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RasGRP4, a New Mast Cell-restricted Ras Guanine Nucleotide-releasing Protein with Calcium- and Diacylglycerol-binding Motifs

IDENTIFICATION OF DEFECTIVE VARIANTS OF THIS SIGNALING PROTEIN IN ASTHMA, MASTOCYTOSIS, AND MAST CELL LEUKEMIA PATIENTS AND DEMONSTRATION OF THE IMPORTANCE OF RasGRP4 IN MAST CELL DEVELOPMENT AND FUNCTION*

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A cDNA was isolated from interleukin 3-developed, mouse bone marrow-derived mast cells (MCs) that contained an insert (designated *mRasGRP4*) that had not been identified in any species at the gene, mRNA, or protein level. By using a homology-based cloning approach, the ~2.6-kb *hRasGRP4* transcript was also isolated from the mononuclear progenitors residing in the peripheral blood of normal individuals. This transcript information was then used to locate the *RasGRP4* gene in the mouse and human genomes, to deduce its exon/intron organization, and then to identify 10 single nucleotide polymorphisms in the human gene that result in 5 amino acid differences. The >15-kb *hRasGRP4* gene consists of 18 exons and resides on a region of chromosome 19q13.1 that had not been sequenced by the Human Genome Project. Human and mouse MCs and their progenitors selectively express RasGRP4, and this new intracellular protein contains all of the domains present in the RasGRP family of guanine nucleotide exchange factors even though it is <50% identical to its closest homolog. Recombinant RasGRP4 can activate H-Ras in a cation-dependent manner. Transfection experiments also suggest that RasGRP4 is a diacylglycerol/phorbol ester receptor. Transcript analysis of an asthma patient, a mastocytosis patient, and the HMC-1 cell line derived from a MC leukemia patient revealed the presence of substantial amounts of non-functional forms of hRasGRP4 due to an inability to remove intron 5 in the precursor transcript. Because only abnormal forms of hRasGRP4 were identified in the HMC-1 cell line, this immature MC progenitor was used to address the function of RasGRP4 in MCs. HMC-1 leukemia cells differentiated and underwent granule maturation when induced to express a normal form of RasGRP4. Thus, RasGRP4 plays an important role in the final stages of MC development.

Mast cells (MCs)[1] release a diverse array of biologically active molecules (including cytokines, chemokines, leukotrienes, prostaglandins, amines, proteoglycans, and proteases) when activated via their high affinity IgE (FcεRI), complement, or protease-activated receptors. MCs play important roles in bacteria infections (1–3) due, in part, to their release of tumor necrosis factor-α (4) and varied granule tryptases (5, 6) that work in concert to induce the extravasation of neutrophils into tissues that kill bacteria. Although these and other findings document that MCs play beneficial immunosurveillance and effector roles in the body, it has been known for some time that MCs also exhibit adverse roles in numerous inflammatory disorders including asthma, chronic urticaria, and systemic anaphylaxis. Recent studies (7–9) have revealed that these effector cells also participate in the pathogenesis of AIDS.

Because MCs play such prominent roles in inflammation, a major effort has been made to identify the regulatory proteins that act relatively early in the varied intracellular signaling pathways of this cell to dampen MC development and/or function. Substantial progress has been made in our understanding of how different populations of MCs signal through their Ig, complement, and cytokine receptors, even though no signal transduction protein has been identified to date that is relatively specific to this cell type. The Ras superfamily of small GTP-binding proteins plays pivotal roles in virtually every cell. GTP activates the varied Ras family members, whereas GDP inactivates them. Guanine nucleotide exchange factors (GEFs) activate these signaling proteins by dissociating bound GDP. All GEFs that have been identified to date in MCs (*e.g.* the Ras GEF Sos (10) and the Rac GEF Vav (11, 12)) are expressed in many other cells. Nevertheless, the fact that a few GEFs that

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF331457, AY040628, AY048119, AY048120, and AY048121.

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[1] The abbreviations used are: MC, mast cell; DAG, diacylglycerol; FcεRI, high affinity IgE receptor; GEF, guanine nucleotide exchange factor; GRP, guanine-releasing protein; IL, interleukin; mBMMC, mouse bone marrow-derived mast cell; PMA, phorbol-12-myristate 13-acetate; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; PBS, phosphate-buffered saline; CPA, carboxypeptidase A.

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are more restricted in their cellular expression have been identified during the last couple of years has raised the possibility that an undiscovered, more restricted GEF might be present in MCs. Three such relatively restricted regulatory proteins are Ras guanine nucleotide releasing protein (RasGRP) 1 (also known as CalDAG-GEFII), RasGRP2 (also known as CalDAG-GEFI), and RasGRP3 (also known as KIAA0846 and CalDAG-GEFIII) (13–21). There is considerable interest in RasGRP1, RasGRP2, and RasGRP3, in part because these homologous GEFs have additional Ca^{2+} and phorbol ester/diacylglycerol (DAG)-binding motifs in their C-terminal domains that implicate their involvement in multiple signaling pathways. T cells express RasGRP1 and targeted disrupted of this GEF in mice results in a marked deficiency of mature $CD4^+/CD8^-$ and $CD4^-/CD8^+$ T cells (22). Thus, at least one RasGRP is of critical importance in cellular differentiation and/or maturation.

Mouse bone marrow-derived MCs (mBMMCs) developed with interleukin (IL) 3 (23–25) are the non-transformed cells that have been most widely used to understand at the molecular level the factors and mechanisms that regulate the proliferation, differentiation, maturation, adherence, cellular senescence, and function of mammalian MCs. For example, mBMMC cDNA libraries have been used by us to clone mouse MC protease-5, mouse MC protease-7, transmembrane tryptase, and serglycin. We also have used mBMMCs to identify some of the key cells and cytokines that regulate the development and function of MCs.

We now describe the cloning of a new GEF from human and mouse MCs and their progenitors. This intracellular protein is designated as RasGRP4 (or CalDAG-GEFIV based on the alternative nomenclature for these proteins) because it is the fourth member of its family. MCs express H-Ras (26), and we show that recombinant RasGRP4 can activate this Ras isoform *in vitro* in a Ca^{2+}- and Mg^{2+}-regulated manner. The cloning of the mouse and human *RasGRP4* cDNAs and genes now provides investigators the opportunity to identify defects in this new signaling protein in MC-dependent diseases. In this regard, we additionally describe single nucleotide/amino acid polymorphisms of the *hRasGRP4* gene, transcript, and protein. We also describe splice variants of the *hRasGRP4* transcript in a mastocytosis patient, an asthma patient, and an immature MC line derived from a MC leukemia patient (27) that encode substantially altered forms of hRasGRP4. Finally, using the latter leukemia MC line, we show that RasGRP4 is a signal transduction protein that participates in the final stages of MC development, most likely by acting downstream of c-*kit*.

EXPERIMENTAL PROCEDURES

Cloning of the mRasGRP4 and hRasGRP4 Transcripts and Genes and Chromosomal Location of the Human Gene—Greater than 2000 clones were arbitrarily isolated and sequenced from an mBMMC cDNA library (28) using standard molecular biology procedures. The BALB/c mBMMCs used to create the library had been cultured for 6 weeks to ensure that no contaminating cell types were present. As assessed by RNA blot analysis, one of the isolated clones corresponded to all but a few hundred nucleotides of the major *mRasGRP4* transcript present in mBMMCs. Thus, a "rapid amplification of cDNA ends" (RACE) approach was carried out with an RLM-RACE kit from Ambion (Austin, TX) to deduce the nucleotide sequence of the missing 5′ portion of the *mRasGRP4* transcript. BALB/c mBMMCs, generated as described previously (25), were the cellular source of the mRNA used in this 5′-RACE reaction. The first PCR was carried out using the outer adapter 5′-GCUGAUGGCGAUGAAUCAACACUGCGUUUGCUGGCUUUGAUGAAA-3′ and the *mRasGRP4*-specific antisense oligonucleotide 5′-CGGAACTCCCAGGTAGTGA-3′. The second nested PCR was carried out using the inner adapter 5′-CGCGGATCCGACACTCGTTTGCTGGCTTTGATGAAA-3′ and the mRasGRP4-specific antisense oligonucleotide 5′-CAGGACTTAGCAGGCTGGAG-3′. Each of the 30 cycles in the PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 55 °C, and a 2.5-min extension step at 72 °C. Multiple amplified products were subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and their inserts were sequenced in both directions using an ABI-377 sequencer and standard methods to deduce the nucleotide sequences of full-length transcripts in mBMMCs.

The site where the *hRasGRP4* gene resides on human chromosome 19q13.1 had not been sequenced in its entirety before we released our *mRasGRP4* and *hRasGRP4* nucleotide sequence data to the public at our GenBank™ sites AF331457, AY040628, AY048119, AY048120, and AY048121. Nevertheless, a genomic fragment was identified near the chromosome 19q13.1 gap site that was homologous to the nucleotide sequence that corresponds to exons 1–9 of the *mRasGRP4* gene. By using primers that correspond to sequences in this human genomic fragment, a 900-bp cDNA was isolated from the mononuclear cells in human peripheral blood that we concluded probably encoded the corresponding portion of the *mRasGRP4* transcript. To deduce the nucleotide sequence of a full-length 2.6-kb *hRasGRP4* transcript, a RACE approach was then carried out with an RLM-RACE kit from Ambion (Austin, TX), and total RNA was obtained from peripheral blood leukocytes. After the mononuclear cells were enriched from peripheral blood using Ficoll-Paque (Amersham Biosciences AB), they were lysed, and total RNA was extracted and purified using Trizol reagent (Invitrogen). Human leukocyte Marathon-Ready™ cDNAs (CLONTECH, Palo Alto, CA) also were used in the identification of the major hRasGRP4 expressed in the human population. The RACE was carried out using the 5′-RACE primer 5′-ACCTGTCGGGCTGTGCCTCA-3′ and the 3′-RACE primer 5′-CAGCACCAAGGCCCTCCTGGAGT-3′. Each of the 30 cycles in the PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 55 °C, and a 2.5-min extension step at 72 °C. Multiple amplified products were subcloned into pcDNA3.1/Directional/V5-His-TOPO (Invitrogen, Carlsbad, CA), and their inserts were sequenced in both directions using an ABI-377 sequencer.

Tissue Distribution of the hRasGRP4 Transcript—Human cDNA libraries (CLONTECH) from adult heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes; from fetal heart, brain, lung, liver, kidney, spleen, thymus, and skeletal muscle; and from peripheral blood mononuclear cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and $CD19^+$ B lymphocytes (before and after lectin activation) were used to evaluate the relative distribution of the *hRasGRP4* transcript in varied adult and fetal human tissues and in varied peripheral blood cells. Each library was created by CLONTECH using pooled poly(A)$^+$ RNA from a large number of people. For example, the leukocyte cDNA library used in this study to clone *hRasGRP4* cDNAs was generated using mRNA pooled from 550 individuals. A blot (CLONTECH) containing ~2 μg of poly(A)$^+$ RNA from varied human tissues also was probed under conditions of high stringency with a radiolabeled 303 bp-probe that corresponds to residues 329–631 in an *hRasGRP4* cDNA.

Four apparently normal individuals, a patient with asthma, a patient with systemic mastocytosis, and a MC line derived from a MC leukemia patient (27) were evaluated individually for the presence of abnormal *hRasGRP4* transcripts. The systemic mastocytosis patient used in our study was a 53-year-old male that had the disease for ~25 years. He presented in 1997 with marked abdominal swelling and extensive lymphadenopathy on a background of long term severe flushing and diarrhea. Physical examination demonstrated extensive skin involvement with pigmentation and erythematous areas on his face trunk and limbs. He had marked ascites hepatomegaly down to the right iliac fossa and marked axillary, inguinal, cervical, and epitrochlear lymphadenopathy. He also had mild splenomegaly. Bone marrow trephine carried out July 1997 demonstrated extensive fibrosis and abnormal megakaryocytes, moderate eosinophilia, and reduced numbers of erythrocytes and granulocytes. Approximately 3% of the cells in the bone marrow biopsy of the patient were MCs that possessed an abnormal morphology. The marrow was hypercellular with moderate infiltration of abnormal primitive cells. These cells did not express CD3, CD15, CD20, CD34, CD45, CD45RO, and κ or λ light chains; they also failed to stain when incubated with periodic acid-Schiff reagent. However, they expressed CD43 and CD117. Analysis of the cells in the bone marrow of the patient on November 1997 revealed that ~30% of them were blast cells, consistent with the development of an acute myeloid leukemia. The patient was given chemotherapy. Although the initial response was good, he had a stormy course over the next 3 years. Eventually, the patient died due to the complications of the leukemia. When the patient was in clinical remission, peripheral blood leukocytes were obtained and frozen for future study. RNA, purified from the isolated cells, was used to evaluate *hRasGRP4* expression.

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   1 AGTTCCTCTTTCCTTCCTAAACTCGGTGGACGCTCCAGCTCCCCGGAGCCTGACAATCTGGTTAGGGAAGGAACCTGATC  80

  81 ACACTCCTGTCAGGAGCCCCCACCCCACCCCCCCCGACCCTCCCCATCCCCTCCAAGAAAC ATG AAC CGG AAA GAC 155
   1                                                                M   N   R   K   D   5

 156 ATC AAA AGG AAG TCT CAT CAG GAA TGC TCT GGG AAG GCA GGA GGG CGG GGT CGG TCA CGT  215
   6 I   K   R   K   S   H   Q   E   C   S   G   K   A   G   G   R   G   R   S   R   25

 216 CAG GCC CGC CGC CAC AAG ACG TGC CCC ACC CCC CGG GAA ATC AGC AAG GTC ATG GCG TCC  275
  26 Q   A   R   R   H   K   T   C   P   T   P   R   E   I   S   K   V   M   A   S   45

 276 ATG AAT CTG GGA GTG CTG AGT GAG AGC AGC TGC AGC GAA GAT GAG CTA TTG GAG GAA TGT  335
  46 M   N   L   G   V   L   S   E   S   S   C   S   E   D   E   L   L   E   E   C   65

 336 ATC CGC TGC TTT GAC TCG GCT GGC AGC CTG CGC CGA GGG GAC CAC ATT CTC AAG ATG GTG  395
  66 I   R   C   F   D   S   A   G   S   L   R   R   G   D   H   I   L   K   M   V   85

 396 CTC ACG ATG CAC AGC TGG GTG CTG CCA TCC TCA GAG CTC GCT GCC CGT CTG CTG ACT TCG  455
  86 L   T   M   H   S   W   V   L   P   S   S   E   L   A   A   R   L   L   T   S   105

 456 TAT CAG AAG GCT GCC AAA GAT GCC CAA GAG CTA AGA CAG CTA CAG ATC TGT TAC TTG GTC  515
 106 Y   Q   K   A   A   K   D   A   Q   E   L   R   Q   L   Q   I   C   Y   L   V   125

 516 AGG TAC TGG CTG ACC CAT CAC CAT GAG GCA GTG CAC CAG GAA CCC CAG CTG GAA GCA GTC  575
 126 R   Y   W   L   T   H   H   H   E   A   V   H   Q   E   P   Q   L   E   A   V   145

 576 ATA AGC CGG TTT TGG ACC ACT GTC GCT CAG GAG GGC AAC ATG GCC CAA AGG AGC CTG GGA  635
 146 I   S   R   F   W   T   T   V   A   Q   E   G   N   M   A   Q   R   S   L   G   165

 636 GAT GCC TCC AGC CTG CTA AGT CCT GGA GGG CCC GGT CCC CCA CCT CCC ATA AGC AGC CCA  695
 166 D   A   S   S   L   L   S   P   G   G   P   G   P   P   P   P   I   S   S   P   185

 696 GGC CTG GGC AAG AAG CGG AAA GTG TCA TTG CTA TTC GAT CAC CTG GAG ACA GAG GAG CTG  755
 186 G   L   G   K   K   R   K   V   S   L   L   F   D   H   L   E   T   E   E   L   205

 756 GCA CAA CAC CTC ACT TAC CTG GAG TTC CGG TCT TTC CAG GCT ATC ACA CCC CAA GAC CTG  815
 206 A   Q   H   L   T   Y   L   E   F   R   S   F   Q   A   I   T   P   Q   D   L   225

