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RGS13 controls G-protein-coupled receptor-evoked responses of human mast cells

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Abstract

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 or LAD2) depleted of RGS13 by specific siRNA or shRNA and HMC-1 cells overexpressing RGS13. Transient RGS13 knockdown in LAD2 cells led to increased degranulation to sphingosine-1-phosphate, but not to antigen/IgE or C3a. Relative to control cells, HMC-1 cells stably expressing an RGS13-specific shRNA had greater Ca^{2+} mobilization in response to several GPCR ligands such as adenosine, C5a, sphingosine-1-phosphate (S-1P), and CXCL12 than wild-type cells. Akt phosphorylation, chemotaxis and cytokine (interleukin 8, IL-8) secretion induced by CXCL12 were also greater in shRGS13-HMC-1 cells compared to control. RGS13 overexpression inhibited CXCL12-evoked Ca^{2+} mobilization, Akt phosphorylation and chemotaxis. These results suggest that RGS13 restricts certain GPCR-mediated biological responses of human mast cells.

Keywords

Signal transduction; Chemokines; Mast cells/basophils

Introduction

Mast cells elicit immediate-type hypersensitivity reactions by degranulating in response to antigen crosslinking of the high affinity receptor for immunoglobulin E (1), FcεRI, 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,

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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 phosphoinositide 3-OH kinase (PI3K) and phospholipase Cγ (PLCγ) induce Ca^{2+} efflux from endoplasmic reticulum (ER). Increased cytosolic Ca^{2+} promotes exocytosis and mast cell degranulation (12). In addition to IgE-antigen crosslinking, a number of compounds including adenosine, prostaglandin E_2, sphingosine-1-phosphate (S-1P), 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 activation independently of IgE-Ag. Adenosine, acting on A_{2b} receptors, or CXCL12, the ligand for the chemokine receptor CXCR4, elicits synthesis of interleukin-8 (IL-8) by human mast cells (17,18).

In contrast to the signaling route induced by IgE-antigen, GPCRs promote exchange of guanosine triphosphate (GTP) on guanosine diphosphate (GDP)-bound G_α subunits of heterotrimeric G proteins ($G_{\alpha\beta\gamma}$), resulting in activation of both the G_α and $G_{\beta\gamma}$ 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 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 signal (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 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 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 shRNA has 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 attenuates both G_αi and G_αq-dependent signaling including chemokine reponses in B cells (27,28). We found that RGS13 attenuated IgE-mediated anaphylaxis of mice and degranulation of bone marrow-derived mast cells (BMMCs). RGS13 attenuated PI3K activation induced by IgE-antigen independently of its GAP activity by interacting with the p85α regulatory subunit that facilitates association of the p110α, β, and δ catalytic subunits with receptor complexes. (29). In contrast, GPCRs activate the p110γ catalytic subunit of PI3 kinase, which is not known to associate with p85. Therefore, we hypothesized that RGS13 should also regulate GPCR-evoked responses of mast cells through its GAP activity. 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 S-1P. 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.

Material and Methods

Cell lines and cell cultures

HMC-1 cells were grown in Iscove's basal medium (IMDM) supplemented with 10% fetal bovine serum, penicillin and streptomycin. The stable transfectants were grown under selection with geneticin (0.4 mg/ml). LAD2 cells were grown in Stem-Pro medium containing Stem-Pro supplement (Invitrogen), human stem cell factor (SCF, 100 ng/ml), and human IL-6 (R& D systems, 100 ng/ml).

Identification of *RGS* genes expressed in MCs

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 polymerase chain reaction (qPCR)

We derived human mast cells by culturing $CD34^+$ cells from adult peripheral blood isolated by magnetic bead selection (Miltenyi Biotech). These cells differentiated into mast cells (determined by morphological criteria) after 6–8 weeks of culture in medium containing 30% FBS (Hyclone), SCF 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, Boston, MA). Basophils were isolated by basophil enrichment magnetic bead separation (Miltenyi Biotec). Monocytes were isolated using Rosettesep Monocyte Enrichment Cocktail (Stemcell Technologies, Vancouver, BC). 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 (ABI, catalogue no. Hs 00243182). 20 ng of total RNA was run per sample in a one step RT-PCR reaction 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 siRNAs (Ambion siRNA ID no. 12298 [GGAACAUUCAGGAACCCAC] or Dharmacon ON-TARGETplus SMARTpool siRNA L-010340-09 [GGAGCACAGUGACGAGAAU] (375 nM) for 48 hrs. in complete Stem-Pro medium using Oligofectamine (Invitrogen) per the manufacturer's instructions. For stable knockdown in HMC-1 cells, seven cassettes consisting of the human U6 RNA polymerase promoter and RGS13-specific target sequences predicted to form a 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 (GGATCCCATCTCTCTAGGAGACTGTGGCTTGATATCCGGCCACAGTCTCCTAGAGAGATTTTTTTCCAAAAGCTT) 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. Stable clones 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-GAAAATTGCTTCACGAAGGGG and Antisense-GCATGTTTGAGTGGGTTCACGAATG. RGS13 expression was evaluated by immunoblotting and immunocytochemistry using rabbit polyclonal anti-RGS13 antibody as described and quantitated by densitometry using Quantity one software (Bio-Rad) (28).

