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The Diacylglycerol Dependent Translocation of Ras Guanine Nucleotide-Releasing Protein 4 (RasGRP4) Inside a Human Mast Cell Line Results in Substantial Phenotypic Changes Including Expression of the Interleukin 13 Receptor IL13Rα2*

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Running Title: RasGRP4-dependent Regulation of Mast Cells

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Ras guanine nucleotide-releasing protein 4 (RasGRP4) is a mast cell (MC) restricted guanine nucleotide exchange factor and diacylglycerol (DAG)/phorbol ester receptor. A RasGRP4-defective variant of the human MC line HMC-1 was used to create stable clones expressing green fluorescent protein-labeled RasGRP4 for monitoring the movement of this protein inside MCs after exposure to phorbol-12-myristate-13-acetate (PMA), and for evaluating the protein's ability to control gene expression. RasGRP4 resided primarily in the cytosol. After exposure to PMA, RasGRP4 quickly translocated to the inner leaflet of the cell's plasma membrane. Fifteen to 30 min later, this signaling protein translocated from the plasma membrane to other intracellular sites. The translocation of RasGRP4 from the cytosol to its varied membrane compartments was found to be highly dependent on Phe^{548} in the protein's C1 DAG/PMA-binding domain. Extracellular signal-regulated kinases 1 and 2 were activated during this translocation process, and c-kit/CD117 was lost from the cell's surface. Transcript-profiling approaches revealed that RasGRP4 profoundly regulated the expression of hundreds of genes in HMC-1 cells. For example, the expression of the transcript that encodes the interleukin (IL) 13 receptor IL-13Rα2 increased 61- to 860-fold in RasGRP4-expressing HMC-1 cells. A marked increase in IL-13Rα2 protein levels also was found. The accumulated data suggest RasGRP4 translocates to varied intracellular compartments via its DAG/PMA-binding domain to regulate signaling pathways that control gene and protein expression in MCs, including the cell's ability to respond to IL-13.

INTRODUCTION

The Ras guanine nucleotide-releasing protein (RasGRP)[2] family of signaling proteins are preferentially expressed in hematopoietic cells where these intracellular proteins play pivotal roles in the final stages of development of numerous immune cells [for review (1)]. Four members of the family have been identified in mice, rats, and humans. RasGRP1 (2) is abundantly expressed in T cells where it facilitates T cell receptor-Ras signaling (3,4). RasGRP1 is required for the final stages of T cell development, and mice lacking this signaling protein develop a lymphoproliferative autoimmune disorder that resembles that seen in patients with systemic lupus erythematosus (5). Dysregulation of RasGRP1 expression also is the eighth leading cause of retroviral induced B- and T-cell leukemia in mice (6,7). RasGRP3 (8,9) is more abundant in B cells where it facilitates B cell receptor-Ras signaling, and RasGRP3-null mice have low levels of IgG2a (10,11). RasGRP2 (12-14) is expressed in megakaryocytes and platelets, and targeted

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disruption of its gene results in defective integrin-dependent signaling in these cells (15).

Culture of mouse bone marrow cells for 3 wk in the presence of interleukin (IL) 3 results in a population of immature cells (16) (designated as mBMMCs) that can give rise to all known populations of tissue mast cells (MCs) when adoptively transferred into MC-deficient $WBB6F_1$-*W/W*^{v} mice (17-19). Sequence analysis of thousands of arbitrarily selected cDNAs from a BALB/c mBMMC cDNA library resulted in the cloning of the 2.3-kb cDNA and 19-kb/18-exon gene that encode mouse RasGRP4 (mRasGRP4) (20). Using homology-based screening approaches, the corresponding cDNAs and genes were then isolated that encode rat RasGRP4 (rRasGRP4) and human RasGRP4 (hRasGRP4) (20,21). Others isolated a hRasGRP4 cDNA from a patient with acute myeloid leukemia (22).

All MCs examined to date contain RasGRP4 mRNA. Although MCs are the only cells in normal tissues so far identified that contain detectable amounts of RasGRP4 protein as assessed immunohistochemically (20,21,23), a population of nongranulated mononuclear cells in mouse and human peripheral blood contains appreciable amounts of hRasGRP4 mRNA (20). Kinetic studies revealed the presence of low levels of the mRasGRP4 transcript in mBMMCs before these cells granulate, and small amounts of RasGRP4 mRNA have been detected in a variety of human and mouse fetal tissues. Cord and peripheral blood-derived human MCs also express hRasGRP4 (Stevens, Boyce, and Krilis, unpublished data). MC-committed progenitors therefore express RasGRP4, and this signaling protein continues to be present as these immune cells complete their maturation and differentiation in tissues. A common hematopoietic progenitor gives rise to macrophages, eosinophils, and MCs (24). Nevertheless, no mature tissue macrophage or peripheral blood eosinophil has been found that contains detectable amounts of RasGRP4 protein (20,23).

The amino acid sequence of the primary isoform of hRasGRP4 in MCs is <50% identical to that of its other family members. However, all RasGRPs contain an N-terminal domain that functions as a guanine nuclear exchange factor and a 50-mer C-terminal C1 domain analogous to that in protein kinases that is a diacylglycerol (DAG)/phorbol ester receptor (1). The preferred small GTP-binding proteins in resting and activated MCs regulated by the signaling protein remain to be identified but recombinant mouse and human RasGRP4 can activate recombinant Ras efficiently in vitro in a cation-dependent manner (20,22).

Using an immunogold localization approach, we previously showed that endogenous mRasGRP4 resides primarily in the cytosol of the splenic MCs in the V3 mastocytosis mouse (23). Contact-inhibited mouse 3T3 fibroblasts that have been artificially forced to express either mouse or human RasGRP4 morphologically resemble normal fibroblasts (20,22,23). However, these transfectants undergo dramatic morphologic changes when exposed to phorbol-12-myristate 13-acetate (PMA), as others found with fibroblast transfectants that have been forced to express RasGRP1, RasGRP2, or RasGRP3. The PMA-treated, RasGRP4-expressing fibroblasts lose their contact inhibition and increase their rate of proliferation. The accumulated data suggest that the C1 domain in its C terminus is used to translocate this signaling protein to undefined compartments inside MCs where its target small GTP-binding proteins reside. In support of this conclusion, a truncated mRasGRP4 transcript isolated from C3H/HeJ mBMMCs encoded a protein that was unresponsive to PMA when expressed in mouse 3T3 fibroblasts (23).

Data from our earlier in vitro studies have led to the conclusion that RasGRP4 probably is important in the intermediate to final stages of MC development. A variant (20) of the hTryptase β^+ HMC-1 cell line established from a patient with a MC leukemia (25) was identified that cannot express biologically active hRasGRP4 due to an inability to remove the U12-dependent intron 3 in its precursor transcript. Many populations of mature human and rodent MCs generate substantial amounts of prostaglandin D_2 (PGD_2) when activated via calcium ionophore (26). RasGRP4-defective HMC-1 cells express very little PGD_2 (27) because of greatly diminished levels of hematopoietic type PGD_2 synthase (28). Using a transfection rescue approach, we induced these hRasGRP4-defective cells to express mRasGRP4 (20). Because the resulting transfectants markedly increased their expression of PGD_2 synthase mRNA and protein and

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preferentially metabolized arachidonic acid to PGD_2 when exposed to calcium ionophore (28), RasGRP4 appears to regulate cyclooxygenase pathways in MCs. In support of these data, attenuation of rRasGRP4 mRNA and protein levels in the RBL-2H3 rat MC line using an RNAi approach led to decreased levels of PGD_2 synthase mRNA and protein (28).

We now describe the creation of new HMC-1 cell clones that contain different levels of green fluorescent protein (GFP)-labeled normal and mutated hRasGRP4. Using these clones, we show that PMA regulates the movement of hRasGRP4 from the cytosol to the plasma membrane and then to varied intracellular compartments. We show that extracellular signal-regulated kinase (ERK) 1 (also known as p44 mitogen-activated protein kinase 3 or MAPK3) and ERK2 (also known as p42 mitogen-activated protein kinase 1 or MAPK1) are activated during this PMA-mediated translocation process and that CD117 is lost from the cell's surface. The mechanisms by which RasGRP4 controls the development and function of MCs remain to be elucidated. Finally, we show that this intracellular signaling protein controls the expression of genes that encode hundreds of proteins in the HMC-1 MC line, including an IL-13 receptor.