 816 CGG GGC TAT GTT TTG CAG GGT TCA GTG AGA GGT TGT CCA GCT CTG GAA GGT TCT GTG GGT  875
 226 R   G   Y   V   L   Q   G   S   V   R   G   C   P   A   L   E   G   S   V   G   245

 876 CTG AGC AAC AGT GTG TCC CGC TGG GTG CAG GTC ATG GTG CTG AGT CGT CCT GGG CCT GCA  935
 246 L   S   N   S   V   S   R   W   V   Q   V   M   V   L   S   R   P   G   P   A   265

 936 CAA CGT GCC CAG GTC CTG GAC AAA TTC ATT CGT GTG GCA CAG AGG CTT CAC CAG CTG CAG  995
 266 Q   R   A   Q   V   L   D   K   F   I   R   V   A   Q   R   L   H   Q   L   Q   285

 996 AAT TTC AAC ACA CTG ATG GCG GTC ACG GGG GGT CTG TGT CAT AGC GCC ATC TCC AGA CTC 1055
 286 N   F   N   T   L   M   A   V   T   G   G   L   C   H   S   A   I   S   R   L   305

1056 AAG GAC TCC CAC GTT CAT CTG AGC CCT GAC AGC ACA AAG GCC CTG CTG GAG CTG ACA GAG 1115
 306 K   D   S   H   V   H   L   S   P   D   S   T   K   A   L   L   E   L   T   E   325

1116 CTC CTC TCA TCC CAC AAC AAC TAT GCC CAC TAC CGC CGC ACC TGG GCC GGC TGT ACT GGC 1175
 326 L   L   S   S   H   N   N   Y   A   H   Y   R   R   T   W   A   G   C   T   G   345

1176 TTC CGG CTG CCA GTA CTG GGT GTG CAC CTC AAG GAC CTG GTA TCT CTA TAT GAG GCT CAT 1235
 346 F   R   L   P   V   L   G   V   H   L   K   D   L   V   S   L   Y   E   A   H   365

1236 CCA GAC AGA TTG CCA GAT GGC CGC CTG CAC CTA CCC AAG CTG AAT AGC CTC TAT CTA CGG 1295
 366 P   D   R   L   P   D   G   R   L   H   L   P   K   L   N   S   L   Y   L   R   385

1296 CTG CAG GAG CTG ATG GCG CTC CAG GGA CAG CAT CCT CCC TGC AGT GCC AAT GAG GAC CTG 1355
 386 L   Q   E   L   M   A   L   Q   G   Q   H   P   P   C   S   A   N   E   D   L   405

1356 CTG CAC CTG CTG ACG CTC TCC CTG GAT CTC TTC TAC ACG GAA GAT GAG ATC TAC GAG CTT 1415
 406 L   H   L   L   T   L   S   L   D   L   F   Y   T   E   D   E   I   Y   E   L   425
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FIG. 1. **Cloning of the *mRasGRP4* cDNA.** Depicted is the nucleotide sequence of the cDNA that corresponds to the predominant ~2.3-kb *mRasGRP4* transcript in BALB/c mBMMCs, as well as the predicted amino acid sequence of its translated product. An alternate form of the *mRasGRP4* transcript also was identified in mBMMCs that lacks the 15-nucleotide sequence that encodes the "VSTGP" sequence in the DAG-binding domain at residues 561–565 of the protein. The initial 163 nucleotides in the depicted cDNA correspond to the 5′-untranslated region and the first eight amino acids in the translated product. Whether or not the 5′-untranslated region is derived from multiple exons remains to be determined. However, preliminary analysis of the *mRasGRP4* gene indicates that the 3′ end of the first coding exon corresponds to residues 118–140 in the depicted cDNA. The subsequent 16 exons correspond to residues 164–348, 349–454, 455–517, 518–649, 650–803, 804–977, 978–1094, 1095–1370, 1371–1451, 1452–1556, 1557–1675, 1676–1835, 1836–1872, 1873–2007, 2008–2117, and 2118–2294, respectively. The *mRasGRP4* cDNA lacks a classical "AATAAA" or "ATTAAA" polyadenylation regulatory site 10–30 residues upstream of its 3′ poly(A) tract. Nevertheless, because "AAAAAA" has weak polyadenylation promoting activity (67), it is presumed that nucleotides 2272–2277 control the polyadenylation of the *mRasGRP4* transcript.

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1416 TCT TAT GCC CGG GAA CCA CGC TGT CCC AAG AGT CTG CCA CCG TCC CCC TTC AGA GCA CCT  1475
 426 S   Y   A   R   E   P   R   C   P   K   S   L   P   P   S   P   F   R   A   P    445

1476 GTG GTG GTA GAG TGG GCC CAG GGT GTG ACA CCA AAG CCA GAC AGC GTG ACT CTG GGT CAG  1535
 446 V   V   V   E   W   A   Q   G   V   T   P   K   P   D   S   V   T   L   G   Q    465

1536 CAT GTG GGA CAG CTG GTG GAG TCC GTG TTC AAG AAC TAC GAC CCA GAA GGC CGT GGC TCC  1595
 466 H   V   G   Q   L   V   E   S   V   F   K   N   Y   D   P   E   G   R   G   S    485

1596 ATC TCT CTG GAG GAC TTT GAG CGG CTG TCA GGC AAC TTC CCG TTC GCC TGC CAT GGG CTT  1655
 486 I   S   L   E   D   F   E   R   L   S   G   N   F   P   F   A   C   H   G   L    505

1656 CAC CCA CCT CCC CGC CAT GGG AGT GGC TCC TTC AGC AGA GAG GAG CTG ACC AAG TAC CTG  1715
 506 H   P   P   P   R   H   G   S   G   S   F   S   R   E   E   L   T   K   Y   L    525

1716 CTC CAT GCC AGT GCC ATC TGC TCC AAG CTG GGC CTG GCC TTC CTG CAC GCC TTC CAG GAG  1775
 526 L   H   A   S   A   I   C   S   K   L   G   L   A   F   L   H   A   F   Q   E    545

1776 GTC ACC TTC CGA AAG CCC ACG TTC TGT CAC AGC TGC AGC GGC TTC GTG AGC ACT GGC CCT  1835
 546 V   T   F   R   K   P   T   F   C   H   S   C   S   G   F   V   S   T   G   P    565

1836 CTC TGG GGG GTC ACC AAG CAA GGC TAT CGC TGT CGG GAC TGT GGG CTG TGT TGT CAC AGA  1895
 566 L   W   G   V   T   K   Q   G   Y   R   C   R   D   C   G   L   C   C   H   R    585

1896 CAC TGC AGG GAT CAA GTG AGA GTG GAG TGT AAG AAG AGG CCA GAG ACT AAG GGT GAC CCG  1955
 586 H   C   R   D   Q   V   R   V   E   C   K   K   R   P   E   T   K   G   D   P    605

1956 GGC CCC CCA GGT GCA CCT GTG CCA GCC ACA TCA CTT CCT CCT GCC AAC TGT GGC TCA GAG  2015
 606 G   P   P   G   A   P   V   P   A   T   S   L   P   P   A   N   C   G   S   E    625

2016 GAA AGT CTG TCC TAT ACA CTC TCC CCG GAT CCG GAG TCT GGT TGC CAC CTT CGC CAT GCT  2075
 626 E   S   L   S   Y   T   L   S   P   D   P   E   S   G   C   H   L   R   H   A    645

2076 TGG ACC CAG ACG GAA TCC TCA CAC TCT TCC TGG GAG CCA GAG GTG GTG CCC TGC CCA GCA  2135
 646 W   T   Q   T   E   S   S   H   S   S   W   E   P   E   V   V   P   C   P   A    665

2136 CGG GTC TTA CCA TCC AGA GCT TCC TCG AAG CCC AGC GTC TGA AGAGCATCTGGAGGCTTTTCCCTT 2201
 666 R   V   L   P   S   R   A   S   S   K   P   S   V   *                          678

2202 CCTCCCCTAACCAACGCCTCCTCCAGCCCTACTCTGCCTCTGACAAAGCACCCACCATCTTTTCCCCTAAAAAAAAGCCG 2281

2282 TTCTTTTGCTGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                  2327
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FIG. 1—*continued*

The asthma patient used in our study was a 32-year-old Caucasian male with a history of asthma, allergic rhinitis, conjunctivitis, and atopic dermatitis. The initial diagnosis of asthma was made at age 7. He used inhaled steroids from age 7 to 12. From age 18 to today, he uses inhaled β2 agonists and inhaled steroids twice per day. He has been hospitalized three times due to the acute exacerbations of severe asthma. The patient has a history of allergic reactions to house dust mites and cats; he often gets asthmatic attacks during upper respiratory tract infections.

RNA, isolated from the HMC-1 cell line and the mononuclear cells of four normal individuals and the above two patients, was converted into cDNA with a reverse transcription (RT) system from Promega (Madison, WI). PCRs were carried out using 5-μl portions of each cDNA preparation (corresponding to ~1 ng cDNA) and 0.4 μM of the sense oligonucleotide 5′-AATGCACCGGAAAAACAGGA-3′ and 0.4 μM of the antisense oligonucleotide 5′-TGAGTCTGGAGATGGCACTG-3′ to generate the 900-bp product that corresponded to exons 1–9 in the *hRasGRP4* transcript. In all instances, the 30 cycles of each PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 55 °C, and a 1-min extension step at 72 °C. As an internal control, samples also were evaluated for the presence of glyceraldehyde-3-phosphate dehydrogenase with the sense and antisense oligonucleotides 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′. Additional PCRs were carried out with the sense oligonucleotide 5′-TGCAGATCTGTCACCTGGTC-3′ and the antisense oligonucleotide 5′-CGGAACTCCAGGTAGGTGAG-3′ and the sense oligonucleotide 5′-CTTCTGACCTCCCAGGCCTG-3′ and the antisense oligonucleotide 5′-GTAGCGGGCGTAGTTGTTGT-3′ to evaluate the expression of the variant 1 and 2 transcripts, respectively, in the region that corresponds to exons 5–6 of the normal *hRasGRP4* transcript.

hRasGRP4 Immunohistochemistry—Analysis of the primary amino acid sequences of the varied RasGRP family members revealed that the N terminus is poorly conserved in this family of GEFs. A computer search also failed to reveal any amino acid sequence in the GenBank™ protein data bases that resembled the 14-mer peptide Met-Asn-Arg-Lys-Asp-Ser-Lys-Arg-Lys-Ser-His-Gln-Glu-Cys residing at residues 1–14 in hRasGRP4. Thus, an anti-peptide approach was used to obtain rabbit antibodies that specifically recognize the N terminus of hRasGRP4. The above synthetic peptide was generated by Affinity Bioreagents (Golden, CO) and coupled to keyhole limpet hemocyanin through the succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate linker with 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-hRasGRP4 antibodies were then purified using a standard peptide affinity chromatography approach.

Immunohistochemistry was carried out on 4% paraformaldehyde/PBS-fixed paraffin-embedded human breast and stomach tissue sections obtained from Imgenex (San Diego, CA) using standard methodologies. Tryptases and chymases are stored in abundance in the secretory granules of human MCs, and the levels of these granule proteins greatly exceed that of any intracellular signaling protein in a MC. Whereas double-staining approaches often are used to identify human MCs in tissues that coexpress these two families of serine proteases (29), such an immunohistochemical approach cannot be used

effectively to identify MCs that express tryptase and hRasGRP4 due to the substantial differences in the levels of these two proteins. Thus, a more reliable serial section approach (30) was used to identify those cells in human breast and stomach that express hRasGRP4.

Generation of Recombinant mRasGRP4 and hRasGR4—A full-length *mRasGRP4* cDNA was constructed using the ~2.1-kb cDNA clone isolated from the mBMMC library and the subsequent 565-bp product generated using the 5′-RACE approach. The latter product was liberated from its vector with *Eco*RI, and then a PCR approach was carried out with the sense oligonucleotide 5′-CACCATGAACCGGAAAGACATCAAA-3′ and the antisense oligonucleotide 5′-CCAAAAC CGGCTTATGACTG-3′ to create a product that corresponds to residues 141–590 in the *mRasGRP4* transcript. The larger fragment was liberated with *Hin*dIII and *Xho*I. A PCR was carried out with the sense oligonucleotide 5′-CATGAATCTGGGAGTGCTGA-3′ and the antisense oligonucleotide 5′-GACGCTGGGCTTCGAGGAAGC-3′. The resulting two PCR products were mixed and then used as templates to generate a single product that corresponds to the entire coding domain (*i.e.* residues 141–2174) of the *mRasGRP4* cDNA. Primers used in this final PCR were the sense oligonucleotide 5′-CACCATGAACCGGAAAGACATCAAA-3′ and the antisense oligonucleotide 5′-GACGCTGGGCTTCGAGGAAGC-3′. Each PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 55 °C, and a 1-min extension step at 72 °C. Only 25 cycles were carried out in these PCRs to minimize the generation of point mutations. In order to detect the recombinant protein and to purify it from the lysates of transfected COS-7 cells and 3T3 fibroblasts, the final PCR product was placed into the mammalian expression vector pcDNA3.1/Directional/V5-His-TOPO (Invitrogen) upstream of the sequences that encode the V5 and His_6 peptides. By using a similar approach with the sense oligonucleotide 5′-CACCATGAACAGAAAAGACAGTAAGAGG-3′ and the antisense oligonucleotide 5′-GGAATCCGGCTTGGAGGATGCAGT-3′, the entire coding domain of an *hRasGRP4* cDNA (generated from one of the 2.1-kb cDNAs we isolated from the human leukocyte library) was subcloned into pcDNA3.1/Directional/V5-His-TOPO.

Vector lacking an *RasGRP4* insert was used as a negative control in these expression experiments. African green monkey, SV40-transformed kidney COS-7 cells (line CRL-1651, ATTC), and 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transient transfections were performed with SuperFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were plated at a density of 2×10^5 cells per well in a 6-well plate 24 h prior to transfection. They were transfected for 2–3 h, trypsinized, and then replated into parallel plates for both immunofluorescence (24-well plates containing 11-mm coverslips) and SDS-PAGE/immunoblot analysis (12-well plates). Conditioned media and cells were collected 24 h post-transfection. mRasGRP4- and hRasGRP4-expressing fibroblasts also were obtained by transfecting Swiss Albino mouse 3T3 fibroblasts with the above expression plasmids. The resulting fibroblasts were cultured in enriched media supplemented with 200–500 ng/ml G418 to increase the percentage of RasGRP4-expressing cells in the culture.

The presence of recombinant protein was evaluated by SDS-PAGE/immunoblotting with anti-V5 antibody (Invitrogen) or the above hRasGRP4-specific antibody. For immunodetection of the recombinant protein, cell, or tissue, lysates were boiled in SDS sample buffer containing β-mercaptoethanol, as were samples of the conditioned media. The resulting soluble proteins were resolved on a 12% polyacrylamide gel (Bio-Rad) and blotted onto polyvinylidene difluoride membranes (Bio-Rad). They were then exposed to Tris-buffered saline containing 0.1% Tween 20, 5% non-fat milk, and 0.5% goat serum to minimize nonspecific binding of the relevant antibody. Treated lysates were exposed to a 5000-fold dilution of a stock solution of mouse anti-V5 antibody or rabbit anti-hRasGRP4 antibody in Tris-buffered saline and 0.1% Tween 20, followed by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (Bio-Rad). Immunoreactive proteins were detected using BioMax MR film (Eastman Kodak) and chemiluminescence kits from Pierce.

Subcellular fractionation studies were carried out to locate where recombinant RasGRP4 resides in the COS-7 cell transfectants. For these experiments, 1×10^7 transfectants were washed three times with Hanks'-buffered salt solution, suspended in 1.4 ml of MIC buffer (4 mM HEPES, pH 7.0, containing 50 mM sucrose, 0.4 mM EDTA, and 0.2 mM dithiothreitol), and sonicated on ice. The resulting lysates were centrifuged at 4 °C for 10 min at ~1,500 × *g* and then at ~10,000 × *g* for another 10 min to remove nuclei and other organelles. The soluble fraction at this step was centrifuged for an additional 30 min at ~100,000 × *g*, and the resulting supernatant (cytosolic fraction) was subjected to SDS-PAGE/immunoblot analysis. The resulting plasma membrane-enriched microsomal fraction was washed once with MIC buffer and then placed in 1 ml of MIC buffer supplemented with 10% deoxycholic acid, 10% Triton X-100, and 30% glycerol. After a 30-min incubation at 4 °C, the detergent-extracted membrane fraction was centrifuged at ~100,000 × *g* for another 30 min. A sample of this supernatant (detergent-extracted microsomal fraction) also was subjected to SDS-PAGE/immunoblot analysis.

