RasGRP4 Is a Novel Ras Activator Isolated from Acute Myeloid Leukemia*

Received for publication, November 28, 2001, and in revised form, February 8, 2002
Published, JBC Papers in Press, March 5, 2002, DOI 10.1074/jbc.M111330200

Gary W. Reuther‡, Que T. Lambert‡, John F. Rebhun¶, Michael A. Caligiuri**‡‡, Lawrence A. Quilliam¶¶, and Channing J. Der‡

From the ‡Lineberger Comprehensive Cancer Center, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7285, the ¶Department of Biochemistry and Molecular Biology, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202, and the **Department of Internal Medicine, The Comprehensive Cancer Center, Ohio State University Columbus, Ohio 43210

Although a number of genetic defects are commonly associated with acute myeloid leukemia (AML), a large percentage of AML cases are cytogenetically normal. This suggests a functional screen for transforming genes is required to identify genetic mutations that are missed by cytogenetic analyses. We utilized a retrovirus-based cDNA expression system to identify transforming genes expressed in cytogenetically normal AML patients. We identified a new member of the Ras guanyl nucleotide-releasing protein (RasGRP) family of Ras guanine nucleotide exchange factors, designating it RasGRP4. Subsequently, cDNA sequences encoding rodent and human RasGRP4 proteins were deposited in GenBank™. RasGRP4 contains the same protein domain structure as other members of the RasGRP family, including a Ras exchange motif, a CDC25 homology domain, a C1/diacylglycerol-binding domain, and putative calcium-binding EF hands. We show that expression of RasGRP4 induces anchorage-independent growth of Rat1 fibroblasts. RasGRP4 is a Ras-specific activator and, interestingly, is highly expressed in peripheral blood leukocytes and myeloid cell lines. Unlike other RasGRP proteins, RasGRP4 is not expressed in the brain or in lymphoid cells. We demonstrated that 32D myeloid cells expressing RasGRP4 have elevated levels of activated Ras compared with control cells, and phorbol 12-myristate 13-acetate (PMA) treatment greatly enhanced Ras activation. PMA induced membrane localization of RasGRP4 and 32D cells expressing RasGRP4 were capable of cytokine-independent proliferation in the presence of PMA. We conclude that RasGRP4 is a member of the RasGRP family of Ras guanine nucleotide exchange factors that may play a role in myeloid cell signaling growth regulation pathways that are responsive to diacylglycerol levels.

Acute myeloid leukemia (AML) is a disease of the hematopoietic cell system in which there is an aberrant accumulation of myeloid cells in the peripheral blood and bone marrow. Chromosomal translocations are common in AML, and the study of these chromosomal abnormalities has led to the identification of a number of AML-associated oncogenes including AML1-Eto, PML-RARα, and CBFβ-MYH11 among others. However, up to 50% of AML cases are cytogenetically normal, suggesting that subtle genetic defects, including point mutations and small deletions, play a causative role in the development of the disease. This suggests that a functional screen of cytogenetically normal AML samples may be an effective approach to identify these mutations.

Most oncogene screening efforts in the past have utilized cell lines as sources of expressed genes and inefficient transfection techniques to deliver these genes to target cells. We have applied a retrovirus-based cDNA expression system that has been used to efficiently screen cDNA libraries representing genes expressed in rodent and human tumor cell lines. We expanded this system to utilize primary tumor samples as sources of expressed oncogenes. This insures that the genes that are surveyed are those expressed in the cancer patient, and it also circumvents any mutations or aberrant expression of genes that may be the result of artificial selection inherent in the establishment and long-term maintenance of cells in culture.

We used this highly efficient retroviral-based cDNA expression system to detect oncogenes in cytogenetically normal AML patients. Recently, we identified an internal deletion mutation in the TrkA receptor tyrosine kinase expressed in a patient with cytogenetically normal AML. This mutation would not have been detected by cytogenetic analyses, demonstrating the credence of this system to identify oncogenes in these AML patients.

