Mast Cells in Airway Hyporesponsive C3H/HeJ Mice Express a Unique Isoform of the Signaling Protein Ras Guanine Nucleotide Releasing Protein 4 That Is Unresponsive to Diacylglycerol and Phorbol Esters

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Mast Cells in Airway Hyporesponsive C3H/HeJ Mice Express a Unique Isoform of the Signaling Protein Ras Guanine Nucleotide Releasing Protein 4 That Is Unresponsive to Diacylglycerol and Phorbol Esters¹

Lixin Li,² Yi Yang,² Guang W. Wong,³ and Richard L. Stevens⁴

cDNAs were recently isolated from BALB/c mouse mast cells (MCs) that encode the new signaling protein mouse Ras guanine nucleotide releasing protein 4 (mRasGRP4). The present study evaluates the expression pattern and biological activity of mRasGRP4 in a variety of mouse strains. As assessed immunohistochemically and by RNA analysis, mRasGRP4 is not coordinately expressed with any of its family members. Normally, mRasGRP4 is an MC-restricted protein in tissues, and kinetic studies revealed that mRasGRP4 is expressed relatively early in developing MCs. The expression of mRasGRP4 in the fetus before granulated MCs become abundant supports the conclusion that RasGRP4 participates in MC-specific differentiation pathways. Functional studies conducted with recombinant material revealed that mRasGRP4 is a cation-dependent, diacylglycerol (DAG)-regulated, guanine nucleotide exchange factor. Immunoelectron microscopic studies revealed that mRasGRP4 resides in either the cytosol or inner leaflet of the plasma membrane of the MC, implying that DAG controls the intracellular movement of this signaling protein in c-kit-stimulated MCs. The mRasGRP4 gene resides on chromosome 7B1 within a site that is prominently linked to baseline airway reactivity in backcrossed C3H/HeJ and A/J mice. A truncated isoform of mRasGRP4 that lacks its DAG-regulatory domain was isolated from C3H/HeJ mouse MCs. Sequence analysis showed that this isoform is the result of defective splicing of the precursor transcript. MCs play a central role in allergic inflammation. The discovery of a novel isoform of mRasGRP4 in hyporesponsive mice suggests that airway reactivity is influenced by RasGRP4-dependent signaling events in pulmonary MCs. The Journal of Immunology, 2003, 171: 390–397.

Mouse bone marrow-derived MCs (mBMMCs)⁵ developed in vitro with IL-3 (1–3) in the presence or absence of c-kit ligand (4, 5) are nontransformed cells that have been widely used in investigations of the factors and mechanisms that regulate the development and function of mammalian MCs (MCs) at the molecular level. We recently sequenced thousands of clones from a BALB/c mBMMC cDNA library to identify transcripts that encode MC-restricted proteins. That transcript hunt resulted in the isolation of cDNAs that contained an ~2.3-kb insert (designated mouse Ras guanine nucleotide releasing protein 4 (mRasGRP4)) that had not been identified in any species at the gene, mRNA, or protein level (6) before the release of our nucleotide sequence data at GenBank in 2001. A homology-based cloning approach was used to isolate the human (6) and rat (7) orthologs of the mRasGRP4 transcript from the mononuclear progenitors residing in peripheral blood and spleen, respectively. The human transcript data were then used to locate the RasGRP4 (hRasGRP4) gene, deduce its exon/intron organization, and identify 10 single nucleotide polymorphisms in its transcript that result in 5 aa differences in its translated protein. The ~17-kb hRasGRP4 gene consists of 18 exons and resides on chromosome 19q13.1. Mature human MCs and their nongranulated white blood cell progenitors express hRasGRP4, and this new intracellular protein contains all of the domains present in the RasGRP family of guanine nucleotide exchange factors (GEFs), even though it is <50% identical with its closest family member.