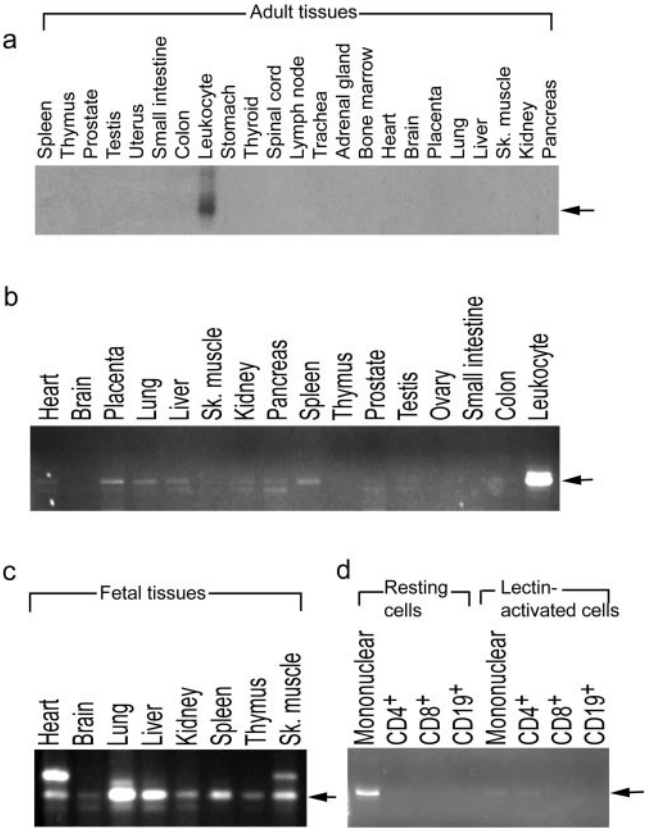

FIG. 2. **Evaluation of *hRasGRP4* mRNA levels in varied fetal and adult tissues and in different populations of peripheral blood mononuclear cells.** Quantitative RNA blot (*a*) and semi-quantitative RT-PCR (*b–d*) approaches were used to evaluate *hRasGRP4* mRNA levels in the indicated adult (*a*, *b*, and *d*) and fetal (*c*) human tissues and cells. The 303-bp probe that corresponds to residues 329–631 in the *hRasGRP4* cDNA was used in the more quantitative RNA blot analysis. The primers used in RT-PCR analyses correspond to sequences residing in exons 1 and 9 of the *hRasGRP4* gene. The expected 900-bp product (*arrow*) is indicated. Because the *hRasGRP4* transcript was abundant in the leukocyte preparation (*a* and *b*), another experiment was carried out in which pooled peripheral blood mononuclear cells from 4 to 36 individuals were sorted by CLONTECH based on their expression of CD4, CD8, and CD19 (*d*). Samples of the resulting cell populations were evaluated for their expression of *hRasGRP4* mRNA immediately after their isolation (*resting*) or after their subsequent exposure to phytohemagglutinin, pokeweed mitogen, and/or concanavalin A (*lectin-activated*). *hRasGRP4* mRNA was detected in unfractionated peripheral blood mononuclear cells but not in T and B lymphocytes. Glyceraldehyde-3-phosphate dehydrogenase-specific primers were used in a similar analysis to confirm the presence of intact RNA in all samples (data not shown).

To evaluate its guanine nucleotide exchange activity inside a living cell and to address whether or not mouse and human RasGRP4 is also a likely phorbol ester receptor, $RasGRP4^-$, $mRasGRP4^+$, and $hRasGRP4^+$ fibroblasts were placed on top of 11-mm glass coverslips in 24-well culture dishes. The attached cells were then exposed to 10 nM phorbol 12-myristate 13-acetate (PMA) (Calbiochem) for 15–30 min at 37 °C. After the coverslips were washed, they were incubated for 10 min in PBS supplemented with 4% paraformaldehyde. The fixative was removed, and the treated cells were immersed in −20 °C methanol for 10 min, rinsed in PBS, and incubated in 5% normal horse serum in PBS for 30 min before the addition of mouse anti-V5 antibody (Invitrogen) and rabbit anti-actin antibody (Sigma). The latter antibody recognizes

the common C-terminal domain of the varied isoforms of actin.

Anti-V5 antibody was used to identify epitope-tagged RasGRP4. Cells were incubated with a 100–500-fold dilution of each primary antibody for 2 h, washed several times in PBS, and then exposed for 1 h to the relevant secondary antibodies (a 200-fold dilution of Cy2-anti-mouse antibody and a 2,000-fold dilution of Cy3–anti-rabbit antibody; Jackson ImmunoResearch, West Grove, PA) in blocking solution supplemented with Hoechst dye 33258 at 50 ng/ml (Sigma). Stained cells were washed extensively with PBS and mounted. The resulting cells were viewed using a Nikon Eclipse 800 microscope; images were digitally captured using a CCD-SPOT RT digital camera and compiled using Adobe Photoshop® software (version 5.5).

Guanine Nucleotide Exchange Assay—ProBond™ nickel-chelating resin (Invitrogen) was used to purify His_6-tagged recombinant hRasGRP4 from the transfectants. As recommended by the manufacturer, $\sim 1 \times 10^7$ transfectants were placed in 4 ml of 20 mM sodium phosphate, pH 7.4, buffer containing 500 mM sodium chloride and multiple protease inhibitors (Roche Diagnostics). Each cell suspension was lysed by two freeze-thaw cycles using liquid nitrogen and a 42 °C water bath. Liberated nuclear DNA was sheared by passing the resulting preparation through an 18-gauge needle four times, and then the cellular debris was removed by a 5-min centrifugation step at 4 °C and at $\sim 14{,}000 \times g$. The resulting hRasGRP4-enriched supernatant was incubated with nickel-charged agarose resin for 1 h at 4 °C to ensure efficient binding of the His_6-tagged recombinant protein. After the equilibration step, the resin was centrifuged in a "Spin" column at $800 \times g$ for 2 min. The non-bound material was discarded, and the column was washed extensively with 500 mM sodium chloride and 20 mM sodium phosphate, pH 6.0, to remove weakly associated protein. Five ml of 50 mM imidazole elution buffer was applied, and the resulting hRasGRP4-enriched eluate was concentrated to ~0.5 ml with a Centriplus-50 (Millipore, Bedford, MA) filtering device having a 50-kDa cut-off membrane.

A modification of the guanine nucleotide exchange assay described by Zheng *et al.* (31) was used to evaluate the predicted function of hRasGRP4. In this assay, 2 μg of recombinant human H-Ras (Oxford Biomedical Research, Oxford, MI) was placed in 60 μl of 100 mM NaCl, 2 mM EDTA, 0.2 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0, supplemented with 100 μM AMP-PNP tetralithium salt (Roche Diagnostics) and 10 μM GDP (Sigma). After a 5-min incubation at room temperature, $MgCl_2$ was added to achieve a final concentration of 5 mM. The solution was then incubated for another 15 min at room temperature to load H-Ras with non-radiolabeled GDP. Twenty μl of the resulting solution was added to 75 μl of reaction buffer (100 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0, supplemented with 100 μM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 2 μM guanosine 5′-γ-^{35}S-triphosphate (~11,000 cpm/pmol; Amersham Biosciences)), followed by 5 μl of purified recombinant hRasGRP4 in the above buffer. The resulting samples were incubated at room temperature generally for 20 min. In a kinetic study with H-Ras, 15-μl aliquots were removed every 5 min and placed in 4 ml of ice-cold termination buffer (100 mM NaCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 8.0). Non-bound radioactivity was removed by filtering each reaction mixture through a 25-mm cellulose nitrate membrane possessing 0.45-μm pores (Whatman). After each filter was washed with 10 ml of ice-cold termination buffer, it was placed in 5 ml of Filtron-X scintillation fluid (National Diagnostics, Atlanta, GA), and the amount of bound radioactivity was quantitated using a Beckman LS5801 machine. For a negative control, purified recombinant hRasGRP4 was boiled for 5 min and then sonicated for 5 min before the guanine nucleotide exchange activity of the denatured protein was assessed.

The ability of $CaCl_2$ (0.2–10 mM) and PMA (3–200 μM) to inhibit the guanine nucleotide exchange activity of recombinant hRasGRP4 also was evaluated. In these competition assays, recombinant H-Ras was preloaded with GDP. $CaCl_2$ or PMA was then added, followed by hRasGRP4 and radiolabeled GTP. The reaction mixtures were then incubated for 20 min at room temperature.

Comparative Protein Structure Modeling—Three-dimensional models of residues 34–445 and 541–597 of normal and variant 2 hRasGRP4 were built using MODELLER (32, 33). Residues 34–445 contain the putative REM and CDC25-like catalytic domains and correspond to 61% of the translated protein. This portion of hRasGRP4 was modeled based on the crystallographic structure of hSos1 (residues 568–1032 of GenBank™ accession number A37488) complexed to H-Ras (Protein Data Bank code 1BKD) (34). Residues 541–597 in hRasGRP4 correspond to its putative DAG-binding domain. This portion of the GEF was modeled based on the NMR structure of the Cys^2 domain in rat protein kinase C-γ (Protein Data Bank code 1TBN) (35). The regions are ~20 and ~33% identical to the corresponding regions in Sos1 and protein kinase C-γ, respectively.

Importance of Normal Isoforms of RasGRP4 in the Differentiation and Granule Maturation of Human MCs—As noted under "Results," the *hRasGRP4* gene was transcribed, but the resulting transcript was not processed correctly in HMC-1 cells. Because a functional form of the signaling protein was not expressed in HMC-1 cells, this MC leukemia cell line was used in an attempt to deduce the function of RasGRP4 in MCs. As also noted under "Results," we identified a number of single nucleotide polymorphisms in the *hRasGRP4* transcript in the human population that, in turn, result in five amino differences in the translated products. Four of these amino acid polymorphisms are non-conservative changes. There is only one *mRasGRP4* allele in the inbred BALB/c mouse. Because it was not obvious which *hRasGRP4* allele should be used in our initial attempt to correct the developmental problem in the HMC-1 cell line, the BALB/c mouse-derived RasGRP4 described under "Results" was used in the transfection experiments. Recombinant RasGRP4 possesses the V5 epitope at its C terminus. Thus, anti-V5 antibody was used to confirm that a functional, non-truncated version of the GEF was expressed in the transfectants. Immunohistochemistry and/or SDS-PAGE/immunoblot approaches were then used with anti-tryptase and anti-chymase antibodies (Chemicon International, Temecula, CA) to evaluate the levels of these neutral proteases in the two populations of cells. A pancreatic carboxypeptidase A (CPA)-derived antibody (Sigma) that also recognizes MC CPA was used to evaluate the levels of this exopeptidase. Finally, standard electron microscopic methodologies were carried out to evaluate the ultrastructure of the HMC-1 cell line before and after transfection.

RESULTS

Cloning of the mRasGRP4 Transcript and Gene—Sequence analysis of >2000 clones arbitrarily selected from a BALB/c mBMMC cDNA library resulted in the identification of a clone that contained a 2.1-kb insert whose nucleotide sequence did not match any sequence in GenBank™ genome and expressed sequence tag data bases. A search of Celera's mouse nucleotide data bases with the resulting product also failed to reveal any intact, completely sequenced gene or transcript that corresponded to the mBMMC-derived cDNA. The fact that the unknown transcript had not been identified in another cell type, coupled with the finding that it was a low abundant transcript in mBMMCs, raised the possibility that it encoded a novel MC-restricted regulatory protein. Thus, a 5′-RACE approach was carried out to deduce the nucleotide sequence of the missing 227-bp portion of the full-length ~2.3-kb cDNA (Fig. 1). Another closely related cDNA was subsequently isolated in this screening approach that was missing 15 nucleotides. Analysis of the exon/intron organization of the *mRasGRP4* gene revealed that the alternate *mRasGRP4* transcript was caused by differentiation splicing of the precursor transcript. The GenBank™ accession numbers for the two isoforms of *mRasGRP4* cloned in this study are AF331457 and AY040628. Preliminary analysis of its gene (see Fig. 1 legend) revealed that the coding portion of the *mRasGRP4* transcript is derived from 17 exons. The similarity of the exon/intron organizations of the *mRasGRP4* and *hRasGRP2* genes supported the nucleotide sequence data that initially suggested mRasGRP4 is a new member of the RasGRP family of GEFs.

Cloning of the hRasGRP4 Transcript, Evaluation of its Expression, Chromosomal Location of its Gene, and Identification of Single Nucleotide Polymorphisms of Its Gene—An extensive search of GenBank™ and Celera's nucleotide and protein data bases failed to reveal any complete human cDNA, gene, or protein that closely resembles that of the *mRasGRP4* transcript, gene, and/or protein. Nevertheless, the 11.5-kb nucleotide sequence residing at one end of the human chromosome 19q13.1-derived BAC clone AC011469 closely resembled that of the 5′-half (*i.e.* exons 1–9 and introns 1–8) of the *mRasGRP4* gene. Although the nucleotide sequence of an overlapping BAC genomic clone corresponding to the missing 3′-half of the *hRasGRP4* gene had not been deposited in GenBank™, these pre-

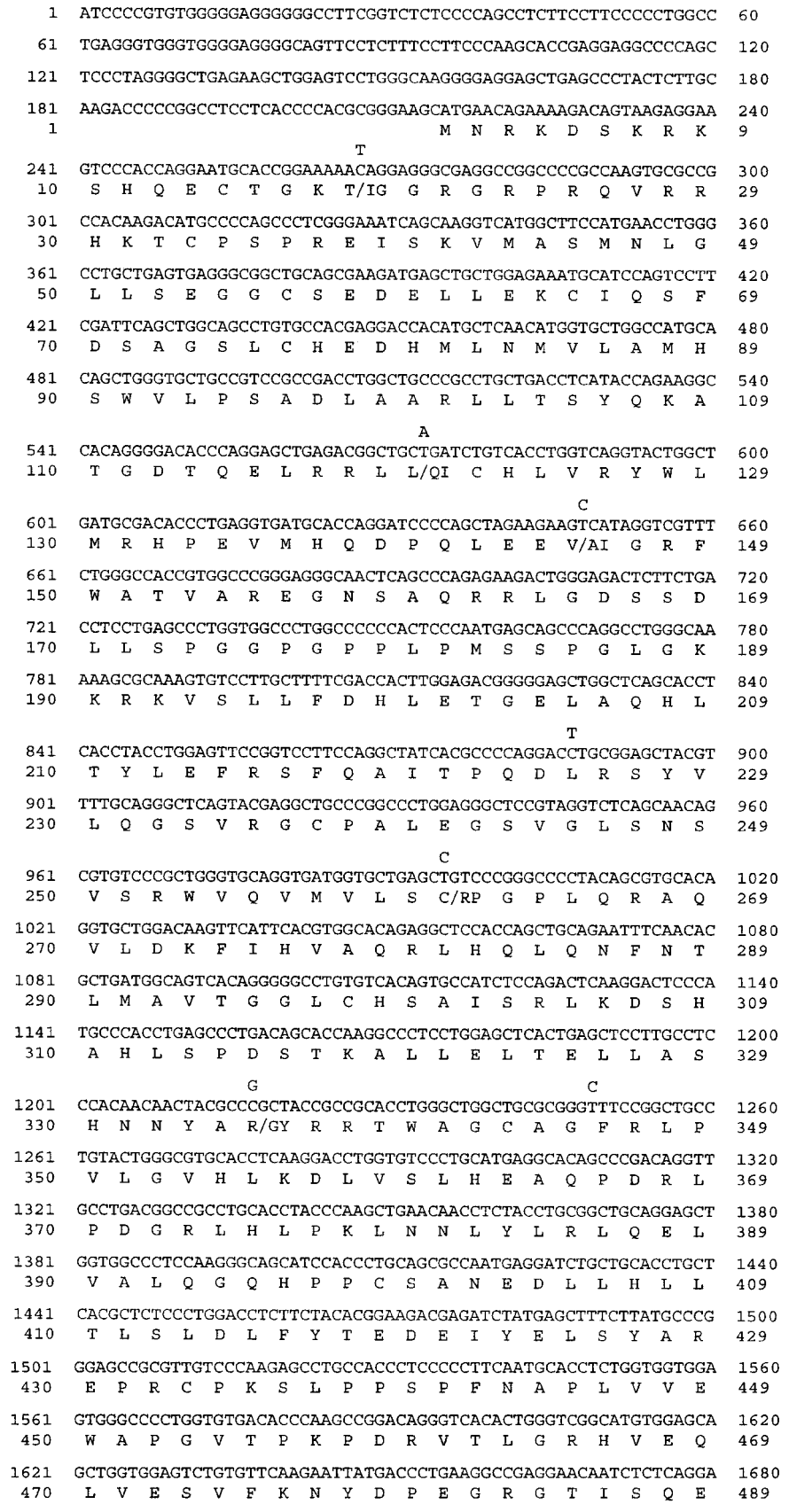

FIG. 3. **Cloning of full-length *hRasGRP4* cDNAs.** Depicted is the nucleotide sequence of the cDNA that corresponds to the major ~2.6-kb *hRasGRP4* transcript present in the human population, as well as the predicted amino acid sequence of its translated product. The 10 indicated nucleotide differences found in the *hRasGRP4* cDNAs that have been sequenced so far are presumed to be allelic polymorphisms of a single *hRasGRP4* gene. The new amino acid is indicated (*e.g.* "T/I" at amino acid residue 18) if the nucleotide change results in a different amino acid. The putative polyadenylation regulatory site is *underlined*.

liminary data raised the possibility that a functional *hRasGRP4* gene exists in the human genome. Based on the chromosomal assignment of BAC clone AC011469, we also concluded that this new gene probably resides ~8 kb downstream of the ryanodine receptor 1 (*RYR1*) gene (GenBank™ LocusLink accession number 6261) within a small region of human chromosome 19q13.1 that had not been sequenced by the Human Genome Project before the release of our nucleotide sequence data (*e.g.* GenBank™ accession number AY048119) to the public on August 19, 2001.