RGS13 overexpression and immunofluorescence

The plasmid encoding HA-RGS13 (pcDNA3.1-HA-RGS13) was obtained from University of Missouri, Rolla, Guthrie Research Institute (cDNA@UMR.edu). 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 cytospin and staining with anti-RGS13 followed by 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 sensitized with biotinylated human IgE (100 ng/ml) for 3-4 hours. Cells were washed with HEPES buffer (10 mM HEPES pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $Na_2HPO_4{\cdot}7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2{\cdot}2H_2O$, 1.3 mM $MgSO_4{\cdot}7H_2O$) containing 0.04% BSA to remove excess IgE followed by aliqotting into individual wells of a 96 well plate in triplicate (10,000 cells/well). Degranulation was triggered in the same buffer with antigen (streptavidin, 1-100 ng/ml) (Sigma) or indicated GPCR agonists (PGE_2 (Calbiochem), 1 μM; adenosine (Sigma), 10 μM; C5a (Sigma), 1 μM; sphingosine-1-phosphate (S-1P) (Sigma), 10-40 μM; complement component C3a (Calbiochem), 5-500 ng/ml) for 30 minutes 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-ME, 10% glycerol, 1% Triton X-100, and a protease-phosphatase inhibitor mix (Roche). Proteins were separated by SDS-PAGE and immunoblotted as indicated. Antibodies 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 Ca^{2+} concentration was measured using FLIPR Calcium 3 assay kit and the FLEXStation II automated fluorimeter according to the manufacturer's instructions (Molecular Devices/MDS Analytical Technologies).

Receptor expression analysis by flow cytometry

FITC- or PE-labeled antibodies specific for human CXCR4, C5aR (CD88), and c-kit or isotype-matched controls were obtained from BD Pharmingen and A_{2b} antibody from Santa Cruz. Flow cytometric analysis was performed on a FACSCalibur (Becton–Dickinson, Mountain View, CA). Data were analyzed by FlowJo software (Tri Star).

IL-8 secretion

IL-8 in cell supernatants was quantitated 24 hrs. after addition of CXCL12 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) per the manufacturer's instructions. Cells were allowed to migrate in the presence of chemokine in the lower chamber for 2 hours followed by quantitation by hemocytometry.

Statistical analysis

Sigma Plot software was used to determine statistical significance by students *t*-test for two groups or analysis of variance (ANOVA) for multiple groups. *P* values <0.05 were considered to be significant.

Results

Mast cells express multiple RGS proteins

Microarray analysis revealed expression of several *RGS* genes in cord blood 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 weeks in culture expressed *RGS13* most abundantly, and *RGS2, 1,* and *17,* and *10* in lesser amounts. 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 ten-fold increase from two to six weeks of culture. IgE-antigen stimulation of cultured human mast cells also increased *RGS13* expression in cord blood mast cells, similar to the upregulation of RGS13 by IgE-antigen stimulation of BMMCs (29). Because RGS13 was specifically expressed in human mast cells compared to 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 or 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 to *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 (Fig. 2B, 3C). RGS abundance is often increased by GPCR agonists whose signaling pathways are then attenuated by the upregulated RGS in a feedback loop (21). C5a and CXCL12 treatment of HMC-1 cells induced RGS13 expression after 24 hours whereas neither adenosine nor 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 eotaxin (30). These results suggested that RGS13 could 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 to 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. Semi-quantitative RT-PCR of *RGS13* mRNA from cells expressing the shRGS13 vector demonstrated at least 75% *RGS13* knockdown by densitometry analysis compared to control (shCTL) cells (Fig. 3A). Immunoblotting confirmed that RGS13 protein was reduced in several transfectants expressing shRGS13 compared to cells expressing the control shRNA (Fig. 3B).

Many R4 RGS proteins display variable intracellular localization depending on the cell type and activation state of the cell (28,31). Previously we detected a green fluorescent protein (GFP)-RGS13 fusion protein in nuclear, membrane, and cytosolic fractions of transfected human embryonic kidney (HEK)293T cells by cell fractionation and immunoblotting with anti-GFP. (27). However, in naïve mouse B lymphocytes and BMMCs, endogenous RGS13 localized predominantly in the cytoplasm (29,32). HMC-1 mast cells had mainly cytosolic and some nuclear RGS13 by immunocytochemistry (Fig. 3C, left panel). shRGS13-transfected cells stained considerably less with the RGS13 antibody (Fig. 3C, 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. $G_{\alpha}q$ induces intracellular Ca^{2+} mobilization by activating PLCβ, which in turn generates inositol 1, 4, 5 trisphosphate (IP_3)(33). Chemokine receptors couple to $G_{\alpha}i$, which presumably initiates IP_3-dependent Ca^{2+} release through $G_{\beta\gamma}$-mediated stimulation of several PLCβ isoforms (34). Since RGS13 binds both $G_{\alpha}i$ and $G_{\alpha}q$ 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 $G_{\alpha}i$ and $G_{\alpha}q$-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 S-1P. We observed a rapid rise in Ca^{2+} concentration in HMC-1 cells treated with these stimuli (Fig. 3D,4A-D). The initial calcium flux was noted within 50 seconds of stimulation (see kinetic tracings). The response to GPCR stimulation of two separate populations of cells expressing RGS13-specific shRNA was equivalent in that both transfectancts had significantly more cytosolic Ca^{2+} than control cells did after exposure to adenosine (Fig. 3D). Since 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 S-1P than cells expressing a control shRNA (Fig. 4A-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. 4A-D). Interestingly, the sustained increase in Ca^{2+}, which is most likely due to influx of extracellular Ca^{2+} (33), was also greater in shRGS13-expressing cells relative to control.

