

RGS13 Controls G Protein-Coupled Receptor-Evoked Responses of Human Mast Cells¹

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IgE-mediated mast cell degranulation and release of vasoactive mediators induced by allergens elicits allergic responses. Although G protein-coupled receptor (GPCR)-induced signals may amplify IgE-dependent degranulation, how GPCR signaling in mast cells is regulated remains incompletely defined. We investigated the role of regulator of G protein signaling (RGS) proteins in the modulation of these pathways in human mast cells. Several RGS proteins were expressed in mast cells including RGS13, which we previously showed inhibited IgE-mediated mast cell degranulation and anaphylaxis in mice. To characterize how RGS13 affects GPCR-mediated functions of human mast cells, we analyzed human mast cell lines (HMC-1 and LAD2) depleted of RGS13 by specific small interfering RNA or short hairpin RNA and HMC-1 cells overexpressing RGS13. Transient RGS13 knockdown in LAD2 cells lead to increased degranulation to sphingosine-1-phosphate but not to IgE-Ag or C3a. Relative to control cells, HMC-1 cells stably expressing RGS13-targeted short hairpin RNA had greater Ca²⁺ mobilization in response to several natural GPCR ligands such as adenosine, C5a, sphingosine-1-phosphate, and CXCL12 than wild-type cells. Akt phosphorylation, chemotaxis, and cytokine (IL-8) secretion induced by CXCL12 were also greater in short hairpin RGS13-HMC-1 cells compared with control. RGS13 overexpression inhibited CXCL12-evoked Ca²⁺ mobilization, Akt phosphorylation and chemotaxis. These results suggest that RGS13 restricts certain GPCR-mediated biological responses of human mast cells. *The Journal of Immunology*, 2008, 181: 7882–7890.

Mast cells elicit immediate-type hypersensitivity reactions by degranulating in response to Ag cross-linking of the high affinity receptor for IgE, FcεRI (1), and releasing proinflammatory mediators (2, 3). Mast cells also function in wound healing and host defense against pathogens through synthesis of cytokines and chemokines (4, 5). Immature mast cell progenitors circulate in the bloodstream and extravasate into tissues where they complete the maturation process (6–8). Mast cell mediators such as histamine, leukotrienes, prostaglandins, and chemokines recruit T lymphocytes and granulocytic inflammatory cells, increase vascular permeability, and elicit smooth muscle contraction (4, 9, 10).

Mast cell degranulation induced by FcεRI involves multiple tyrosine phosphorylation events including activation of the Src family kinase Lyn, which in turn phosphorylates and activates Syk kinase (11). Subsequent activation of PI3K and phospholipase Cγ

(PLCγ)⁶ induce Ca²⁺ efflux from the endoplasmic reticulum. Increased cytosolic Ca²⁺ promotes exocytosis and mast cell degranulation (12). In addition to IgE-Ag cross-linking, a number of physiological ligands including adenosine, prostaglandin E₂, sphingosine-1-phosphate (S1P), complement components C3a and C5a, and chemokines (13–16) that bind cognate G protein-coupled receptors (GPCRs) may either potentiate FcεRI-dependent mediator release or in some cases induce mast cell activation independently of IgE-Ag. Adenosine, acting on A_{2b} receptors, or CXCL12, the ligand for the chemokine receptor CXCR4, elicit synthesis of IL-8 by human mast cells (17, 18).

In contrast to the signaling route induced by IgE-Ag, GPCRs promote exchange of GTP on GDP-bound G_α subunits of heterotrimeric G proteins (G_{αβγ}), resulting in activation of both the G_α and G_{βγ} subunits (19). These G protein components stimulate distinct downstream effectors, including PLCβ and PI3Kγ, which may mediate GPCR-induced mast cell degranulation (13, 20). Members of the regulator of G protein signaling (RGS) protein family, which number greater than 30 in mammalian cells, impair G protein-dependent signaling through their GTPase accelerating (GAP) activity on G_α subunits, which hastens G protein deactivation (21). In some instances, RGS proteins may inhibit G protein-effector interactions to disrupt the downstream signaling (22). The highly conserved RGS domain mediates binding to G_α subunits and GAP activity (23).

We investigated the function of the RGS R4/B subfamily in human mast cells, which includes RGS1–5, 8, 13, 16, 18, and 21, because many of these proteins are enriched in hematopoietic cells. Studies of gene-targeted mice and RNA interference have begun to

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⁶ Abbreviations used in this paper: PLC, phospholipase C; S1P, sphingosine-1-phosphate; GPCR, G protein-coupled receptor; RGS, regulator of G protein signaling; shRNA, short hairpin RNA; BMMC, bone marrow-derived mast cell; siRNA, small interfering RNA.

elucidate the physiological function(s) of individual R4 RGS proteins in some organs (21). For example, studies of *Rgs1*^{-/-} mice and human cell lines expressing RGS1-specific short hairpin RNA (shRNA) have revealed that RGS1 controls B lymphocyte homing to lymph nodes and motility within the lymph node microenvironment by regulating G_αi2 signaling elicited by chemokines (24–26).

RGS13 is an R4 subfamily member that impairs both G_αi and G_αq-dependent signaling including chemokine responses in B cells (27, 28). Previously, we found that RGS13 unexpectedly attenuated IgE-mediated anaphylaxis of mice and degranulation of bone marrow-derived mast cells (BMMCs). This novel function of RGS13 was independent of its GAP activity. RGS13 reduced PI3K activation induced by IgE-Ag by physically interacting with the p85 α regulatory subunit of PI3K that associates with p110 α , β , and δ catalytic subunits. RGS13 appeared to block the association of PI3K with receptor complexes (29). In contrast to Fc ϵ RI, GPCRs activate the p110 γ catalytic subunit of PI3K, which does not associate with p85. Therefore, we hypothesized that RGS13 could also regulate GPCR-evoked responses of mast cells through its GAP activity or antagonism of G protein effectors. Knockdown of endogenous RGS13 in human mastocytoma HMC-1 cells enhanced their responsiveness to several GPCR ligands including CXCL12 and adenosine, resulting in increased chemotaxis and cytokine production. Transient knockdown of RGS13 in LAD2 cells increased degranulation to S1P. These data suggest that RGS13 may control the intensity of mast cell-driven allergic inflammation induced by certain serum and tissue factors independently of IgE.

Materials and Methods

Cell lines and cell cultures

HMC-1 cells were grown in IMDM supplemented with 10% FBS, penicillin, and streptomycin. The stable transfectants were grown under selection with 0.4 mg/ml geneticin. LAD2 cells were grown in Stem-Pro medium containing Stem-Pro supplement (Invitrogen), 100 ng/ml human stem cell factor (R&D Systems), and 100 ng/ml IL-6 (PeproTech).