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1681  GGACTTTGAGCGACTCTCGGGCAATTTTCCCTTCGCCTGCCATGGGCTTCACCCACCCCC  1740
 490   D  F  E  R  L  S  G  N  F  P  F  A  C  H  G  L  H  P  P  P   509

1741  ACGCCAGGGGAGAGGATCCTTCAGCAGAGAGGAGCTGACAGGGTACCTGCTCCGGGCCAG  1800
 510   R  Q  G  R  G  S  F  S  R  E  E  L  T  G  Y  L  L  R  A  S   529

      T     C
1801  CGCCATTTGCTCCAAGTTGGGCCTGGCCTTCCTGCACACCTTCCATGAGGTCACCTTCCG  1860
 530   A  I  C  S  K  L  G  L  A  F  L  H  T  F  H  E  V  T  F  R   549

1861  AAAGCCTACCTTCTGCGACAGCTGCAGTGGCTTCCTCTGGGGTGTCACCAAGCAAGGCTA  1920
 550   K  P  T  F  C  D  S  C  S  G  F  L  W  G  V  T  K  Q  G  Y   569

                                                      C
1921  CCGCTGTCGGGAGTGCGGGCTGTGTTGCCACAAACACTGCAGAGATCAGGTGAAGGTAGA  1980
 570   R  C  R  E  C  G  L  C  C  H  K  H  C  R  D  Q  V  K  V  E   589

1981  ATGTAAGAAGAGGCCAGGGGCCAAGGGCGATGCAGGACCCCCCGGAGCTCCTGTCCCATC  2040
 590   C  K  K  R  P  G  A  K  G  D  A  G  P  P  G  A  P  V  P  S   609

2041  CACACCAGCTCCCCATGCCAGCTGTGGCTCCGAGGAAAATCACTCCTACACGCTATCCCT  2100
 610   T  P  A  P  H  A  S  C  G  S  E  E  N  H  S  Y  T  L  S  L   629

2101  GGAGCCTGAGACTGGGTGCCAGCTTCGCCATGCCTGGACCCAGACTGAATCCCCACACCC  2160
 630   E  P  E  T  G  C  Q  L  R  H  A  W  T  Q  T  E  S  P  H  P   649

2161  TTCCTGGGAAACAGATACGGTCCCCTGCCCGGTGATGGACCCACCATCAACTGCATCCTC  2220
 650   S  W  E  T  D  T  V  P  C  P  V  M  D  P  P  S  T  A  S  S   669

2221  CAAGCCGGATTCCTAGACATCTCTTGGTCTCCTCTCTTCCTCACTCCCTTCCCCCAGTCA  2280
 670   K  P  D  S  *                                                673

2281  GTCCTGAGTCCTGCCGTCAGACTCTGGCAGGGCTCCCAGAGGAGCATCAACTCCAGGAGA  2340

2341  ACAGGGATTTCTATCAATGTCATTCACTGCTGCATCCCCAGTGCCTAGAACAGGGCTGGC  2400

2401  AGGTGGTAGGCGCCTGACAGATGCTTGTCTGATGCCTGAATGTCTCCTTACCCATGCCCA  2460

2461  CGGCACAGGATAGATGTGCTATAGGGCAAAGAACTTTGAGGGTCGAGCAGCAGGGGCCCA  2520

2521  TTCAGTCCCAGGGAGCAGAGACCCCCCCAACCCCTTCACAAACCCCAACACCCCTGACTT  2580

2581  GGCCCCCACAGAGAGAGGTCCTACAGCTGTCATAAATTAAATTTATTCTCTGGA         2634
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FIG. 3—*continued*

Primers corresponding to the nucleotide sequences residing in exons 1 and 9 of the suspected *hRasGRP4* gene were used in RT-PCR (Fig. 2, *b–d*) approaches to determine whether or not this putative new human gene is transcribed *in vivo.* Because the expected transcript was detected in nearly every examined human fetal tissue, the novel human gene we identified on chromosome 19q13.1 is transcribed *in vivo*. In addition, it is transcribed relatively early in human development. The level of the *hRasGRP4* transcript was more abundant in fetal lung than in heart, brain, liver, kidney, spleen, thymus, or muscle. Although the expected 900-bp PCR product was detected in cDNA libraries generated from a number of adult human tissues, the amount of *hRasGRP4* mRNA in these tissue samples was considerably less than that in fetal lung (Fig. 2*c*).

Different sized RT-PCR fragments were occasionally seen in the tissue and blood samples pooled from many individuals. As noted below, sequence analysis of the corresponding transcripts in the mononuclear cells of a systemic mastocytosis patient and an asthma patient revealed that these larger and smaller RT-PCR products are the result of differential splicing of the precursor transcript. The levels of *hRasGRP4* mRNA were below detection in resting or lectin-activated $CD19^+$ B lymphocytes, $CD4^+$ T lymphocytes, or $CD8^+$ T lymphocytes (Fig. 2*d*). The levels of *hRasGRP4* mRNA also were below detection in lectin-treated mononuclear cells. Nevertheless, the steady-state levels of the *hRasGRP4* transcript were sufficiently high enough in the non-lectin-treated mononuclear leukocytes to detect the transcript by routine blot analysis (Fig. 2*a*).

By using pooled mRNA obtained in varied 3′- and 5′-RACE approaches, eventually we were able to deduce the nucleotide sequence of the primary full-length *hRasGRP4* transcript in the mononuclear leukocytes of most normal individuals (Fig. 3). The GenBank™ accession number for the major *hRasGRP4* cDNA present in the human population is AY048119. Sequence analysis of multiple subcloned *hRasGRP4* cDNAs revealed 10 single nucleotide differences in the eight analyzed clones that ultimately resulted in five amino acid differences in the translated products. It is presumed that these nucleotide differences are the result of minor allelic polymorphisms of a single *hRasGRP4* gene in the human genome rather than distinct genes that are >99% identical. The primary *hRasGRP4* transcript is ~300 bp larger than the primary *mRasGRP4* transcript due to a somewhat longer 3′-untranslated region. However, the rest of

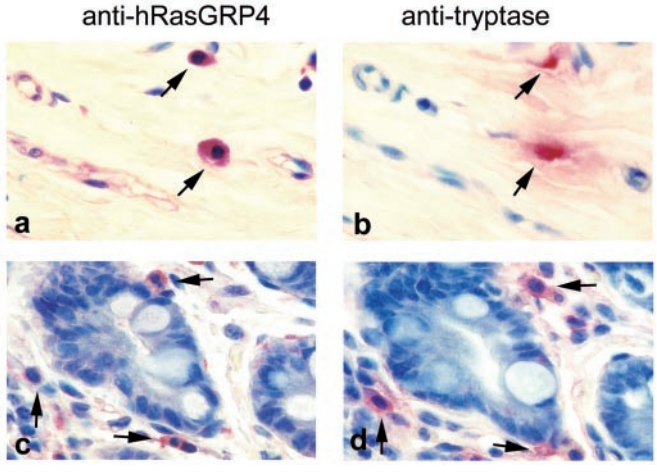

FIG. 4. **Immunohistochemistry.** Serial sections of human breast (*a* and *b*) and stomach (*c* and *d*) were stained with anti-hRasGRP4 antibody (*a* and *c*) or anti-tryptase antibody (*b* and *d*). The *red* reaction product (*arrows*) indicates the presence of hRasGRP4 or tryptase depending on the antibody used in the analysis.

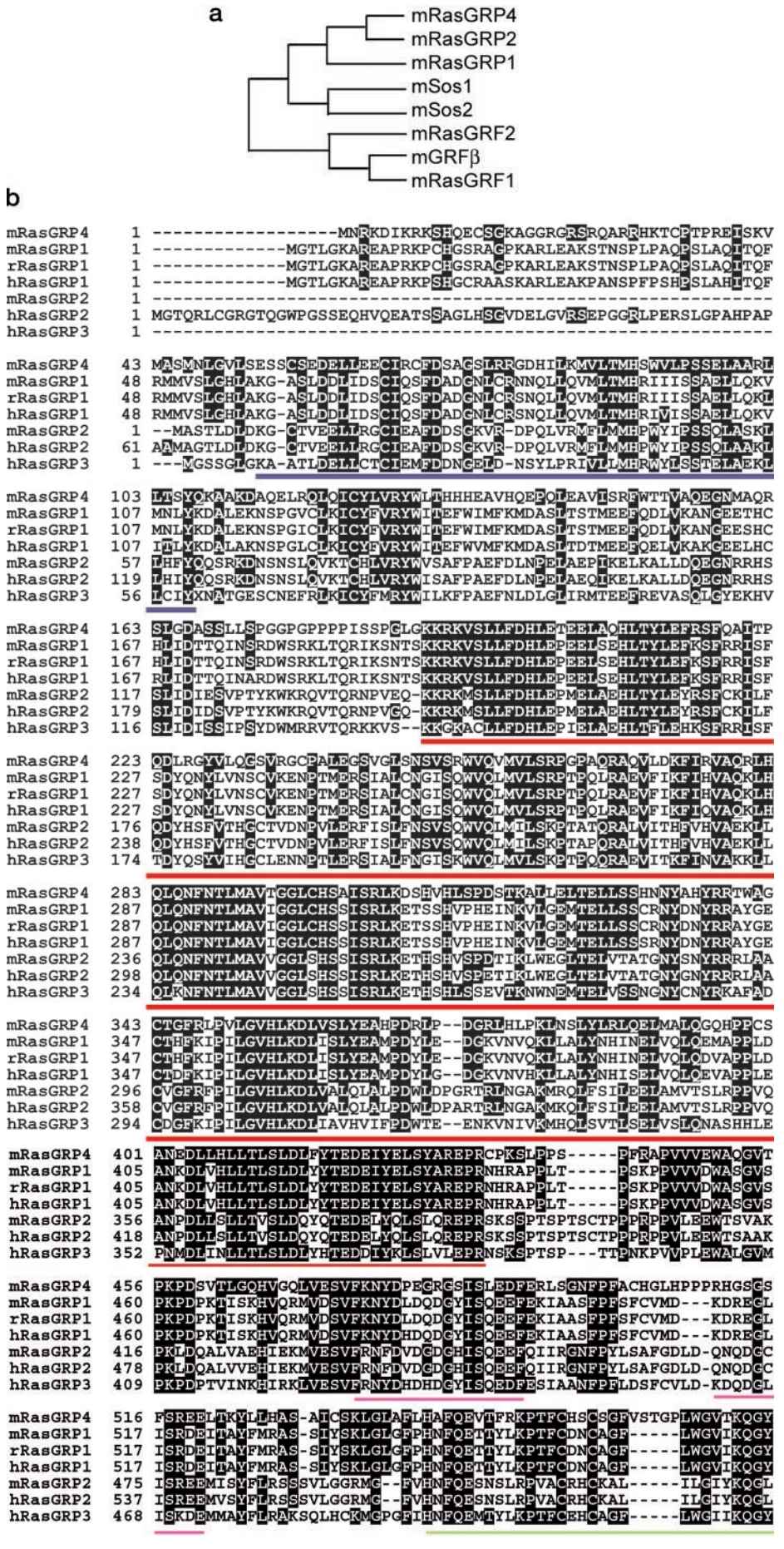

FIG. 5. **Comparison of the amino acid sequences of mouse and human RasGRP4 with mouse, rat, and human RasGRP1, RasGRP2, and RasGRP3.** *a*, dendrogram comparing mRasGRP4 with its closely related proteins was generated by the GCG program "Distances" using the unweighted pair group with arithmetic mean algorithm (UPGMA). *b*, comparison of the amino acid sequences of mRasGRP4 with those of mRasGRP1, rRasGRP1, hRasGRP1, mRasGRP2, hRasGRP2, and hRasGRP3. The amino acid sequences of the previously cloned members of this family of GEFs were extracted from GenBank™. *Gaps* (-) are indicated. The putative REM domains (*blue*), CDC25-like catalytic domains (*red*), EF hands of the Ca^{2+}-binding domains (*yellow* or *purple*), and the phorbol ester/DAG-binding domains (*green*) of the RasGRPs are highlighted. *c*, comparison of the amino acid sequences of mRasGRP4 and varied allelic forms of hRasGRP4. The five residues that differ in the allelic variants of hRasGRP4 are indicated at positions 18, 120, 145, 261, and 335.

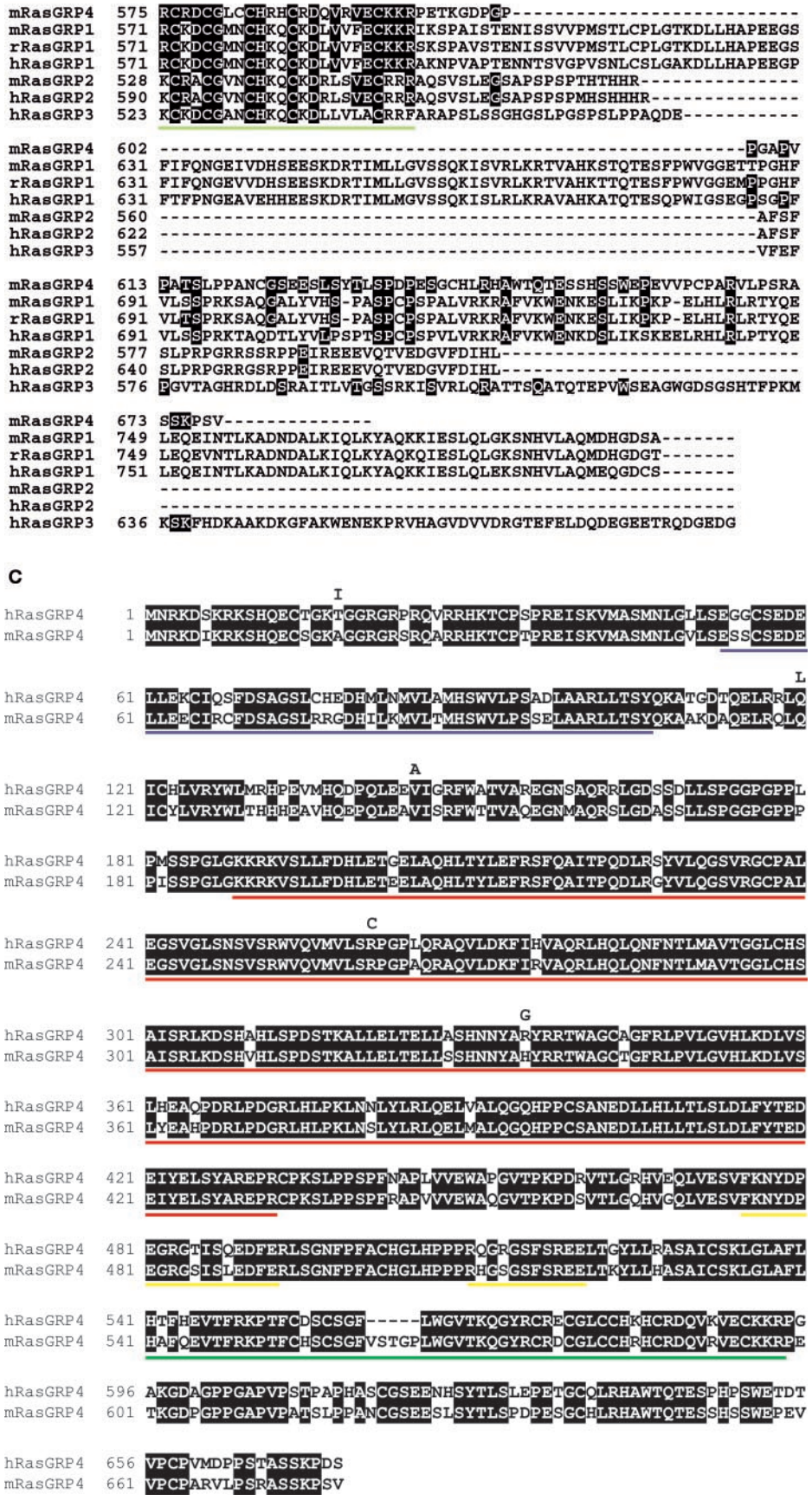

FIG. 5—*continued*

the *hRasGRP4* transcript is >80% identical to that of the *mRasGRP4* transcript.

