RasGRP4 Regulates the Expression of Prostaglandin D₂ in Human and Rat Mast Cell Lines*

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Mast cells (MCs) are a major source of prostaglandin (PG) D₂ in connective tissues, and the expression of this eicosanoid has been linked to asthma and other inflammatory disorders. While it is known that the surface receptor c-kit controls PGD₂ expression in MCs by regulating the levels of a synthase that converts PGH₂ to PGD₂, the intracellular signaling proteins that act downstream of c-kit in this cyclooxygenase pathway have not been identified. We recently cloned a new cation-dependent, guanine nucleotide exchange factor/phorbol ester receptor (designated RasGRP4) that is required for the efficient expression of granule proteases in the human MC line HMC-1. GeneChip analysis of ∼12,600 transcripts in RasGRP4⁺ and RasGRP4⁻ HMC-1 cells revealed a >100-fold difference in the levels of hematopoietic PGD₂ synthase mRNA. No other transcript in the eicosanoid pathway was influenced by RasGRP4 in a comparable manner. As assessed by SDS-PAGE immunoblot analysis, RasGRP4⁺ HMC-1 cells contained substantial amounts of PGD₂ synthase protein. RasGRP4⁺ MCs also produced ∼15-fold more PGD₂ than did RasGRP4⁻ MCs when both cell populations were activated by calcium ionophore. The induced transcript is therefore translated, and substantial amounts of functional PGD₂ synthase accumulate in RasGRP4⁺ MCs. In support of the conclusion that RasGRP4 controls PGD₂ expression in MCs, inhibition of RasGRP4 expression in the rat MC line RBL-2H3 using a siRNA approach resulted in low levels of PGD₂ synthase protein.

Activated human and rodent mast cells (MCs) generate and release substantial amounts of prostaglandin (PG) D₂, and many of the vasodilation and hemodynamic problems that occur in patients with systemic mastocytosis are thought to be caused by the excessive production of this eicosanoid. PGD₂ is a neuromodulator/sleep-inducing factor in the central nervous system. In peripheral tissues, PGD₂ inhibits platelet aggregation (2) but activates eosinophils. PGD₂ is a potent chemotactic factor for eosinophils (3), and PGD₂-treated eosinophils increase their calcium mobilization, actin polymerization, and surface expression of CD11b (4, 5). This eicosanoid also enhances the rate of apoptosis of eosinophils if these granulocytes are cultured for −20 h in the absence of a viability-enhancing cytokine such as interleukin (IL) 5 (6). Pulmonary MCs play important roles in the initiation and/or progression of asthma, and substantial amounts of PGD₂ are released into the lungs during asthma attacks (7, 8). The observation that patients with asthma undergo bronchoconstriction when they inhale PGD₂ (9) documents the pathologic consequences of high levels of PGD₂ in the lung. PGD₂ exerts its biological actions via two seven-transmembrane, G protein-coupled receptors (designated PTGDR/DP and GPR44/CRTH2) (10–12). Targeted disruption of the PTGDR gene in the mouse leads to a marked reduction in antigen-induced airway reactivity to acetylcholine (13), thereby supporting the earlier inhalation studies in humans and dogs that implicated an adversarial role for PGD₂ in the lung.

In the cyclooxygenase pathway that ultimately leads to PGD₂ expression, liberated arachidonic acid is converted to PGG₂ and then to PGH₂, PG endoperoxide H synthase (PGHS) 1 (also known as cyclooxygenase 1) and PGHS-2 (also known as cyclooxygenase 2) are both able to carry out this two-step biosynthetic process. The resulting precursor eicosanoid is then metabolized by terminal synthases to form PGD₂, PGE₂, PGF₂α, PGJ₂/prostacyclin, and thromboxane A₂. Two PGD₂ synthases have been identified in mice, rats, and humans (14, 15). The brain enzyme is a glutathione-independent member of the lipocalin family of proteins. The distinct hematopoietic enzyme that is expressed in MCs (16) is a sigma-class, glutathione S-transferase family member.