Identification of RGS genes expressed in mast cells

Total RNA from various cell lines was isolated using the RNeasy mini kit (Qiagen), followed by DNase treatment. cDNA was generated from RNA using the Superscript RT II reverse transcription kit (Invitrogen). Specific primers designed for the various RGS genes are listed in Table I.

Real-time quantitative PCR

We derived human mast cells by culturing CD34⁺ cells from cord blood or adult peripheral blood isolated by magnetic bead selection (Miltenyi Biotec). Contaminating cells were mostly macrophages, which were removed with anti-CD11c beads. The remainder of the cells differentiated into mast cells (routinely >95% pure as determined by morphological criteria) after 6–8 wk of culture in medium containing 30% FBS (HyClone), stem cell factor and GM-CSF (100 ng/ml and 10 pg/ml, respectively; R&D Systems) and 2–4% of a 20-fold concentrate of conditioned medium derived from the immortalized MCM B cell line. Mononuclear cells were obtained from buffy coat byproducts from blood component donors (Massachusetts General Hospital). Basophils were isolated by basophil enrichment magnetic bead separation (Miltenyi Biotec). Monocytes were isolated using RosetteSep monocyte enrichment cocktail (StemCell Technologies). Monocyte-derived dendritic cells were cultured in the presence of 10 ng/ml hGM-CSF and hIL-4 (R&D Systems) for 5–7 days. RNA was prepared from cultured mast cells or isolated blood cell subsets. RNA from B cells and resting and activated T cells (pooled from multiple donors) was obtained from Clontech. Primers and probes for human *RGS13* were purchased from Applied Biosystems (catalog no. Hs 00243182). Total RNA (20 ng) was run per sample in a quantitative RT-PCR with Taqman One Step RT-PCR master mix. Data were normalized to *GAPDH* expression, and absolute quantitation was based on a standard curve of human mast cell RNA. Primers for *GAPDH* were forward: ACACCCACTCCTCCACCTTTG, reverse: CATACCAGGAAATGAGCTTGACAA, and probe: CTGGCATTGCCCTCAACGACCA.

RNA interference

To achieve transient knockdown of RGS13, LAD2 cells were transfected with either of 2 duplex small interfering RNAs (siRNAs) (Ambion siRNA ID no. 12298 (GGAACAUUCAGGAACCCAC) or Dharmacon ON-TARGETplus SMARTpool siRNA L-010340–09 (GGAGCAGUGACGAGAAU) (375 nM) for 48 h. In complete StemPro medium using Oligofectamine (Invitrogen) per the manufacturer's instructions. For stable knockdown of RGS13 in HMC-1 cells, seven cassettes consisting of the human U6 RNA polymerase promoter and RGS13-specific target sequences predicted to form shRNA were generated by PCR and first tested for their ability to knockdown endogenous RGS13 in Ramos B lymphocytes by immunoassay. The double-stranded oligonucleotide sequence most effective in reducing RGS13 content (GGATCCCATCTCTCTAGAGACTGTGGCTTGATATCCGGCCA-CAGTCTCTAGAGAGATTTTTTCCAAAAGCTT) was subcloned into the pRNAT-U6.1/Neo expression vector (GenScript). This construct or pRNAT-U6.1 containing a scrambled shRNA insert was electroporated into HMC-1 cells as described previously (30). In brief, cells were harvested, washed, and resuspended in PBS at a density of 10⁷ cells/ml. A mixture of 320 μ l of cell suspension and 30 μ l of DNA solution in 4-mm-wide cuvettes (Bio-Rad) was exposed to an electric pulse of 380V/960 microfarads, provided by a Gene Pulser electroporation device (Bio-Rad). After electroporation, the transfected cells were cultured with 6 ml of medium. Stable transfectants were selected by resistance to neomycin followed by limiting dilution. *RGS13* knockdown was assessed by RT-PCR from total RNA with the *RGS13*-specific primers: sense-GAAATGCTTTCACGAAGGGG and antisense-GCATGTTGAGTGGGTTACGAATG. RGS13 expression was evaluated by immunoblotting and immunocytochemistry using rabbit polyclonal anti-RGS13 Ab as described (28).

RGS13 overexpression and immunofluorescence

The plasmid encoding HA-RGS13 (pcDNA3.1-HA-RGS13) was obtained from University of Missouri Rolla, Guthrie Research Institute (Rolla, MO). This construct or the empty pcDNA3.1 vector was electroporated in HMC-1 cells to generate populations of RGS13-overexpressing transfectants or vector control cells by selection with neomycin followed by limiting dilution as for shRNA-expressing cells. For immunofluorescence studies, cells were fixed in cold acetone/methanol followed by sequential staining with anti-RGS13 and Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes/Invitrogen). Confocal images were obtained using a Leica SP2 laser scanning confocal microscope.

Degranulation

LAD2 cells were sensitized with biotinylated human IgE (100 ng/ml) for 3–4 h. Cells were washed with HEPES buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·2H₂O, 1.3 mM MgSO₄·7H₂O) containing 0.04% BSA to remove excess IgE followed by aliquoting into individual wells of a 96-well plate in triplicate (10,000 cells/well). Degranulation was triggered in the same buffer with Ag (streptavidin, 1–100 ng/ml) (Sigma-Aldrich) or indicated GPCR agonists: 1 μ M PGE₂ (Calbiochem); 10 μ M adenosine (Sigma-Aldrich); 1 μ M C5a (Sigma-Aldrich); 10–40 μ M S1P (Sigma-Aldrich); 5–500 ng/ml complement component C3a (Calbiochem) for 30 min at 37°C. Degranulation was monitored by the release of β -hexosaminidase into the supernatants and calculated as a percentage of the total content (cells and media) after cell activation.

Phosphorylation studies

Serum-starved HMC-1 cells were stimulated for indicated times with agonist followed by lysis in NuPAGE LDS sample buffer (Invitrogen) containing 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM β -mercaptoethanol, 10% glycerol, 1% Triton X-100, and a protease-phosphatase inhibitor mix (Roche). Proteins were separated by SDS-PAGE and immunoblotted as indicated. Abs used were purchased as follows: HA (clone 12CA5; Roche); β -actin (Sigma-Aldrich); pAkt (Thr308) or pAkt (Ser473), Akt (Cell Signaling Technology).

Calcium mobilization

Cells were plated overnight in a 96-well plate containing serum-free medium in triplicate, and intracellular Ca²⁺ concentration was measured using FLIPR calcium 3 assay kit and the FLEXStation II automated fluorometer according to the manufacturer's instructions (Molecular Devices/MDS Analytical Technologies).