Four members of the RasGRP family of GEFs have been identified in mice, rats, and humans (6–17). Interest in the RasGRPs has increased because of their oncogenic activity and restricted cellular expression. In this regard, fibroblasts do not express RasGRP4 (6). Nevertheless, if these mesenchymal cells are transiently forced to express hRasGRP4, the resulting transfectants lose their contact inhibition, undergo dramatic morphologic changes, and increase their rate of division when exposed to low doses of PMA. Copeland and coworkers (18, 19) noted that the spontaneous insertion of leukemia retrovirus into the mRasGRP1 or mRasGRP2 genes often results in leukemia. The hRasGRP4 gene resides at chromosome 19q13.1 (6), and breakpoint alterations at or near this site often lead to leukemia. The human MC-1 (HMC-1) cell line (20) was established from a patient with an MC leukemia (21). Although the hRasGRP4 gene is transcribed in HMC-1 cells, these transformed cells preferentially produce abnormal forms of hRasGRP4 due to an inability to remove introns.
3 and 5 in the precursor transcript (6). When HMC-1 cells were induced to express a normal form of RasGRP4 via a transfection approach, the resulting cells underwent granule differentiation and maturation. They also increased their expression of prostaglandin D2 synthase >100-fold (22). T cells express RasGRP1, and targeted disruption of this GEF’s gene in mice results in a marked deficiency of mature CD4+CD8− and CD4+/CD8+ T cells (23). In a similar manner, it now appears that RasGRP4 regulates the final stages of MC differentiation and maturation, including what protease and lipid mediators MCs produce.

In the present study, we evaluated the expression pattern and function of mRasGRP4 in mouse strains that differ in their baseline airway reactivity to methacholine. The discovery that the MCs in hyporesponsive C3H/HeJ mice (24) preferentially express a truncated isoform of mRasGRP4 that is unresponsive to diacylglycerol (DAG) and PMA suggests that some MC-dependent disorders are influenced by the expression of abnormal isoforms of RasGRP4 in mature MCs and/or their progenitors.

Materials and Methods

Chromosomal location of the mRasGRP4 gene and identification of an abnormal isoform of mRasGRP4 in C3H/HeJ mBMMCs

A fluorescent in situ hybridization (FISH) technique was used to determine the chromosomal location of the mRasGRP4 gene in the BALB/c mouse strain. Preliminary studies revealed that the mRasGRP4 gene resided in the bacterial artificial chromosome mouse genomic clone F1231 (Incyte Genomics, St. Louis, MO). Slides containing normal metaphase chromosomes derived from mouse embryonic fibroblasts therefore were incubated with the digoxigenin-dUTP-labeled clone F1231 in the presence of 50% formamide, 10% dextran sulfate, 2× SSC, and sheared mouse DNA. After this hybridization step, the slides were incubated with fluoresceinated anti-digoxigenin Ab and counterstained with 4,6-diamidino-2-phenylindole. The data obtained indicated that the mRasGRP4 gene resides at, or near, a site that controls baseline airway reactivity in backcrossed A/J and C3H/HeJ mice (24). Thus, mBMMCs were generated from A/J and C3H/HeJ mice by culturing their bone marrow progenitors for 2–7 wk in IL-3-enriched medium in the presence or absence of c-kit ligand. Primers corresponding to various regions in the BALB/c mRasGRP4 transcript were then used in an RT-PCR approach to isolate cDNAs that correspond to the prominent mRasGRP4 transcripts in A/J and C3H/HeJ mBMMCs. The resulting products were subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced using standard molecular biology procedures.