In this study, we describe the isolation of a novel transforming protein expressed in a patient with AML that exhibits homology to the RasGRP family of Ras activators. We have designated it RasGRP4 and have initiated its characterization and possible role in myeloid cell oncogenesis. During the course and review of our studies, R. L. Stevens and colleagues deposited sequences in GenBank™ that encode for the same protein, which they also designated RasGRP4.²
Experimental Procedures

Northern Blot Analysis—A human multiple tissue mRNA Northern blot (BD Biosciences/CLONTECH) was probed with the manufacturer’s instructions with the open reading frame of RasGRP4 radiolabeled by random priming.

DNA Plasmids—Wild-type RasGRP4 was cloned by PCR from reverse-transcribed total RNA of human peripheral blood leukocytes using primers designed from sequence information from the AML-derived RasGRP4 cDNA. This sequence has been assigned the GenBank™ accession number AF448437. RasGRP4 cDNA and the AML-derived RasGRP4 cDNA were cloned into pBabeuro-PA, a retrovector vector designed to place a Hpa epitope tag coding sequence at the 5′-end of the cDNA, as PCR-amplified EcoRI fragments. These cDNAs were also cloned into pALFA, a modified version of pALFA designed to introduce an HA epitope tag at the 5′-end of the cDNA. All plasmid inserts were confirmed by DNA sequencing. Full-length human RasGRP3 was kindly provided by Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). The EcoRVXbaI fragment of RasGRP3 was subcloned into pcDNA3. RasGRP2 was constructed from two ESI clones (GenBank™ accession numbers a055643 and t78563) as described (7). This construct creates the nonmyristoylated, Rap-specific form of RasGRP2 (8). A BamHI site was incorporated immediately 5′ of the initiating methionine, and the cDNA was subcloned into the BamHI and NotI sites of pcDNA3. Rlf was kindly provided by Douglas Andres (University of Kentucky, Lexington, Kentucky). pFLAG-CMV2-Rap1A and pFLAG-CMV2-Ha-Ras have been used previously (9).

Library Construction and Screening—A cDNA library of the genes expressed in the leukocytes of a cytogenetically normal AML patient was constructed in the pCTV1B vector exactly as we have described previously (5). The screen for and isolation of transforming genes was also performed exactly as described previously (5).

Retroviral Production and Cell Culture—Ectropic retrovirus was made in 293T cells using the pVpack retroviral production system (Strategene). 293T cells and Rat1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). 32D (clone 3 mouse myeloid progenitor cells were grown in RPMI supplemented with 10% FBS and 10% WEHI3B conditioned serum (FBS). 32D (clone 3) mouse myeloid progenitor cells were grown in RPMI supplemented with 10% FBS and 10% WEHI3B conditioned medium as a source of IL-3 (10). Stable cell lines were generated by retroviral infection as previously described (5). Purumycin (Sigma) was used at a concentration of 1 μg/ml to select stable cell lines, and stable lines were maintained under drug selection. PMA (Calbiochem) was used at a final concentration of 100 nM in all experiments. 32D cells expressing H-Ras(G1L) have been described previously (5). Soft agar assays were performed using single-cell suspensions in agar (5 × 10^5 Rat1 cells/60-mm dish) as described previously (11).

Measurement of Cellular GTPase Activation—Activation of Ras was measured utilizing a GST fusion protein containing the Ras-binding domain (RBD) of Ras-f1 as described (12). For experiments with Rat1 cells, 7 × 10^5 cells were plated in 100-mm dishes. Twenty-four hours later the cells were depleted of serum for 18 h and then treated with 100 nM PMA or 0.1% MeSO for 30 min prior to harvesting. For experiments with 32D cells, 3 × 10^5 cells were plated in 10 ml of growth medium and grown overnight. Cells were then washed twice with RPMI only and incubated in 10 ml of RPMI in conical tubes for 1 h before treatment with 100 nM PMA or 0.1% dimethyl sulfoxide (MeSO) for 30 min. Transient activation of GTPases in 293T cells has been described previously (9).