Receptor expression analysis by flow cytometry

FITC- or PE-labeled Abs specific for human CXCR4, C5aR (CD88), and *c-kit* or isotype-matched controls were obtained from BD Pharmingen and A₂₁ Ab from Santa Cruz Biotechnology. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences). Data were analyzed by FlowJo software (Tri Star).

IL-8 secretion

IL-8 in cell supernatants was quantitated 24 h after addition of CXCL12 (100 ng/ml) using the DuoSet ELISA development system according to the manufacturer's instructions (R&D Systems).

Chemotaxis

Chemotaxis was analyzed using 8- μ m pore size 96-well ChemoTx system (NeuroProbe) per the manufacturer's instructions. Cells were allowed to migrate in the presence of chemokine in the lower chamber for 2 h followed by quantitation by hemocytometry.

Statistical analysis

Sigma Plot software was used to determine statistical significance by Student's *t* test for two groups or ANOVA for multiple groups. Values of *p* < 0.05 were considered significant. Immunoblots were quantitated by densitometry using Quantity one software (Bio-Rad).

Results

Mast cells express multiple RGS proteins

Microarray analysis revealed expression of several *RGS* genes in cord blood-derived human mast cells, including (in decreasing amounts) *RGS19*, *13*, *2*, *17*, *1*, *10*, and *1*, whereas mast cells derived from peripheral blood progenitor cells after 6 wk in culture expressed *RGS13* most abundantly, and *RGS2*, *1*, and *17*, and *10* in lesser amounts (data not shown). Quantitative PCR of RNA from peripheral blood cell subsets showed that *RGS13* mRNA was much more abundant in mast cells than other hematopoietic cells including basophils, monocytes, B and T lymphocytes, and dendritic cells (Fig. 1). In addition, the microarray analysis showed that older mast cells expressed more *RGS13* than immature mast cells, with almost a 10-fold increase from 2 to 6 wk of culture. IgE-Ag stimulation of cultured human mast cells also increased *RGS13* expression in cord blood-derived mast cells, similar to the up-regulation of *RGS13* by IgE-Ag stimulation of BMMCs (29). Because *RGS13* was preferentially expressed in human mast cells compared with other hematopoietic cell types, we evaluated *RGS13* further as a potential regulator of mast cell-dependent allergic inflammation.

The difficulty in manipulating gene expression by transfection of primary human mast cells as well as their long maturation process

and limited lifespan lead us to use LAD-2 and HMC-1 mast cell lines to examine the role of *RGS13* in GPCR-mediated signaling in human mast cells. We first analyzed expression of the R4 *RGS* family members in HMC-1 cells using gene-specific primers. RT-PCR demonstrated that *RGS5*, *10*, and *13* were relatively abundant in HMC-1 cells compared with *RGS1*, *2*, *3*, and *16*, and *RGS4* was not detected (Fig. 2A). We also identified *RGS13* protein in unstimulated HMC-1 cells by immunoblotting and immunohistochemistry (Figs. 2B and 3C). *RGS* abundance is often increased by GPCR agonists whose signaling pathways are then attenuated by the up-regulated *RGS* protein in a feedback loop (21). C5a and CXCL12 treatment of HMC-1 cells induced *RGS13* expression after 24 h whereas neither adenosine nor CCL11 (eotaxin) had much effect on *RGS13* content (Fig. 2A and data not shown). The latter finding can be partially explained by the fact that HMC-1 cells express low CCR3, the receptor for CCL11 (31). These results suggested that *RGS13* may regulate, among others, both C5aR (CD88) and CXCR4-evoked signaling pathways in mast cells.

Reduction of *RGS13* expression in HMC-1 cells

We used RNA interference to permanently reduce *RGS13* content and thereby determine how *RGS13* controls GPCR-induced signals in HMC-1 cells. Transfection of cassettes containing the human U6 RNA polymerase promoter, which drove expression of shRNA sequences, in Ramos B lymphocytes, a Burkitt lymphoma cell line with high endogenous *RGS13* expression (24), revealed that *RGS13* was amenable to RNA silencing. One sequence in particular reduced *RGS13* in Ramos cells more than 80% compared with either a control shRNA containing a scrambled sequence or a luciferase-specific sequence (data not shown). We subcloned this shRNA oligonucleotide into a plasmid vector containing the U6 promoter and electroporated the construct into HMC-1 cells. We isolated several individual cell populations by antibiotic resistance followed by limiting dilution. Semiquantitative RT-PCR of *RGS13* mRNA from cells expressing the sh*RGS13* vector demonstrated at least 75% *RGS13* knockdown by densitometry analysis compared with control (shCTL) cells (Fig. 3A). Immunoblotting confirmed that *RGS13* protein was reduced in two transfectants expressing sh*RGS13* compared with cells expressing the scrambled control shRNA (Fig. 3B).

Many R4 *RGS* proteins display variable intracellular localization depending on the cell type and activation state of the cell (28, 32). Previously we detected a GFP-*RGS13* fusion protein in nuclear, membrane, and cytosolic fractions of transfected HEK293T

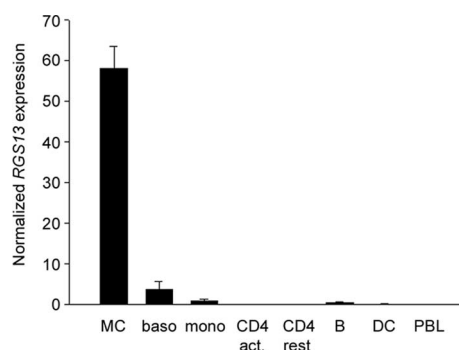


FIGURE 1. *RGS13* expression in human hematopoietic cells. Quantitative real-time PCR of relative *RGS13* expression in RNA derived from various human hematopoietic cell subsets compared with RNA from cultured mast cells. Data are the mean $\pm$ SD of *RGS13* expression normalized to *GAPDH* in RNA from 1 to 2 donors (or multiple donors pooled into a single sample for B and T cell RNA) assayed in two separate experiments.

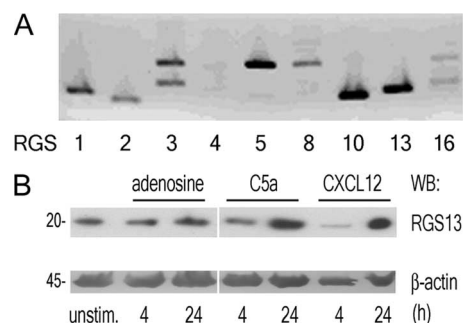


FIGURE 2. *RGS13* expression in HMC-1 cells. **A**, RNA from HMC-1 cells reverse transcribed and subject to PCR using *RGS*-specific primers as outlined in Table I. **B**, HMC-1 cells were left untreated or stimulated with adenosine, complement component C5a, or CXCL12 for the indicated times. Lysates were prepared and evaluated by immunoblotting with anti-*RGS13* Ab or anti- β -actin Ab to assess protein loading. Data are representative of three similar experiments.