No genomic fragment was detected in the GenBank™ genome data base that corresponded to the 3′ end of the *hRasGRP4* cDNA. Probing of Celera's human genome data base with the full-length *hRasGRP4* cDNA also failed to reveal an

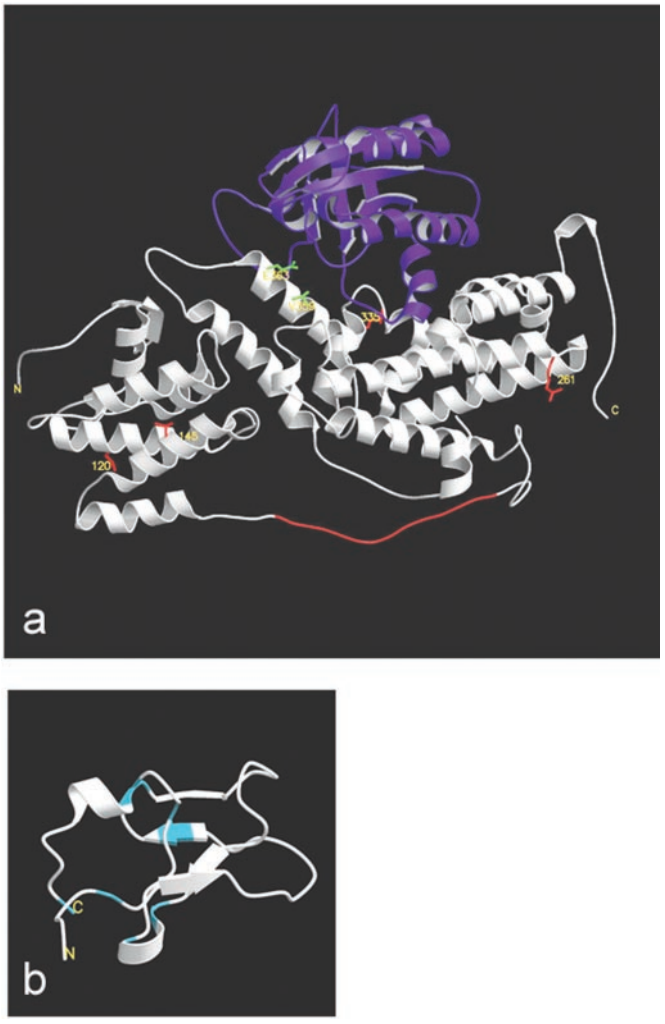

FIG. 6. **Three-dimensional models of domains in hRasGRP4.** *a*, schematic representation of the three-dimensional model of residues 34–445 of hRasGRP4 (*gray*) bound to H-Ras (*blue*). Shown in *ball* and *stick* (*green*) representation are the two residues (Val^{359} and Glu^{363}) in hRasGRP4 corresponding to the residues in hSos-1 that are absolutely essential for its interaction with H-Ras. The 14-residue peptide that is lost in the variant 2 transcript isolated from the asthma patient is highlighted (*red*). Four allelic differences in hRasGRP4 have been noted at residues 120, 145, 261, and 335 (*yellow*). The fifth allelic difference occurs outside of the modeled region at residue 18. *b*, schematic representation of the three-dimensional model of the DAG-binding domain of hRasGRP4 (residues 537–590). The conserved His and Cys residues in DAG-binding proteins are indicated (*blue*). All representations were rendered using Molscript (68).

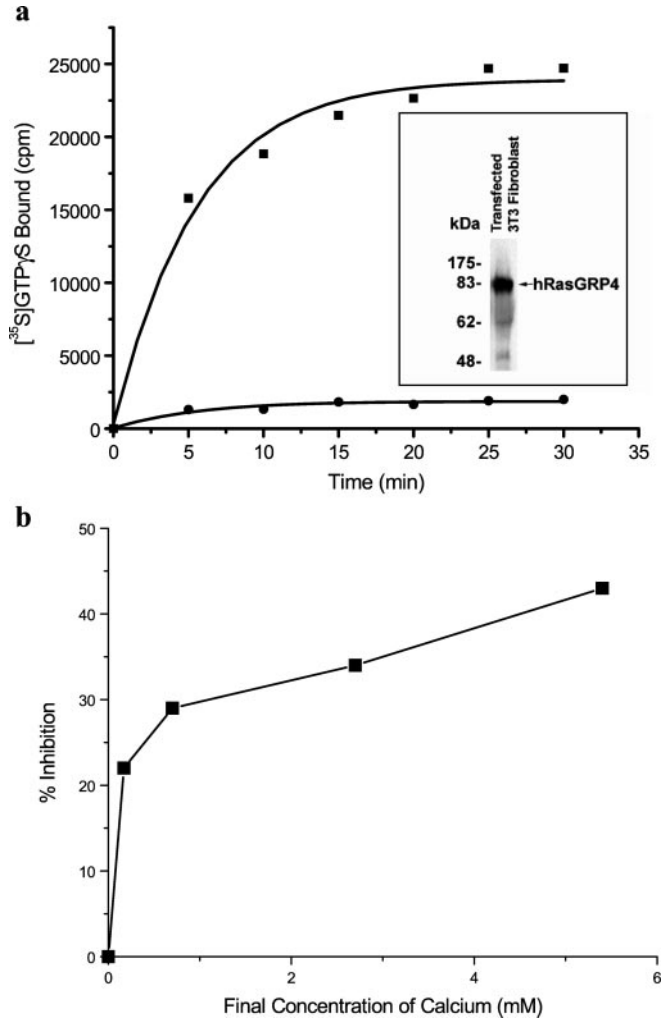

FIG. 7. **Generation of recombinant hRasGRP4 in COS-7 cells and fibroblasts, and evaluation of its guanine nucleotide exchange activity.** *a*, purified native (■) and heat-denatured (●) recombinant hRasGRP4 were evaluated for their ability to transfer radiolabeled GTP to GDP-loaded H-Ras in a kinetic manner at room temperature. The level of immunoreactive hRasGRP4 in the conditioned media of the expressing cells always was below detection (data not shown). The *inset* shows the SDS-PAGE/immunoblot analysis of lysates of fibroblasts transfected with the *hRasGRP4* construct; the depicted blot was probed with the anti-V5 antibody that recognizes the C-terminal epitope tag. Molecular weight markers are shown on the *left*. Recombinant hRasGRP4 is ~10 kDa larger than the native protein due to the epitope tag. *b*, the ability of purified recombinant hRasGRP4 to transfer radiolabeled GTP to GDP-loaded recombinant H-Ras was then compared after a 20-min incubation at room temperature in the presence of the indicated amounts of $CaCl_2$.

intact *hRasGRP4* gene in their data base. Nevertheless, a genomic fragment (designated GA2KMHMR58UU) was identified in Celera's data base that corresponded to the 3′ end of exon 10 to exon 18 of the *hRasGRP4* gene. By using a PCR approach, we were able to deduce the missing portion of the *hRasGRP4* gene. The *hRasGRP4* gene is >15 kb in size and consists of 18 exons. Like the mouse gene, the coding portion of the transcript is derived from 17 exons. Exon 18 corresponds to the 3′-untranslated region. Exons 1–18 correspond to residues 1–237, 238–422, 423–530, 531–591, 592–723, 724–877, 878–1051, 1052–1168, 1169–1444, 1445–1525, 1526–1630, 1631–1750, 1751–1894, 1895–1931, 1932–2066, 2067–2189, 2190–2272, and 2273-end, respectively, in the cDNA depicted in Fig. 3. The release of our *hRasGRP4* cDNA to the public domain eventually enabled the Human Genome Project to fill in the missing gap on chromosome 19q13.1 in the fall of 2001.

Evaluation of the Structure of RasGRP4 Protein—As assessed immunohistochemically using antibodies directed against the predicted N terminus of the translated product, the *hRasGRP4* transcript was selectively converted into protein in the $tryptase^+$ MCs that reside in varied normal human tissues (Fig. 4). Every $tryptase^+$ MC in the interlobular connective tissue of human breast and the mucosa layer of human stomach contained hRasGRP4 protein. Similar immunohistochemical data were obtained in the mouse with a different antibody. Thus, RasGRP4 is selectively expressed in mature MCs and their progenitors in humans and mice.

The nucleotide sequences of the isolated cDNAs indicate that mouse and human RasGRP4 exist as 673–678-residue, ~75-kDa proteins. Mouse and human RasGRP4 have a <50% amino acid sequence identity with mouse, rat, and human RasGRP1, RasGRP2, and RasGRP3 (Fig. 5). As found for other cytosolic proteins, RasGRP4 lacks a hydrophobic signal peptide at its N terminus. A Kyte-Doolittle hydropathy analysis also failed to

reveal an extended hydrophobic domain in the primary amino acid sequence of the protein. Immunoreactive mRasGRP4 and hRasGRP4 were not constitutively secreted from the COS-7 cell and fibroblast transfectants, and immunoreactive mRasGRP4 was not detected in extracellular matrices adjacent to tissue MCs. Thus, RasGRP4 is an intracellular protein.

A dendrogram comparison of the nine most related mouse proteins in this superfamily revealed that mRasGRP4 is slightly more similar to mRasGRP2 than to mRasGRP1 (Fig. 5*a*). The amino acid sequence of mRasGRP3 has not yet been deposited in GenBank™. Nevertheless, mRasGRP4 is only distantly related to hRasGRP3. mRasGRP4 also lacks the extended C-terminal domains present in mRasGRP1, rRasGRP1, hRasGRP1, and hRasGRP3 (Fig. 5*b*). The putative REM, CDC25-like catalytic, Ca^{2+}-binding EF hands, and phorbol ester/DAG-binding domains of the varied RasGRPs are highlighted in Fig. 5*b*. All of these domains are present in mouse and human RasGRP4 at the expected locations in its primary amino acid sequence. The most conserved region is the CDC25-like catalytic domain. For example, residues 405–433 in the CDC25-like catalytic domains of mRasGRP4 and mRasGRP1 differ only in two amino acid residues, and even these differences are minimal (*i.e.* Leu → Val and Phe → Tyr). The amino acid sequence of hRasGRP4 is ~85% identical to that of mRasGRP4 (Fig. 5*c*). Except for the C-terminal 21 amino acids where the degree of sequence identity drops to 52%, the high degree of conservation extends throughout the entire length of the protein.

Residues 34–445 of hRasGRP4 (Fig. 6*a*) are predicted to resemble the two α-helical domains of hSos-1 with 6 and 11 helices in the first and second domains, respectively. Mimicking hSos-1, the eighth α-helix in the CDC25-like catalytic domain of hRasGRP4 is predicted to interact with H-Ras. The residues involved in H-Ras interaction are generally conserved between hRasGRP4 and hSos1. The three-dimensional model of residues 537–590 of hRasGRP4 (Fig. 6*b*) also closely resembles that of the putative DAG-binding domain of protein kinase Cγ despite the poor degree of primary amino acid sequence identity. The minor allelic differences identified so far in hRasGRP4 are not expected to grossly alter its three-dimensional structure, nor are they likely to affect its interaction with H-Ras or DAG greatly.

Guanine Nucleotide Exchange Activity of Recombinant RasGRP4 and Increased Sensitivity of RasGRP4-expressing Fibroblasts to PMA—Bioengineered forms of mouse and human RasGRP4 were expressed in COS-7 cells and fibroblasts that contained the V5 and His_6 peptides at their C termini. The latter epitope tag was added so that each recombinant protein could be identified and purified. Although substantial amounts of immunoreactive RasGRP4 were always recovered in the soluble cytosolic portion of the lysates of the transfectants, a portion of the recombinant protein was consistently recovered in the microsomal fractions. Thus, RasGRP4 does not reside in a single intracellular compartment in either transfected cell type.

As the three-dimensional model of residues 34–445 predicted, purified recombinant hRasGRP4 was able to transfer γ-^{35}S-GTP to GDP-loaded H-Ras in a catalytic manner (Fig. 7*a*). The first EF hand in rRasGRP1 functions as the primary Ca^{2+}-binding site of this protein (14), and the critical residues that form the "regulatory EF hand" in rRasGRP1 and other Ca^{2+}-binding proteins (36) are present in mouse and human RasGRP4 at the expected locations. Whether or not Ca^{2+} binds specifically to this domain remains to be determined using a site-directed mutagenesis approach. Nevertheless, 1 mM Ca^{2+} was sufficient to inhibit significantly the ability of recombinant hRasGRP4 to transfer GTP to GDP-loaded H-Ras even if 5 mM Mg^{2+} was present in the reaction buffer (Fig. 7*b*).

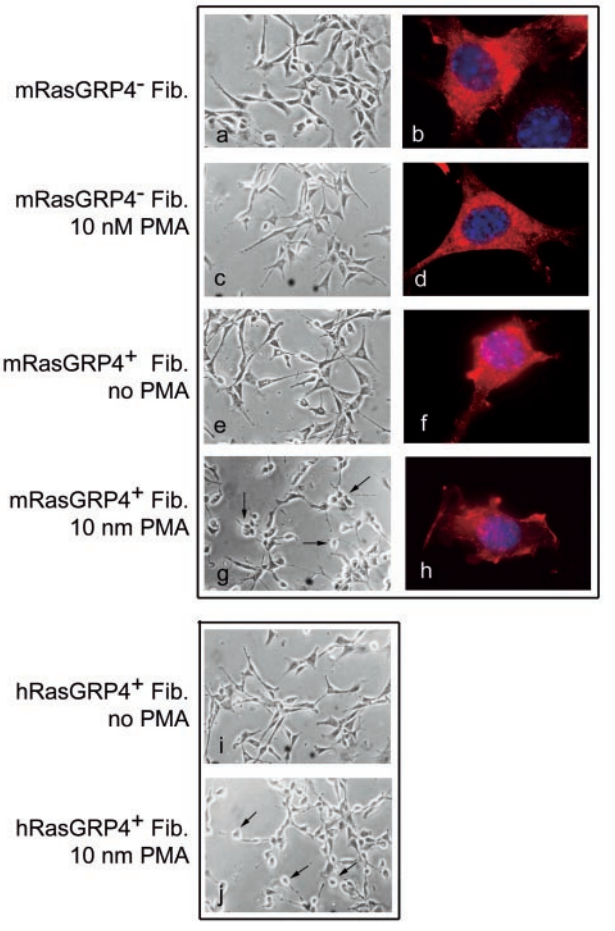

FIG. 8. **Morphology of normal and RasGRP4-expressing fibroblasts before and after exposure to PMA.** Normal RasGRP4$^-$ fibroblasts (*Fib.*) (*a–d*), mRasGRP4$^+$ fibroblast transfectants (*e–h*), and hRasGRP4$^+$ fibroblast transfectants (*i–j*) were evaluated before (*a*, *b*, *e*, *f*, and *i*) and after a 15-min exposure at 37 °C to 10 nM PMA (*c*, *d*, *g*, *h*, and *j*). At the light level (*a*, *e*, and *i*), all populations of fibroblasts are extremely adherent to plastic culture dishes before PMA treatment and form prominent focal adhesions and extended membrane projections. RasGRP4$^-$ fibroblasts do not undergo noticeable morphologic changes when exposed to low levels of PMA for a brief period (*c*). However, many of the mRasGRP4$^+$ fibroblasts (*arrows* in *g*) and hRasGRP4$^+$ fibroblasts (*arrows* in *j*) quickly round up when similarly treated. As assessed immunohistochemically, actin (*red*) redistributes in those mRasGRP4$^+$ fibroblasts that have been exposed to PMA (*h*). DNA stains *blue* in these immunohistochemical assays. Approximately 80% of the fibroblasts shown in *panel g* express mRasGRP4 as assessed immunohistochemically with anti-V5 antibody (data not shown). Similar findings were obtained in a second experiment with mRasGRP4.