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 A_{2B} receptors (the major adenosine receptor subtype expressed in HMC-1 cells leading to Ca^{2+} 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 Ca^{2+} 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 other than Ca^{2+} which are 'downstream' of $G_{\alpha}i$ activation induced by CXCL12. 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).

Since cytosolic Ca^{2+} 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 (phorbol 12-myristate 13-acetate, 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 Ca^{2+} and Akt activity also promote cytokine synthesis in mast cells by activating transcription factors including NFκB and nuclear factor of activated T cells (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 hrs. RGS13-deficient HMC-1 cells secreted significantly higher quantities of IL-8 than control cells after CXCL12 treatment (Fig. 6D). In contrast, the Ca^{2+} 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 Ca^{2+} signaling and chemotaxis

To determine if 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-expressing cells using the RGS13 antibody (Fig. 7C).

We then examined the Ca^{2+} increase of two individual cell populations expressing HA-RGS13 induced by CXCL12 compared to vector-transfected cells. These 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 one of the transfectants overexpressing RGS13 in more detail. These cells had reduced CXCL12-evoked Ca^{2+} flux (Fig. 8B), Akt phosphorylation (Fig. 8C), and chemotaxis (Fig. 8D) In contrast, we observed equivalent migration of the control and HA-RGS13-expressing cells 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 mast cell line to analyze degranulation by measuring release of the granule protein β-hexosaminidase. As expected, these cells degranulated in response to Ag, C3a, and S-1P, but not to C5a, PGE2, or adenosine (Fig. 9A) (43). We used duplex siRNAs to knockdown RGS13 transiently essentially as previously described (44) Such treatment resulted in a 70-90% reduction of RGS13 protein 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 (Figs. 9C-D). By contrast, RGS13-depleted cells degranulated significantly more to S-1P (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 to other hematopoietic cells and tissues. Depletion of RGS13 in the human mastocytoma cell line HMC-1 by RNA interference enhanced GCPR-evoked signaling induced by several ligands including adenosine, S-1P, C5a, and CXCL12. Accordingly, HMC-1 cells with reduced RGS13 expression migrated more to a CXCL12 gradient than control cells did. CXCR4 stimulation promotes $G_{\beta\gamma}$ release from G_{α}i-GTP. Free $G_{\beta\gamma}$ activates PLCβ, resulting in intracellular Ca^{2+} mobilization, and induces Akt phosphorylation by stimulating PI3Kγ. Thus, the absence of RGS13 would be predicted to increase the lifetime of G_{α}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. Finally, LAD2 mast cells with reduced RGS13 expression degranulated more to S-1P, but not in response to IgE-Ag or C3a.

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 (GRKs) and other kinases (e.g. PKA) leads to internalization and downregulation 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_{α}o and G_{α}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, since 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. Since most cells express more than one RGS protein, eradication of each RGS individually would be required to resolve whether functional redundancy exists. Elimination of specific RGS family members by gene-targeting or RNAi has indicated that these proteins may control the amplitude of G-protein-dependent signaling in some hematopoietic cells. MO7e megakaryocytic cells with reduced RGS16 expression had greater chemotaxis to CXCL12 than control cells did. In contrast, poor migration of germinal center T lymphocytes

to CXCL12 has been associated with high expression of RGS13 and RGS16 in this cell population (55). However, the function of RGS proteins in human mast cells had not been explored.

Similar to other recent studies utilizing RNAi 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}s for adenosine, C5a, and CXCL12 were not reduced in shRGS13 cells compared to control. Rather, RGS13 deficiency enhanced the magnitude of the response (agonist efficacy), particularly at high agonist concentrations. These findings are surprising since RGS overexpression decreases the potency of agonists when GTPase activity, which immediately follows GPCR activation, is measured in cell membranes (56). 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, since their abundance in unactivated cells is often quite low relative to GPCRs and G proteins (48,57). 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.

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 that we did not achieve 100% knockdown of RGS13 acutely, and this residual RGS13 might suffice to inhibit IgE-mediated degranulation. Alternatively, dysregulated signaling components in LAD2 cells could mitigate the loss of RGS13. For example, these cells have been shown to have constitutively active substrates of the PI3 kinase pathway (mTOR1) compared to primary human mast cells, which may reflect higher basal PI3 kinase activity (58). Similarly, although stable knockdown of RGS13 in HMC-1 cells seemed to enhance signaling to all GPCRs tested, RGS13 depletion selectively increased degranulation to S-1P but not C3a. This result suggests that RGS13 does not effectively regulate C3a signaling in LAD2 cells. Current studies are aimed at achieving more stable and complete RGS13 silencing in LAD2 cells as well as determining 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 (41,59,60). 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. Interestingly, 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 uninflammed 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$^{W\text{-}sh/W\text{-}sh}$, *W-sash*) mice are ongoing to delineate how RGS13 controls mast cell migration.

Although RGS13-depleted HMC1 cells had more basal Akt phosphorylation, these cells did not migrate more in the absence of chemokine (chemokinesis). Chemokines promote Akt phosphorylation through activation of PI3Kγ, 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 PI3Kγ (61) 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. However, similar to RGS13-deficient cells, the P3Kγ-mutant macrophages migrated more than wild type cells in response to several chemoattractants. Thus, mechanisms leading to chemokinesis 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 hours). 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 chemokinesis under these assay conditions. Since 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 (62,63), 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 mast cells, RGS13 might one day represent a useful target for therapeutic intervention of allergic inflammatory diseases.