EXPERIMENTAL PROCEDURES

Generation of HMC-1 Cell Clones Containing Varying Amounts of GFP-labeled hRasGRP4—To monitor the intracellular movement of hRasGRP4 inside a human MC, we placed the coding domain of a cDNA that encodes biologically active hRasGRP4 (see GenBank accession number AY048119) (20) in pcDNA3.1/CT-GFP-TOPO using the CT-GFP Fusion TOPO TA expression kit (Invitrogen). This expression system uses a cloning strategy that places PCR products in the vector to generate bioengineered fusion proteins that contain GFP at their C termini. The expected fusion protein produced by the transfectants is ~30 kDa larger than wild-type ~75-kDa hRasGRP4 because of the covalently linked reporter protein. Crameri and coworkers described the modified *GFP* gene (namely Cycle 3 GFP) in this vector (29). We chose cDNA3.1/CT-GFP-TOPO for our studies because Cycle 3 GFP can be expressed in most mammalian cells. Moreover, the reporter often results in a >40-fold increase in fluorescence intensity relative to that of replicate transfectants that express recombinant proteins labeled with wild-type GFP. The sense oligonucleotide 5′-CAGATGAACAGAAAAGACAGTAAGAGG -3′ and the antisense oligonucleotide 5′-CGGAATCCGGCTTGGAGGATGCAGT-3′ were used in the PCR approach to generate the coding domain of the signaling protein, namely residues 215-2233 in the full-length cDNA. The resulting ~2.1-kb cDNA was purified by agarose gel electrophoresis and its nucleotide sequence was determined to confirm the absent mutations. The hRasGRP4-expression vector was generated with the CT-GFP Fusion TOPO TA expression kit using the methodology recommended by Invitrogen. The TOP10 strain of *E. coli* (Invitrogen) was transformed with the CT-GFP mock vector and the newly created hRasGRP4-CT-GFP vector using the One Shot (Invitrogen) chemical transformation procedure. A standard PCR/gel approach was then used with the T7 sense and GFP antisense oligonucleotides present in the CT-GFP Fusion TOPO TA expression kit to identify those bacterial colonies containing the appropriate construct.

The RasGRP4-defective variant (20) of the HMC-1 cell line (25) was maintained in Iscove's modified Dulbecco's medium (GIBCO Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air. Since no transfection study of HMC-1 cells had been done with Nucleofector methodology before our studies were initiated, we carried out preliminary transfection experiments with Amaxa's Nucleofect-II device. We used several Nucleofection programs, as well as 2 μg of pmaxGFP control vector DNA and solution T, which are both present in the VCA-1002 Cell Line Nucleofector T kit (Amaxa). Approximately 40% of the resulting HMC-1 cells in our initial control transfection experiments contained GFP, as assessed by fluorescent microscopy, when the T20 program of the Nucleofect II device was used (data not shown). Thus, 10^6 HMC-1 cells in their log phase of growth were transfected with Nucleofection and 5 μg of linearized CT-GFP vector DNA with or without the hRasGRP4 insert. The resulting transfectants were grown as described above except for the addition of

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geneticin (GIBCO Life Technologies) (0.5 or 1.25 mg/ml depending on the cell density) to the culture medium on day 2 post-transfection to induce apoptosis of the non-transfectants. The two doses of the antibiotic were selected on the basis of the results of geneticin-induced death curve experiments carried out on HMC-1 cells cultured at low and high densities.

Once we had obtained sufficient numbers of hRasGRP4-expressing HMC-1 cells, we performed a limiting dilution clonal assay in which cells were suspended at a density of ~5 cells/ml in culture medium supplemented with 0.5 mg/ml geneticin. One hundred microliters of this cell suspension was added to each chamber of flat-bottomed 96-well plates (Greiner Bio-One). After 4 d of culture, those wells that contained a single clone of cells were identified. These wells were reexamined by fluorescence microscopy at d 14 to identify clones that differed in their intracellular levels of GFP-labeled hRasGRP4. To confirm that the selected clones maintained their levels of hRasGRP4-GFP, each was subjected to a second limiting dilution cloning step.

Generation of HMC-1 Cells that Express a Ser^{548} Mutant of hRasGRP4—Residue 548 in hRasGRP4 is Phe, and this amino acid is the eighth amino acid in the signaling protein's putative DAG/PMA-binding domain. The corresponding amino acid in hRasGRP1, hRasGRP2, and hRasGRP3 is Tyr, Ser, and Tyr, respectively. Because it has been concluded that this residue plays a critical role in the DAG/PMA-dependent translocation of other RasGRPs (30), a site-directed mutagenesis approach was used to convert Phe^{548} in hRasGRP4-GFP to Ser. The sense oligonucleotide 5'-CACACCTTCCATGAGGTCACC**TCG**CGAA AGCCTACCTTCTGCGACAG-3', the antisense oligonuleotide 5'-CTGTCGCAGAAGGTAGGCTTTCG**CGA**GG TGACCTCATGGAAGGTGTG-3', and the QuiKchange II XL mutagenesis kit (Strategene) were employed to mutate nucleotides 1856 to 1858 in the expression construct noted above that encodes normal hRasGRP4-GFP. Plasmid transformation was performed following *Dpn*I digestion of the template DNA. Colonies were screened by PCR and the hRasGRP4-GFP cDNAs in the resulting clones were sequenced to confirm the presence of the desired mutation. After transformation for 7 d, the transfectants were washed with phosphate buffered saline (PBS) and sorted for GFP expression using a MoFlo MLS Flow Cytometer (Cytomation, Fort Collins, CO) equipped with a 488 nm argon-ion laser. Sorted cells were then expanded in culture medium supplemented with 500 ng/ml geneticin to obtain the desired transfectants that express the Ser^{548} mutant of hRasGRP4-GFP.

Evaluation of hRasGRP4 Expression in HMC-1 Cell Transfectants—The *RasGRP4* gene is transcribed in the starting HMC-1 cell line used in our study, but these human leukemic MCs cannot express functional RasGRP4 protein due to an inability to remove intron 3 from its precursor transcript (20). Thus, reverse transcriptase (RT) PCR and SDS-PAGE immunoblot approaches were used to confirm that our newly generated HMC-1 cell clones contain appreciable amounts of biologically active hRasGRP4 mRNA and protein.

For transcript analysis, total RNA was extracted from the parental HMC-1 cell line and the transfectants with the QIAamp RNA Blood Mini Kit (Qiagen) which uses spin-column technology. A semi-quantitative, SuperScript one-step RT-PCR was carried out with the Platinum Taq kit (Invitrogen) and the sense oligonucleotide 5′-AGAGGAAGTCCCACCAGGAA-3′ and the antisense oligonucleotide 5′-CGGAACTCCAGGTAGGTAGGTGAG-3′ which correspond to nucleotide sequences in exons 2 and 6 of the hRasGRP4 gene, respectively. For each sample, 100 to 500 ng of total RNA was placed in RT-containing buffer and incubated for 30 min at 50ºC. After a 2-min heat-denaturation step at 94ºC, each of the 30 cycles of the subsequent PCR steps consisted of a 2-min denaturing step at 94ºC, a 30-s annealing step at 59ºC, and a 1-min extension step at 72ºC. The resulting PCR products were heated at 72ºC for 10 min and then electrophoresed in 1.5% agarose gels. As internal controls, the same RNA samples also were evaluated in this semiquantitative RT-PCR analysis for their levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA using the oligonucleotides 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