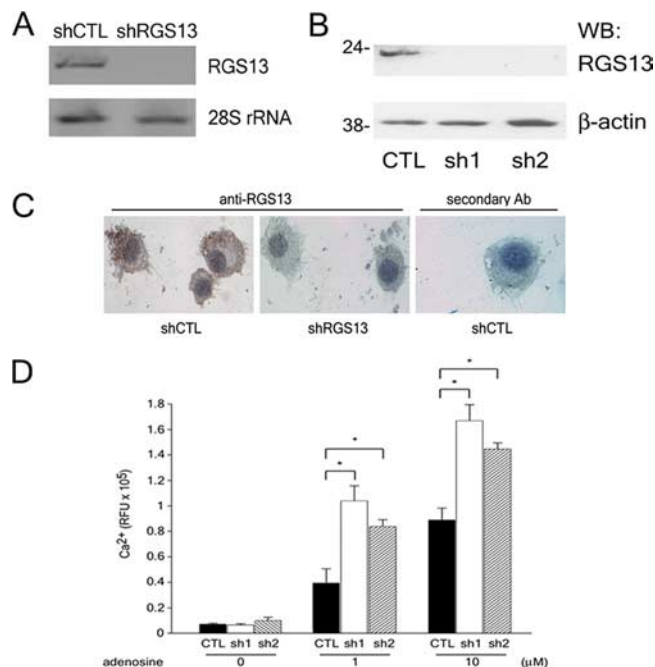


FIGURE 3. Knockdown of endogenous RGS13 in HMC-1 cells by RNA interference. *A–C*, HMC-1 cells expressing a plasmid vector containing a scrambled shRNA oligonucleotide (shCTL) or an RGS13-specific shRNA oligonucleotide (shRGS13) were evaluated for RGS13 expression by RT-PCR (*A*), immunoblot (*B*), or immunocytochemistry (*C*). shCTL cells were stained with secondary Ab alone as a control for anti-RGS13 immunostaining (*C*, right panel). *D*, Cells expressing control shRNA or two individual populations expressing RGS13-specific shRNA (sh1 and sh2) were loaded with a calcium-sensing dye, followed by stimulation with the indicated concentrations of adenosine and measurement of intracellular Ca^{2+} concentration by fluorometry (mean $\pm$ SEM, maximum-minimum value of relative fluorescence units (RFU) obtained from three independent experiments; (*, $p < 0.02$, one-way ANOVA).

cells by cell fractionation and immunoblotting with anti-GFP (27). However, immunofluorescent microscopy of B lymphocytes and BMMCs showed localization of RGS13 predominantly in the cytoplasm (28, 29). HMC-1 mast cells had mainly cytosolic and some nuclear RGS13 by immunocytochemistry (Fig. 3*C*, left panel). shRGS13-transfected cells stained considerably less with the RGS13 Ab (Fig. 3*C*, middle panel), which confirmed the reduced RGS13 expression suggested by immunoblot analysis.

RGS13 knockdown enhances GPCR-evoked calcium mobilization in HMC-1 cells

A general property of chemokine receptor signaling is the rise in intracellular Ca^{2+} observed upon agonist stimulation. $\text{G}_{\alpha q}$ induces intracellular Ca^{2+} mobilization by activating $\text{PLC}\beta$, which in turn generates inositol 1,4,5-trisphosphate (IP_3) (33). Chemokine receptors couple to $\text{G}_{\alpha i}$, which presumably initiates IP_3 -dependent Ca^{2+} release through $\text{G}_{\beta\gamma}$ -mediated stimulation of several $\text{PLC}\beta$ isoforms (34). Because RGS13 binds both $\text{G}_{\alpha i}$ and $\text{G}_{\alpha q}$ and inhibits GPCR signaling associated with these G proteins (27, 28), we reasoned that reducing RGS13 expression in HMC-1 cells would lead to greater Ca^{2+} influx induced by $\text{G}_{\alpha i}$ and $\text{G}_{\alpha q}$ -coupled receptors. To test this hypothesis, we loaded cells with Ca^{2+} -sensing fluorescent dye and measured accumulation of intracellular Ca^{2+} after stimulation with various GPCR agonists including CXCL12, adenosine, C5a, and S1P. We observed a rapid rise in Ca^{2+} concentration in HMC-1 cells treated with these stimuli (Figs. 3*D* and 4*A–D*). The initial calcium flux was noted within

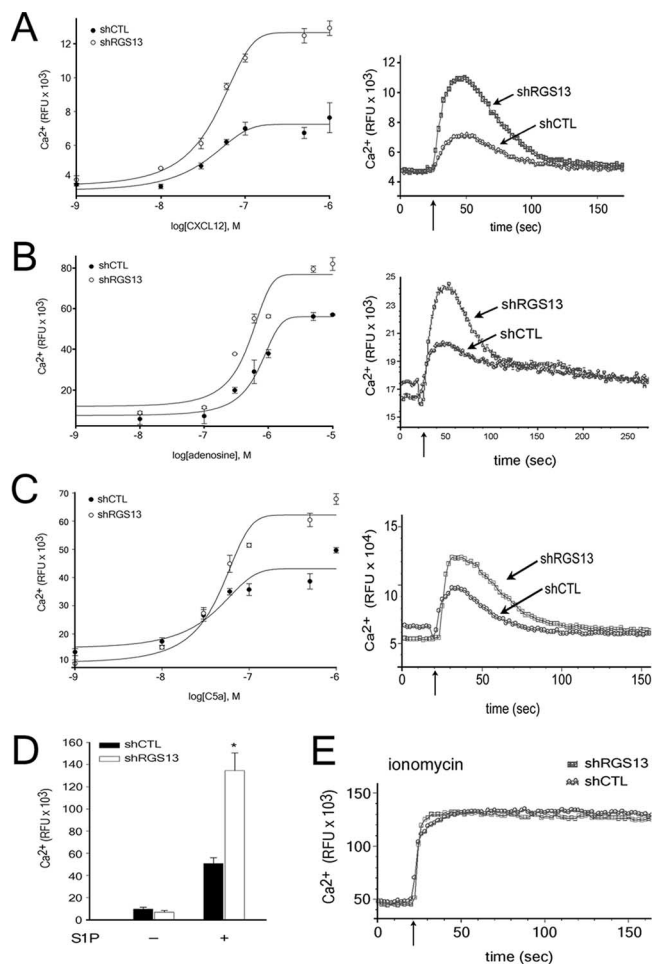


FIGURE 4. RGS13-depleted HMC-1 cells have enhanced Ca^{2+} mobilization. shCTL or shRGS13 cells were loaded with a calcium-sensing dye, followed by stimulation with various concentrations of CXCL12 (*A*), adenosine (*B*), C5a (*C*), 100 μM S1P (*D*), or 5 μM ionomycin (*E*), and measurement of intracellular Ca^{2+} concentration by fluorometry. Each data point represents the mean $\pm$ SEM (maximum-minimum value of relative fluorescence units; RFU) obtained from three to four independent experiments (*, $p = 0.004$, one-way ANOVA). Representative kinetic tracings of intracellular Ca^{2+} after stimulation with each agonist are shown on the right. Time of stimulus addition is indicated by an arrow.