PGH₂ can be metabolized inside cells to thromboxane A₂ and to a variety of PGs. Thus, the amount of PGD₂ produced by an FeR R- or calcium ionophore-activated MC is determined in a large part by the amount of PGD₂ synthase protein in the cell. MCs are heterogeneous in terms of what eicosanoids they produce. c-kit is a member of the type III receptor tyrosine kinase family. PGD₂-expressing MCs contain abundant amounts of c-kit on their surfaces, and Murakami et al. (17) noted that c-kit ligand (KL) somehow regulates the levels of PGD₂ synthase in mouse MCs. To a lesser extent, IL-3 and IL-10 also influence the expression of PGD₂ synthase in MCs. Treatment of human megakaryocytic cell lines with phorbol esters results in a 2–5-fold increase in the levels of PGD₂ synthase mRNA (18, 19). While these findings suggest that one or more diacylglycerol/phorbol ester-responsive proteins play an important role in the expression of PGD₂ synthase in hematopoietic cells, the intracellular proteins that act downstream of c-kit and other membrane receptors to control the levels of PGD₂ synthase in MCs have not been identified.

We recently cloned a new member of the Ras guanine nucleo-

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†The abbreviations used are: MC, mast cell; mBMMC; mouse bone marrow-derived MC; IL, interleukin; LT, leukotriene; PG, prostaglandin; PGHS, PG endoperoxide H synthase; RT, reverse transcriptase; RBL, rat basophilic leukemia; TNF-α, tumor necrosis factor α; KL, c-kit ligand; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA.

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otide-releasing protein (RasGRP) family of intracellular signaling proteins (20). In contrast to the other three members of its family, RasGRP4 normally is restricted to mature MCs and their circulating progenitors. RasGRP4 functions as a cation-dependent, guanine nucleotide exchange factor. It also is a diacylglycerol/phorbol ester receptor that appears to act downstream of c-Akt. The hRasGRP4 gene resides on chromosome 19q13.1 (20) in the vicinity of a site that has been linked to bronchial hyperresponsiveness (21, 22). RasGRP1 is essential for the final stages of T-cell development (23). Although human MCs do not express RasGRP1, RasGRP2, or RasGRP3, transfection studies carried out with the RasGRP4-defective HMC-1 cell line derived from a patient with a MC leukemia suggests that RasGRP4 is required for the final stages of MC development (20). Thus, at least two members of the RasGRP family of signaling proteins appear to control cellular differentiation and maturation. We previously noted that RasGRP4 influences the storage of varied neutral proteases in the secretory granules of a MC line. We now report that RasGRP4 also controls what eicosanoids this immune cell produces.

**EXPERIMENTAL PROCEDURES**

**Transcript Analysis of RasGRP4**- and RasGRP4**+** HMC-1 Cells—RasGRP4**+** and RasGRP4**+** HMC-1 cells (20) were cultured in enriched medium (Iscove’s modified Dulbecco’s medium (BioWhittaker) containing 10% heat-inactivated fetal calf serum (Sigma), 2 mgl−1-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μM monothioglycerol (Sigma) with or without 200–500 μg/ml G418 in the absence of human cytokines. Total RNA was isolated from the two populations of cells with TRIzol (Invitrogen), and comparative transcript profiling was carried out at the Gene Array Technology Center (Brigham and Women’s Hospital, Boston, MA) with HG-U95A GeneChips (Affymetrix, Santa Clara, CA) and the experimental protocol recommended by Affymetrix. Each GeneChip contains ~12,600 probe sets. In these analyses, 8 μg of total RNA from RasGRP4**+** and RasGRP4**+** HMC-1 cells were reverse-transcribed using the GeneChip T7-oligo(dT) promoter primer kit. Biotinylated complementary cRNAs, generated from the resulting cDNAs, were fragmented and incubated with the GeneChips for 16 h. The resulting GeneChips were incubated with streptavidin-

![Image](https://example.com/image.png)