Tissue distribution of the mRasGRP4 transcript
cDNA libraries (Clontech, Palo Alto, CA) from adult mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis from day 7 to day 17 embryonic stages were used to evaluate relative distribution of mRasGRP4 transcript in normal mice. Each tissue library was created using pooled RNA from mice ranging in age from 8 to 12 wk. Each embryo library was derived using pooled RNA from Swiss Webster/National Institutes of Health mouse embryos of a fixed age. PCRs were conducted using 50-μl portions of each library (corresponding to ~1 ng of cDNA) and 0.4 μM of the oligonucleotides 5′-CATGAACTCTGGGAGATG GCTGA-3′ and 5′-CAGGACTTACGACGCTTGAG-3′ to generate the mRasGRP4 product. Similar approaches were used to determine whether BALB/c and C57BL/6 mBMMCs express mRasGRP1 and mRasGRP2. In these studies, the oligonucleotides 5′-GCAAGCTTCTGGGAGATG GCTGA-3′ and 5′-ATGGTTGAGTCTCTTATGCG-3′ were used to evaluate mRasGRP1 expression, whereas the oligonucleotides 5′-CACA GCTAAGGCAGCATTTT-3′ and 5′-ATGGCTGAAAGCTGGCTCAT-3′ were used to evaluate mRasGRP2 expression.

Blot analyses were conducted using total RNA isolated from BALB/c mBMMCs, the mouse MC lines V3 and CL.05/C7.1, the rat MC lines RBL-1 and RBL-2H3, the mouse myelomonocytic/macrophage cell line RAW264.7, and WEHI-3, the mouse fibroblast cell line 3T3, and the mouse T cell hybridoma MTC-1 (American Type Culture Collection, Manassas, VA). The resulting blots were probed under standard conditions of high stringency with a 197-bp probe that corresponds to residues 1978–2174 in BALB/c mRasGRP4. The V3, CL.05/C7.1, and RBL cell lines were examined because they are well-described IL-3-independent MC lines that have been maintained in culture for years. The RBL cell lines have also been used by many to study FcεRI-mediated signaling events.

The two myelomonocytic/macrophage lines were examined because macrophages and MCs originate from a common progenitor in the bone marrow (25). The WEHI-3 cell line also resembles mBMMCs in its dependence on IL-3. The 3T3 cell line was examined because this fibroblast line was used in the transfection experiments noted below to evaluate the role of mRasGRP4 in proliferation and activation of 3T3 cells. Finally, the T cell hybridoma was examined because mRasGRP1 was cloned from a T cell hybridoma and because this GEF is essential for T cell development and function in the mouse (23, 26).

Mouse RasGRP4 immunohistochemistry

Analysis of the primary amino acid sequences of mouse and human RasGRP1, RasGRP2, RasGRP3, and RasGRP4 revealed that the N terminus of each family of GEFs is highly conserved in its ability to bind DAG and also failed to reveal any amino acid sequence in variant protein databases that resembled the 12-mer peptide Arg-Lys-Asp-Ile-Lys-Arg-Lys-Ser-His-Gln- Glu-Cys residing at residues 3–14 in BALB/c mRasGRP4. Thus, an anti-peptide approach was used to obtain rabbit Abs that recognize this novel peptide sequence. The synthetic peptide was generated by Genemed Synthesis (San Francisco, CA) and then was coupled to keyhole limpet hemocyanin through the thiol group of the C-terminal Cys in the peptide. A rabbit was immunized four times with the peptide conjugate (0.5 mg/immunization) over a 60-day period. The resulting anti-mRasGRP4 Abs were then purified using a standard peptide-affinity chromatography approach.

