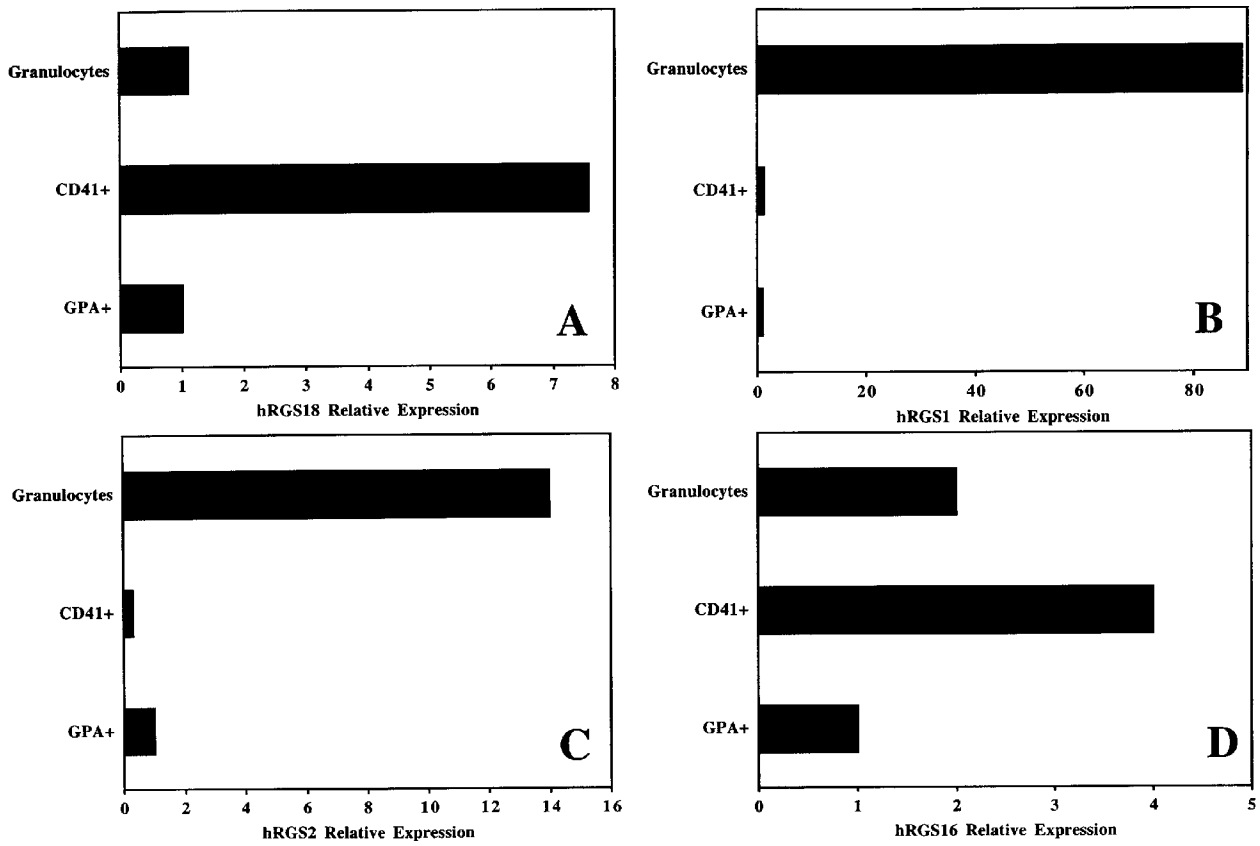

Figure 4 RGS18 is highly expressed in human megakaryocytes

RT-QPCR analysis was used to determine the average relative mRNA expression of human (**A**) RGS18, (**B**) RGS1, (**C**) RGS2 and (**D**) RGS16 in granulocytes, and *ex vivo* megakaryocyte ($CD41^+$) and erythroid glycophorin A^+ (GPA^+) day-14 cultures. The amount of cDNA added for RT-QPCR was normalized by the simultaneous amplification of the respective RGS and β2-microglobulin. All reactions were performed in duplicate and are relative to the no-tissue control.