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The presence of ~105-kDa biologically active hRasGRP4-GFP protein was then evaluated by an SDS-PAGE immunoblotting assay with an antibody to GFP (Invitrogen). In these experiments, cell lysates were boiled in SDS-sample buffer containing β-mercaptoethanol. The resulting solubilized proteins were resolved on precast NuPAGE 4 to 12% Bis-Tris gels (Invitrogen), and the separated proteins were transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences/Amersham Biosciences). GFP-labeled positope protein (Invitrogen) was used as a positive control. The resulting protein blots were exposed to Tris-HCl-buffered saline containing 0.1% Tween-20 and 5% non-fat milk to minimize nonspecific binding of the primary and secondary antibodies. The treated blots were then exposed to a 1000-fold dilution of the rabbit anti-GFP antibody (Molecular Probes) in the above blocking buffer for 1 h at room temperature, followed by a 1000-fold dilution of horseradish peroxidase-labeled goat anti-rabbit antibodies (DakoCytomation). Immunoreactive proteins were detected with Hyperfilm ECL radiographic film (GE Healthcare Bio-Sciences/Amersham Biosciences) after the blots were exposed to ECL developing reagent.

hRasGRP4-GFP Translocation and Co-localization Experiments—PMA stimulation of the varied HMC-1 cell clones was performed in 96-well plates. GFP- and hRasGRP4-GFP-expressing HMC-1 cells were placed in serum-free medium before exposure to PMA. Cell number and viability were determined before the HMC-1 cells were exposed to 1 to 1000 nM PMA (Sigma-Aldrich) in 0.003 to 0.01% dimethyl sulfoxide (DMSO) for 10, 30, and 60 min. The treated cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed again with PBS. All slides were treated with ImmunO (immuno-fluore mounting medium; MP Biomedicals, Aurora, Ohio). Confocal microscopy was performed with the Leica laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) DM IRE2 TCS SP2 AOBS using a 100x/1.4-0.7 PL APO oil immersion objective. GFP and hRasGRP4-GRP were detected using a 488 nm Ar laser. Each image represents a single 'Z' optical section. The images were prepared as figures using Adobe Photoshop and Illustrator CS.

hRasGRP4-induced Phosphorylation of ERK1 and ERK2 in HMC-1 Cells—Control HMC-1 cells and their transfectants were evaluated before and after PMA exposure to determine if hRasGRP4 regulates the phosphorylation of ERK1 and/or ERK2. For the PMA studies, GFP- and hRasGRP4-GFP-expressing HMC-1 cells were placed in serum-free medium and then were exposed for 15 min to varied amounts of PMA at 37ºC. PMA stimulation was performed in Microtest 96-well V-bottom plates (Sarstedt) at 10^6 cells/well. A number of inhibitors [e.g., GF109203X/bisindoylmaleimide I (31), Go6976, and Ro318220] of PKC isozymes have been developed and used in many signaling studies. For example, Zheng and coworkers (32) used Go6976 and Ro318220 to demonstrate that RasGRP3 is phosphorylated by PKCθ in cultured Ramos B cells. In our study, HMC-1 cells were exposed to 10 μM GF109203X (Sigma-Aldrich) in DMSO for 30 min before encountering PMA to minimize the contribution of PKC in these ERK1/ERK2 activation experiments. The GF109203X-treated cells were centrifuged at 1200 rpm for 5 min at 4ºC, and the resulting cell pellets were resuspended in culture medium without PMA or with 1, 10, 100, or 1000 nM concentrations of the phorbol ester for 15 min at 37ºC. Treated cells were centrifuged, washed once in ice-cold Dulbecco's PBS, and lysed by the addition of 60 μl of 0.5% deoxycholate, 0.1% SDS, 1% Triton X, 1% Sigma-Aldrich's protease inhibitor solution, 1 mM sodium orthovanadate, 150 mM NaCl, and 50 mM Tris-HCl, pH 8. Samples were mixed for 30 sec and centrifuged at 14,000 rpm for 15 min at 4ºC. Sixty microliters of each soluble fraction was diluted in Invitrogen's 4x NuPAGE sample buffer containing β-mercaptoethanol and heated at ~85ºC for 4 min. Samples were subjected to SDS-PAGE, the separated proteins were transferred onto nitrocellulose membranes, and the resulting protein blots were probed with an antibody to phospho-ERK1/ERK2 (Cell Signaling Technology). Once the expression data were obtained, the anti-phospho-ERK1/ERK2 antibody was removed by a 1-h incubation at 60ºC in 2% SDS, 100 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 6.7. The stripped blots were then reprobed using an antibody that recognizes all

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forms of ERK1 and ERK2 (Cell Signaling Technology), followed by an antibody that recognizes β-actin (Sigma-Aldrich).

FACS Analysis of HMC-1 Cells for CD117 Expression—To detect and quantitate surface and intracellular levels of the tyrosine kinase receptor CD117, HMC-1 cells (10^6 cells/ml) were exposed 10 h to 0.003% DMSO with or without 30 nM PMA. The treated cells were washed three times with ice-cold FACS buffer (PBS containing 0.5% BSA and 0.1% sodium azide) and then incubated with phycoerythrin-conjugated mouse anti-human CD117 monoclonal antibody (Miltenyi Biotec) (1:11 dilution, 5 x 10^6 cells/ml, 50 μl vol) in the dark at 4ºC for 45 min before or after cell permeabilization. For the permeabilization step, the control and PMA-treated HMC-1 cells were resuspended in 100 μl of Cytofix/Cytoperm solution (BD Biosciences), incubated for 20 min at 4ºC, and washed with Perm/Wash buffer (BD Biosciences). Permeabilized and non-permeabilized stained cells were examined on a FACS Calibur Flow Cytometer (BD Biosciences). Phycoerythrin and GFP excitation was at 545 and 489 nm and detection was at 575 and 508 nm, respectively, using the company's CellQuest software.

Transcript Profiling of HMC-1 Cells that Differ in their hRasGRP4 Levels—Microarray screening approaches were used to identify candidate hRasGRP4-regulated transcripts in HMC-1 cells. As noted in RESULTS, dose-response and kinetic experiments revealed translocation of hRasGRP4-GFP to different membrane compartments when the transfectants were exposed to 30, 100, or 1000 nm PMA for various time intervals up to 1 h. Thus, total RNA was isolated as described above from ~8 x 10^6 GFP-expressing HMC-1 cells and ~8 x 10^6 low and high RasGRP4-GFP expressing HMC-1 cells before and after the three populations of cells were exposed to 30 nM PMA in 0.003% DMSO. RNA purity was initially assessed on the basis of their ratios of absorbance at 260 and 280 nm (A260:A280 ratio). Aliquots of the six samples were then subjected to gel electrophoresis to confirm the presence of intact 18S and 28S rRNA. The One-Cycle Eukaryotic Target Labeling Assay Protocol recommended by Affymetrix was used by the Clive and Vera Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia) to generate the biotin-labeled cRNA targets that were hybridized to six Affymetrix "HG-U133 Plus 2.0" GeneChips. Before the microarray analyses, the amount and quality of the generated cRNAs also were examined on an Agilent 2100 Bioanalyzer. After microarray image acquisition, the GeneSpring Analysis Platform (Silicon Genetics) was used to identify differentially regulated genes in the transfected cells. Microarray data were normalized to the 50th percentile value for each transcript, after which a transcript was considered consistently up-regulated or down-regulated if the difference in expression in both populations of hRasGRP4-transfected cells in each experiment was at least twofold or half that of the mock GFP-transfected cells, respectively. Varied house keeping transcripts (e.g., β-actin and GAPDH mRNA) contained ~40,000 units of signal in these microarray experiments (see Supplemental Tables 1 and 6). Thus, to minimize background noise and false positives, a threshold value of 400 units of signal was arbitrarily selected which means the transcript of interest had to be present in at least one population of HMC-1 cells at a level corresponding to ≥1% of the level of the β-actin and GAPDH transcripts.