50 s of stimulation (see kinetic tracings). The response of two separate populations of cells expressing RGS13-specific shRNA to GPCR stimulation was equivalent in that both transfectants had significantly more cytosolic Ca^{2+} than control cells did after exposure to adenosine (Fig. 3*D*). Because the two transfectants behaved similarly, we examined the Ca^{2+} response of one of the transfectants to a range of concentrations of adenosine and various other agonists. This cell population also had greater Ca^{2+} responses to CXCL12, C5a, and S1P than cells expressing a control shRNA (Fig. 4, *A–D*). The difference in Ca^{2+} concentration between shRGS13 cells and control cells was most evident at higher agonist concentrations whereas the EC_{50} for each agonist was not substantially reduced by the loss of RGS13 (Fig. 4, *A–D*).

In contrast to GPCR-evoked responses, the receptor-independent increase in Ca^{2+} induced by the Ca^{2+} ionophore ionomycin was similar in control and shRGS13 HMC-1 cells (Fig. 4*E*). To exclude the possibility that altered agonist presentation due to greater cell surface receptor abundance could account for the enhanced GPCR responses of shRGS13-expressing HMC-1 cells, we

analyzed receptor expression by flow cytometry. Surface expression of CXCR4 (the receptor for CXCL12), C5aR (CD88), and adenosine A_{2B} receptors (the major adenosine receptor subtype expressed in HMC-1 cells leading to Ca²⁺ mobilization) (35) was similar in control and shRGS13 cells as was expression of the receptor tyrosine kinase *c-kit* (Fig. 5). Together these results suggested that augmented G protein-dependent signaling rather than greater receptor abundance or total cellular Ca²⁺ content could underlie the observed abnormalities in GPCR-evoked responses in cells with reduced RGS13 expression.

RGS13 abundance affects CXCL12-induced chemotaxis and cytokine secretion

To determine how physiological responses of HMC-1 cells were affected by the loss of RGS13, we first examined the activity of effectors induced by CXCL12 other than Ca²⁺ which are “downstream” of G_{αi} activation. Chemokine receptors elicit rapid increases in the activity of Erk and Akt kinases through phosphorylation (36). We observed Akt phosphorylation in response to CXCL12, and both basal and CXCL12-evoked Akt activation were greater in HMC-1 cells with reduced RGS13 expression relative to control (Fig. 6A).

Because cytosolic Ca²⁺ and PI3K activation (which is reflected by Akt phosphorylation) are important for cell migration (37), we examined how RGS13 knockdown affected chemotaxis of HMC-1 cells in Transwell assays. We observed dose-dependent chemotaxis of cells to CXCL12 with maximal migration elicited by a CXCL12 concentration of 2.5 nM (Fig. 6B). shRGS13 HMC-1 cells migrated more to the optimal concentrations of CXCL12 than control cells did (Fig. 6B). In contrast, chemotaxis induced by a GPCR-independent stimulus (PMA) was similar in control and RGS13 knockdown cells (Fig. 6C). No cell migration was observed in the absence of chemokine or in the presence of equivalent concentrations of chemokine in the upper and lower chambers (chemokinesis).

GPCR-mediated increases in intracellular Ca²⁺ and Akt activity also promote cytokine synthesis in mast cells by activating transcription factors including NFκB and NFAT (38–40). In HMC-1 cells, CXCL12 stimulation leads to selective production of IL-8 (41). We examined CXCL12-induced IL-8 secretion in HMC-1

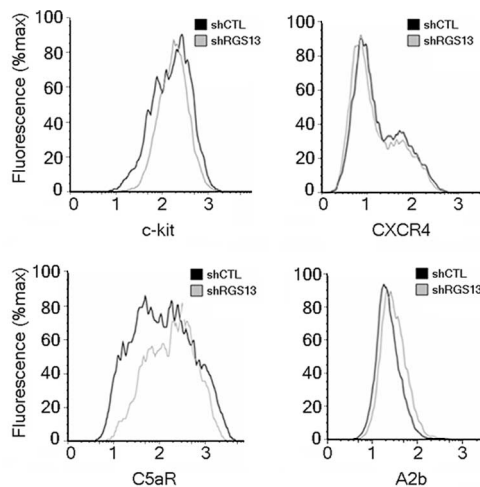


FIGURE 5. Surface receptor expression in control or shRGS13 HMC-1 cells. shCTL or shRGS13 HMC-1 cells were evaluated for surface receptor expression by flow cytometry as indicated. Data are presented as the mean fluorescence intensity from a single experiment representative of three similar experiments.