The phorbol ester/DAG-binding, C1 domain in protein kinase C is ~50 residues in length and possesses the motif of $HX_{12}CX_2CX_{13/14}CX_2CX_4HX_2CX_7C$, where H is His, C is Cys, and *X* is any other amino acid. Because these residues are present in mouse and human RasGRP4 and because the three-dimensional model predicts that residues 537–590 resemble a C1-like domain, the possibility that RasGRP4 is a phorbol ester/DAG receptor also was tested experimentally. As noted in Fig. 8, RasGRP4-expressing fibroblasts underwent dramatic morphologic changes when exposed to low levels of PMA for only 15 min.

Isolation of Abnormal hRasGRP4 Transcripts in an Asthma Patient and a Mastocytosis Patient—Primers corresponding to nucleotide sequences in exons 1 and 17 of the *hRasGRP4* gene were used to generate cDNAs that correspond to the coding

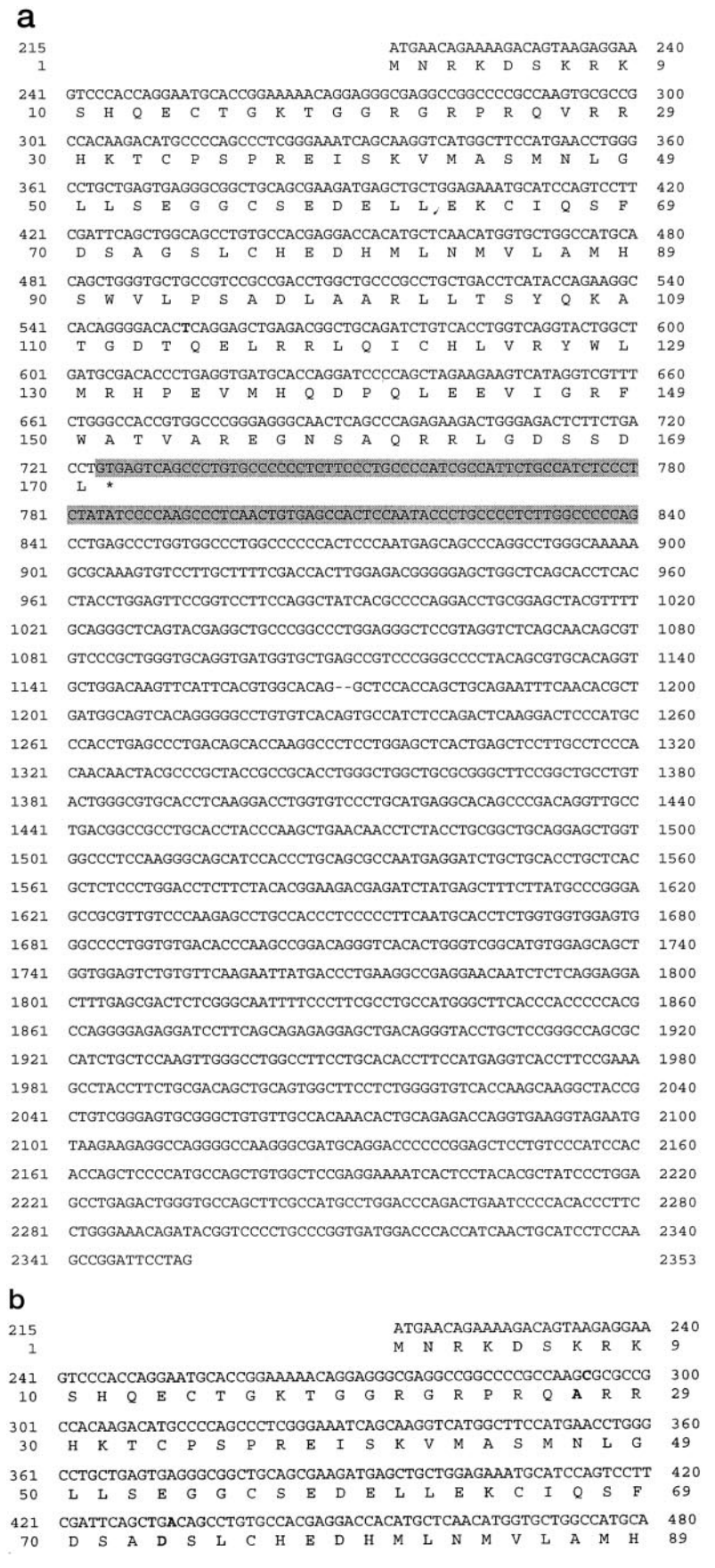

FIG. 9. **Isolation of two aberrant *hRasGRP4* cDNAs from an asthma patient and a mastocytosis patient that encode truncated proteins.** *a*, aberrant *hRasGRP4* cDNA (designated variant 1) was isolated from an asthma patient and a mastocytosis patient that contained a 117-bp insertion. Sequence analysis of the cDNA and the *hRasGRP4* gene revealed variant 1 was caused by a failure of the hRasGRP4-expressing cell to remove intron 5 (*shaded region*) in the precursor transcript. The 117-bp insertion (*shaded sequence*) causes an early translation-termination codon (*). One of the two sequenced variant 1 transcripts isolated from the asthma patient possessed an additional problem at the intron 7/exon 8 splice site that resulted in the loss of the indicated 2 nucleotides (*dashes* at residue 1168) in the newly formed 3′-untranslated region. *b*, a different aberrant *hRasGRP4* cDNA (designated variant 2) was isolated from the same asthma patient. Variant 2 encodes a truncated form of hRasGRP4 that lacks the Leu-Ser-Pro-Gly-Gly-Pro-Gly-Pro-Pro-Leu-Pro-Met-Ser-Ser sequence that precedes the CDC25-like catalytic domain in the normal protein. Analysis of the hRasGRP4 gene revealed that variant 2 is caused by a failure of the hRasGRP4-expressing cell to use the normal intron 5/exon 6 splice site to remove intron 5 in the precursor transcript. The use of a cryptic splice site in the middle of exon 6 to eliminate the intron and a portion of exon 6 results in the production of a truncated hRasGRP4. The area of the transcript and protein that is affected by this post-transcriptional splicing event is indicated (#). The minor nucleotide differences noted in the variant 1 and 2 cDNAs relative to the *hRasGRP4* cDNA shown in Fig. 3 are in *boldface*. The 5′-untranslated regions of the variant 1 and 2 cDNAs were not deduced. Nevertheless, it is assumed that the initial 215 nucleotides in these transcripts correspond to those in the normal *hRasGRP4* transcript. *c*, RT-PCR analyses were carried out using different primer sets to evaluate the expression of the variant 1 (*top panel*) and variant 2 (*bottom panel*) forms of the *hRasGRP4* transcript in a pooled leukocyte preparation derived from 550 individuals and in the MC progenitors residing in the blood of four normal individuals, a patient with asthma, and a patient with systemic mastocytosis. *d*, shown is a schematic representation of the splicing events that result in the generation of the normal *hRasGRP4* transcript and its two abnormal variants in the asthma patient.

domains of the forms of hRasGRP4 that are expressed in the MC-committed progenitors residing in the peripheral blood of an asthma patient. Five of the eight arbitrarily subcloned cDNAs from this patient corresponded, with minor differences, to the normal *hRasGRP4* cDNA depicted in Fig. 3. However, two of the cDNAs (designated variant 1) were 117 nucleotides

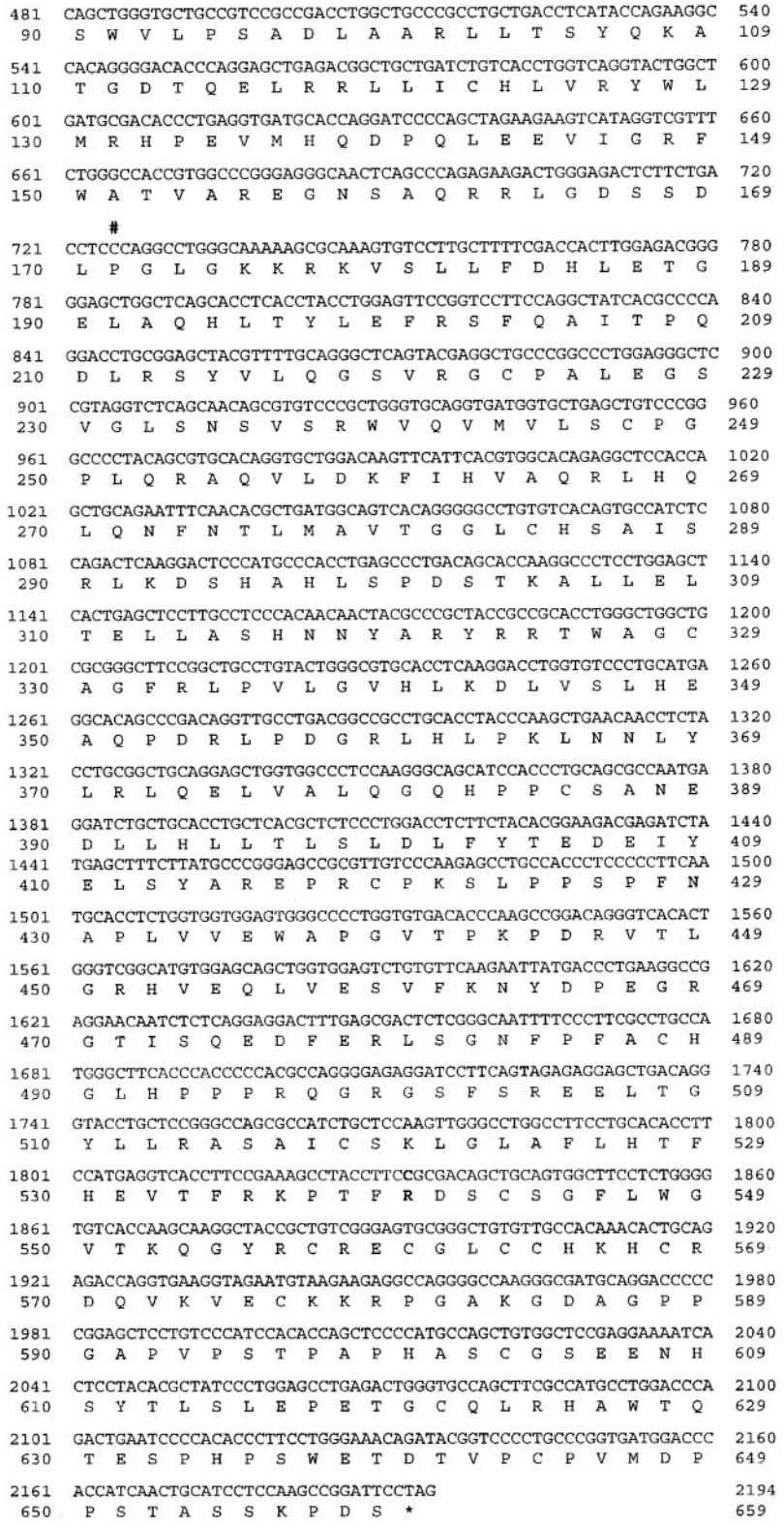

FIG. 9—*continued*

larger in size. Sequence analysis revealed that variant 1 was created by a failure of the hRasGRP4-expressing cell to remove intron 5 from the precursor transcript (Fig. 9, *a* and *d*). The failure to remove this single intron results in the formation of a premature translation-termination codon in the expressed transcript. Assuming the normal translation-initiation site is used, the resulting 170-residue protein will not be able to activate H-Ras because it lacks ~75% of its primary amino acid sequence, including the entire CDC25-like catalytic domain.

One of the eight arbitrarily subcloned cDNAs from the asthma patient possessed a 42-residue deletion (Fig. 9, *b* and *d*) rather than a 117-bp insertion. This isoform (designated variant 2) also was caused by a failure of the hRasGRP4-expressing cell in this patient to properly remove intron 5. Because the normal intron 5/exon 6 splice site was not used in the maturation of the precursor human transcript, a cryptic splice site residing 42 nucleotides within exon 6 was employed to remove intron 5 during the maturation of the variant 2 transcript. The

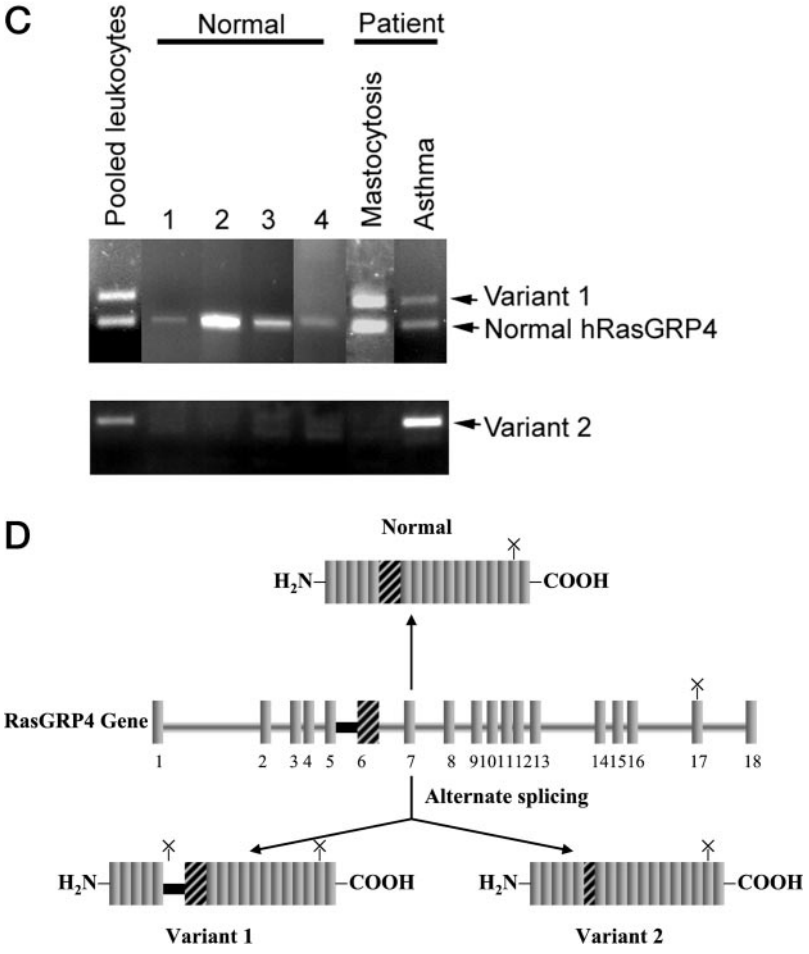

FIG. 9—*continued*

open reading frame of the truncated variant 2 *hRasGRP4* transcript remains intact. However, the resulting protein lacks the 14-mer sequence that links the two major helical domains within the N-terminal segment (Fig. 6*a*). The GenBank™ accession numbers for the variant 1 and 2 *hRasGRP4* transcripts are AY048120 and AY048121, respectively.