Quantitation of the Levels of the GATA-1 and IL13Rα2 Transcripts and Protein in HMC-1 Cells, and Evaluation of IL-13-dependent Signaling in HMC-1 Cells—As noted in RESULTS, the Affymetrix gene-profiling data suggested substantial changes in the levels of the transcripts that encode the hematopoietic-restricted transcription factor GATA-1 and the IL-13 receptor IL13αR2 in HMC-1 cells that differed in their RasGRP4 levels. We therefore carried out kinetic experiments in which GFP-expressing HMC-1 cells and low- and high-hRasGRP4 expressing HMC-1 cells (10^6 cells/ml) were exposed to 100 nM PMA in 0.01% DMSO for 4 to 20 h to confirm and extend these findings. RNA was extracted from replicate cultures before and after exposure to PMA. Real-time quantitative polymerase chain reaction (qPCR) assays were performed using the Rotor-Gene 3000 Analyzer (Corbett Research) and the dsDNA-binding fluorescent dye SYBR green. Shown in Table 1 are the nucleotide sequences of the primers and the annealing temperatures used to amplify the desired regions in the four transcripts analyzed in these

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qPCR assays. All amplifications were performed with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen), 200 nM primers, and 50 pg to 50 ng of cDNA template. The thermal cycling program consisted of an initial denaturation step at 95ºC for 75 s, followed by up to 40 cycles of PCR. Each PCR cycle consisted of a 45-s denaturing step at 95ºC, a 30-s annealing step at varying temperatures, and a 30-s extension step at 73ºC. Fluorescence readings were taken during the extension step of each cycle. Melting-curve analyses also were performed to ensure the amplification of the expected product. Reactions with no RT or no template were included as negative controls. Samples were run in duplicate from three independent experiments for each transcript. Transcript levels in each sample were normalized relative to that of the housekeeping transcripts that encode GAPDH and hypoxanthine guanine phosphoribosyltransferase I (HPRTI).

SDS-PAGE immunoblot analyses also were carried out to assess the hRasGRP4-dependent expression of IL13Rα2 protein in HMC-1 cells before and after these cells encountered PMA. In these experiments, GFP- and hRasGRP4-GFP-expressing (clone C721) HMC-1 cells were untreated or were exposed to 30 nM PMA in 0.003% DMSO for 6, 12, and 20 h. Protein blots of lysates of the resulting cells were probed with goat anti-human IL13Rα2 antibodies (R&D Systems) followed by rabbit anti-goat HRP-conjugated secondary antibodies.

IL-13-induced Phosphorylation of Signal Transducer and Activator of Transcription 6 (STAT6) in GFP- and hRasGRP4-GFP-expressing HMC-1 Cells–Following serum starvation for 2 h, GFP- and hRasGRP4-GFP-expressing HMC-1 cells (10^6 cells/well) were treated with either medium or 100 nM PMA (Sigma) for 5 h at 37ºC. Cells were then placed in OPTI-MEM (Invitrogen GIBCO) medium without or with 50 ng/ml IL-13 for an additional 1 h at 37ºC. For SDS-PAGE immunoblot analysis of phospho-STAT6 (pSTAT6) levels, the treated cells were washed with ice-cold PBS and lysed by the addition of 30 µl of ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM sodium orthovanadate, and 100 µl/ml of a protease inhibitor cocktail that contained 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. For SDS-PAGE immunoblot analysis of IL-13Rα2 levels, the treated cells were washed with ice-cold PBS and lysed by the addition of 60 µl ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, and 100 µl/ml of the protease inhibitor cocktail. The resulting cell lysates were centrifuged at 500 x g for 10 min at 4ºC, and the protein concentration of each sample was determined using the BCA protein assay kit. In each experiment, ~20 µg of protein were subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Amersham). After a 1-h exposure to 5% (w/v) skim milk in TBST buffer (137 mM NaCl, 0.1% Tween-20, and 25 mM Tris-HCl, pH 7.4), each blot was incubated for 1 h at room temperature with either rabbit anti-human pSTAT6 polyclonal antibody (R & D Systems) in TBST or goat anti-human IL13Rα2 polyclonal antibody (R & D Systems). The blots were washed three times with TBST; they were then incubated for 1 h at room temperature with either horseradish peroxidase-labeled goat anti-rabbit antibody (DakoCytomation) or horseradish peroxidase labeled rabbit anti-goat IgG (DakoCytomation). The SDS-PAGE immunoblots finally were developed using Amersham's Enhanced Chemiluminescence Detection System according to the manufacture's instructions.

RESULTS

Generation of hRasGRP4-GFP Expressing HMC-1 Cells—Using the pcDNA3.1/CT-GFP-TOPO expression vector, we generated eight stable HMC-1 cell clones that expressed ~105-kDa hRasGRP4-GFP (Fig. 1*A*). For negative controls, we also generated two stable HMC-1 cell clones that expressed just 30-kDa GFP. Relative to their levels of β-actin, five of the hRasGRP4-GFP-expressing clones (designated as C711, C712, C713, C714, and C721) contained considerably more of the intracellular signaling protein than did clones C611, C612, and C613 (Fig. 1*B*). For example, clone C721 contained ~6 fold more immunoreactive hRasGRP4-GFP protein than did clone C611. Because low molecular weight GFP-labeled products were not detected in any of the cell lysates, it is unlikely that the different intracellular levels of hRasGRP4-GFP protein in

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the clones are the result of variable rates of proteolysis of the translated product. As assessed by real-time qPCR analysis (data not shown), clone C721 contained ~4-fold more hRasGRP4-GFP mRNA than did clone C611. Thus, much of the difference in hRasGRP4-GFP protein levels in the low- and high-expresser clones appears to be the result of variable amounts of the expression construct and its transcript in each clone.

PMA-induced Translocation of hRasGRP4-GFP Inside HMC-1 Cells—GFP resided primarily in the cytosol of the GFP-expressing clones, and the subcellular distribution of this reporter protein did not change after 60 min of exposure of these control cells to 100 nM or 1 μM PMA (data not shown). In contrast, PMA altered the distribution of wild-type hRasGRP4-GFP in HMC-1 cells in dose- and time-dependent manners (Fig. 2). Before exposure to PMA, wild-type hRasGRP4-GFP and its Ser^{548} mutant resided primarily in the cell's cytosol (Fig 2). When the transfectants that were expressing wild-type hRasGRP4-GFP were exposed to 30, 100 (Fig. 2), or 1000 nM PMA, the signaling protein quickly translocated to the plasma membrane. It then translocated from the plasma membrane to the nuclear membrane and an undefined intracellular site. In contrast, the Ser^{548} mutant of this signaling protein failed to translocate to any membrane compartment when its expressing cells encountered PMA.

PMA-dependent Activation of hRasGRP4 in HMC-1 Cells Leads to Increased Phosphorylation of ERK1/ERK2 and Increased Internalization of CD117—ERK1 and ERK2 often participate in Ras-dependent signaling pathways, and the PMA dependent stimulation of RasGRP3-expressing HEK-293 cells results in increased phosphorylation of both kinases (33). On the basis of these data, we evaluated the phosphorylation status of ERK1 and ERK2 in GFP- and hRasGRP4-GFP-expressing HMC-1 cells before and after PMA exposure. In these experiments, replicate cells were exposed to the protein kinase C (PKC) inhibitor GF109203X prior to PMA treatment to minimize the PKC-mediated phosphorylation of the kinases. As noted in Figures 3*A* and 3*B*, essentially all of the PMA-dependent phosphorylation of ERK1 and ERK2 that takes place in HMC-1 cells that lack hRasGRP4 appears to be mediated by PKCs. hRasGRP4-expressing HMC-1 constitutively contained three- to four-fold more phosphorylated ERK1 and ERK2 than control cells (Fig. 3*C*). In addition, these cells were able to increase their intracellular levels of phosphorylated ERK1/ERK2 more than twofold even when exposed to GF109203X before PMA stimulation (Fig. 3, *C* to *F*).

No substantial difference was noted when non-permeablized and permeablized $hRasGRP4^-$ and $hRasGRP4^+$ HMC-1 cells were exposed to DMSO, stained with phycoerythrin-conjugated anti-CD117 antibody, and then analyzed by a fluorescence-activated cell sorter (FACS) (Fig. 4). Based on these data, hRasGRP4 does not regulate the constitutive expression of CD117 on the surface of resting HMC-1 cells. While there was a modest decrease in the levels of surface CD117 when $hRasGRP4^-$ HMC-1 cells encountered PMA, the loss of this tyrosine kinase receptor from the plasma membrane was much greater in the PMA-treated $hRasGRP4^+$ cells. Analysis of the latter population of cells when permeabilized revealed increased internalization of the surface receptor.