Subcellular Fractionation—For subcellular fractionation, cells were cultured and treated the same as for the experiments that measured cellular GTPase activation (see above). SLP100 subcellular fractionation was performed essentially as described except the protein in each fraction was resuspended in 2× sample buffer (20 m NaPO4 pH 7.0, 20% glycerol, 10% β-mercaptoethanol, 0.2 mg/ml diithiothreitol, and 0.02% bromphenol blue) following acetone precipitation (13). Equivalent proportions of S100 and P100 were analyzed by immunoblotting.

Immunoblot Analyses—Immunoblot analyses were performed using single-cell suspensions in agar (5 × 10^5 Rat1 cells/60-mm dish) as described previously (11).

RESULTS AND DISCUSSION

Isolation of a Transforming Gene That Encodes a New RasGRP Family Member—To identify transforming genes in cytogenetically normal AML, we created and screened cDNA libraries from primary patient samples. We utilized both Rat1 fibroblasts and Rat intestinal epithelial-1 cells as biological screening assays for genes that induce cellular transformation. We have determined previously that these lines are useful for identifying transforming genes because they are efficiently infected by retrovirus and have a very low rate of spontaneous transformation (5). We independently identified a 2293-bp cDNA twice as a gene that induces Rat1 cell transformation. Standard nucleotide BLAST data base searching identified the cDNA as a previously unidentified gene on human chromosome 19. The cDNA contains an open reading frame that encodes a 673-amino acid protein. An NCBI BLAST search indicated that the protein encoded by the cDNA is homologous to members of the Ras guanyl nucleotide-releasing protein (RasGRP) family of Ras guanine nucleotide exchange factors (6). ClustalW alignments indicate that RasGRP4 shows 39% identity to RasGRP2, 38% identity to RasGRP1, and 36% identity to RasGRP3 over the entire length of the proteins. Therefore, we have designated this protein RasGRP4. Compared with data base genomic sequence as well as two RasGRP4 cDNAs that were isolated by reverse transcription-polymerase chain reaction (PCR) from normal individuals, the AML-derived RasGRP4 cDNA contains a point mutation at codon 620 that changes a glutamic acid to a lysine in the carboxyl terminus of the protein. The cDNA and amino acid sequences of wild-type RasGRP4 are shown in Fig. 1A.

RasGRP4 exhibits a similar domain structure to the three human members of the RasGRP protein family (Fig. 1B) (6). There is a central CDC25 homology domain that is known to exert the catalytic function of guanine nucleotide exchange factors (GEFs) for Ras family proteins (Ras, R-Ras, Rap, and Raf proteins). A phylogenetic tree indicating the relationship of the CDC25 homology domain of RasGRP4 to the CDC25 homology domains of other human Ras, Rap, or Raf exchange factors is shown in Fig. 1C. The strongest sequence similarity is seen with the CDC25 homology domains of RasGRP proteins. Amino-terminal to the CDC25 homology domain is a Ras exchange motif that is present in other Ras exchange factors and is believed to play a critical role of CDC25 catalytic activity in vitro but not in vivo.

Carboxyl terminal to the CDC25 homology domain is a C1 domain, a cysteine-rich domain that binds diacylglycerol in RasGRP and protein kinase C family proteins (14). Diacylglycerol has been shown to regulate RasGRP2 subcellular localization and catalytic activity (8, 15–17). Finally, other RasGRP family members contain two calcium-binding EF hands between the CDC25 homology and C1 domains. This region of RasGRP4, however, shows only weak homology to these domains. This weak homology is in part due to a stretch of three prolines in the second putative EF hand, which may alter its ability to bind calcium. These prolines are not present in the EF hands of the other RasGRP proteins. It has been postulated that intracellular calcium can regulate the activity of RasGRP proteins through the EF hands (8, 18). However, the effect of calcium on RasGRP protein activity remains unclear. Finally, unlike RasGRP2, RasGRP4 does not contain a myristylation signal sequence at its amino terminus, indicating that RasGRP4 is not lipid modified in this manner (8).
RasGRP4 Tissue Expression Is Distinct from Other RasGRP Family Members—Previous studies determined that RasGRP1, RasGRP2, and RasGRP3 show overlapping, but distinct tissue patterns of gene expression (15, 16, 18–20). Our Northern blot analysis indicates that RasGRP4 is expressed highly in peripheral blood leukocytes (Fig. 1D). Significantly lower transcript levels are seen in other tissues including heart, skeletal muscle, spleen, liver, placenta, and lung. An ∼4-kb message is seen in these tissues. A multiple tissue expression array probed with the cDNA for RasGRP4 indicated the highest expression of RasGRP4 in bone marrow and peripheral blood leukocytes and lower expression in other tissues. This expression pattern is distinct from that described for the other RasGRPs (15, 16, 18–20). In particular, we did not detect RasGRP4 message in the brain, whereas the other RasGRP transcripts and proteins