An RT-PCR approach was next used to evaluate the prevalence of both abnormal *hRasGRP4* transcripts in the general population and in an additional mastocytosis patient. As noted in Fig. 9*c* using different primer sets, the levels of the variant 1 and 2 transcripts were below detection in the MC progenitors residing in the blood of four normal individuals. Nevertheless, aberrant *hRasGRP4* transcripts were detected in the pooled leukocyte preparation derived from 550 individuals. Variant 1 mRNA was found in the mastocytosis patient, but the level of the variant 2 transcript was below detection.

None of the eight *hRasGRP4* cDNAs isolated and sequenced from the asthma patient and none of the *hRasGRP4* cDNAs isolated and sequenced from the leukocyte preparation generated from multiple individuals lacked an entire coding exon. These preliminary data suggest that the loss of an entire coding exon in the *hRasGRP4* transcript is a rare event, if it ever occurs, in the MC-committed progenitors circulating in the blood of normal individuals. Nevertheless, removal of any combination of exons 7–16 (except exons 14 and 15) will result in a truncated transcript that remains in the correct reading frame relative to the normal translation-termination codon. Thus, further studies are needed to address whether or not differential exon splicing of the precursor *hRasGRP4* transcript occurs in the mature MCs that develop in different tissue sites of normal individuals.

Evaluation of hRasGRP4 Expression and Function in the HMC-1 Cell Line—The immature HMC-1 cell line was derived by Butterfield *et al.* (27) in 1988 from a patient with a MC leukemia. The identification of defective variants of *hRasGRP4* mRNA in a mastocytosis patient (Fig. 9*c*) raised the possibility that the HMC-1 cell line might also express abnormal variants of hRasGRP4. Thus, *hRasGRP4* expression at the mRNA level was next evaluated in this cell line. By using various primers sets, we discovered that the *hRasGRP4* gene is transcribed in HMC-1 cells (Fig. 10). However, only abnormal variants of *hRasGRP4* are expressed in this transformed cell line. As noted in Fig. 10, a new isoform of *hRasGRP4* (designated variant 3) that is closely related to the variant 1 isoform was isolated from HMC-1 cells. Sequence analysis of this RT-PCR product revealed that the larger sized transcript was caused by a failure to remove intron 5 and additionally intron 3 in the precursor transcript. The failure to remove these two introns results in a translation-termination codon that occurs earlier in variant 3 than that in variant 1. Thus, even if translated, variant 3 also would be unable to activate any Ras family member. A premature translation-termination codon at a similar location in a tryptase transcript results in its rapid degradation in C57BL/6 mouse mBMMCs by the nonsense-mediated pathway (37). If the defective *hRasGRP4* transcript is being catabolized nearly as fast as it is being generated as we suspect, this would account for its lower levels in the HMC-1 cell line.

HMC-1 cells are poorly granulated, blast-like leukemia cells (Fig. 11*i*) that fail to express detectable amounts of MC chymase (Fig. 11*e*) or CPA (Fig. 11*g*) protein. Because HMC-1 cells only express small amounts of β-tryptase (Fig. 11, *b* and *c*), this cell line became an attractive MC-committed progenitor to

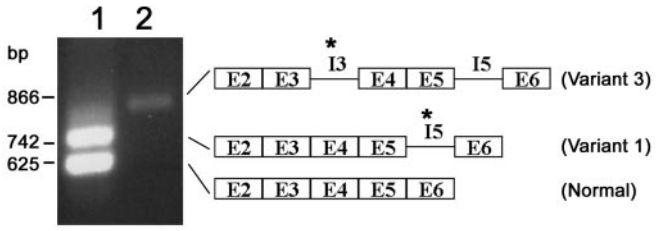

FIG. 10. ***hRasGRP4* expression in the MC leukemia cell line HMC-1.** Oligonucleotides corresponding to sequences in exon 2 (*E2*) and exon 6 (*E6*) of the *hRasGRP4* gene were used in an RT-PCR approach to evaluate the extent of processing of the *hRasGRP4* precursor transcript in the HMC-1 cell line (*lane 2*) and spleen of 22 pooled Caucasian fetuses (ages 20–33, CLONTECH) (*lane 1*). The normal 625-bp, properly processed portion of the transcript was detected in the pooled spleen sample, as well as the 742-bp variant 1 transcript that contains intron 5 (*I5*). In contrast, the normal *hRasGRP4* transcript and the variant 1 transcript were not detected in the HMC-1 cell line. Rather an 866-bp form (variant 3) was detected in the HMC-1 cell line whose nucleotide sequence revealed that it contained both intron 3 (*I3*) and intron 5 (*I5*). The failure to remove these introns causes a premature translation-termination codon (*).

begin to address the function of RasGRP4 in a more natural setting than occurs in a transfected fibroblast line. Thus, using an expression/transfection approach, HMC-1 cells were induced to express a normal, biologically active form of mRasGRP4 (Fig. 11*a*). As noted in Fig. 11, the resulting transfectants underwent dramatic morphologic changes (Fig. 11*j*) and increased their levels of tryptase substantially (Fig. 11, *b* and *d*). The transfectants also began expressing MC chymase (Fig. 11*f*) and CPA (Fig. 11*h*).

DISCUSSION

We describe a new mouse and human cation-dependent, GEF/phorbol ester receptor (designated RasGRP4) that is selectively expressed in MCs and their progenitors. The *hRasGRP4* gene is >15 kb in size, consists of 18 exons, and resides on chromosome 19q13.1. Because the same four *RasGRP* genes are present in the mouse and human genomes, the varied members of its family apparently evolved >100 million years ago before the divergence of mice and humans. The *hRasGRP1*, *hRasGRP2*, and *hRasGRP3* genes reside on chromosomes 15, 15, and 2, respectively (17, 20). It now appears that an ancestral *RasGRP*-like gene duplicated twice, and the resulting three genes translocated to distinct chromosomes. The new gene that segregated to the genomic fragment that eventually became human chromosome 19 failed to duplicate again and became *RasGRP4* in both species. A similar situation occurred for the *RasGRP3* gene that eventually developed on human chromosome 2. However, the ancestral *RasGRP*-like gene that segregated to the genomic fragment that eventually became human chromosome 15 duplicated one more time to become the *RasGRP1* and *RasGRP2* genes. The resulting four genes then underwent substantial amino acid divergence to create the final *RasGRP1, RasGRP2, RasGRP3*, and *RasGRP4* genes in both species. All four genes were then maintained throughout the evolution of mice and humans. The biological significance of these evolutionary events is that most RasGRP data obtained in mice should be relevant to humans. In addition, mRasGRP4 should be active in human MCs and hRasGRP4 should be active in mouse MCs.

As assessed by RNA blot (Fig. 2*a*), RT-PCR (Fig. 2, *b–d*), and immunohistochemical (Fig. 4) analyses, RasGRP4 is an ~75-kDa intracellular protein that is selectively expressed in mature MCs and their progenitors. When the four RasGRPs were compared (Fig. 5), the least conserved regions were found to be the N and C termini. The N terminus of hRasGRP2 is myristoylated and palmitoylated at Gly^2 and Cys^7, respectively (19).

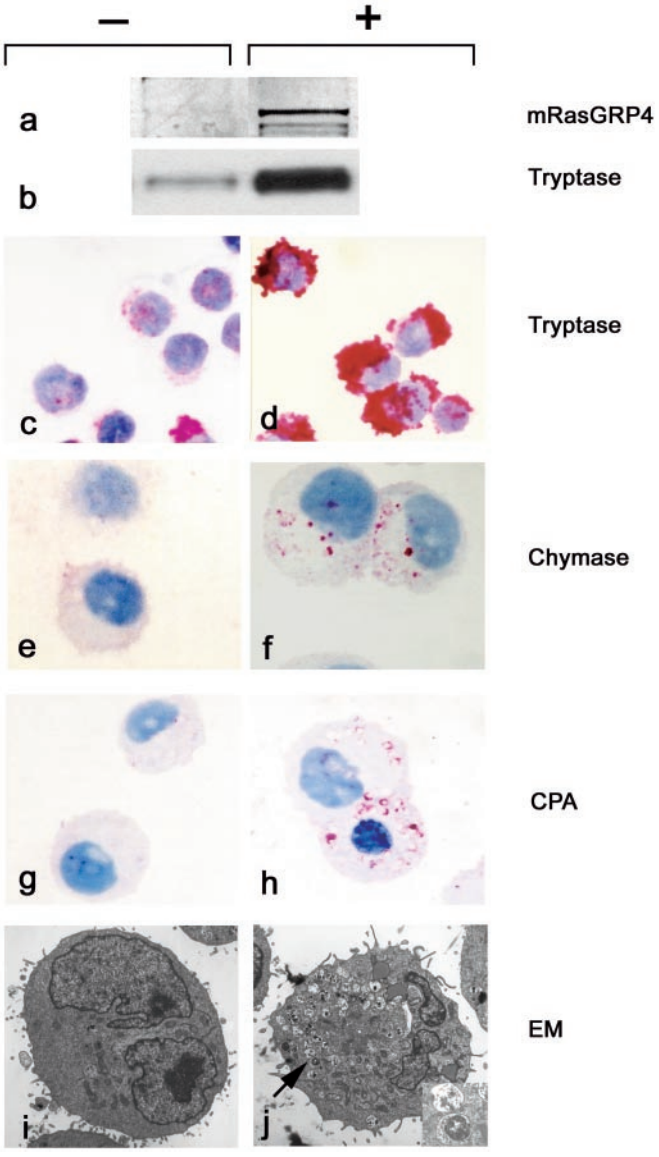

FIG. 11. **Granulation of HMC-1 cells and their mRasGRP4 transfectants.** Because only non-functional forms of hRasGRP4 are present in HMC-1 cells, a transfection approach was used to force this immature human MC line to express a functional form of the GEF (*a*). As assessed immunohistochemically (*c–h*) and by SDS-PAGE-immunoblot analysis (*b*), the mRasGRP4-expressing transfectants (+) contained substantially more tryptase (*b–d*), chymase (*e* and *f*), and CPA (*g* and *h*) in their granules than the non-transfectants (−). Although granules are rarely found in HMC-1 cells (*i*), many granules that contain electron dense material are found in the transfectants (*j*). Shown in the *inset* in the *lower right j* is a higher magnification of two typical granules in the transfectants. Although the transfectant depicted in the EM shown in *j* is more typical of the cells in the mRasGRP4-expressing cultures, ~10% of the transfectants contain granules that are nearly completely filled with electron dense material (data not shown).

Although RasGRP4 has a conserved Cys at residue 14, residues 2 and 7 in this GEF are Asn and Lys, respectively (Figs. 1, 3, and 5). Not only does the N terminus of RasGRP4 fail to resemble that of RasGRP2, its amino acid sequence also does not resemble that found in other palmitoylated and myristoylated G proteins (38, 39). Thus, RasGRP4 and RasGRP2 appear to differ in at least one important structural feature.

The *Saccharomyces cerevisiae* cell division cycle 25 (CDC25) protein is required for the function of adenylate cyclase in yeast, and CDC25 promotes the exchange of guanine nucleotides bound to Ras (40, 41). Yeast replication is dependent on

CDC25, and inactivation of this GEF results in cell cycle arrest in G_1. The most conserved region within RasGRP1 and RasGRP4 corresponds to the catalytic domain of CDC25 (Fig. 5). A comparative three-dimensional model of residues 34–445 of hRasGRP4 (Fig. 6*a*) predicted that the overall structure of this region resembles that of hSos1 even though the sequence identity is only ~20%. The key residues in hSos1 needed to interact with H-Ras are all present in RasGRP4 when it is folded. These data therefore suggested that RasGRP4 probably functions in MCs as a GEF. As noted in Fig. 7, recombinant RasGRP4 is indeed able to activate H-Ras *in vitro*. MCs express varied members of the Ras superfamily of GTP-binding proteins (12, 26, 42–47). However, because recombinant hRasGRP3 is able to transfer GTP to GDP-loaded H-Ras, R-Ras, and Rap1 *in vitro* (18), it remains to be determined which Ras family members RasGRP4 prefers to interact with inside a living MC.

Immunoelectron microscopy revealed that a substantial portion of the RasGRP4 present in the splenic mouse MCs resides on the cytosolic side of the plasma membrane of the cell. Although this finding strongly implies that RasGRP4 participates in early signaling events at the plasma membrane of the MC, immunoreactive mRasGRP4 also was found in the cytoplasm. Subcellular fractionation studies of the mRasGRP4 and hRasGRP4 transfectants confirmed these ultrastructural findings. Based on these data, some unknown intracellular factor or post-translational modification event must regulate the movement of RasGRP4 from the cytoplasm to the inner leaflet of the plasma membrane of the MC. Activation of MCs via FcεRI or c-*kit* results in the rapid generation of DAG, and the generation of this lipid has been linked to the morphological changes that occur in MCs. Mouse, rat, and human RasGRP1, RasGRP2, and RasGRP3 have C1-like domains, and recombinant hRasGRP3 expressed in *E. coli* can bind 12-[^{3}H]phorbol 13-dibutyrate efficiently in the presence of phospholipids (20). We are unaware of any group that has expressed a truncated RasGRP lacking its C1 domain to map precisely the location of its phorbol ester/DAG-binding site. Nevertheless, the data reported in numerous studies (19, 20, 48, 49) have led to the conclusion that DAG is required for the efficient movement of the varied RasGRPs from their cytosolic compartment to the plasma membrane.

The primary consequence of increased levels of DAG in MCs is thought to be activation of varied protein kinase C isoforms (50). However, the facts that RasGRP4 contains a potential DAG-binding domain (Fig. 6*b*) that is relatively conserved (Fig. 5) and that PMA treatment of RasGRP4-expressing fibroblasts results in dramatic morphologic changes (Fig. 8) now suggest that DAG and varied phorbol esters also regulate the movement of RasGRP4 to the plasma membrane and that this translocation process ultimately contributes to the membrane ruffling and spreading seen in activated MCs. Although the putative DAG-binding domain in RasGRP4 appears to be functionally important in the context of a living cell (Fig. 8), PMA does not enhance or suppress the guanine nucleotide exchange activity of the major form of RasGRP4 expressed in MCs, at least in one *in vitro* assay. A similar finding has been reported for recombinant hRasGRP2 (19).

The specific receptor-mediated signaling pathway(s) in MCs that depends on RasGRP4 remains to be determined experimentally. All mature MCs appear to express FcεRI, c-*kit*, and RasGRP4. Although RasGRP4 could participate in FcεRI-mediated signaling pathways, our data suggest an important role for our intracellular protein in c-*kit*-mediated signaling. c-*kit* is the only cytokine and adherence receptor that is absolutely essential for the development of mature MCs (51, 52), and MCs undergo substantial morphological changes when they bind to c-*kit* ligand$^+$ mesenchymal cells (53). The finding that RasGRP4-expressing fibroblasts undergo dramatic morphologic changes when exposed to PMA (Fig. 8) now suggests that RasGRP4 helps regulate the morphological changes that occur when MCs are activated via c-*kit*. W/W^v mice are MC-deficient (51) because of a genetic abnormality in c-*kit* (54). This mouse strain contains normal numbers of MC-committed progenitors in its bone marrow, and these progenitors readily target to connective tissue sites. However, the committed progenitors cannot develop into mature MCs because of the genetic defect in the tyrosine kinase domain of c-*kit* that resides inside MCs and participates in signal transduction pathways. Interestingly, Gordon and Galli (55) noted that substantial numbers of mature MCs develop in the skin of W/W^v mice following exposure to PMA. This finding suggested that an undefined PMA-dependent signaling protein acts downstream of c-*kit* in mouse MC development.

