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


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.
elucidate the physiological function(s) of individual R4 GRS 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_{12} signaling elicited by chemokines (24–26).

RGS13 is an R4 subfamily member that impairs both G_{q} and G_{i2} 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-By physical interacting with the p85α regulatory subunit of PI3K that associates with p110α, β, and δ catalytic subunits. RGS13 appeared to block the association of PI3K with receptor complexes (29). In contrast to FceRI, GPCRs activate the p110γ 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 cyto- tokine production. Transient knockdown of RGS13 in LAD2 cells increased degranulation to SIP. 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, peni- cillin, and streptomycin. The stable transfectants were grown under selection with 0.4 mg/ml genetin. 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 Bio- tec). Counting 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-1 cell line. Mononuclear cell subsets 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 from purified mast cells or isolated 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 pur- chased 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: AACCACCTCTCCCTAC- CTTTG, reverse: CATACCGGAATAGACTGTGAA, and probe: CTTGCATTTGCCCTCAACGACCA.

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 (GGAACAUUCGGAAGCCCA) or Dharmacon ON-TAR- GETplus SMARTpool siRNA t-010340–09 (GGAACACAGUGAC- GAGAU)) (375 nM) for 48 h in complete StemPro medium using Oli- gofettamite (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 sequence-sequences predicted to form shRNA were generated by PCR and first tested for their ability to knockdown endogenous RGS13 in Ramos B lymphocytes by immunoblot. The double-stranded oligonucleotide sequence most effective in reducing RGS13 content (GATTTCCACTTCCTGAGA-CGACTGTGCTTATAGCAGCA-A) was subcloned into the pRNAT-U6.1/Neo expression vector (GenScript). This construct or pRNA-U6.1 containing a scrambled shRNA insert was electroporated into HMC-1 cells as described previously (30). In brief, cells were har- vested, washed, and resuspended in PBS at a density of 10^7 cells/ml. A mixture of 320 μl of cell suspension and 30 μl of DNA solution in 4-xm- 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 me- dium. Stable transfectants were selected by resistance to neomycin fol- lowed by limiting dilution. RGS13 knockdown was assessed by RT-PCR from total RNA with the PEC-3-specific primer: sense-GAAAATTGCT- TCACCAGGGGG and antisense-GCATGTTGTGAGTGGGTTCAC- GAATG. RGS13 expression was evaluated by immunoblotting and immu- nocytochemistry 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 lim- iting 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 NaHPO4, 7.5 mM, 5.6 mM glucose, 1.8 mM CaCl2, 3H2O, 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 indi- cated GPCR agonists: 1 μM PGE2 (Calbiochem); 10 μM adenosine (Sig- ma-Aldrich); 1 μM C5a (Sigma-Aldrich); 10–40 μM S1P (Sigma-Ald- rich); 5–500 ng/ml complement component C5a (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); B-actin (Sigma-Aldrich); pAkt (Thr308) or pAkt (Ser473), Akt (Cell Sig- naling Technology).

Calcium mobilization

Cells were plated overnight in a 96-well plate containing serum-free me- dium in triplicate, and intracellular Ca^{2+} 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 Miltenyi Biotec, respectively. 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-µm pore size 96-well ChemoTx system (NeuroProbe) according to 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 cloned this shRNA oligonucleotide into a plasmid vector containing the U6 promoter and electroporated the construct into Ramos cells. We isolated several individual cell populations by antibiotic resistance followed by limiting dilution. Semi-quantitative RT-PCR of RGS13 mRNA from cells expressing the shRGS13 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 shRGS13 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 cells.
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, left panel). shRGS13-transfected cells stained considerably less with the RGS13 Ab (Fig. 3, 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. G$_{q}$ couples intracellular Ca$^{2+}$ mobilization by activating PLC$\beta$, which in turn generates inositol 1,4,5-trisphosphate (IP$_3$) (33). Chemokine receptors couple to G$_{q}$, which presumably initiates IP$_3$-dependent Ca$^{2+}$ release through G$_{q}$-mediated stimulation of several PLC$\beta$ isoforms (34). Because RGS13 binds both G$_{q}$ and G$_{q}$ 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 G$_{q}$ and G$_{q}$-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. 3D and 4A–D). The initial calcium flux was noted within 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. 3D). 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. 4A). 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. 4E). 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 A2AR 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 Gs,i 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 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-RGS1. Immunoblot analysis with anti-RGS1 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).
degranulated to C3a and S1P, but not to C5a, PGE2, or adenosine cells degranulated in response to IgE-Ag. In addition, the cells (43). We used duplex siRNAs to knockdown RGS13 (Fig. 9A) (43). We used duplex siRNAs to knockdown RGS13

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. 8A). 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. 8B), Akt phosphorylation (Fig. 8C), and chemotaxis (Fig. 8D). In contrast, control and HA-RGS13-expressing cells migrated equivalently to PMA (Fig. 8E). 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 \(\beta\)-hexosaminidase. As expected, these cells degranulated in response to IgE-Ag. In addition, the cells degranulated to C3a and S1P, but not to C5a, PGE\(_2\), or adenosine (Fig. 9A) (43). We used duplex siRNAs to knockdown RGS13 transiently essentially as previously described (44). Such treatment resulted in a \(\sim 70\%\) reduction of RGS13 protein quantities by immunoblot analysis (Fig. 9B). 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. 9E).

**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.
CXCR4 stimulation by CXCL12 promotes $G_{\text{i}}, G_{\text{i}}$, release from $G_{\text{i}}, G_{\text{i}}$,-GTP. Free $G_{\text{i}}, G_{\text{i}}$, activates PLC$\beta$, resulting in intracellular Ca$^{2+}$ mobilization, and induces Akt phosphorylation by stimulating PI3K. Thus, the absence of RGS13 would be predicted to increase the lifetime of $G_{\text{i}}, G_{\text{i}}, G_{\text{i}}$,-GTP, thereby promoting effector activation by expanding the pool of free $G_{\text{i}}, G_{\text{i}}$ (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 $G_{\text{o}}, G_{\text{o}}, G_{\text{o}}$, and $G_{\text{q}}, G_{\text{q}}, G_{\text{q}}, G_{\text{q}}, G_{\text{q}}, G_{\text{q}}, G_{\text{q}}, G_{\text{q}}$, 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 $G_{\text{s}}$ 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 $G_{\text{s}}$ 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 $G_{\text{s}}$ 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 rest-
ing conditions (29). Thus, RGS13 could primarily regulate che-
mokin receptors other than CXCR4 in murine mast cells. Alter-
natively, other chemokin receptors may be more important in
maintaining mast cell numbers in uninflamed mouse tissues. Fur-
ther studies of the chemotactic properties of Rgs13−/− BMMCs in
vitro as well as their homing into tissues in vivo after transfer into
mass cell-deficient (Kit+/-W-sash, W-sash) mice are ongoing to de-
lineate 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, which leads to PI3 accumulation at the
plasma membrane of the cell’s leading edge. Our results bear re-
ssemblance to studies of macrophages expressing constitutively ac-
Dive RGS13 and plasma membrane-associated PI3 in the absence of chemoa-
tractant than wild-type counterparts, yet they exhibited equivalent
motility under resting conditions. Similar to RGS13-deficient cells,
the PI3Kγ-mutant macrophages migrated more than wild type
cells in response to several chemottractants. Thus, mechanisms
mediating motility in the absence of chemokine may differ signif-
icantly 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 neutro-
phils and eosinophils to inflammatory sites (64, 65), the regulation
function in the recruitment of inflammatory cells such as neutro-
phil-/basophil-specific transcriptional regulation of human L-histidine decar-
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