3 G. W. Reuther, Q. T. Lambert, and C. J. Der, unpublished observations.
are expressed highly in this organ (16, 18–20). While RasGRP1 is expressed in lymphoid cells but not myeloid cells (15), high expression of RasGRP4 in peripheral blood leukocytes and its isolation from myeloid cells suggests that RasGRP4 may have a distinct role in these cells. Indeed, Northern analysis using hematopoietic cell lines indicated that RasGRP4 is highly expressed in myeloid cells compared with lymphoid cells.3 While RasGRP1 plays a role in the activation of Ras in response to T-cell receptor signaling (21, 22), RasGRP4 may function in a myeloid-specific signaling pathway required for proper development and function of cells of this hematopoietic lineage.

To verify that RasGRP4 was the basis for the transformation seen in our library screen, we established Rat1 fibroblasts stably infected with a retrovirus expression vector encoding a hemagglutinin (HA) epitope-tagged version of RasGRP4. In contrast to cells infected with the empty vector, the Rat1 cells stably expressing the AML-mutated RasGRP4, or control vector (V) were starved of serum for 18 h, and the amount of activated GTP-bound Ras was determined utilizing a GST-RafRBD pull-down assay. The amount of bound, activated Ras-GTP in the pull-downs and total Ras in lysates were visualized by immunoblotting with pan-Ras antibodies (right panels). C, RasGRP4 activates Ras but not Rap in 293T cells. 293T cells were transfected with expression plasmids (750 ng) encoding empty vector, Rif, RasGRP2, RasGRP3, or RasGRP4, together with FLAG epitope-tagged Rap1a or H-Ras (750 ng). After 24 h, cells were starved of serum for 18 h, and activation of Rap1a and H-Ras was determined by pull-down assays utilizing GST-RalGDSRBD and GST-RafRBD, respectively. The amount of bound, activated Rap1a-GTP and H-Ras-GTP in the pull-down assays and total transfected Rap1a and H-Ras in lysates were identified by immunoblotting with anti-FLAG antibodies. D, PMA induces morphological transformation of Rat1 cells expressing RasGRP4. Rat1 fibroblasts stably expressing RasGRP4 or control vector were treated with dimethyl sulfoxide (DMSO) vehicle or 100 nM PMA, and cell morphology was photographed under phase contrast microscopy after 48 h.