For immunohistochemistry at the light level, peritoneal cavity cellular exudates from BALB/c mice and skin biopsies from BALB/c and C57BL/6 mice were snap frozen in liquid nitrogen and then incubated for 4 h at room temperature in 4% paraformaldehyde and 0.1 M sodium phosphate (pH 7.6). They were then washed twice with PBS containing 2% DMSO. The spleen of the V3 mastocytosis mouse was examined because this organ contains large numbers of v-abl-immortalized MCs 2 wk after the adoptive transfer of the V3 MC line into the tail vein (27). Each specimen was dehydrated and embedded in plastic with the JB-4 kit from Polysciences (Warrington, PA). Sections were cut on a Reichert-Jung Suprantic Microtome (Leica, Deerfield, IL) with glass knives and then were picked up on glass slides. The slides were incubated sequentially for 15 min at 37°C in 2 mM CaCl2 containing 0.025% trypsin, for 15 min at room temperature in PBS containing 0.1% BSA, for 10 min at 37°C in PBS containing 0.05% Tween 20 and 1% normal goat serum, and then overnight at 4°C in buffer containing purified rabbit anti-mRasGRP4 Ab. Samples were washed, incubated for 1 h at room temperature in buffer containing biotin-labeled goat anti-rabbit IgG, washed twice in 0.1% BSA and 0.05% Tween 20 in PBS, incubated for 1 h at room temperature in Vectastain ABC-AP reagent (Vector Laboratories, Burlingame, CA), and then incubated for 15 min at room temperature in the presence of an alkaline phosphatase substrate solution. The tissue sections were then counterstained with Gill’s hematoxylin and covered with Immu-Mount (Shandon, Pittsburgh, PA). To ensure that most of the mRasGRP4 epitope was not lost and/or destroyed during the plastic-embedding step and to minimize background-staining, non-plastic-embedded frozen sections of tongue also were reacted with anti-mRasGRP4 Ab.

Ultrathin frozen sections of a spleen from a V3 mastocytosis mouse were processed for immunoelectron microscopy to determine where mRasGRP4 resides inside MCs. Tissue biopsies (2 mm3) were fixed overnight in PBS containing 4% paraformaldehyde, infiltrated for ~30 min at room temperature in PBS containing 2.3 M sucrose and 0.15 M glycine, mounted, and frozen in liquid nitrogen. Ultrathin sections were cut at −120°C with a cryo-diamond knife and a Reichert Ultracut-S Microtome, picked up with a loop dipped in a 1:1 mixture of 2.3 M sucrose and 2% methylcellulose, transferred to a formvar-coated copper grid, and incubated for 15 min in PBS containing 1% BSA to minimize nonspecific binding of the rabbit Abs. The treated grids were then placed in PBS containing 1% BSA and a 1/50 dilution of affinity-purified rabbit anti-mRasGRP4 Ab. After washing four times with PBS, each section was exposed to 10 nm of protein A-gold (University Medical Center, Utrecht, The Netherlands) in PBS containing 1% BSA for 30 min. Treated sections were washed again, exposed to 1% uranyl acetate, and finally examined in a JEOL 1200EX electron microscope (Peabody, MA).

Generation of recombinant BALB/c and C3H/HeJ mRasGRP4

The cDNA that encodes the prominent isoform of mRasGRP4 present in C3H/HeJ mBMMCs was inserted into the mammalian expression vector pcDNA3.1/Direcional/V5-His-TOPO (Invitrogen) upstream of the sequences that encode the V5 and 6xHis peptides. Recombinant C3H/HeJ mRasGRP4 was generated in transiently transfected COS-7 cells and 3T3 fibroblasts, as previously described for the generation of recombinant mRasGRP4 (6). To evaluate the functional importance of the DAG-binding
domain in mRasGRP4, we next evaluated fibroblasts that express the BALB/c or C3H/HeJ isoforms of this protein for their susceptibility to 10 nM PMA. To determine whether or not BALB/c mRasGRP4 is a cation-regulated GEF, we monitored its ability to transfer guanosine 5'-(γ-35)S triphosphate to GDP-loaded H-Ras, as previously described for hRasGRP4 (6). We then evaluated the ability of CaCl$_2$ (0.2–10 mM), PMA (3–200 μM), and affinity-purified rabbit peptide Abs directed against mRasGRP4 (10 μg) or an irrelevant protein (human eosinophil serine protease 1; 10 μg) to inhibit the guanine nucleotide exchange activity of recombinant mRasGRP4. In the latter competition assays, recombinant H-Ras was preloaded with GDP. CaCl$_2$, FMA, or Ab was then added, followed by recombinant mRasGRP4 and radiolabeled GTP.