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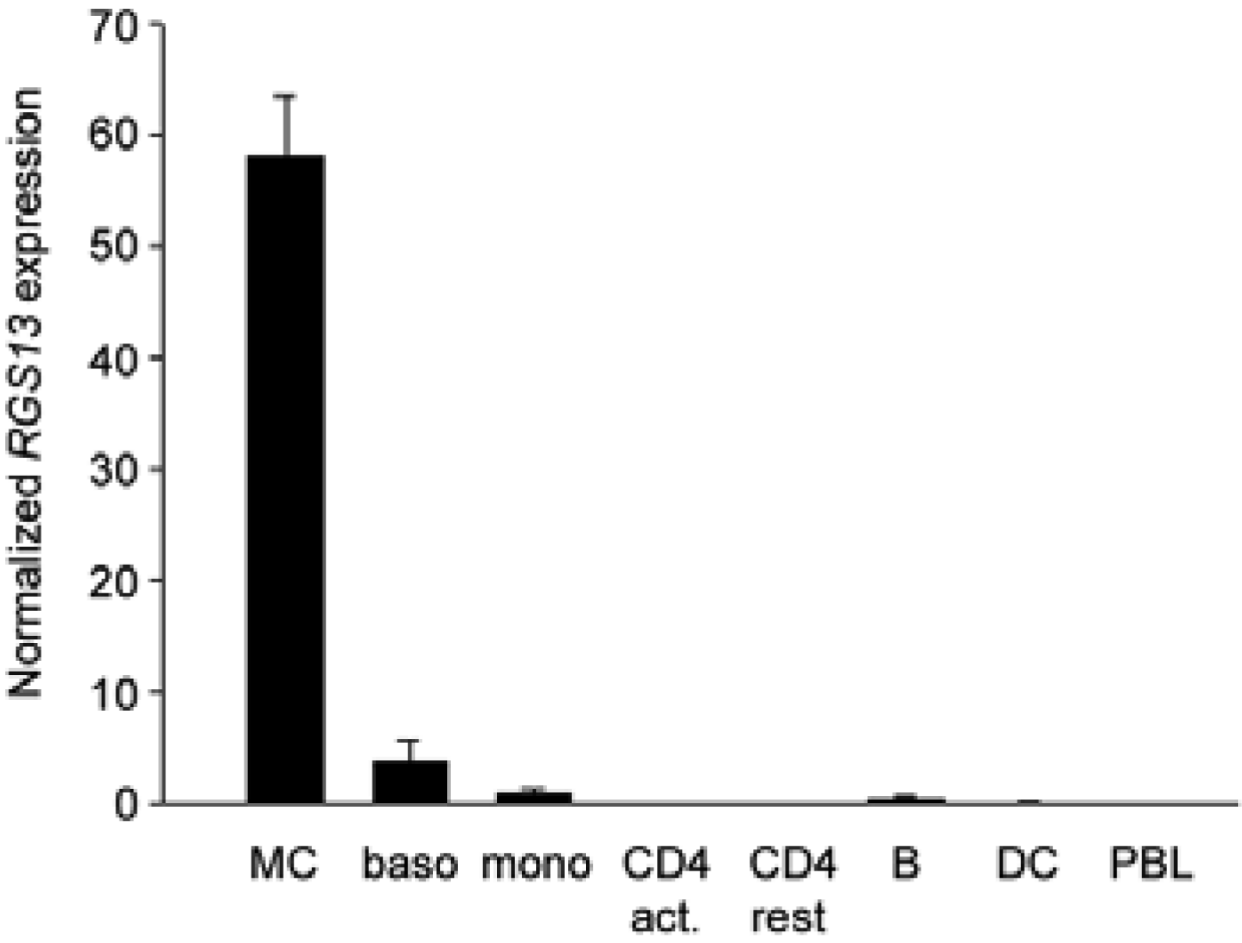

Figure 1. RGS13 expression in human hematopoietic cells

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

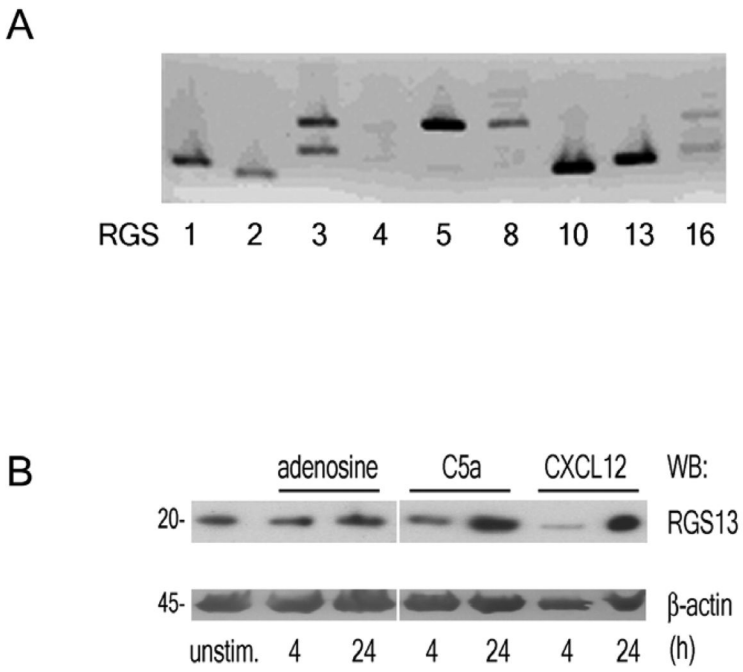

Figure 2. RGS13 expression in HMC-1 cells

(**A**) RNA from HMC-1 cells reverse transcribed and subject to PCR using RGS-specific primers as outlined in Table 1. (**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 antibody or anti-β-actin antibody to assess protein loading. Data are representative of 3 similar experiments.