Human asthma is a polygenic disorder that is additionally influenced by environmental factors. It is generally accepted that pulmonary MCs play an important role in the initiation and/or progression of this disease. Thus, an intense effort has been made during the last decade to understand the varied signaling pathways that regulate the development and function of pulmonary MCs. We discovered that the *hRasGRP4* gene resides at chromosome 19q13.1. Interestingly, gene-linkage studies carried out by others have revealed a human asthma susceptibility locus ($p = 0.0013$) at chromosome 19q13.1 in Caucasians (56). The fact that many cytokine precursor transcripts undergo alternative splicing to produce receptor antagonists rather than receptor activators (57–60) documents the importance of post-transcriptional mechanisms in allergic asthma. Thus, we looked for the expression of altered forms of hRasGRP4 that would be caused by differential splicing of the precursor transcript. Although some of the single amino acid polymorphisms noted in Figs. 3 and 5*c* could result in forms of the GEF that differ slightly in their ability to activate H-Ras, we searched for the expression of *hRasGRP4* transcripts in an asthma patient that encodes more severely altered proteins. Sequence analysis of eight arbitrarily subcloned *hRasGRP4* cDNAs from an asthma patient revealed the presence of two transcripts that contained a 117-nucleotide insertion due to a failure of the hRasGRP4-expressing MC progenitor to remove intron 5 from the precursor transcript (Fig. 9, *a* and *d*). No point mutation was noted at the exon 5/intron 5 or intron 5/exon 6 boundaries of the gene. Thus, the variant 1 transcript appears to be caused by an unprecedented post-transcriptional mechanism. Although the mechanism that hinders removal of intron 5 in this patient remains to be determined, the functional consequences of the post-transcriptional event are clear. The aberrant splicing event results in the expression of a 170-residue, non-functional protein.

Another abnormal *hRasGRP4* cDNA was identified in the asthma patient that also was caused by a failure to properly remove intron 5. In contrast to the variant 1 transcript, the variant 2 transcript possessed a 42-nucleotide deletion (Fig. 9, *b* and *d*). When translated, variant 2 should encode a 659-residue protein that lacks the Pro-rich, 14-mer sequence immediately preceding the CDC25-like catalytic domain. The three-dimensional model (Fig. 6*a*) predicts that the deleted sequence is an extended loop in the CDC25-like catalytic domain opposite the face that interacts with H-Ras, linking one helical domain with another. The loss of this "rigid" Pro-rich, 14-mer sequence undoubtedly will affect the interaction of the two helical domains within the N-terminal segment of hRasGRP4. Because the first helical region is postulated to stabilize the second region that interacts with H-Ras (34), disruption of

the structure of the loop linking the two domains should adversely affect the ability of hRasGRP4 to transfer GTP to H-Ras. As noted in Fig. 9*c*, these two aberrantly spliced transcripts were not found in the MC progenitors in the blood of four normal individuals. The mouse counterparts of these two abnormal forms of hRasGRP4 also were not found in BALB/c mBMMCs. Nevertheless, preliminary RT-PCR analysis of 12 other asthma patients revealed that the expression of the nonfunctional, variant 1 form of hRasGRP4 is a common occurrence in this patient group.[2] Surprisingly, variant 1 is more prevalent in the human population than variant 2 (Figs. 9*c* and 10) even though the former is the more severely altered form of hRasGRP4. Although it is possible that the expression of nonfunctional forms of hRasGRP4 in multiple asthma patients is coincidental, the gene-linkage studies are suggestive of an adverse role for hRasGRP4 in the development of asthma in some patients.

The inbred BXH2 and AKXD13 mouse strains are particularly susceptible to leukemia viruses, and Li *et al.* (61) noted that spontaneous retroviral insertion into the *RasGRP1* gene in these strains often results in myeloid, B cell, and T cell leukemia. The *hRasGRP4* gene resides at chromosome 19q13.1. As noted at the "Mitelman Data base of Chromosome Aberrations in Cancer" at the NCI web site (cgap.nci.nih.gov/Chromosome/Mitelman), breakpoint alterations at chromosome 19q13.1 often lead to leukemia. Systemic mastocytosis and MC leukemia are heterogeneous disorders that result in the production of excessive numbers of MCs. The genetic abnormality that occurs in most systemic mastocytosis patients has not been deduced. Nevertheless, many mastocytosis patients possess a mutation in the intracellular domain of c-*kit* that causes their tissue MCs to be in a heightened state of activation (62, 63). Interestingly, this gain-in-function mutation was first described in the HMC-1 cell line (64) established in 1988 from a patient with a MC leukemia (27). The identification of transcripts that encode substantially altered forms of hRasGRP4 in the HMC-1 cell line (Fig. 10) and in a patient with systemic mastocytosis (Fig. 9) could be another coincidence. However, we recently identified the same defect in two other systemic mastocytosis patients.[3] Interestingly, one of these patients only produces variant 1. The cumulative data therefore raise the additional possibility that the expression and intracellular accumulation of substantial amounts of aberrant forms of hRasGRP4 (particularly variants 1 and 3) in human MCs and their progenitors is a contributing factor in the development of systemic mastocytosis and/or MC leukemia.

Its precise role, if any, in systemic mastocytosis and MC leukemia remain to be determined experimentally. Nevertheless, the fact that HMC-1 cells do not express a normal, biologically active form of hRasGRP4 (Fig. 10) implies that this GEF is not essential for the early stages of MC commitment. Despite this finding, we were intrigued by the fact that HMC-1 cells are so immature that they do not even express the high affinity IgE receptor on their surfaces (27). Unlike cutaneous MCs, HMC-1 cells also lack electron dense granules and do not contain detectable amounts of MC chymase or CPA protein (Fig. 11, *e*, *g*, and *i*). Although HMC-1 cells expresses a β-tryptase (65), the amount of this neutral protease in the secretory granules of the cell is extremely low (Fig. 11, *b* and *c*) relative to a normal tissue MC (66) and the immature MC populations that we and others routinely generate in our varied culture systems.

Mature MCs are rarely seen in the tissues of W/W^v and Sl/Sl^d mice that possess genetic defects in c-*kit* and its ligand, respectively. Thus, c-*kit*-dependent signaling events are essential for the final stages of MC development. Despite the fact that HMC-1 cells possess a gain-in-function mutation in c-*kit*, these cells exhibit a blast-like appearance. To explain this inconsistency, we concluded that HMC-1 cells probably have a defect downstream of c-*kit* that prevents these cells from differentiating and maturing. Because HMC-1 cells express only abnormal forms of hRasGRP4, we concluded that this cell line might be useful for deducing the function of RasGRP4 in MCs. When HMC-1 cells were induced to express a normal form of mRasGRP4, the resulting transfectants contained many electron dense granules (Fig. 11*j*). Because the transfectants began expressing MC chymase (Fig. 11*f*) and CPA (Fig. 11*h*) and also increased their granule tryptase content dramatically (Fig. 11, *b* and *d*), RasGRP4 appears to play a very important role in the final stages of MC differentiation and maturation.

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[2] L. Li and R. L. Stevens, manuscript in preparation.

[3] L. Li, L. Escribano, and R. L. Stevens, manuscript in preparation.

REFERENCES

1. Malaviya, R., Ikeda, T., Ross, E., and Abraham, S. N. (1996) *Nature* **381,** 77–80
2. Echtenacher, B., Männel, D. N., and Hültner, L. (1996) *Nature* **381,** 75–77
3. Prodeus, A. P., Zhou, X., Maurer, M., Galli, S. J., and Carroll, M. C. (1997) *Nature* **390,** 172–175
4. Wershil, B. K., Wang, Z. S., Gordon, J. R., and Galli, S. J. (1991) *J. Clin. Invest.* **87,** 446–453
5. Huang, C., Friend, D. S., Qiu, W. T., Wong, G. W., Morales, G., Hunt, J., and Stevens, R. L. (1998) *J. Immunol.* **160,** 1910–1919
6. Huang, C., De Sanctis, G. T., O'Brien, P. J., Mizgerd, J. P., Friend, D. S., Drazen, J. M., Brass, L. F., and Stevens, R. L. (2001) *J. Biol. Chem.* **276,** 26276–26284
7. Patella, V., Florio, G., Petraroli, A., and Marone, G. (2000) *J. Immunol.* **164,** 589–595
8. Marone, G., Florio, G., Petraroli, A., and De Paulis, A. (2001) *J. Allergy Clin. Immunol.* **107,** 22–30
9. Li, Y., Li, L., Wadley, R., Reddel, S. W., Qi, J. C., Archis, C., Collins, A., Clark, E., Cooley, M., Kouts, S., Naif, H. M., Alali, M., Cunningham, A., Wong, G. W., Stevens, R. L., and Krilis, S. A. (2001) *Blood* **97,** 3484–3490
10. Turner, H., Reif, K., Rivera, J., and Cantrell, D. A. (1995) *J. Biol. Chem.* **270,** 9500–9506
11. Song, J. S., Haleem-Smith, H., Arudchandran, R., Gomez, J., Scott, P. M., Mill, J. F., Tan, T. H., and Rivera, J. (1999) *J. Immunol.* **163,** 802–810
12. Song, J. S., Gomez, J., Stancato, L. F., and Rivera, J. (1996) *J. Biol. Chem.* **271,** 26962–26970
13. Kedra, D., Seroussi, E., Fransson, I., Trifunovic, J., Clark, M., Lagercrantz, J., Blennow, E., Mehlin, H., and Dumanski, J. (1997) *Hum. Genet.* **100,** 611–619
14. Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) *Science* **280,** 1082–1086
15. Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13278–13283
16. Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Hirosawa, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1998) *DNA Res.* **5,** 355–364
17. Bottorff, D., Ebinu, J., and Stone, J. C. (1999) *Mamm. Genome* **10,** 358–361
18. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K., and Matsuda, M. (2000) *J. Biol. Chem.* **275,** 25488–25493
19. Clyde-Smith, J., Silins, G., Gartside, M., Grimmond, S., Etheridge, M., Apolloni, A., Hayward, N., and Hancock, J. F. (2000) *J. Biol. Chem.* **275,** 32260–32267
20. Lorenzo, P. S., Kung, J. W., Bottorff, D. A., Garfield, S. H., Stone, J. C., and Blumberg, P. M. (2001) *Cancer Res.* **61,** 943–949
21. Rebhun, J. F., Castro, A. F., and Quilliam, L. A. (2000) *J. Biol. Chem.* **275,** 34901–34908
22. Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., and Stone, J. C. (2000) *Nat. Immunol.* **1,** 317–321
23. Razin, E., Cordon-Cardo, C., and Good, R. A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2559–2561
24. Schrader, J. W., Lewis, S. J., Clark-Lewis, I., and Culvenor, J. G. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 323–327
25. Razin, E., Ihle, J. N., Seldin, D., Mencia-Huerta, J. M., Katz, H. R., LeBlanc, P. A., Hein, A., Caulfield, J. P., Austin, K. F., and Stevens, R. L. (1984) *J. Immunol.* **132,** 1479–1486
26. Graham, T. E., Pfeiffer, J. R., Lee, R. J., Kusewitt, D. F., Martinez, A. M., Foutz, T., Wilson, B. S., and Oliver, J. M. (1998) *J. Immunol.* **161,** 6733–6744
27. Butterfield, J. H., Weiler, D., Dewald, G., and Gleich, G. J. (1988) *Leuk. Res.* **12,** 345–355
28. Lam, B. K., Penrose, J. F., Rokach, J., Xu, K., Baldasaro, M. H., and Austin, K. F. (1996) *Eur. J. Biochem.* **238,** 606–612

29. Irani, A. A., Schechter, N. M., Craig, S. S., DeBlois, G., and Schwartz, L. B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4464–4468
30. Friend, D. S., Ghildyal, N., Austin, K. F., Gurish, M. F., Matsumoto, R., and Stevens, R. L. (1996) *J. Cell Biol.* **135,** 279–290
31. Zheng, Y., Hart, M. J., and Cerione, R. A. (1995) *Methods Enzymol.* **256,** 77–84
32. Šali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234,** 779–815
33. Šali, A., and Overington, J. P. (1994) *Protein Sci.* **3,** 1582–1596
34. Boriack-Sjodin, P. A., Margarit, S. M., Bar-Sagi, D., and Kuriyan, J. (1998) *Nature* **394,** 337–343
35. Xu, R. X., Pawelczyk, T., Xia, T. H., and Brown, S. C. (1997) *Biochemistry* **36,** 10709–10717
36. Rashidi, H. H., Bauer, M., Patterson, J., and Smith, D. W. (1999) *J. Mol. Microbiol. Biotechnol.* **1,** 175–182
37. Hunt, J. E., Stevens, R. L., Austin, K. F., Zhang, J., Xia, Z., and Ghildyal, N. (1996) *J. Biol. Chem.* **271,** 2851–2855
38. Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P., and Gokel, G. W. (1991) *J. Biol. Chem.* **266,** 8647–8650
39. Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995) *J. Biol. Chem.* **270,** 503–506
40. Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987) *Cell* **48,** 789–799
41. Jones, S., Vignais, M. L., and Broach, J. R. (1991) *Mol. Cell. Biol.* **11,** 2641–2646
42. Satoh, T., Nakafuku, M., Miyajima, A., and Kaziro, Y. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3314–3318
43. Oberhauser, A. F., Balan, V., Fernandez-Badilla, C. L., and Fernandez, J. M. (1994) *FEBS Lett.* **339,** 171–174
44. Turner, H., Gomez, M., McKenzie, E., Kirchem, A., Lennard, A., and Cantrell, D. A. (1998) *J. Exp. Med.* **188,** 527–537
45. Ehrhardt, G. R., Leslie, K. B., Lee, F., Wieler, J. S., and Schrader, J. W. (1999) *Blood* **94,** 2433–2444
46. Tuvim, M. J., Adachi, R., Chocano, J. F., Moore, R. H., Lampert, R. M., Zera, E., Romero, E., Knoll, B. J., and Dickey, B. F. (1999) *Am. J. Respir. Cell Mol. Biol.* **20,** 79–89
47. Yang, F. C., Kapur, R., King, A. J., Tao, W., Kim, C., Borneo, J., Breese, R., Marshall, M., Dinauer, M. C., and Williams, D. A. (2000) *Immunity* **12,** 557–568
48. Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J., and Kay, R. J. (1998) *Mol. Cell. Biol.* **18,** 6995–7008
49. Kazanietz, M. G. (2000) *Mol. Carcinog.* **28,** 5–11
50. Swann, P. G., Odom, S., and Rivera, J. (1999) in *Signal Transduction in Mast Cells and Basophils* (Razin, E., and Rivera, J., eds) pp 152–170, Springer Press, New York
51. Kitamura, Y., Go, S., and Hatanaka, K. (1978) *Blood* **52,** 447–452
52. Kitamura, Y., and Go, S. (1979) *Blood* **53,** 492–497
53. Levi-Schaffer, F., Dayton, E. T., Austin, K. F., Hein, A., Caulfield, J. P., Gravallese, P. M., Liu, F. T., and Stevens, R. L. (1987) *J. Immunol.* **139,** 3431–3441
54. Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988) *Cell* **55,** 185–192
55. Gordon, J. R., and Galli, S. J. (1990) *Blood* **75,** 1637–1645
56. Collaborative Study on the Genetics of Asthma (1997) *Nat. Genet.* **15,** 389–392
57. Atamas, S. P., Choi, J., Yurovsky, V. V., and White, B. (1996) *J. Immunol.* **156,** 435–441
58. Klein, S. C., Golverdingen, J. G., Bouwens, A. G., Tilanus, M. G., and de Weger, R. A. (1995) *Immunogenetics* **41,** 57
59. Tsytsikov, V. N., Yurovsky, V. V., Atamas, S. P., Alms, W. J., and White, B. (1996) *J. Biol. Chem.* **271,** 23055–23060
60. Atamas, S. P. (1997) *Life Sci.* **61,** 1105–1112
61. Li, J., Shen, H., Himmel, K. L., Dupuy, A. J., Largaespada, D. A., Nakamura, T., Shaughnessy, J. D., Jr., Jenkins, N. A., and Copeland, N. G. (1999) *Nat. Genet.* **23,** 348–353
62. Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10560–10564
63. Longley, B. J., Tyrrell, L., Lu, S. Z., Ma, Y. S., Langley, K., Ding, T. G., Duffy, T., Jacobs, P., Tang, L. H., and Modlin, I. (1996) *Nat. Genet.* **12,** 312–314
64. Furitsu, T., Tsujimura, T., Tono, T., Ikeda, H., Kitayama, H., Koshimizu, U., Sugahara, H., Butterfield, J. H., Ashman, L. K., and Kanayama, Y. (1993) *J. Clin. Invest.* **92,** 1736–1744
65. Butterfield, J. H., Weiler, D. A., Hunt, L. W., Wynn, S. R., and Roche, P. C. (1990) *J. Leukocyte Biol.* **47,** 409–419
66. Schwartz, L. B., Lewis, R. A., and Austin, K. F. (1981) *J. Biol. Chem.* **256,** 11939–11943
67. Wickens, M. (1990) *Trends Biochem. Sci.* **15,** 277–281
68. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950