Transcript Profiling of HMC-1 Cells that Differ in their hRasGRP4 Levels—Using a transfection approach, we previously induced hRasGRP4-defective HMC-1 cells to express mRasGRP4 (28). The amino acid sequences of mouse and human RasGRP4 are ~20% dissimilar (20). Because species-dependent differences in this signaling protein could exist and because better GeneChips have recently been developed in the last five years, we compared transcript expression in hRasGRP4-deficient HMC-1 cells and low and high RasGRP4-expressing HMC-1 cells before (Supplemental Tables 1-5) and after (Supplemental Tables 6 to 10) the three populations of cells encountered PMA. In agreement with the results from our earlier study of mRasGRP4-expressing HMC-1 cells (28), $hRasGRP4^+$ HMC-1 cells constitutively contained much more PGD_2 synthase mRNA than did $hRasGRP4^-$ cells (Supplemental Tables 1-3). However, our GeneChip data also indicated hundreds of other transcripts were substantially increased in our hRasGRP4-GFP expressing clones[3] relative to the GFP-expressing control cells, whereas hundreds of other transcripts were substantially decreased (Supplemental Tables 4 and 5). For example, the level of the transcript that

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encodes the α2 chain of the IL-13 receptor (IL13αR2) was 61-860-fold higher in the hRasGRP4-GFP-expressing HMC-1 cell clones relative to that in the GFP-expressing cells in this microarray analysis. In contrast, the level of the transcript that encodes the transcription factor GATA-1 was 5.3-49-fold higher in the control cells than the hRasGRP4-expressing HMC-1 cells.

Real-time qPCR analyses confirmed that hRasGRP4-expressing HMC-1 cells contained substantially less GATA-1 mRNA (data not shown) and more IL13Rα2 mRNA (Fig. 5) than did hRasGRP4-defective HMC-1 cells. Treatment of the three populations of HMC-1 cells with PMA also revealed a striking time-dependent change in the hRasGRP4-dependent expression of IL-13Rα2 mRNA (Fig. 5 and Supplemental Tables 6-10) and protein (Fig. 6). In regard to receptor levels, the amount of IL-13αR2 protein was below detection in GFP-expressing HMC-1 cells before and after these cells encountered PMA as assessed by SDS-PAGE immunoblot analysis. In contrast, IL13Rα2 protein was detected in hRasGRP4-GFP-expressing HMC-1 cells and the levels of this cytokine receptor increased after these cells encountered PMA for 6 h (Fig. 6, *A* and *B*). Thus, the hRasGRP4-mediated increase in IL13Rα2 mRNA levels leads to substantial changes in its translated product.

IL-13-dependent Phosphorylation of STAT6 in GFP- and hRasGRP4-GFP expressing HMC-1 Cells Before and After Exposure to PMA— Although the levels of IL13Rα2 mRNA and protein were below detection in GFP-expressing HMC-1 cells, these cells contained substantial amounts of IL13Rα1 mRNA before and after exposure to PMA (Supplemental Tables 1 and 6). Because GFP-expressing HMC-1 cells increased their levels of pSTAT6 when they encountered this cytokine (Fig. 6*C*, lanes 1 and 3), it is likely that the IL13Rα1 transcript is translated and the expressed receptor is functional. The levels of pSTAT6 were unchanged when these cells encountered PMA (Fig. 6*C*, lanes 3 and 4). Thus, PMA does not adversely influence IL-13/IL13Rα1-dependent signaling in hRasGRP4-defective HMC-1 cells. The levels of the IL13Rα1 transcript modestly increased in the hRasGRP4-GFP-expressing HMC-1 cells (Supplemental Tables 1 and 6) and the levels of pSTAT6 increased when these cells encountered IL-13 (Fig. 6*C*, lanes 3 and 7), consistent with an activating role for IL13Rα1 in MCs and other cell types. Although the levels of the IL13Rα2 transcript markedly increased in the hRasGRP4-GFP-expressing transfectants (Supplemental Tables 1-3 and 6-9), high expression of IL13Rα2 protein only occurred when these cells encountered PMA (Fig. 6, *A* and *B,* lanes 6 and 8). hRasGRP4-GFP-expressing HMC-1 cells significantly decreased their ability to respond to IL-13 if these cells were exposed to PMA before the cytokine $p<0.025$ ($n = 4$) (Fig. 6*C,* lanes 7 and 8). In contrast, the levels of pSTAT6 in the GFP-expressing HMC-1 cells were not significantly different (Fig. 6*C,* lanes 3 and 4; $p = 0.245$, $n = 4$).

DISCUSSION

Using an immunogold localization approach, we previously demonstrated that endogenous RasGRP4 resides primarily in the cytosol of the splenic MCs in the V3 mastocytosis mouse (23). Mouse, rat, and human RasGRP4 contain classical DAG/phorbol ester-binding C1 domains at their C-termini, and mouse and human RasGRP4-expressing fibroblasts undergo substantial morphologic changes when exposed to PMA (20,22). The identification of a hRasGRP4-defective variant (20) of the hTryptase β^+ HMC-1 MC line (25) gave us the opportunity to generate stable cell clones that differed in their intracellular levels of hRasGRP4-GFP to evaluate the PMA-dependent movement of the human signaling protein in a more physiologically relevant cell line than fibroblasts, COS-1 cells, or CHO cells which normally never express RasGRP4.

Although mBMMCs contain much more mRasGRP4 than mRasGRP1 (20), Liu and coworkers (34) concluded that mRasGRP1 is needed for the FcεRI-dependent release of β-hexosaminidase, tumor necrosis factor-α, IL-3, and IL-4 from this non-transformed population of MCs. Another advantage of using the hRasGRP4-defective HMC-1 cell line to study hRasGRP4-dependent signaling events is that this transfectable MC cell line does not express significant amounts of hRasGRP1, hRasGRP2, or hRasGRP3 (Supplemental Table 1). Thus, one does not have to worry about the contributions of other RasGRPs in experiments carried out on this human MC line. We now describe the creation of eight stable HMC-1 cell clones that contain

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different amounts of hRasGRP4-GFP (Fig. 1). Two HMC-1 cell clones also were generated that contain only GFP, as well as HMC-1 cell clones that express the Ser^{548} mutant of hRasGRP4-GFP.

Many non-transformed populations of RasGRP4-expressing MCs respond to PMA. We therefore evaluated the ability of PMA to induce translocation of hRasGRP4-GFP to different intracellular compartments in our HMC-1 cell clones. GFP resided primarily in the cytosol of the control cell clones and the subcellular distribution of GFP did not change after these cells were exposed to PMA. In contrast, GFP-labeled hRasGRP4 quickly translocated from the cytosol to the inner leaflet of the plasma membrane when the transfectants that expressed the signaling protein encountered 30, 100 (Fig. 2), or 1000 nM PMA.

MC development is highly dependent on stem cell factor/kit ligand and its tyrosine kinase receptor CD117. The $WBB6F_1$-*W/W*^{v} mouse is MC deficient (35) due to a point mutation in the CD117 gene (36,37). The signaling pathways at the plasma membrane regulated by RasGRP4 remain to be identified. Nevertheless, the observation that RasGRP4 is a cytosolic signaling protein that translocates to the inner leaflet of the plasma membrane in PMA-treated HMC-1 cells (Fig. 2) and regulates gene expression in the transfectants (20,28) (Figs. 5, 6, and S1, and Supplemental Tables 1-10) suggests that RasGRP4 participates in MC development by acting downstream of CD117 and upstream of Ras. The observation that substantially more CD117 is lost from the surface of PMA-treated hRasGRP4$^+$ HMC-1 cells than PMA-treated hRasGRP4$^-$ HMC-1 cells (Fig. 4) is consistent with a likely role for this signaling protein in CD117-dependent signaling after the guanine nucleotide exchange factor binds DAG and reaches the inner leaflet of the plasma membrane (Fig. 2). Supporting this conclusion is the ability of PMA to rescue the genetic defect in the CD117-pathway in the cutaneous MCs of the $WBB6F_1$-*W/W*^{v} mouse (38). In addition, stem cell factor/kit ligand treatment of IL-3-developed mBMMCs results in increased expression of PGD_2 synthase (39) as occurs in human (Supplemental Tables 1-10) and mouse (28) RasGRP4$^+$ HMC-1 cells before and after exposure to PMA.