Results and Discussion
Expression of mRasGRP4 at the transcript and protein levels

Mouse RasGRP4 cDNAs have been isolated from 6-wk-old BALB/c mBMMCs (6). Nevertheless, the timing of expression of this new signaling protein in MCs, its preferred location inside an MC, whether it is abundant in other cell types, and whether it is coordinately expressed with its other family members all remained to be determined. These issues must be addressed before investigators can interpret the relevance of mRasGRP4 data to humans. As assessed by RT-PCR (Fig. 1), RNA blot (Fig. 2), and immunohistochemistry (Fig. 3), mRasGRP4 is highly restricted to mature MCs and their immature circulating progenitors in normal mice. As reported by others, the mRasGRP1 and mRasGRP2 transcripts are abundant in mouse brain (Fig. 1a). The levels of the mRasGRP1 and mRasGRP2 transcripts are below detection by a similar RT-PCR approach in BALB/c and C57BL/6 mBMMCs (Fig. 1a). It is now apparent from these data that mRasGRP4 is not coordinately expressed with its other family members. The inability to detect hRasGRP1, hRasGRP2, and hRasGRP3 transcripts in the RasGRP4$^+$ human MC line HMC-1 by GeneChip analysis (L. Li, Y. Yang, and R. L. Stevens, unpublished observations) supports this conclusion. Although the mRasGRP4 transcript was initially cloned from BALB/c mBMMCs, the strain-dependent expression of granule proteases in mouse MCs (28–30) raised the possibility that the transcript had not been cloned in the 1990s because mRasGRP4 is selectively expressed in BALB/c mice. Thus, C57BL/6 mBMMCs and Swiss Webster mouse embryos were examined. The presence of the appropriate RT-PCR product in C57BL/6 mBMMCs (Fig. 1a) and Swiss Webster mouse embryos (Fig. 1b) now indicates that mRasGRP4 is not restricted to the BALB/c mouse. As noted below, mRasGRP4 also is expressed in A/J and C3H/HeJ mBMMCs.

When a blot containing RNA isolated from varied tissues was probed under conditions of high stringency, the level of mRasGRP4 mRNA was below detection in all examined tissues (data not shown). These data are consistent with our earlier cDNA library data indicating that the steady-state level of the mRasGRP4 transcript in MCs is substantially lower than that of its protease transcripts (6). Nevertheless, when a more-sensitive RT-PCR approach was used, low levels of the mRasGRP4 transcript were detected in nearly every tissue of the BALB/c mouse, including the cells in peritoneal cavity exudates (Fig. 1b). Although the levels of the mRasGRP4 transcript were almost below detection in day 7 mouse embryos, this transcript was readily detected in mouse embryos after day 11. The liver and kidney of the adult mouse contain considerably fewer MCs than do the heart, lung, or spleen. Toluidine blue-positive MCs also are more prominent in day 11 to day 14 mouse embryos than in day 7 embryos (L. Li and R. L. Stevens, unpublished data). Thus, the presence of the mRasGRP4 transcript in embryos and adult tissues is correlated with MC numbers.
mBMMCs than in the less mature MC lines supports the above RT-PCR data suggesting that mRasGRP4 is expressed at virtually all stages in the development of MCs in mice. Subsequent kinetic analysis of the mRasGRP4 transcript levels in the BALB/c mBMMC cultures (Fig. 2b) and immunohistochemical analysis of tissue-residing mature MCs (Fig. 3) confirmed this conclusion. Mouse RasGRP4 mRNA was readily detected at the 2-wk time point before most of the MC-committed progenitors in the cultures had granulated (Fig. 2b). In contrast with the transiently expressed mouse MC protease-7 transcript (30), mRasGRP4 mRNA was readily detected in the 5- to 7-wk-old cultures. Whereas the mRasGRP4 cDNA was initially isolated from an IL-3-dependent population of nontransformed MCs, the mRasGRP4 transcript was detected in three different rodent MC lines that are not dependent on IL-3 for their viability (Fig. 2a).