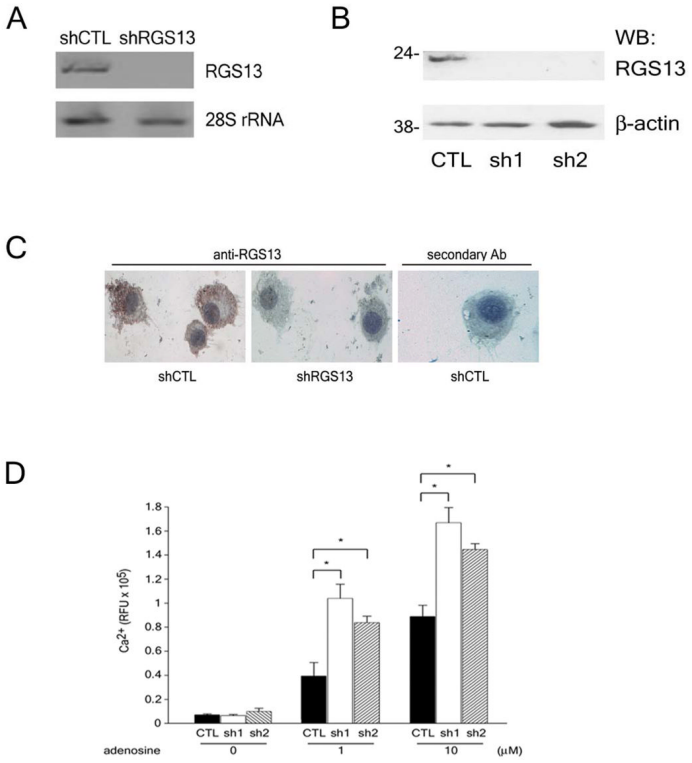

Figure 3. Knockdown of endogenous RGS13 in HMC-1 cells by RNA interference

(**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 antibody alone as a control for anti-RGS13 immunostaining (C, right panel). (**D**) Two populations of cells expressing control or shRGS13 were loaded with a calcium-sensing dye, followed by stimulation with the indicated concentrations of adenosine and measurement of intracellular Ca^{2+} concentration by fluorimetry (mean ± S.E.M. [maximum-minimum value of relative fluorescence units [RFU] obtained from 3 independent experiments (*P<0.02, 1-way ANOVA).

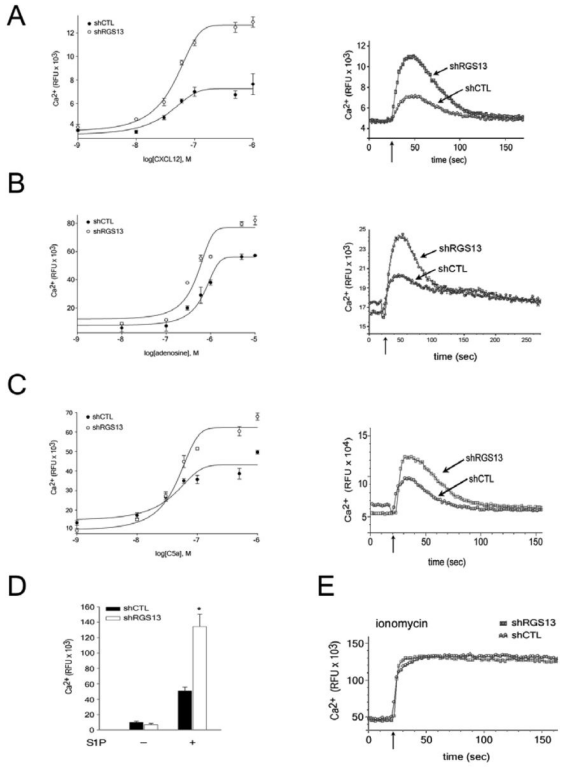

Figure 4. RGS13-depleted HMC-1 cells have enhanced Ca^{2+} mobilization

shCTL or shRGS13 cells were loaded with a calcium-sensing dye, follwed by stimulation with various concentrations of adenosine (**A**), C5a (**B**), CXCL12 (**C**), 100 PM sphingosine 1-phosphate (S-1P) (**D**) or ionomycin (5 μM, **E**) and measurement of intracellular Ca^{2+} concentration by fluorimetry. Each data point represents the mean ± S.E.M. (maximum–minimum value of relative fluorescence units [RFU]) obtained from 3-4 independent experiments (**P*=0.004, 1-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.