Others reported that RasGRP1, RasGRP2, and RasGRP3 are able to translocate from the cytosol to the plasma membrane in phorbol ester-treated cells but only RasGRP1 and RasGRP3 are able to eventually translocate to the Golgi complex (30,40-42). The mechanism was not deduced in their study but Caloca and coworkers (30) concluded that RasGRP2 is unable to translocate to the Golgi because the eighth residue in its 50-mer C1 domain is Ser rather than Tyr as found in RasGRP1 and RasGRP3. Of the 50 residues in the C1 domains of hRasGRP1 and hRasGRP4, 16 are different. For example, the eighth residue in the C1 domains of mouse, rat, and human RasGRP4 is Phe rather than Tyr or Ser. We converted Phe^{548} in hRasGRP4-GFP to Ser analogous to that in the C1 domain of hRasGRP2 to evaluate the ability of the mutated signaling protein to translocate to different compartments in PMA-treated HMC-1 cells. Surprisingly, our Ser^{548} mutant could not even reach the plasma membrane when the transfectants were exposed to PMA (Fig. 2). Irie and coworkers (43) reported that ~50-mer synthetic peptides corresponding to the C1 domains of RasGRP1, RasGRP3, and RasGRP4 bind to radiolabeled phorbol-12,13-dibutyrate in vitro considerably better than the peptide that corresponds to the C1 domain of RasGRP2. While our data and that of Irie and coworkers (43) suggest that a Phe/Tyr aromatic residue at position 8 in the C1 domain is of critical importance in the ability of RasGRP1, RasGRP3, and RasGRP4 to recognize DAG and phorbol esters, unexplained is how RasGRP2 can translocate to any membrane in response to phorbol esters if its C1 domain cannot recognize DAG and phorbol esters. Also unexplained is the mechanisms and factors that control the translocation of RasGRP4 from the plasma membrane to other intracellular membrane compartments. In this regard, Okamura and coworkers (44) reported that the 127-mer amino acid sequence C-terminal of the DAG/PMA-binding C1 domain in hRasGRP3 helps regulate the intracellular movement of this signaling protein in CHO-K1 cell transfectants. Although the corresponding domain in hRasGRP4 is very different, the RasGRP3 data raise the possibility that other regions in RasGRP4 probably contribute to its redistribution in activated MCs.

ERK1 and ERK2 often participate in Ras-dependent signaling pathways, and hRasGRPs

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1 to 3 constitutively activate ERK1 and ERK2 in transfected human 293T embryonic kidney cells (8). GFP- and hRasGRP4-GFP-expressing HMC-1 cells therefore were evaluated before and after PMA exposure to determine if hRasGRP4 regulates the phosphorylation of these two kinases. hRasGRP4-GFP-expressing HMC-1 cells constitutively contained more phosphorylated ERK1 and ERK2 than did hRasGRP4-deficient HMC-1 cells. The transfectants also increased their intracellular levels of phosphorylated ERK1/ERK2 after PMA stimulation (Fig. 3). The accumulated data suggest the activation of ERK1 and ERK2 in MCs before and after exposure to PMA is mediated in part by hRasGRP4. These data also suggest that some of the earlier observed effects of PMA on MCs actually are mediated by RasGRP4 rather than PKC.

We used a transfection approach in an earlier study to create noncloned HMC-1 cells that expressed BALB/c mRasGRP4 (20). In that study, we placed the mouse signaling protein rather than its human ortholog in the hRasGRP4-defective cell line because it was not apparent to us at that time which splice or allelic variant of hRasGRP4 we identified should be used in the rescue approach to correct the deficiency in the signaling protein. In the present study, we placed a biologically active isoform of hRasGRP4 in HMC-1 cells and then cloned the transfectants to obtain cell lines that differed in their levels of the human signaling protein (Fig. 1). We then used an Affymetrix GeneChip approach to identify candidate transcripts regulated by hRasGRP4 in negative or positive manners.

hRasGRP4$^-$ and hRasGRP4$^+$ HMC-1 cells had similar amounts of CD117 mRNA (Supplemental Table 1) and similar amounts of CD117 protein on their surfaces (Fig. 4). Both populations of cells also contained substantial amounts of hTryptase β mRNA (Supplemental Table 1). Thus, not all MC-restricted genes are regulated by RasGRP4. Nevertheless, as noted in Supplemental Tables 1-10, we discovered that the levels of hundreds of transcripts were altered in HMC-1 cells that differed in their expression of hRasGRP4. Amazingly, some of these transcripts altered their expression >1000 fold. In this regard, most human tissue MCs metabolize arachidonic acid preferentially to PGD_2 via the cyclooxygenase pathway (26). Macchina and coworkers discovered that HMC-1 cells do not generate significant amounts of PGD_2 even though they produce large amounts of leukotrienes and thromboxane A2 when exposed to calcium ionophore (27). We previously showed the inability of these cells to produce PGD_2 was caused by a hRasGRP4-dependent defect in the expression of PGD_2 synthase (28). As with mRasGRP4-expressing HMC-1 cells, the level of the PGD_2 synthase transcript was markedly increased in the hRasGRP4-GFP-expressing HMC-1 cells relative to that of the parental cell line, and only nine transcripts increased their absolute levels higher than that of the PGD_2 synthase transcript (Supplemental Table 3). Of the many transcripts whose expressions were markedly altered in the transfectants, we focused our attention on IL13Rα2 (Figs. 5 and 6) because only two other transcripts increased their levels in RasGRP4-expressing HMC-1 cells more than the IL13Rα2 transcript (Supplemental Table 2) and because of the perceived importance of IL-13 and its receptors in varied pathologic states, including MC-dependent inflammation of the lung.

IL-13 is pleiotropic immunoregulatory cytokine produced by activated mouse T_H2 cells (45) and numerous populations of MCs (46-49). This ~12-kDa cytokine plays a prominent role in asthma and other inflammatory disorders, and its effects are mediated via surface receptors that consist of the α subunit of the IL-4 receptor (IL4Rα) linked to the IL-13-specific IL13Rα1 or IL13Rα2 subunit [for review (50)]. IL-13 binds to IL13Rα2/IL4Rα receptor complexes with high affinity (51). Although it has been reported that IL13Rα2 is needed for the IL-13-mediated expression of transforming growth factor β1 in cultured macrophages (52), IL13Rα2-expressing cells generally are substantially less responsive to IL-13 than are IL13Rα1-expressing cells (53,54). IL-13 is rapidly internalized when bound to IL13Rα2 (51), and IL13Rα2-null mice are more sensitive than wild-type mice to IL-13 (55). The accumulated data have led to the conclusion that IL13Rα2 functions in most cell types as a critical decoy/inhibitory receptor for IL-13-dependent activation of cells. Kaur and coworkers (56) showed that in vivo-differentiated MCs from human lung also express IL13Rα1, and Lorentz and coworkers (57) showed that normal human intestinal MCs express both IL13Rα1 and

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IL13Rα2. Not only do canine MC lines express IL13Rα1 and IL13Rα2, the transcript that encodes canine IL13Rα2 was first cloned from a mastocytoma cell line (58). It therefore has been known for some time that many mammalian MCs express both types of IL-13 receptors. Unexplained is why varied populations of MCs are considerably less responsive to IL-13 than to IL-4 (57,59). Assuming the presence of IL13Rα2 is the reason why MCs are poorly responsive to IL-13, it is important to identify the factors and mechanisms that control the expression of this inhibitory receptor in MCs.