**Fig. 2.** RasGRP4 transforms Rat1 fibroblasts and activates Ras in Rat1 fibroblasts and 293T cells. A, RasGRP4 transforms Rat1 fibroblasts. Rat1 cells were generated to express RasGRP4 by retroviral infection. Cells stably expressing RasGRP4 readily formed colonies of proliferating cells in soft agar (lower panel), whereas control cells expressing the empty vector did not (upper panel). B, RasGRP4 expression activates Ras in Rat1 fibroblasts. Expression of wild-type RasGRP4 (WT) and the AML-mutated RasGRP4 (A) in Rat1 fibroblasts was detected by immunoblot analyses of total cell lysates with anti-HA monoclonal antibodies (left panel). The molecular masses of the protein markers are indicated in kilodaltons. Rat1 fibroblasts stably expressing wild-type RasGRP4, the AML-mutated RasGRP4, or control vector (V) were starved of serum for 18 h, and the amount of activated GTP-bound Ras was determined utilizing a GST-RafRBD pull-down assay. The amount of bound, activated Ras-GTP in the pull-downs and total Ras in lysates were visualized by immunoblotting with pan-Ras antibodies (right panels). C, RasGRP4 activates Ras but not Rap in 293T cells. 293T cells were transfected with expression plasmids (750 ng) encoding empty vector, Rif, RasGRP2, RasGRP3, or RasGRP4, together with FLAG epitope-tagged Rap1a or H-Ras (750 ng). After 24 h, cells were starved of serum for 18 h, and activation of Rap1a and H-Ras was determined by pull-down assays utilizing GST-RalGDSRBD and GST-RafRBD, respectively. The amount of bound, activated Rap1a-GTP and H-Ras-GTP in the pull-down assays and total transfected Rap1a and H-Ras in lysates were identified by immunoblotting with anti-FLAG antibodies. D, PMA induces morphological transformation of Rat1 cells expressing RasGRP4. Rat1 fibroblasts stably expressing RasGRP4 or control vector were treated with dimethyl sulfoxide (DMSO) vehicle or 100 nM PMA, and cell morphology was photographed under phase contrast microscopy after 48 h.
and a GST fusion to the RBD of RalGDS to pull down activated Rap (9, 24) (Fig. 2C). In agreement with previous observations, we found that RasGRF3 activated both Ras and Rap1, whereas the Rap-specific short form of RasGRF2 (i.e. CalDAG-GEF1) activated Rap1 and not Ras. In contrast, transient expression of RasGRF4 activated Ras but did not activate Rap1 (Fig. 2C). Expression of Rif, a guanine nucleotide exchange factor for the Ral GTPase (25), did not activate Ras or Rap1. Thus, RasGRF4 acts as a Ras-specific GEF and, like other RasGRFs, is not an activator of Rap.4

PMA Promotes RasGRF4 Membrane Association and GEF Activity—Diacylglycerol or phorbol ester interaction with the C1 domain has been shown to promote membrane association and GEF activity of the three known RasGRF proteins (8, 15–17). To determine whether RasGRF4 function is modulated by an increase in diacylglycerol, we determined whether treatment with the diacylglycerol analog, PMA, would enhance the transforming activity of RasGRF4 when expressed in Rat1 cells. PMA treatment induced morphological transformation of Rat1 cells expressing RasGRF4 but not vector control cells (Fig. 2D), suggesting that PMA further stimulated RasGRF4 activity. This morphologic transformation is similar to that caused by expression of constitutively activated mutants of Ras in Rat1 cells (26, 27). PMA treatment did not significantly increase the ability of Rat1 fibroblasts expressing RasGRF4 to grow in soft agar.5 However, these cells grew quite efficiently in soft agar (1500 agar colonies/5000 cells plated) in the absence of PMA, and thus PMA could only increase soft agar growth by 3-fold at most. Taken together, we interpret these results to suggest that a lower threshold of Ras activation is sufficient to promote anchorage-independent growth but not morphologic transformation. Therefore, although PMA treatment to further activate Ras did not greatly enhance growth in soft agar, it did raise the threshold level of Ras to that required for morphologic transformation.

Previous studies have shown that PMA stimulated RasGRF function by promoting enhanced plasma membrane association (8, 15–17). Because RasGRF4 mRNA is expressed highly in peripheral blood leukocytes and was isolated from a myeloid leukemia, we evaluated the ability of PMA to regulate Ras-GRF4 subcellular location and activity in 32D mouse myeloid progenitor cells (28, 29). For these analyses, we established 32D cells stably expressing HA epitope-tagged RasGRF4 and then determined whether PMA treatment also promoted membrane association of this exchange factor (Fig. 3A). We treated 32D cells expressing RasGRF4 with PMA for 30 min and performed subcellular fractionation. PMA treatment induced a translocation of RasGRF4 from the S100 soluble fraction to the P100 membrane-containing fraction (Fig. 3B). Identical results were seen in Rat1 cells, with translocation occurring within 5 min of PMA treatment.3