**TABLE 1**

| Transcript and its GenBank™ accession number | Affymetrix ID number | Fold change |
|--------------------------------------------|---------------------|-------------|
| PGD2 synthase, hematopoietic (AF150241)    | 35,523              | 107.0       |
| 15-Lipoxygenase, type 1 (M23982)           | 34,836              | 6.2         |
| Thromboxane A synthase 1 (D34925)         | 33,777              | 4.5         |
| 15-Lipoxygenase, type 2 (U78294)          | 37,430              | 3.4         |
| 12-Lipoxygenase (M62982)                  | 35,124              | 1.8         |
| PGE2 synthase 1 (AF010316)                | 38,131              | 1.8         |
| PGE2 synthase (AKR1C3) (D17793)           | 37,599              | 1.4         |
| Hydroxyprostaglandin dehydrogenase 15-(NAD) (X82460) | 37,322 | 1.4 |
| PGH2 synthase 2/COX2 (U04636)             | 1069                | 1.4         |
| PGH2 synthase 1/COX1 (M59979)             | 37,969              | 1.3         |
| 5-Lipoxygenase (J03600)                   | 307                 | 1.2         |
| LTA4 synthase (U50138)                    | 39,968              | 1.2         |
| PGD2 synthase, brain (M98539)             | 216                 | 1.1         |
| PGH2/prostacyclin synthase (D38145)       | 759                 | 1.0         |
| LTβ, hydroxylase (D12620)                 | 1305                | 1.0         |
| ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (L05628) | 1896 | 0.9 |
| 5-Lipoxygenase-activating protein/FLAP (AI806222) | 37,099 | 0.9 |
| 12-Lipoxygenase, 12R type (AF038461)      | 33,029              | 0.9         |
| LTA4, hydrolyase (J03459)                 | 38,081              | 0.8         |
| Microsomal glutathione S-transferase 2 (U77604) | 820 | -1.6 |

*The fold change* values represent the levels of the indicated transcripts in RasGRP4**+** HMC-1 cells relative to that in RasGRP4**+** HMC-1 cells. In each case, data were normalized to the ubiquitously expressed transcripts that encode β-actin and glyceraldehyde-3-phosphate dehydrogenase.
RasGRP4-dependent Regulation of PGD<sub>2</sub>

Calcium Ionophore Activation of RasGRP4<sup>+</sup> and RasGRP4<sup>+</sup> HMC-1 Cells—RasGRP4<sup>+</sup> and RasGRP4<sup>+</sup> HMC-1 cells were washed, suspended at a concentration of 10<sup>6</sup> cells/ml in calcium/magnesium-free phosphate-buffered saline, and stimulated with 0.5 μM calcium ionophore A23187 (Sigma) at 37 °C for 30 min as done in other eicosanoid studies of MCs (26). The generated eicosanoids PGD<sub>2</sub>, PGE<sub>2</sub>, and leukotriene C<sub>4</sub> (LTC<sub>4</sub>) in the supernatants were quantitated using the relevant ELISA kits (Cayman Chemical). Each reaction was read at 450 nm using an ELISA plate reader (Molecular Device). Data are given as mean ± S.D. Significance was defined as p < 0.05 by the Student’s t test.