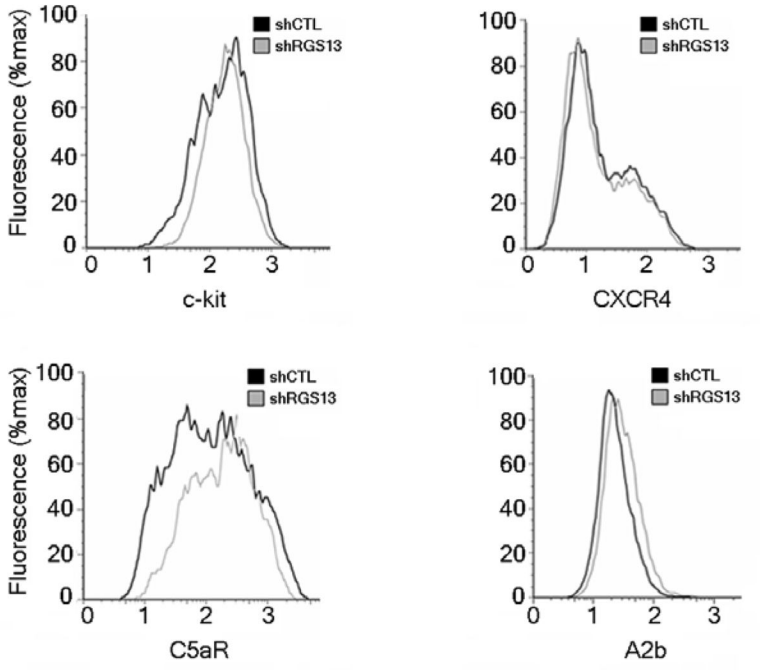

Figure 5. Surface receptor expression in control or shRGS13 HMC-1 cells

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.

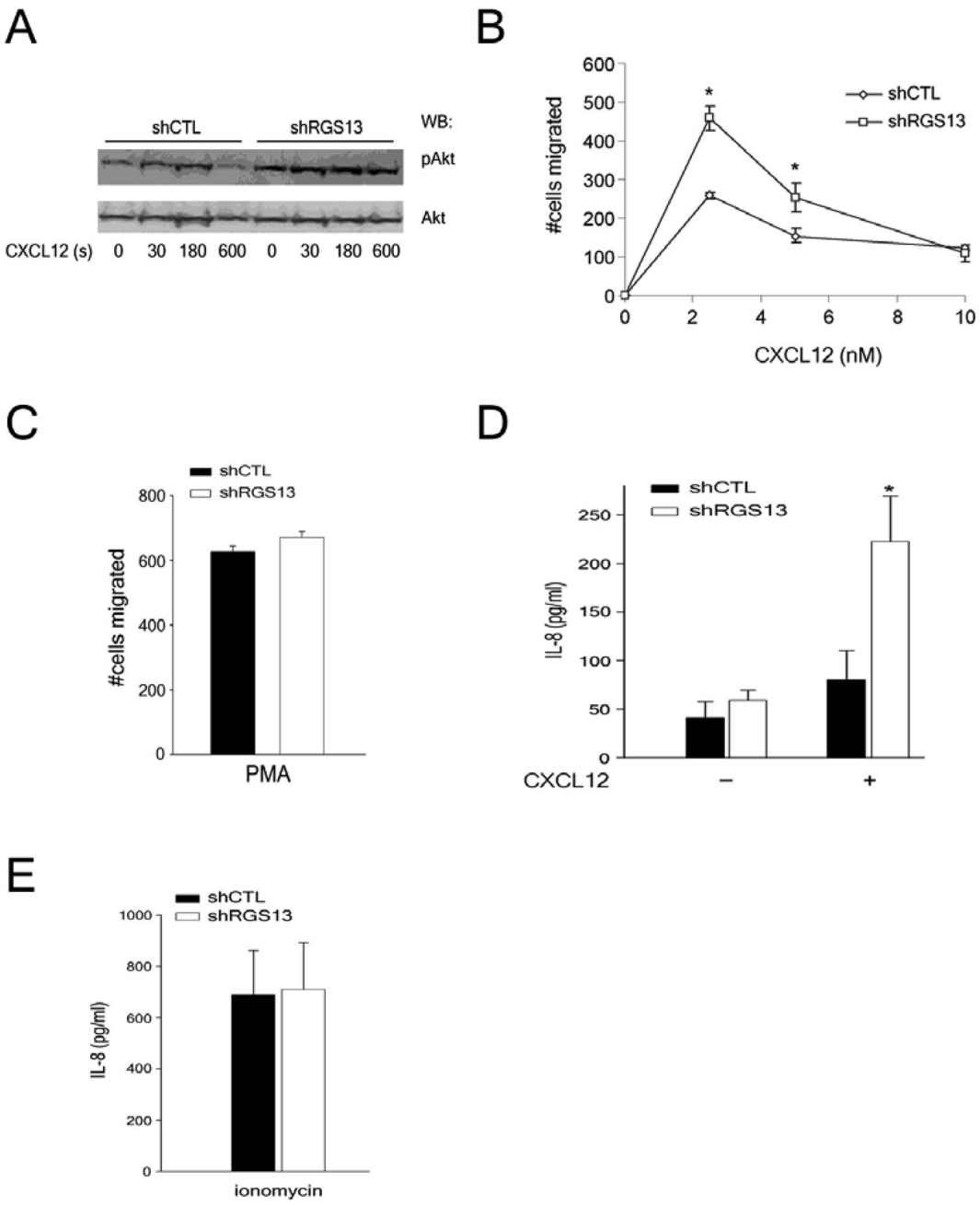

Figure 6. Reduced RGS13 expression enhances CXCL12-induced signaling and physiological responses of HMC-1 cells

(**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 antibody against phosphoAkt(Thr308) (top panel). The total amount of Akt in each sample was determined by immunoblotting with anti-Akt (bottom panel). Data are representative of 3 similar experiments. (**B-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 hrs. incubation, cells in the lower chamber were counted by hemocytometry (**B**: **P*=0.004, 2-way ANOVA,

n=3). (**D-E**) Enhanced CXCL12-elicited cytokine synthesis in HMC-1 cells depleted of RGS13. Cells were left untreated or exposed to CXCL12 (**D**) or ionomycin (5 μM) (**E**) for 24 hrs. IL-8 in cell supernatants was measured by ELISA (**B-D**: Bar graphs show mean ± S.E.M of 3 experiments; (**D**: **P*=0.001, 1-way ANOVA).