At the cellular level, the endogenous in vivo-differentiated mature MCs that reside in the tongue (Fig. 3, a and b), skin (Fig. 3, c and d), and peritoneal cavity (Fig. 3, e and f) of the BALB/c mouse contain high levels of mRasGRP4 protein. The exogenous v-abl-immortalized MCs that develop in the spleen of the V3 mastocytosis mouse also contain substantial amounts of mRasGRP4 protein (Fig. 3, g and h). The accumulated data indicate that mRasGRP4 is preferentially expressed in MCs in most mouse tissues. Preliminary immunohistochemical data suggest a similar MC-restricted pattern of expression of this protein in rats (7) and humans (6). Thus, the MC-restricted expression of this newly discovered protein apparently has been conserved for >100 million years of evolution. Mouse RasGRP4 is also abundantly expressed in the circulating nongranulated white blood cells that give rise to mature MCs. MCs and macrophages originate from a common progenitor in the bone marrow (25). Surprisingly, the level of mRasGRP4 protein was below detection in the macrophages that reside in numerous tissues. The level of mRasGRP4 mRNA also was below detection in two myelomonocytic/macrophage cell lines (Fig. 2a). Thus, the common MC/macroage progenitor in the bone marrow must cease expression of mRasGRP4 as it completes its final stages of differentiation into a tissue macrophage.

**Evaluation of the structure and biological activity of mRasGRP4 protein**

Analysis of the nucleotide sequence of the predominant cDNA in BALB/c mBMMCs predicted that mRasGRP4 exists in vivo as a 678-residue, ~75-kDa protein. SDS-PAGE immunoblot analysis of the peritoneal cavity cellular exudates (Fig. 4) confirmed that the native protein is indeed ~75 kDa in this.

![FIGURE 3. Immunohistochemistry. Frozen 5-µm sections of tongue (a and b) or plastic-embedded 1.5-µm serial sections of skin (c and d), spleen (g and h), or a peritoneal cavity-derived cell pellet (e and f) were stained at the light level with toluidine blue (a, c, e, and g) or with anti-mRasGRP4 Ab (b, d, f, and h). The spleen was from a V3 mastocytosis mouse. Arrows and arrowheads point to MCs. As noted in a and b, the number of toluidine blue-positive cells in the tongue of a normal BALB/c mouse is comparable to the number of mRasGRP4-expressing cells. Although background staining is greater in tissue samples embedded in plastic, more conclusive evidence that mature MCs selectively express mRasGRP4 was obtained with serially sectioned tissue and cell pellets (c–h). Frozen sections (100 nm) of spleen also were stained with anti-mRasGRP4 Ab followed by gold-labeled secondary Ab to identify the two primary locations of mRasGRP4 inside V3 MCs (i). At the ultrastructural level, mRasGRP4 resides primarily in the cytoplasm (+) or plasma membrane (+). Immunoreactive mRasGRP4 was not detected in the nucleus (N) or secretory granules (data not shown) of these MCs.

At the cellular level, the mRasGRP4 transcript was detected in mBMMCs, two well-described mouse MC lines, and two well-described rat MC lines (Fig. 2a). In contrast, the levels of the ~2.3-kb transcript were below detection by blot analysis in two myelomonocytic/macroage cell lines, a fibroblast line, and a T cell hybridoma. The fact that the steady-state level of the mRasGRP4 transcript was actually higher in primary BALB/c

![FIGURE 4. SDS-PAGE immunoblot analysis of native mRasGRP4. The proteins in the detergent extract of the cells isolated from the peritoneal cavity of a BALB/c mouse were subjected to SDS-PAGE immunoblot analysis to evaluate the size of mRasGRP4 in MCs that have been differentiated in vivo. The arrow on the right points to the immunoreactive 75-kDa protein present in the sample.](http://www.jimmunol.org/).
mouse strain. The failure to detect low-molecular mass forms of mRasGRP4 implies that little, if any, of the initially translated product is proteolytically processed into smaller forms that lack the Ca\textsuperscript{2+}- and/or DAG-binding motifs in BALB/c mice.