siRNA-mediated Inhibition of RasGRP4 Expression in RBL-2H3 Cells—A siRNA approach similar to that described by Elbashir et al. (27) was used to evaluate the consequences of decreased expression of PGD<sub>2</sub> synthase mRNA in the rat MC line RBL-2H3. The coding sequence of rat RasGRP4 (28) was scanned to identify a gene-specific 21-nucleotide sequence downstream of an “AA” sequence that possesses a 55% GC content. A BLAST search confirmed that the selected sequence (corresponding to residues 27–47 in GenBank<sup>™</sup> accession number AF465263) is not present in another transcript in GenBank<sup>™</sup> databases. The RasGRP4-specific oligonucleotide 5′-GUCUCAUCAGGAGUCCUGGdTdT-3′ and its corresponding oligonucleotide 5′-CCAGGACGUGAUCUGACAGdTdT-3′ were synthesized and purified (Dharmacon Research, Lafayette, CO) and then annealed to form the final siRNA duplex with its 3′ overhangs. The resulting siRNA duplex was introduced into RBL-2H3 cells (line CRL-2236; American Type Culture Collection, Manassas, VA) using a liposome transfection approach. Liposome/siRNA complexes were formed at room temperature using 3 μl of 20 μM siRNA, 2 μl of LipofectAMINE™ 2000 (Invitrogen), and 100 μl of Opti-MEM I serum-free culture medium (Invitrogen). The resulting solution was added dropwise to each culture dish containing ~5 x 10<sup>6</sup> adherent MCs. The cells were incubated 3–4 h at 37 °C. One ml of serum-enriched medium was then added, and the cells were cultured for an additional 24–48 h. The transiently transfected cells were harvested, and the levels of PGD<sub>2</sub> synthase and β-actin protein were measured using the above SDS-PAGE immunoblot approach. In these assays, each protein blot was incubated ~17 h with anti-PGD<sub>2</sub> synthase antibody and then for 1 h with the anti-β-actin antibody (Sigma) before final development.

RESULTS AND DISCUSSION

All nontransformed rodent and human MCs that have been examined to date preferentially metabolize arachidonic acid via the cyclooxygenase pathway to PGD<sub>2</sub> rather than to PGE<sub>2</sub>. Nevertheless, Macchia et al. (29) discovered that HMC-1 cells produce ~20-fold more PGE<sub>2</sub> than PGD<sub>2</sub>. This surprising finding allowed us to use the c-kit<sup>+</sup> HMC-1 cell line to further elucidate the intracellular signaling pathways that control PGD<sub>2</sub> production in MCs. Transcript analysis (Fig. 1) revealed that the failure of HMC-1 cells to generate large amounts of PGD<sub>2</sub> is a consequence of a low rate of transcription of the PGD<sub>2</sub> synthase gene and/or a high rate of catabolism of its transcript.

The RasGRP4 transcript was initially cloned from IL-3-developed mouse bone marrow-derived MCs (mBMMCs). While all mouse, rat, and human MCs appear to express RasGRP4 mRNA and/or protein, the amount of RasGRP4 protein in a...
mouse peritoneal MC greatly exceeds that in a mBMMC as assessed by SDS-PAGE immunoblot analysis. Calcium ionophore- or FceRI-activated mBMMCs produce ~25-fold more LTC₄ than PGD₂, whereas peritoneal MCs activated in a similar manner produce ~40-fold more PGD₂ than LTC₄ (1, 26). The cumulative data raised the possibility that RasGRP4 regulates arachidonic acid metabolism in MCs. Thus, we evaluated whether or not RasGRP4 controls PGD₂ and/or LTC₄ expression in HMC-1 and RBL-2H3 cells.

**Comparative transcript analysis of RasGRP4** and RasGRP⁴⁺ HMC-1 cells using an Affymetrix GeneChip approach revealed a dramatic difference in the steady-state levels of the transcript that encodes hematopoietic PGD₂ synthase in the two populations of cells (Table 1). RasGRP⁴⁺ HMC-1 cells contained >100-fold more PGD₂ synthase mRNA than did the starting population of HMC-1 cells that express nonfunctional forms of RasGRP4. No transcript was induced to a comparable level, including the transcripts that encode brain-type PGD₂ synthase and LTC₄ synthase. Table 1 shows profile data relating to the levels of the transcripts that encode different proteins that participate in arachidonic acid metabolism. The PGD₂ synthase GeneChip data were confirmed by real-time RT-PCR (Fig. 1A) and by semiquantitative RT-PCR (Fig. 1B) analyses in three separate populations of RasGRP4-expressing cells. In a control experiment, HMC-1 cells transfected with the expression vector pcDNA3.1 lacking the RasGRP4 cDNA contained barely detectable amounts of PGD₂ synthase transcript (data not shown).