2B). RGS18 was highly expressed in K562 (chronic myelogenous leukaemia) and Jurkat (acute T cell leukaemia line) cells, whereas negligible expression was detected in HEK-293 (transformed embryonal kidney line) and HL60 (pro-myelocytic line) cell-culture lines (Figure 2B). mRNA from embryonic day-14.5 mice was analysed to extend the observation of the relatively high RGS18 mRNA levels in fetal human liver. RGS18 expression was only observed in the fetal mouse liver (Figure 2C), which is the principal organ for haematopoiesis at this stage of fetal development [1]. RGS18 expression was not detected in the rest of the mouse embryo, whereas the opposite expression pattern was observed for RGS16 (Figure 2C).

Expression pattern of RGSs in progenitor, myeloerythroid and lymphoid blood lineages

The relative expression levels of RGSs 1, 2, 16 and 18 in human leucocyte antigen populations were estimated by RT-QPCR to determine if any of these RGSs exhibited a blood-lineage-specific expression pattern. These RGSs were screened because they have been reported previously to be expressed in leucocytes [15,16, 19,21,33].

RGS18 exhibited high levels of relative expression in $CD34^+$ haematopoietic stem cells (Figure 3A), and myeloerythroid lineage granulocyte and glycophorin A^+ (erythroid) cells (Figure 4A). However, the highest levels of RGS18 were observed in $CD41^+$ (megakaryocytes) cells which exhibited approximately 6-fold higher levels as compared with other myeloerythroid lineages (Figure 4A). No RGS18 expression was detected in naïve lymphoid cells, activated T helper-1 and-2 cells (Figure 3A) or activated $CD4^+$ and $CD19^+$ cells (results not shown). Conversely, RGS1 was expressed more highly in the lymphoid lineage, with the highest levels detected in $CD19^+$ cells (B lineage cells) (Figures 3B and 4B). Lower RGS1 expression levels were observed in naïve or activated T cells (Figure 3B). In myeloerythroid cells, negligible RGS1 expression was observed in $CD14^+$ (myelomonocytic lineage), $CD41^+$ and erythroid cells, although expression was detected in granulocytes, which was comparable with that observed in T cells (Figures 3B and 4B). RGS2 was most highly expressed in $CD14^+$ cells, with lower levels observed in other myeloerythroid lineages (Figures 3C and 4C). RGS2 was also expressed at low levels in naïve lymphoid cells, but not in T helper-1 and -2 activated cells (Figure 3C). Haematopoietic stem cells also exhibited low RGS1 and RGS2 expression levels (Figure 3). In contrast with the other RGSs screened, negligible RGS16 expression levels were observed in haematopoietic stem cells (Figure 3D). Furthermore, high RGS16 expression levels were detected in both myeloerythroid and lymphoid lineages, with the highest expression levels observed in activated T helper-1 and -2, $CD19^+$ and $CD41^+$ cells (Figures 3D and 4D).

RGS18 modulates G_α-signalling pathways in yeast and mammalian cells

The yeast pheromone-response assay was used to determine if RGS18 contained a functional RGS domain that could modulate

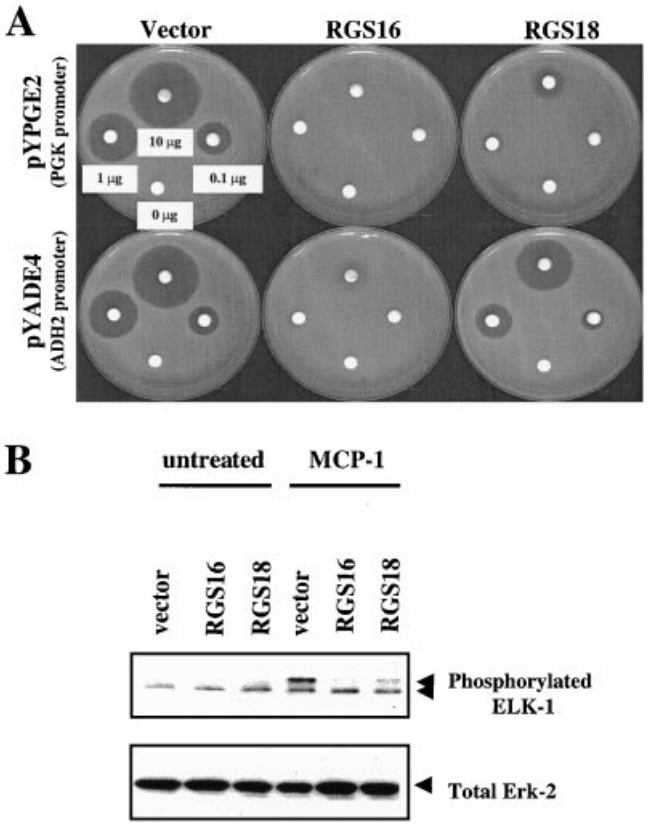

Figure 5 Overexpression of human RGS18 inhibits G_{α}-signalling pathways in yeast and mammalian cells