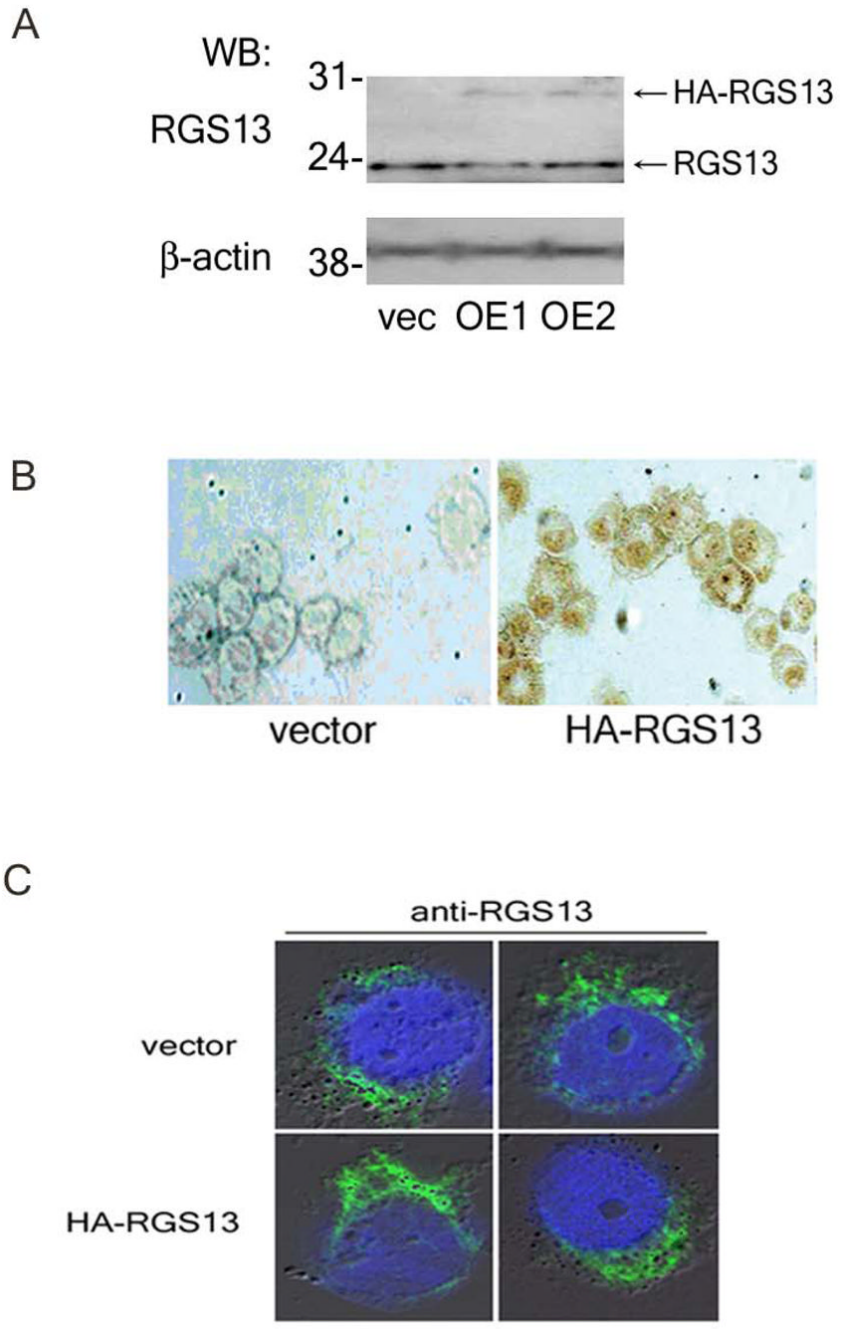

Figure 7. Overexpression and localization of RGS13 in HMC-1 cells

(**A**) Two individual transfectants expressing HA-RGS13 were analyzed by immunoblotting with anti-RGS13 antibody and compared to cells transfected with empty vector. Ectopically expressed RGS13 could be differentiated from endogenous protein by its different migration, most likely due to the fusion of 3 HA tags to RGS13. (B) Localization of HARGS13 by immunocytochemistry. Vector-or HA-RGS13 expressing cells were stained with anti-HA (**B**) or anti-RGS13 antibody (**C**) followed by cytospin and microscopy (confocal images in (**C**). RGS13 staining is green; nuclei are stained in blue with 4′-6-diamidino-2-phenylindole (DAPI).