Because the levels of a transcript do not always correlate with the levels of its translated product, an SDS-PAGE immunoblot approach was used to compare the levels of PGD₂ synthase protein in RasGRP4⁺ and RasGRP⁴⁺ HMC-1 cells. The amount of PGD₂ synthase protein in RasGRP4⁺ HMC-1 cells was nearly below detection (Fig. 2). In contrast, RasGRP⁴⁺ HMC-1 cells contained substantial amounts of an intracellular 25-kDa protein that was recognized by the anti-PGD₂ synthase antibody. The induced PGD₂ synthase transcript is therefore translated and the appropriately sized biosynthetic enzyme accumulates in the transfectants. As assessed by SDS-PAGE immunoblot analysis, RasGRP4 did not induce HMC-1 cells to increase their accumulation of PGH₁, PGE₂ synthase, or 5-lipoxygenase protein (Fig. 2). Thus, RasGRP4 induces a selective accumulation of PGD₂ synthase mRNA and protein in this MC line.

As assessed by ELISA, calcium ionophore-activated RasGRP⁴⁺ HMC-1 cells produced 12–20-fold more PGD₂ (p < 0.05) than did RasGRP⁴⁻ HMC-1 cells (Fig. 3). The levels of PGE₂ and LTC₄ were modestly increased and decreased, respectively, in the calcium ionophore-treated RasGRP⁴⁺ cells. However, the variations in the amounts of these eicosanoids were not statistically significant. The fact that HMC-1 cells express nonfunctional forms of RasGRP4 indicates that RasGRP4 is not essential in the early stages of MC development, including the c-kit/KL-mediated proliferation of its progenitors. Nevertheless, the observation that HMC-1 cells are unable to granulate (20) and to produce substantial amounts of PGD₂ (Fig. 3) implies that RasGRP4 is required for the efficient expression of the cassette of genes that encode a number of the granule and lipid mediators of MC. The siRNA data obtained from transiently transfected RBL cells (Fig. 4) support this conclusion. RBL cells contain PGD₂ synthase protein, and these rat MCs (30) produce substantial amounts of PGD₂ when exposed to calcium ionophore (31). RBL-2H3 cells also contain RasGRP⁴⁺ mRNA.² Thus, a siRNA approach was used to evaluate the consequences of decreased expression of RasGRP4 in RBL-2H3 cells. As noted in Fig. 4, inhibition of RasGRP4 expression in the MC line resulted in a transient (~12–48 h) inhibition of PGD₂ synthase expression. As far as we are aware, no one has examined eicosanoid production in transgenic mice that lack RasGRP1 or in cultured cells that have been induced to express varied forms of the other RasGRP family members. Nevertheless, the finding that RasGRP4 regulates PGD₂ expression in two populations of cultured MCs raises the possibility that RasGRP1, RasGRP2, and/or RasGRP3 regulate eicosanoid production in other cell types.

**Earlier in vitro studies suggested that KL is required for maximal expression of PGD₂ synthase in mouse MCs.** HMC-1 cells are able to proliferate in the absence of exogenous human cytokines, because these transformed cells possess an activating mutation in c-kit (32). The inability of HMC-1 cells to produce large amounts of PGD₂ supports the conclusion that RasGRP4 acts downstream of c-kit. Murakami et al. (17) identified a number of cytokines that influence the KL-mediated expression of PGD₂ synthase in cultured mouse MCs either in a positive or negative manner. As assessed by GeneChip analysis (data not shown), HMC-1 cells express the transcripts that encode the surface receptors for IL-4, IL-10, IL-13, and KL. This MC line also expresses three distinct receptors that recognize TNF-α and its family members. RasGRP⁴⁺ and RasGRP⁴⁺ HMC-1 cells were therefore cultured for 5 days in the presence of varied combinations of IL-3, IL-4, IL-10, IL-13, KL, and TNF-α. None of these cytokines were able to induce PGD₂ synthase expression in RasGRP⁴⁺ HMC-1 cells (data not shown). In addition, none of these cytokines were able to inhibit the expression of PGD₂ synthase in RasGRP⁴⁺ HMC-1 cells. These data imply that RasGRP4 is the dominant intracellular signaling protein that controls PGD₂ expression in MCs no matter what extracellular cytokine environment this immune cell encounters in tissues.

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