(**A**) Yeast pheromone-response assay: pYPGE2 or pYADE4 constructs containing the open reading frame of RGS16 or RGS18 were transformed into the *SST2*-deficient strain MF41 (*MAT*α, *ura3–52*, *trp1*Δ*1*, *leu2*–3,*112*, *lys2–801*, sst2::URA3). Sterile paper disks were spotted with 0, 0.1, 1 or 10 μg of synthetic α-factor. The diameter of the halos of growth arrested cells surrounding the filter disks containing α-factor were photographed and measured after 24 h of growth. The data are representative of two independent experiments. (**B**) MAPK assay: HEK-293 cells stably expressing the human CCR2 receptor were transiently transfected with pCDNA3.1, pcDNA3.1-human RGS16 or pcDNA3.1-human RGS18 constructs. Transfection efficiencies $> 80\%$ were routinely obtained as judged by β-galactosidase staining. The cells were incubated for 24 h in media containing 0.5% (v/v) fetal bovine serum, then for an additional 24 h in media containing no fetal bovine serum. The cells were treated with 5 ng/ml monocyte chemotactic protein-1 for 10 min and lysed. Active MAPK was immunoprecipitated from cell extracts, incubated with Elk-1 (residues 307–428) fusion protein, and phosphorylation of this protein was measured by immunoblotting with a polyclonal phospho-Elk-1 (Ser^{383}) antibody and chemiluminescence detection. Total cell extracts were immunoblotted with a monoclonal antibody to total p42/Erk-2 for normalization of protein loading. The data are representative of three independent experiments.

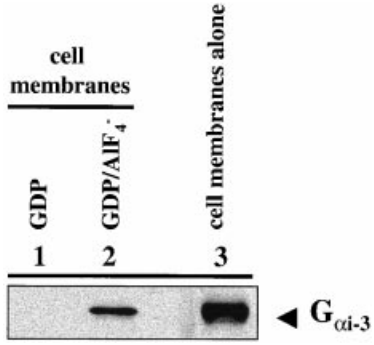

Figure 6 RGS18 interacts specifically with the $G_{\alpha i\text{-}3}$ protein in K562 cell-membrane extracts

The RSG–G-protein binding assay was performed by pre-incubating K562 cell membranes with either GDP or GDP·AlF_4^-, then with bead-attached glutathione S-transferase–human RGS18 protein. Bound protein was eluted and analysed by immunoblotting with antibodies specific for individual G_{α} proteins. Cell-membrane extracts were also immunoblotted to determine the endogeneous levels of the different G_{α} proteins analysed.

a G_{α}-signalling pathway. This assay was used because previous studies [34,35] have shown that mammalian RGSs can complement the yeast pheromone-sensitive phenotype caused by mutations in the RGS gene, *SST2*. *SST2* mutations cause increased sensitivity and defective desensitization induced by α-factor mating pheromone, in which yeast lacking the *SST2* gene product fail to resume growth after exposure to α-factor, whereas *SST2* overexpression markedly enhances the recovery rate from this pheromone-induced arrest.

SST2-deficient yeast transformed with pYPGE2 alone resulted in a growth-inhibition zone of 3.5 cm in diameter in the presence of 10 μg of α-factor (Figure 5A). This phenotype was complemented by the overexpression of either RGS18/pYPGE2 or RGS16/pYPGE2, in which decreased zones of inhibition were observed (Figure 5A). Lower levels of complementation were observed when RGS18 or RGS16 were under the control of the *ADH2* (alcohol dehydrogenase 2) promoter in the vector pYADE4 (Figure 5A). Since the glucose-activated *PGK* (phosphoglycerate kinase) promoter is reported to be 17 times stronger than the *ADH2* promoter [25], these results suggested that α-factor resistance conferred by RGS18 or RGS16 expression is a function of their RGS expression levels.