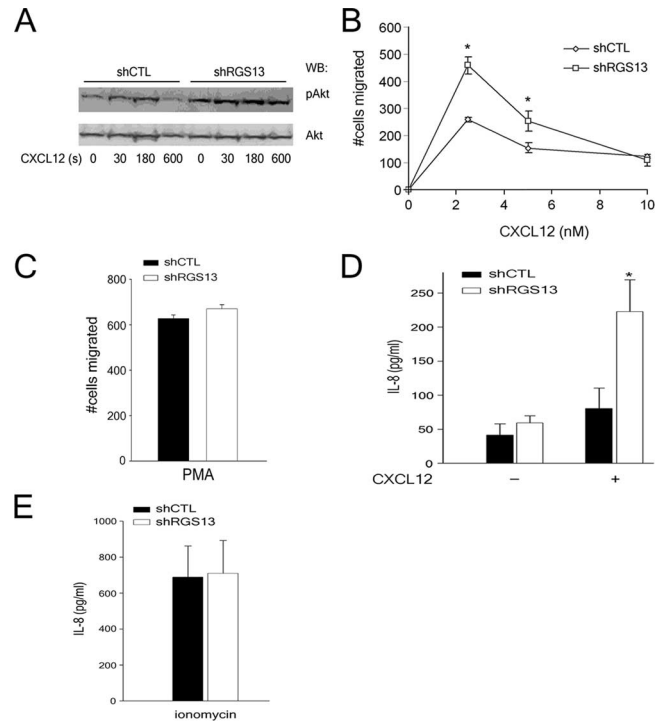


FIGURE 6. Reduced RGS13 expression enhances CXCL12-induced signaling and physiological responses of HMC-1 cells. *A*, Akt phosphorylation was evaluated in lysates of shCTL or shRGS13 HMC-1 cells left untreated or stimulated with CXCL12 for the indicated times by immunoblotting with an Ab against phospho-Akt (Thr308) (top panel). The total amount of Akt in each sample was determined by immunoblotting with anti-Akt (bottom panel). Data are representative of three similar experiments. *B* and *C*, RGS13 knockdown enhances chemotaxis of HMC-1 cells to CXCL12. shCTL, or shRGS13 cells were exposed to various concentrations of CXCL12 (*B*) or a single concentration of PMA (100 nM) (*C*) in a Transwell assay. After 2 h of incubation, cells in the lower chamber were counted by hemocytometry (*B*, *, $p = 0.004$, two-way ANOVA, $n = 3$). *D* and *E*, Enhanced CXCL12-elicited cytokine synthesis in HMC-1 cells depleted of RGS13. Cells were left untreated or exposed to 100 ng/ml CXCL12 (*D*) or 5 μ M ionomycin (*E*) for 24 h. IL-8 in cell supernatants was measured by ELISA (*B*–*D*, bar graphs show mean $\pm$ SEM of three experiments (*D*, *, $p = 0.001$, one-way ANOVA).

cells by quantifying IL-8 in cell supernatants after stimulation with CXCL12 for 24 h. RGS13-deficient HMC-1 cells secreted significantly higher quantities of IL-8 than control cells after CXCL12 treatment (Fig. 6D). In contrast, the Ca²⁺ ionophore ionomycin induced similar IL-8 production in control and shRGS13 cells (Fig. 6E). Thus, the reduction in RGS13 expression in HMC-1 cells augmented CXCL12-evoked chemotaxis and cytokine production.

RGS13 overexpression inhibits CXCL12-induced signaling and chemotaxis

To determine whether RGS13 overexpression in HMC-1 cells resulted in the opposite phenotype of cells with reduced RGS13 expression, we generated HMC-1 cells that stably express HA-RGS13. Immunoblot analysis with anti-RGS13 showed that these transfectants had more RGS13 than vector-transfected cells (Fig. 7A). Similar to the localization of endogenous RGS13, HA-RGS13 was present in the cytoplasm and nucleus of HMC-1 cells but not in cells transfected with empty vector (Fig. 7B). By immunofluorescence and confocal microscopy, we observed similar staining of both vector- and HA-RGS13-transfected cells using the RGS13 Ab (Fig. 7C).

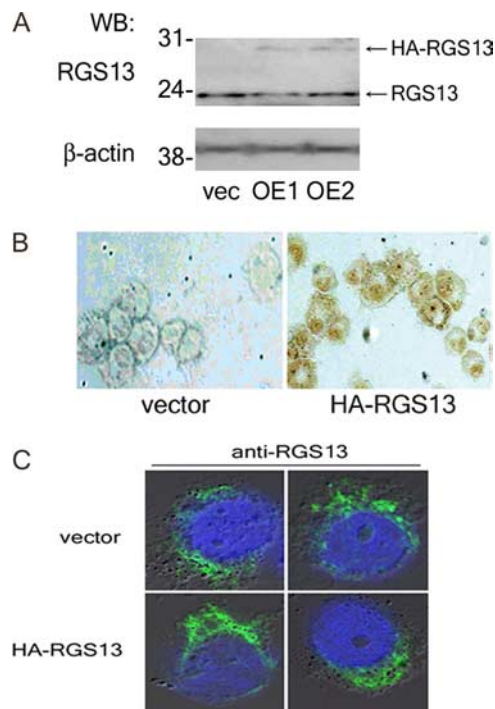


FIGURE 7. Overexpression and localization of RGS13 in HMC-1 cells. *A*, Two individual transfectants expressing HA-RGS13 (OE1 and OE2) were analyzed by immunoblotting with anti-RGS13 Ab and compared with cells transfected with empty vector (vec). Ectopically expressed RGS13 could be differentiated from endogenous protein by its different migration due to the fusion of 3 HA tags to RGS13. *B*, Localization of HA-RGS13 by immunocytochemistry. Vector- or HA-RGS13-expressing cells were stained with anti-HA (*B*) or anti-RGS13 Ab (*C*) followed by microscopy (confocal images in *C*). In *C*, RGS13 staining is green; nuclei are stained in blue with 4'-6-diamidino-2-phenylindole.

We then examined the Ca^{2+} increase induced by CXCL12 of two individual cell populations expressing HA-RGS13 compared with vector-transfected cells. These two transfectants, which expressed roughly equivalent amounts of HA-RGS13, had reduced Ca^{2+} mobilization after adenosine treatment but similar responses to ionomycin (Fig. 8*A*). We then examined events induced by CXCL12 in more detail in one of the transfectants overexpressing RGS13. These cells had reduced CXCL12-evoked Ca^{2+} flux (Fig. 8*B*), Akt phosphorylation (Fig. 8*C*), and chemotaxis (Fig. 8*D*). In contrast, control and HA-RGS13-expressing cells migrated equivalently to PMA (Fig. 8*E*). Collectively, these results indicate that cellular quantities of RGS13 strongly influence physiological responses of mast cells elicited by chemokine stimulation.

Effect of RGS13 knockdown on GPCR-induced mast cell degranulation

Although HMC-1 cells were useful to determine the role of RGS13 in regulating GPCR signaling in mast cells, they were not a suitable substrate for degranulation studies. These cells are derived from immature mast cell leukemia and lack abundant granules and a functional IgE receptor (42). For this reason, we used the LAD2 human mast cell line to analyze degranulation by measuring release of the granule protein β -hexosaminidase. As expected, these cells degranulated in response to IgE-Ag. In addition, the cells degranulated to C3a and S1P, but not to C5a, PGE₂, or adenosine (Fig. 9*A*) (43). We used duplex siRNAs to knockdown RGS13 transiently essentially as previously described (44). Such treatment resulted in a ~70% reduction of RGS13 protein quantities by im-