GFP-expressing, hRasGRP4-defective HMC-1 cells lacked IL13Rα2 mRNA and protein (Supplemental Tables 1 and 6, and Figs. 5 and 6) but contained significant amounts (>1170 mRNA units) of IL13Rα1 mRNA before and after exposure to PMA. Because GFP-expressing HMC-1 cells increased their levels of pSTAT6 when they encountered IL-13, these hRasGRP4-defective cells apparently are IL-13 responsive due to their IL13Rα1 receptors. Although the levels of the IL13Rα2 transcript markedly increased in the hRasGRP4-GFP-expressing transfectants, these cells did not accumulate high levels of IL13Rα2 protein until they encountered PMA (Fig. 6). The reason why these cells do not contain large amounts of IL13Rα2 protein despite having substantial amounts of its transcript remains to be determined. However, it is possible that a PMA/protein kinase C-dependent pathway prevents the rapid catabolism of this cytokine receptor in MCs. Whatever the reason, hRasGRP4-GFP/IL13Rα2-expressing HMC-1 cells significantly decreased their ability to respond to IL-13 when exposed to PMA (Fig. 6). The accumulated data suggest that MCs use hRasGRP4 to control their levels of IL13Rα2 in order to dampen IL-13/IL13Rα1-mediated signaling events in this immune cell. Thus, our new data support that in previous studies which have led to the conclusion that RasGRP4 helps control the development, phenotype, and function of MCs.

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FOOTNOTES

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[2] The abbreviations used are: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HPRTI, hypoxanthine guanine phosphoribosyltransferase-I; IL, interleukin; mBMMC, mouse bone marrow-derived MC; MC, mast cell; PBS, phosphate-buffered saline; PGD_2, prostaglandin D_2, PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; STAT6, signal transducer and activator of transcription 6; pSTAT6, phospho-STAT6; qPCR, quantitative polymerase chain-reaction; RasGRP, Ras guanine releasing protein; and RT, reverse transcriptase.

[3] The *240862_at* probe set on the *HG-U133 Plus 2.0* GeneChip used in our study was designed by Affymetrix to recognize the wild-type hRasGRP4 transcript. The failure to detect the transcript in the hRasGRP4-GFP expressing HMC-1 cell transfectants as noted in the Supplemental Tables is a technical artifact due to the removal of the transcript's 3' untranslated region in the expression construct which is the target nucleotide sequence used in Affymetrix's probe set.

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FIGURE LEGENDS

FIG. 1. **Immunoblot Analysis of GFP- and hRasGRP4-GFP-expressing clones of HMC-1 cells**. *A*, Lysates of two clones of HMC-1 cells that expressed GFP (lanes 2 and 3) and eight clones that expressed hRasGRP4-GFP (lanes 4-11) were evaluated by SDS-PAGE immunoblot analysis for their expression of GFP (upper panel) and β-actin (lower panel). For a positive control for the GFP immunoblot analysis, 55-kDa GFP-labeled positope protein was placed in lane 1. As expected, an ~30-kDa immunoreactive protein was present in each GFP-transfectant and an ~105-kDa immunoreactive protein was present in each hRasGRP4-GFP transfectant. *B*, The Quantity One–Version 4.1.1 software package and the Versadoc Imaging System Conversion Plate (Biorad) were used to compare the protein levels of hRasGRP4-GFP in the eight clones relative to that of β-actin. The obtained data revealed substantial differences in the levels of hRasGRP4-GFP in some of the transfectants. The low-expresser clone C611 and the high-expresser clone C721 were used in most follow-up experiments.

FIG. 2. **PMA-induced translocation of hRasGRP4-GFP inside HMC-1 cells**. HMC-1 cells were induced to express wild-type hRasGRP4-GFP (left panels) or its Ser^{548} mutant (right panels). The resulting two populations of cells were examined by confocal microscopy before (upper panels) and after (bottom panels) a 15-min exposure to 100 nM PMA. In the untreated cells, hRasGRP4-GFP resided primarily in the cell's cytoplasm (asterisk). After PMA treatment, the wild-type signaling protein translocated to the inner leaflet of the plasma membrane (thick arrows), to a perinuclear site (thin arrow), and to an undefined intracellular compartment (line). The Ser^{548} mutant of hRasGRP4-GFP failed to translocate to any membrane compartment in response to PMA. Bar: 10 μm.

FIG. 3. **Phosphorylation of ERK1 and ERK2 in control- and hRasGRP4-GFP-expressing HMC-1 cells before and after exposure to PMA.** Non-transfected HMC-1 cells (*A*), GFP-expressing HMC-1 cells (*B*), and hRasGRP4-GFP-expressing HMC-1 cells (clone C721) (*C*) were exposed for 15 min to 0.01% DMSO without or with 1, 10, 100, and 1000 nM PMA. Replicate cells were exposed to 10 μM GF109203X in DMSO for 30 min before the addition of PMA. Phosphorylated ERK1/ERK2 (pERK1/pERK2) and total ERK1/ERK2 were measured by SDS-PAGE immunoblot analyses using the relevant antibodies. For hRasGRP4-GFP- and GFP-expressing cells, the levels of pERK1/pERK2 and total ERK1/ERK2 in the presence of GF109203X and PMA were measured by densitometry and the ratio of phosphorylated:total ERK1/ERK2 was determined in each instance (*D*). For hRasGRP4-GFP-expressing HMC-1 cells, this ratio was expressed relative to that of untreated cells (*E*). To reduce basal ERK1/ERK2 activation, the above experiment was repeated with the low hRasGRP4-GFP clone C611, which resulted in greater ERK1/ERK2 activation in cells exposed to 100 and 1000 nM PMA (*F*). Shown are the mean data ± standard error of the mean (SEM) for three experiments carried out on different weeks on different batches of the clones.

FIG. 4. **Surface and intracellular levels of CD117.** $hRasGRP4^-$ (*A* and *C*) and $hRasGRP4^+$ (*B* and *D*) HMC-1 cells were exposed to DMSO with (red curves) or without (green curves) PMA. Non-permeablized (*A* and *B*) and permeablized (*C* and *D*) cells were then stained with phycoerythrin-conjugated anti-CD117 antibody, and analyzed by FACS.

FIG. 5. **Evaluation of IL13Rα2 mRNA levels in HMC-1 cells that differ in their hRasGRP4 expression before and after exposure of the cells to PMA.** HMC-1 cell clones that contain low levels of hRasGRP4-GFP (clone C611), high levels of hRasGRP4-GFP (clone C721), or just GFP (Mock cells) were cultured for up to 20 h in the presence of 0.01% DMSO without (-) or with 100 nM PMA (+). Total RNA was isolated from the different populations of cells and real-time qPCR analyses were carried out with the IL13Rα2, GAPDH, and HPRTI primer sets. Shown are the levels of IL13Rα2 mRNA (mean ± SEM, n = 3) in the treated cells relative to the GAPDH and HPRTI transcripts (internal control).

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FIG. 6. **Evaluation of IL13Rα2 protein levels in HMC-1 cells that differ in their hRasGRP4 expression before and after exposure of the cells to PMA, and evaluation of IL-13-dependent phosphorylation of STAT6 in these cells.** GFP-expressing HMC-1 cells (lanes 1-4) and hRasGRP4-GFP expressing HMC-1 cells (lanes 5-8) were untreated (lanes 1 and 5), or were exposed to PMA (lanes 2 and 6), IL-13 (lanes 3 and 7), or PMA followed by IL-13 (lanes 4 and 8). The obtained raw SDS-PAGE immunoblot data, the β-actin corrected IL13Rα2 data, and the β-actin corrected pSTAT6 data are shown in *A*, *B,* and *C*, respectively. Data are expressed as mean ± SD (n = 4). NS = not significant; * = $p<0.025$.