Because MCs express H-Ras (31), we tried to evaluate whether recombinant mRasGRP4 can activate this Ras isoform in vitro. Purified recombinant mRasGRP4 was able to transfer radiolabeled GTP to H-Ras in a kinetic manner (Fig. 5a). The inhibition of transfer of GTP to H-Ras by anti-mRasGRP4 Ab in this functional assay (Fig. 5b) confirmed that the guanine nucleotide exchange activities of the varied protein preparations originate from recombinant mRasGRP4 rather than from a GEF contaminant constitutively produced by COS-7 cells and fibroblasts. Although the amino acid sequences of mRasGRP4 and hRasGRP4 differ at 94 residues, recombinant hRasGRP4 is also able to activate Ras effectively (6). Thus, the 94 residues that are altered during the evolution of this signaling protein in mice and humans (including the 12 differences that reside in the cell division cycle 25-like domain) apparently do not adversely affect its guanine nucleotide exchange activity. Mouse RasGRP4 has a putative Ca\textsuperscript{2+}-binding domain analogous to that of the other members of its family, and 2 mM Ca\textsuperscript{2+} (n = 3) dominantly inhibits the ability of recombinant mRasGRP4 to transfer GTP to GDP-loaded H-Ras, even if 5 mM Mg\textsuperscript{2+} is present in the reaction buffer (Fig. 5c). Although Ras-specific GEFs are often activated by Ca\textsuperscript{2+} (32), Ca\textsuperscript{2+} also dominantly inhibits the guanine nucleotide exchange activity of hRasGRP1 (6) and hRasGRP2 (14). Thus, the inhibitory effects of Ca\textsuperscript{2+} may be a general feature of the RasGRP family of GEFs.

Location of the mRasGRP4 gene at a site on chromosome 7 that regulates airway reactivity in backcrossed C3H/HeJ and A/J mice and isolation of a novel isoform of mRasGRP4 in C3H/HeJ mBMMCs

Although the BALB/c mRasGRP4 gene was isolated and its exon/intron organization was deduced in our earlier study (6), its chromosomal location had not been determined before we submitted our sequence data to GenBank. FISH analysis revealed that the BALB/c mRasGRP4 gene resides on chromosome 7 at a position ~20 cM from the centromere (Fig. 6). This site approximately corresponds to 7B1. In support of this finding, the Human and Mouse Genome Projects recently concluded, based on analysis of their bacterial artificial chromosome and cosm id genomic clones, that mouse chromosome 7B1 corresponds to the region of human
chromosome 19q13.1, where we previously showed that hRas-GRP4 resides (6). In the C57BL/6 mouse genome, the mRasGRP4 gene resides 20.8 cM from the centromere end of chromosome 7.

Asthma is a polygenic disorder that is additionally influenced by varied environmental factors. Many genes (e.g., those that encode IL-4, IL-5, IL-12, IL-13, IL-4Rα, TIM1, bradykinin, ADAM (a disintegrin and metalloprotease) member 33, FcεRIβ, tryptase, leukotriene C₄ synthase, prostaglandin D₂ synthase, and complement 5) have been implicated in this airway disorder. De Sanctis et al. (24) noted that baseline airway reactivity to methacholine in