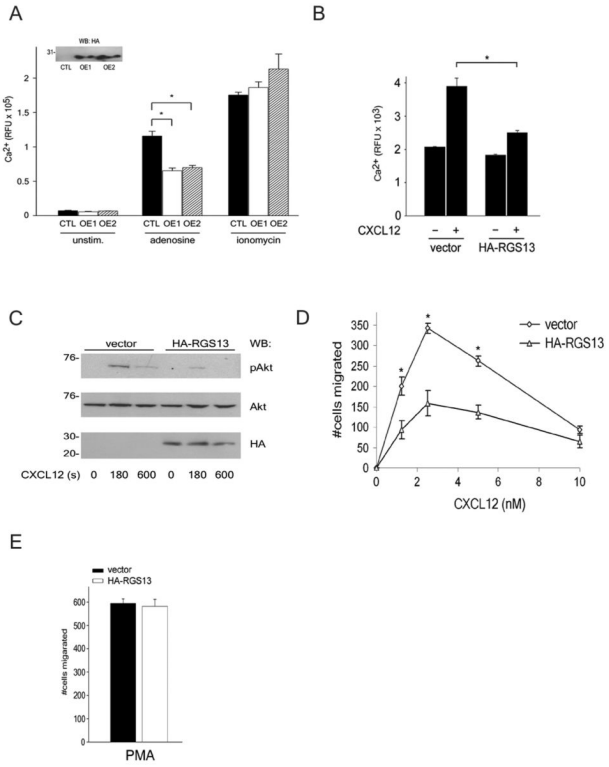

Figure 8. RGS13 overexpression inhibits CXCL12-evoked chemotaxis and cytokine production by HMC-1 cells

(**A**) Vector control or two individual HMC-1 transfectants expressing HA-RGS13 were loaded with a Ca^{2+}-sensing dye followed by exposure to medium alone or adenosine and measurement of intracellular Ca^{2+} by fluorimetry. Data represent 3 independent experiments (**P*=0.01, 1-way ANOVA). HA-RGS13 expression is shown in the immunoblot (inset) using anti-HA antibody (**B-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 3 independent experiments (**P*=0.006, paired *t*-test). (**D-E**) Cells expressing empty vector or HARGS13 were exposed to various concentrations of CXCL12 (**D**) or PMA (100 nM) (**E**) in Transwell plates. After 2 hrs. the number of cells migrating through the filter was quantitated by hemocytometry. Graphs show the mean ± S.E.M. of 3 independent experiments (**P*=0.001, 2-way ANOVA).

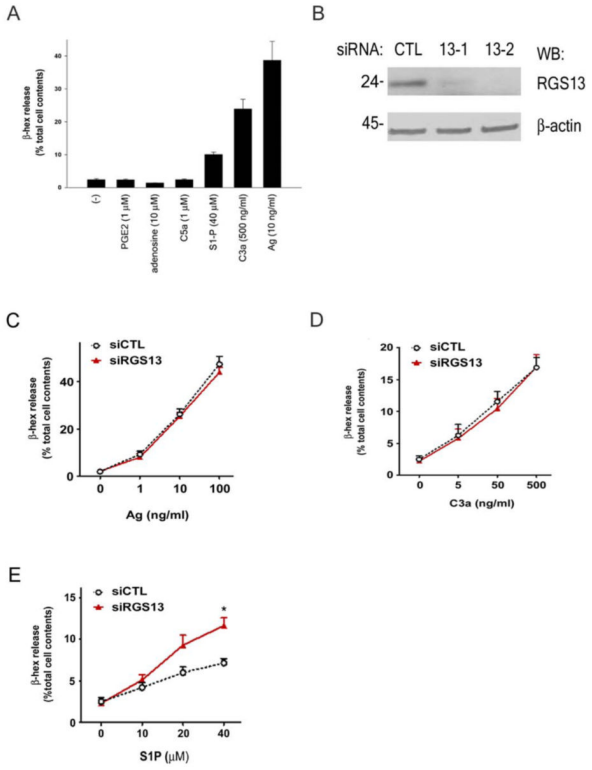

Figure 9. Effect of RGS13 knockdown on mast cell degranulation

(**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 were transfected into LAD2 cells for 48 hrs. followed by measurement of RGS13 protein amounts by immunoprecipitation followed by immunoblotting using anti-RGS13 antibodies. Relative quantification by densitometry of RGS13 amounts compared to control siRNA (set as 1) is indicated. (**CE**) LAD2 cells expressing control (CTL) or RGS13-specific siRNA were sensitized for 3-4 hours with IgE and then challenged with the indicated GPCR agonist or Ag for 30 minutes.. Degranulation data are presented as mean ± S.E.M. of 5 separate experiments conducted in duplicate (**P* < 0.01, CTL *v*. RGS13 siRNA, unpaired Student's *t-test*).

Table I

PCR primers

Gene	'Upstream' and 'downstream' primers (5′ to 3′)	PCR Product (bp)
RGS1	ATTGAGTTCTGGCTGGCTTG and GGGGGTTGGTGCTTTAATCT	171
RGS2	CGAGGAGAAGCGAGAAAAGA and TTCCTCAGGAGAAGGCTTGA	151
RGS3	TATTCGGACCTGCTGCTCTT and AGGCACCAGCACACACTCTCTT	209
RGS4	CCAGAGAGTGAGCCAAGAGG and ATCTTTTTGGCCTTGGGACT	198
RGS5	AAGATGGCTGAGAAGGCAAA and TCAGGGCATGGATTCTTTTC	163
RGS8	TTAACCCGAAGCCTCTCTGA and GGTTGGGTTTGTCTGGAAGA	160
RGS10	GGCTCAACGAGAAGATCCTG and CAGTTTGAGCATCAGGCAAA	174
RGS13	CTAAGAGGCCCCCTTCAAAC and TAGGTTTCACATGCCATCCA	163
RGS16	GATCCGATCAGCTACCAAGC and TCCTTCTCCATCAGGGTACG	189