MAPK activity was measured in HEK-293 cells stably expressing the chemokine receptor CCR2 for determining the effect of RGS18 transfection on mammalian $G_{\alpha i}$-linked intracellular signalling pathways. Relative to untreated controls, a decrease in MAPK activity was observed in monocyte chemotactic protein-1-stimulated HEK-293/CCR2 cells transiently transfected with RGS18 or RGS16 as compared with vector alone (Figure 5B). The presence of a higher-molecular-mass band upon chemokine treatment could be due to hyperphosphorylation of Ser^{383} and Ser^{389} in Elk-1 [36], a change in its net charge, or some conformational change retained under the electrophoresis conditions used. A similar decrease in MAPK activity was also observed in stromal-cell-derived factor-1α-stimulated HEK-293/CXCR4 cells transiently transfected RGS18 or RGS16 (results not shown).

Interaction of RGS18 with K562 cell-membrane-derived G_{α} subunits

The RGS–G_{α}-protein binding assay [37] was used to identify the G_{α} subunits from membrane extracts with which RGS18 can interact specifically. Membranes were prepared from the human chronic myelogenous leukaemia cell line K562. This cell line was used because it is of myeloid origin and allowed for sufficient isolation of membranes for this study. $G_{\alpha i\text{-}3}$ was the only subunit detected to interact with bead-attached glutathione S-transferase–RGS18 (Figure 6). Importantly, this interaction only occurred in the presence of GDP and AlF_4^- (Figure 6), suggesting that this RGS has a high affinity for the $G_{\alpha i\text{-}3}$ subunit in its transition state [14]. The inability to detect an interaction between $G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$, $G_{\alpha o}$ or $G_{\alpha q}$ with RGS18 was most likely due to their low endogenous levels in K562 cells (results not shown).

DISCUSSION

This study has described the characterization of RGS18, which was found to be myeloerythroid-specific and highly expressed in megakaryocytes. Structurally, RGS18 was characterized by its relatively small molecular mass and unique short sequences that flanked a C-terminal RGS domain. Mammalian RGSs have been grouped into five subfamilies (RZ, R4, R7, R12 and RA) on the basis of sequence identity among their RGS domains [12]. Sequence comparison of its RGS domain placed RGS18 within the R4 subfamily. The amino acid structure of RGS18 was consistent with other R4 subfamily members [12] in terms of an RGS domain with relatively short stretches of flanking amino acids, an N-terminal amphipathic helix and the absence of other known subdomains. All genes in the R4 family (except for RGS3) co-segregate in a tight cluster on mouse chromosome 1, with RGS18 located in a microcluster most closely linked with RGSs 1 and 2 (N. Jenkins and T. M. Wilkie, unpublished work).

RGS18 is most likely to be membrane-associated due to the presence of a highly hydrophobic region and a cluster of basic residues within its first 33 amino acids. This amino-acid arrangement is characteristic of an amphipathic helix, and might be important for the receptor-selective binding of RGS18. Zeng et al. [38] showed that the analogous N-terminal domains in RGSs 4 and 16 confer receptor-selective inhibition of G-protein signalling, and proposed that this region mediates high-affinity interaction with $G_{\alpha q}$-coupled receptors. Furthermore, the presence of this N-terminal polybasic domain has been shown to be important in RGS plasma membrane localization by a mechanism likely to involve electrostatic interactions between RGS basic residues and plasma membrane anionic phospholipids [32,39]. The covalent attachment of lipids to cysteine residues is another mechanism by which proteins can be associated with the plasma membrane [32]. RGS18 contains one of the two cysteine residues shown to be palmitoylated in RGS4, although this lipid modification is not required for RGS plasma membrane targeting [32,39].

To understand the biological role of RGS18, an extensive mRNA expression analysis was performed on fetal and adult tissues, as well as progenitor and mature cell populations representing different blood lineages. mRNA expression analysis suggested that RGS18 has an important role in haematopoietic stem-cell progenitor, myeloerythroid lineage and megakaryocyte development. RGS18 was highly expressed in mature cells of the myeloerythroid lineages, with the highest levels of expression observed in megakaryocytes. Conversely, no expression was detected in mature lymphoid lineage cells. RGS18 was also highly expressed in tissues that are the sites for haematopoiesis during different developmental stages [1], including fetal liver and adult bone marrow and spleen. Further support for a role in haematopoiesis was suggested by the expression of RGS18 in $CD34^+$ progenitor cells.