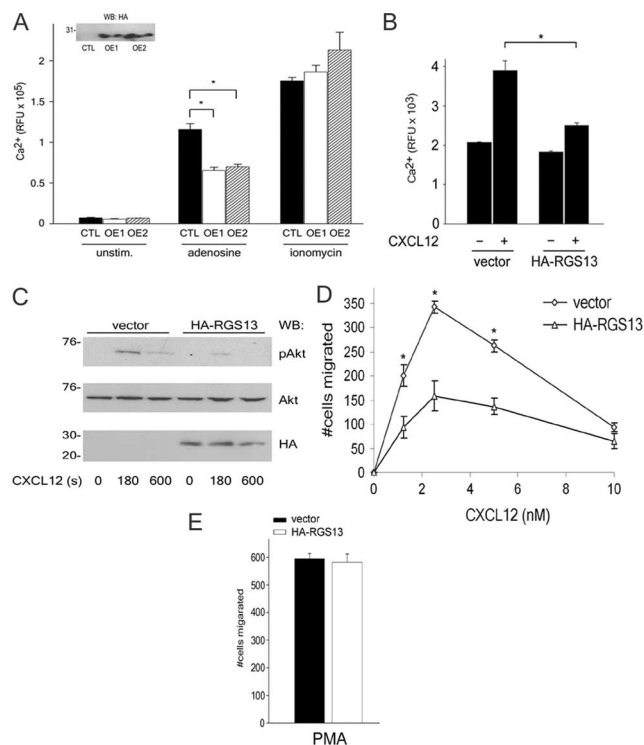


FIGURE 8. RGS13 overexpression inhibits CXCL12-evoked chemotaxis and cytokine production by HMC-1 cells. *A*, Vector control or two individual HMC-1 transfectants expressing HA-RGS13 (OE1 and OE2) were loaded with a Ca^{2+} -sensing dye followed by exposure to medium alone or adenosine and measurement of intracellular Ca^{2+} by fluorometry. Data represent three independent experiments (*, $p \leq 0.01$, one-way ANOVA). HA-RGS13 expression by immunoblot analysis using anti-HA Ab is shown (*inset*). *B* and *C*, Vector- or HA-RGS13-transfected cells were treated with medium alone or CXCL12 followed by measurement of intracellular Ca^{2+} (*B*) or Akt phosphorylation (*C*). Data represent three independent experiments (*, $p = 0.006$, paired *t* test). *D* and *E*, Cells expressing empty vector or HA-RGS13 were exposed to various concentrations of CXCL12 (*D*) or PMA (100 nM) (*E*) in Transwell plates. After 2 h, the number of cells migrating through the filter was quantitated by hemocytometry. Graphs show the mean $\pm$ SEM of three independent experiments (*, $p = 0.001$, two-way ANOVA).

munoblot analysis (Fig. 9*B*). Interestingly, LAD2 cells expressing RGS13 siRNA degranulated to the same degree as cells expressing a scrambled siRNA after either Ag or C3a stimulation (Fig. 9, *C* and *D*). By contrast, RGS13-depleted LAD2 cells degranulated significantly more to S1P (Fig. 9*E*).

Discussion

The main finding of our studies is that RGS13 controls biological responses of mast cells to GPCR stimulation. We demonstrated that human mast cells express multiple RGS proteins, of which RGS13 is among the most abundant. In contrast to the widespread expression of several other RGS proteins of the R4 subfamily such as RGS2, 3, and 16 (45–47), RGS13 appears to be selectively enriched in human mast cells compared with other hematopoietic cells and tissues. Depletion of RGS13 in the human mastocytoma cell line HMC-1 by RNA interference enhanced GPCR-evoked signaling induced by several ligands including adenosine, S1P, C5a, and CXCL12. Accordingly, HMC-1 cells with reduced RGS13 expression migrated more to a CXCL12 gradient than control cells did. Finally, LAD2 mast cells with reduced RGS13 expression degranulated more to S1P.

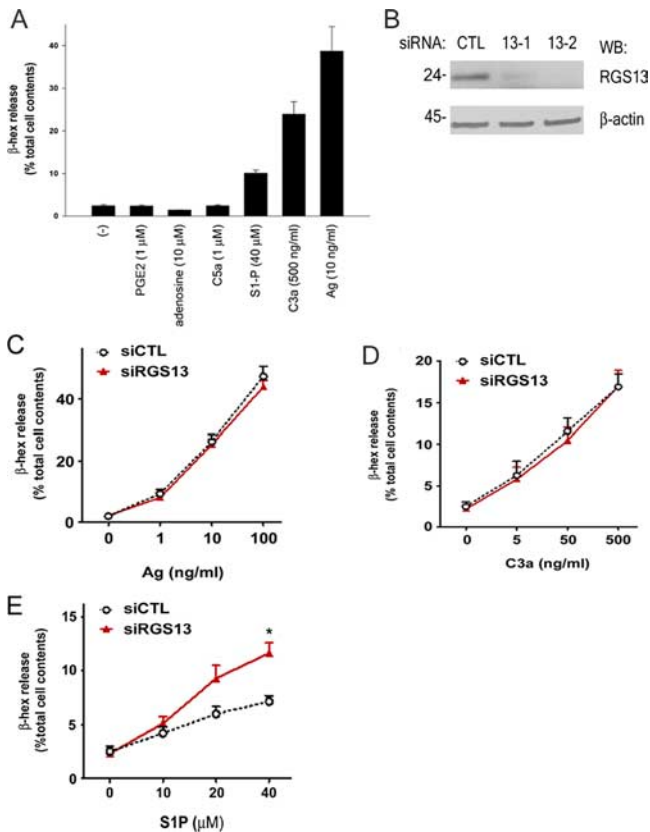


FIGURE 9. Effect of RGS13 knockdown on mast cell degranulation. *A*, Degranulation of LAD2 cells after stimulation with IgE-Ag or various GPCR agonists was monitored by the release of β -hexosaminidase. *B*, Duplex siRNAs (13-1 and 13-2) were transfected into LAD2 cells for 48 h, followed by measurement of RGS13 protein quantities by immunoprecipitation and immunoblotting using anti-RGS13 Abs. *C–E*, LAD2 cells expressing control (CTL) or RGS13-specific siRNA were sensitized for 3–4 h with IgE and then challenged with the indicated GPCR agonist or Ag for 30 min. Degranulation data are presented as mean $\pm$ SEM of five separate experiments conducted in duplicate (*, $p < 0.01$, CTL vs RGS13 siRNA, ANOVA).