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Table 1
Transcript Expression
Shown are the sequences and annealing temperatures of the primers used for real-time qPCR analysis of the GATA1, IL13Rα2, HPRT1, and GAPDH transcripts in GFP- and hRasGRP4-GFP expressing HMC-1 cell clones

Primer name	Primer sequence	Annealing Temperature
GATA1 Forward	5′-CCAGCTTCCTGGAGACTTTG-3′	60ºC
GATA1 Reverse	5′-CAGGGAGTGATGAAGGCAGT-3′	
IL13Rα2 Forward	5′-ACGGAATTTGGAGTGAGTGG-3′	55ºC
IL13Rα2 Reverse	5′-TGGTAGCCAGAAACGTAGCA-3′	
HPRT1 Forward	5′-GACCAGTCAACAGGGGACAT-3′	60ºC
HPRT1 Reverse	5′-CCTGACCAAGGAAAGCAAAG-3′	
GAPDH Forward	5′-AATCCCATCACCATCTTCCA-3′	60ºC
GAPDH Reverse	5′-TGGACTCCACGACGTACTCA-3′	

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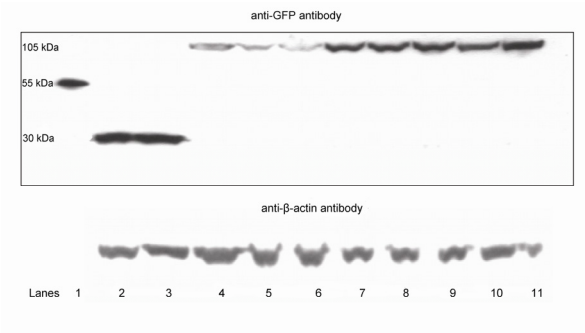

Figure 1A

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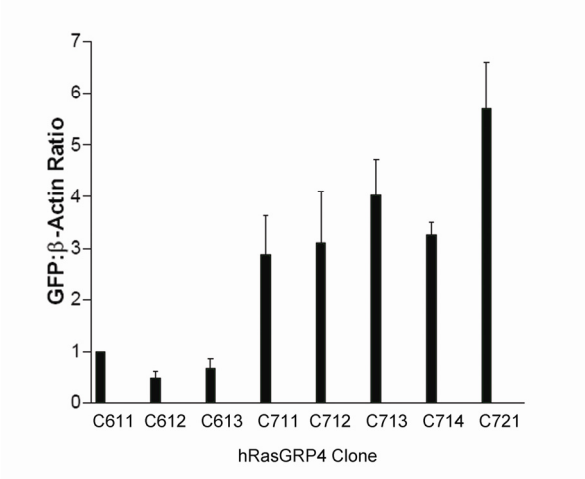

Figure 1B

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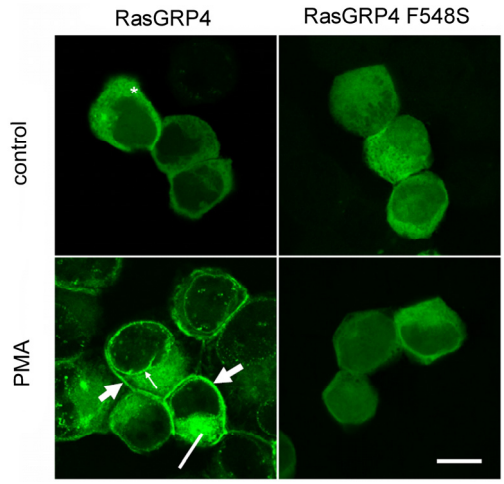

Figure 2

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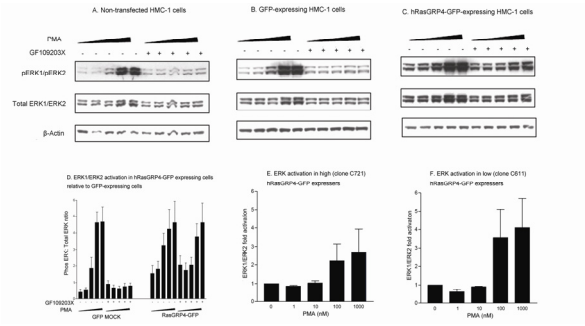

Figure 3

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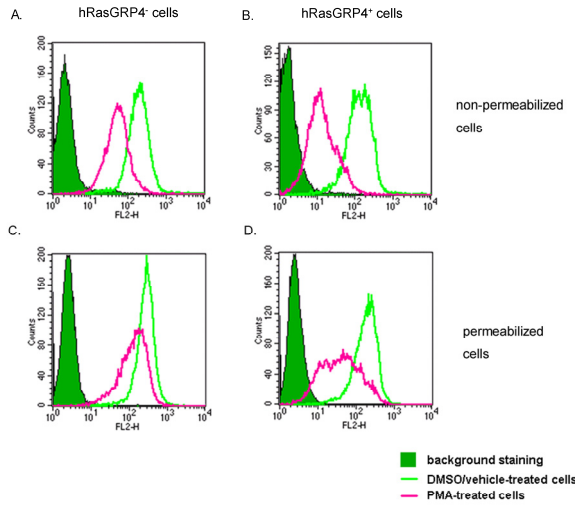

Figure 4

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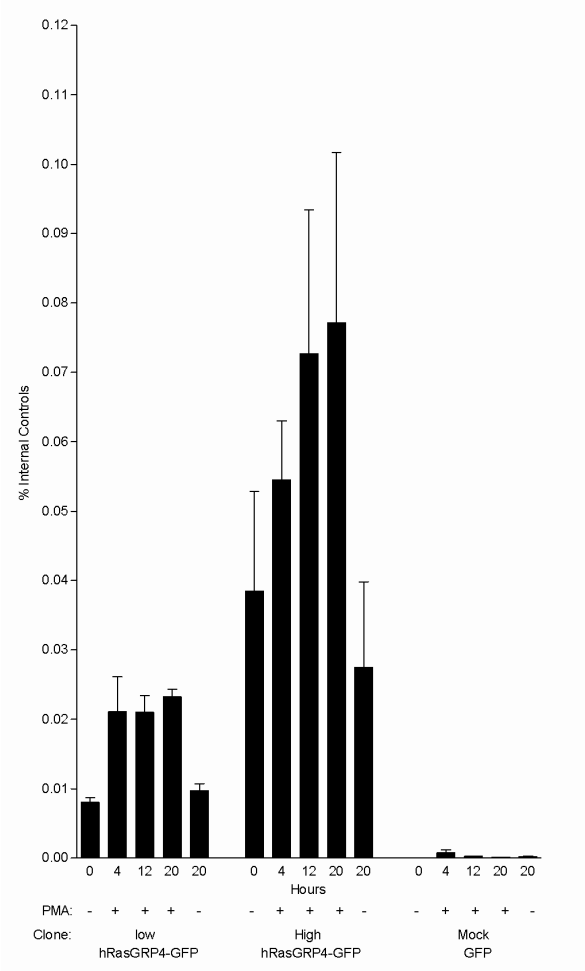

Figure 5

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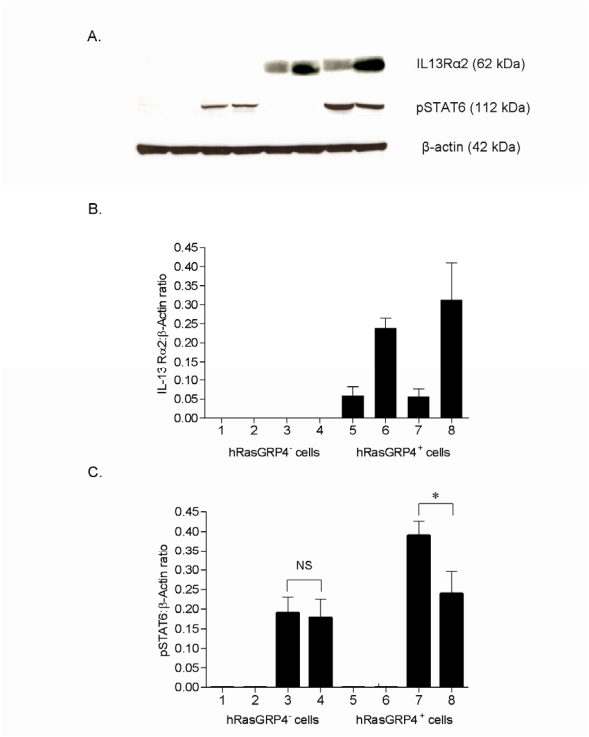

Figure 6

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