The expression pattern of RGS18 suggested that it has an important role in megakaryocyte biology. Megakaryocyte generation is a complex process that is dependent on the interaction of haematopoietic progenitor cells, growth factors and stromal elements [40]. The development of megakaryocytes can be divided into several stages, including proliferation of $CD34^+$ haematopoietic stem cells into rapidly proliferating megakaryoblasts and formation of morphologically identifiable megakaryocytes, which undergo nuclear endoreplication, followed by platelet shedding [40,41]. Thrombopoietin has been shown to be the primary regulator of megakaryocyte development and platelet production [40,42–44], although megakaryocytopoiesis can also be stimulated by several other cytokines [45–48].

Chemokines are also involved in the regulation of megakaryocytopoiesis by acting as either enhancers or inhibitors of this process. Several studies have shown that stromal-derived factor-1α can induce migration of progenitor and mature megakaryocytes, enhance adhesion of mature marrow megakaryocytes to the endothelium, induce intracellular calcium mobilization and actin polymerization, and act in concert with thrombopoietin to enhance colony-forming unit-megakaryocyte proliferation [41,49]. On the other hand, inhibitory effects on megakaryocyte and platelet development have been observed for other chemokines, including platelet factor 4, interleukin-8, macrophage inhibitory proteins-1α and -1β, and neutrophil-activating peptide-2 [50]. In regard to platelet activation, stromal-derived factor-1α and macrophage-derived chemokine can act as weak platelet agonists and enhance the response of platelets to ADP, adrenaline (epinephrine) or 5-hydroxytryptamine (serotonin).

Chemokine receptors belong to the GPCR family of proteins, and RGSs are known inhibitors of chemokine receptor-signalling pathways. Overexpression of RGS proteins in cultured cells impairs chemokine receptor-signalling pathways, including MAPK activation and calcium mobilization, and leads to inhibition of chemotaxis and adhesion [11,17,18,20,21,34]. Hence RGS18 might modulate the response of megakaryocytes and platelets to agonists or antagonists of chemokine receptors. Furthermore, the majority of chemokine receptors couple via $G_{\alpha i}$ proteins [11]. RGS18 was implicated in $G_{\alpha i}$-linked pathways because RGS18 could specifically bind and inhibit $G_{\alpha i}$ signalling pathways in both yeast and mammalian cells. Indeed, transplantation of bone marrow retrovirally infected with antisense RGS18 into lethally irradiated mice suggested that inhibition of this RGS *in vivo* has a significant effect on the reconstitution of platelet levels at about week-2 post-transplantation (D. Yowe, unpublished work). During this period, mice that were transplanted with bone-marrow cells expressing antisense mouse RGS18 exhibited approx. 40% higher platelet counts as compared with vector control, with no differences observed in the platelet volume or other leucocyte blood parameters (D. Yowe, unpublished work). These data suggested that RGS18 may be involved in GPCR pathways that are agonistic for platelet production, such as the stromal-derived factor-1α/CXCR4 pathway, because inhibition of RGS18 *in vivo* led to enhanced platelet levels.

The mRNA expression pattern of other RGSs was evaluated to understand their role in haematopoiesis in relation to RGS18, and to determine if they also exhibited lineage-specific expression patterns. RGSs 1, 2 and 16 were expressed in both myeloerythroid and lymphoid lineages, and at low levels in haematopoietic stem cells and megakaryocytes relative to granulocytes. These data suggested that the expression pattern of RGS18 is restricted uniquely to the myeloerythroid lineage, and is most highly expressed in megakaryocytes, at least among the RGSs studied.

The biological role(s) of RGSs in haematopoiesis are largely unknown. However, the results concerned with mRNA expression in this study and others [15–17,19,21,33] have implicated several RGSs as being important to the function of haematopoietic stem, myeloid and lymphoid lineage cells. Furthermore, the expression pattern of some RGSs, such as RGS18, are highly restricted to specific haematopoietic lineages. This suggested that certain RGSs may have a more dominant role in the regulation of specific blood cells. RGSs are known modulators of GPCR pathways. A large number of GPCRs have been implicated in haematopoiesis, including several chemokine receptors, thrombin, thromboxane A_2 and ADP. Hence RGSs probably have an important role in modulating the response of blood cells to ligands for these receptors during different stages of haematopoiesis.

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