**FIGURE 7.** Analysis of the mRasGRP4 transcript in C3H/HeJ mBMMCs. RT-PCR analyses were conducted on A/J and C3H/HeJ mBMMCs. As noted, the predominant mRasGRP4 transcript present in the MCs developed from C3H/HeJ mice is slightly smaller than the corresponding transcript in A/J mBMMCs. G3PDH was used as a control. Transfectants that expressed the C3H/HeJ isoform of mRasGRP4 maintain their extended/spindle morphology (bottom right panel, arrow) when exposed to PMA. The predominant mRasGRP4 isoform expressed in C3H/HeJ mBMMCs was subcloned and characterized. Sequence analysis revealed that the transcript lacks 22 nucleotides because of the preferential use of a cryptic splice site in the middle of exon 15. This RNA editing event results in a unique isoform that lacks the regulatory, C-terminal DAG-binding domain. The expressed protein also contains five different amino acids at its C terminus.
inbred C3H/HeJ and A/J mice is dominantly influenced by an unidentified gene on mouse chromosome 7B1 (20–22 cM from the centromere). This site was not identified in backcrossed A/J and C57BL/6 mice (33). Thus, the C3H/HeJ mouse differs from other mouse strains in that it apparently contains a defective gene on chromosome 7B1 that causes it to be hyperresponsive to methacholine. The putative chromosome 7B1 gene that controls baseline airway reactivity in mice remains to be identified. Nevertheless, human gene–linkage studies (34, 35) have identified a comparable site on human chromosome 19q13.1 that is associated with increased airway reactivity in asthma patients.

MCs regulate allergic inflammation and play important immunoregulatory roles in the lung. Because RasGRPs4 influence the final stages of MC development, including what lipid (22) and protease (6) mediators this cell produces, De Sanctis et al. (24) raised the possibility that an unusual isoform of mRasGRP4 might be expressed in the MCs of C3H/HeJ mice. RT–PCR analysis (Fig. 7) indicated that the predominant mRasGRP4 transcript present in the MCs developed from C3H/HeJ mice is slightly smaller in size than the corresponding transcripts in A/J and BALB/c mBMMCs. Sequence analysis showed that the truncated transcript lacks 22 nucleotides due to the preferential use of a cryptic splice site in the middle of exon 15. This RNA editing event results in a premature translation–termination codon. The resulting 578-residue isoform also contains five different amino acids (i.e., TAGIK) at its C terminus. The complete nucleotide and amino acid sequences of this new mRasGRP4 isoform can be viewed at GenBank accession numberAY196476. The 3′UTR fibroblasts that express BALB/c mRasGRP4 undergo dramatic morphologic changes when exposed to 10 nM PMA (Ref. 6 and Fig. 7, upper light micrograph). This rounding-up morphologic feature does not occur in the transfec-
tants that have been induced to express the mRasGRP4 isoform present in C3H/HeJ mBMMCs (Fig. 7, lower light micrograph).

Data obtained from previous in vitro studies suggested that hRasGRP4 acts downstream of the surface receptor c-kit (6, 22). Nevertheless, the intracellular locations of mouse, rat, and human RasGRPs4 have not been deduced. Immunoelectron microscopy revealed that a substantial portion of the mRasGRP4 present in V3 MCs resides on the cytosolic side of the plasma membrane (Fig. 3i). Although this finding implies that mRasGRP4 participates in early signaling events at the MC’s plasma membrane, immunoreactive mRasGRP4 also was found in the cytoplasm. Subcellular fractionation studies of the transfectants confirmed both findings. The accumulated data suggest that some unknown intracellular factor or posttranslational modification event regulates the movement of mRasGRP4 from the cytoplasm to the inner leaflet of the MC’s plasma membrane. Phospholipase C isoforms are activated when c-kit encounters its ligand at the MC’s plasma membrane. This activation event results in the rapid generation of DAG. The phospholipase C-mediated production of DAG has been linked to the morphological changes that occur in c-kit-activated MCs. All normal variants of mouse, rat, and human RasGRPs have a 50-mer domain in their C-terminal portions that is weakly homologous to the C1, DAG-binding domain in protein kinase C. Recombinant hRasGRP3 can bind 12-[^3H]phorbol-13-butyrate efficiently in the presence of phospholipids (15). Thus, it has been speculated that the interaction with DAG or PMA is needed for the efficient movement of the varied RasGRPs from their cytosolic compart-
ments to the plasma membrane (14, 15, 36, 37). Although the location of the DAG-binding site has not been deduced experimentally in any RasGRP, it has been assumed that the site is the C1-like sequence that resides in the C-terminal domain of these GEFs. The finding that transfectants that have been induced to express the C3H/HeJ isoform of mRasGRP4 are unresponsive to

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