CXCR4 stimulation by CXCL12 promotes $G_{\beta\gamma}$ release from $G_{\alpha i}$ -GTP. Free $G_{\beta\gamma}$ activates PLC β , resulting in intracellular Ca^{2+} mobilization, and induces Akt phosphorylation by stimulating PI3K γ . Thus, the absence of RGS13 would be predicted to increase the lifetime of $G_{\alpha i}$ -GTP, thereby promoting effector activation by expanding the pool of free $G_{\beta\gamma}$ (48). Consistent with the importance of Ca^{2+} and Akt in cytokine gene transcription in mast cells, we observed augmented CXCR4-mediated Ca^{2+} mobilization and Akt phosphorylation in HMC-1 cells with reduced RGS13 expression, which was accompanied by more IL-8 production.

In general, several molecular components are thought to control the robustness of GPCR-elicited signal transduction. Phosphorylation of receptors by G protein-coupled receptor kinases and other kinases (e.g., PKA) leads to internalization and down-regulation of receptors (49–51). In contrast, proteins of the RGS family promote adaptation to an external stimulus by increasing G protein deactivation through their GAP activity (52). The introduction of a mutation in $G_{\alpha o}$ and $G_{\alpha i2}$ rendering these G proteins insensitive to RGS binding resulted in markedly increased potency and efficacy of GPCR agonists in cardiomyocytes and neuronal cells (53, 54), which supports the physiological relevance of RGS GAP activity. However, because RGS proteins exhibit promiscuous G protein binding and GAP activity in vitro, this approach does not allow

identification of the RGS protein(s) that specifically regulate the GPCR in question. Because most cells express more than one RGS protein, eradication of each RGS individually would be required to resolve whether functional redundancy exists. Finally, because RGS proteins can impair GPCR signaling through GAP-independent mechanisms such as effector antagonism (55) and possibly GPCR binding (56), future studies such as overexpression of GAP-inactive mutants may be useful to determine the relative importance of RGS13 GAP activity for its attenuation of GPCR-evoked responses in mast cells.

Similar to other recent studies using RNA interference or cells from *Rgs* knockout mice (24), RGS13 knockdown in mast cells did not increase agonist potency to raise intracellular Ca^{2+} (i.e., shift the dose-response curve to the left) as the EC_{50} values for adenosine, C5a, and CXCL12 were not reduced in shRGS13 cells compared with control. Rather, RGS13 deficiency enhanced the magnitude of the response (agonist efficacy), particularly at high agonist concentrations. These findings are surprising because RGS overexpression decreases the potency of agonists when GTPase activity, which immediately follows GPCR activation, is measured in cell membranes (57). Conversely, several GPCR ligands more potently induce effector activation (channel opening or pheromone-induced gene expression in yeast) in cells expressing RGS-insensitive G_{α} subunits (48).

Stoichiometry of RGSs, GPCRs, and G proteins may contribute to these discrepancies. At lower agonist concentrations, multiple RGS proteins in a given cell with similar GAP activity may compensate for the loss of one family member. When agonist presentation increases at higher ligand concentrations, the total pool of RGS proteins available to deactivate G proteins at a particular GPCR may become limiting, because their abundance in unactivated cells is often quite low relative to GPCRs and G proteins (48, 58). Only at higher agonist concentrations might the loss of one RGS protein such as RGS13 become apparent. By contrast, elimination of all RGS activity rendered by RGS-resistant substitutions in G_{α} subunits may expand the pool of activated G proteins, and thus increase signaling output, at all concentrations. Conversely, since RGS proteins act catalytically and are able to increase the GTPase activity of G_{α} subunits by at least 100-fold (59), low basal RGS expression might be expected as higher quantities of RGS proteins could set a high threshold for any signaling to occur.

Although BMMCs derived from mice with a germline deletion of *Rgs13* degranulated much more to IgE-Ag than wild-type cells, we saw essentially equivalent Ag-induced degranulation of LAD2 cells expressing RGS13-specific or control siRNAs. One possibility to explain this discrepancy might be dysregulated signaling components in LAD2 cells that could mitigate the loss of RGS13. For example, these cells have constitutively active substrates of the PI3K pathway (mTOR1) compared with primary human mast cells, which may reflect higher basal PI3K activity (60). Future studies are aimed at permanent extinction of RGS13 expression in LAD2 cells and the effect of siRNA on primary human mast cells to provide further insight into these findings.

The chemokine CXCL12 may recruit mast cells to tissues under basal conditions (41, 61, 62). Our microarray analysis demonstrated greater RGS13 expression in human mast cells as they matured. Thus, the relatively low abundance of RGS13 in immature mast cell progenitors may promote homing and migration into tissues by allowing efficient chemokine signaling. By contrast, in mature tissue-embedded mast cells, greater quantities of cellular RGS13 could restrict chemokine responses, thus providing a “stop” signal for further migration. We observed normal tissue

mast cell numbers in several organs of *Rgs13*^{-/-} mice under resting conditions (29). Thus, RGS13 could primarily regulate chemokine receptors other than CXCR4 in murine mast cells. Alternatively, other chemokine receptors may be more important in maintaining mast cell numbers in uninflamed mouse tissues. Further studies of the chemotactic properties of *Rgs13*^{-/-} BMMCs in vitro as well as their homing into tissues in vivo after transfer into mast cell-deficient (*Kit*^{W-sh/W-sh}, *W-sash*) mice are ongoing to delineate how RGS13 controls mast cell migration.

Although RGS13-depleted HMC-1 cells had more basal Akt phosphorylation than control cells, they did not have increased basal motility. Chemokines promote Akt phosphorylation through activation of PI3K γ , which leads to PIP₃ accumulation at the plasma membrane of the cell's leading edge. Our results bear resemblance to studies of macrophages expressing constitutively active PI3K γ (63). Such cells had more basal Akt phosphorylation and plasma membrane-associated PIP₃ in the absence of chemoattractant than wild-type counterparts, yet they exhibited equivalent motility under resting conditions. Similar to RGS13-deficient cells, the PI3K γ -mutant macrophages migrated more than wild type cells in response to several chemoattractants. Thus, mechanisms mediating motility in the absence of chemokine may differ significantly from signaling pathways evoked by a chemotactic gradient. An alternative explanation for our findings might be related to the relatively short period of time the cells were exposed to chemokine (2 h). We observed no Transwell migration of wild type or mutant cells in the absence of chemokine, suggesting that we may have been unable to detect subtle differences in basal motility under these assay conditions.

Because IL-8 and other cytokines secreted by mast cells have a function in the recruitment of inflammatory cells such as neutrophils and eosinophils to inflammatory sites (64, 65), the regulation of IL-8 synthesis by RGS13 provides insight into the signaling pathways induced by chemokines. As it seems to regulate chemotaxis, degranulation, and cytokine production in human mast cells, RGS13 might one day represent a useful target for therapeutic intervention of allergic inflammatory diseases.

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Disclosures

S. Rao and K. H. Nocka were employees of UCB Pharma, Inc. at the time that microarray data were collected.

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