We next determined whether this increase in membrane association coincided with enhanced Ras activation in 32D cells expressing RasGRF4. 32D cells expressing RasGRF4 exhibited elevated basal Ras-GTP levels when compared with control empty vector-infected cells (Fig 3C). This level of activated Ras was greatly enhanced by PMA treatment, whereas PMA alone did not further activate Ras in control cells (Fig. 3C). Rap1 was not activated under these conditions,3 consistent with the inability of RasGRF4 to activate Rap1 in 293T cells (Fig. 2C). The elevated basal levels of Ras activation in cells expressing RasGRF4 is likely due to the significant amount of RasGRF4 that is already associated with membranes (Fig. 3B). Identical results were obtained with the wild-type and AML-mutated forms of RasGRF4, suggesting that the AML-derived mutation does not alter the ability of RasGRF4 to translocate to membranes and activate Ras in response to PMA treatment (Fig. 3, B and C). These data indicate that RasGRF4, like other Ras-GRF family members, has a functional C1 diacylglycerol-binding domain that regulates the activity of the CDC25 domain. Thus, RasGRF4 likely plays a role downstream of extracellular stimuli that elicit elevated diacylglycerol levels, such as those that stimulate protein-coupled receptor or receptor tyrosine kinase activation of phospholipase C β or γ, respectively (30). RasGRF4 Activation by PMA Promotes Cytokine-independent Growth of 32D Myeloid Cells—Finally, because had we identified RasGRF4 as a transforming protein, we determined whether RasGRF4 was capable of altering the growth properties of myeloid cells. To evaluate this possibility, we analyzed the ability of RasGRF4 to promote cytokine-independent growth of 32D cells. 32D cells require interleukin-3 (IL-3) for survival and growth (28, 29, 31). Removal of IL-3 from 32D cells

4 J. F. Rebhun and L. A. Quilliam, unpublished observations.
induces a rapid and complete apoptotic response resulting in all cells dying within 48 to 72 h. Expression of several leukemia-associated oncogenes (e.g. Bcr-Abl, Flt3) promotes the growth and survival of these cells in the absence of IL-3 (32, 33). Expression of RasGRP4 in 32D cells did not alter the rate of cell death in response to IL-3 withdrawal. However, treatment of these cells with PMA resulted in an increase in cell viability5 and promoted 32D cell growth in the absence of IL-3 (Fig. 4, B and C). This IL-3 independence is reversible, as removal of PMA from the growth medium resulted in apoptosis of the culture.5 PMA did not affect the survival or growth of control cells in the absence of IL-3 (Fig. 4A). Thus, PMA-mediated activation of RasGRP4 can promote growth transformation of mouse myeloid cells.

Our observation that activation of RasGRP4 can promote IL-3-independent growth of 32D cells was unexpected, because we and others had found that expression of activated versions of Ras in 32D cells does not induce IL-3-independent growth, although activated Ras can inhibit apoptosis in these cells (34, 35). PMA treatment of 32D cells expressing constitutively activated H-Ras(61L) did not promote IL-3 independence, suggesting that the effect of PMA on RasGRP4 cells is not merely a cooperation between Ras activation and other signals activated by PMA (Fig. 4D). Similar results were obtained with 32D cells expressing activated K-Ras(12V) or N-Ras(12D).5 This suggests that aberrant activation of RasGRP4 in myeloid cells may promote the transformation of myeloid cells to a growth-factor-independent state more efficiently than activated alleles of Ras itself. How might this occur? One possibility is that RasGRP4 activation likely causes concurrent activation of multiple Ras isoforms, and possibly R-Ras family proteins, hence causing a quantitatively stronger and qualitatively distinct activation of signaling pathways from those activated by a single mutated Ras protein. Alternatively, it is possible that RasGRP4 activation of Ras, causing the rapid GDP/GTP cycling of Ras, has different downstream signaling consequences than are caused by GTPase-deficient, chronically GTP-bound activated Ras. Evidence for this latter possibility is provided from observations that fast GDP/GTP-cycling mutants of Rho small GTPases show greater transforming potency than GTPase-deficient mutants (36). To test this possibility, we expressed two different Ras mutants, H-Ras(117E) and H-Ras(119H), in 32D cells. Although these mutants have an increased rate of cycling between GDP/GTP binding and are highly transforming (37), they were unable to transform 32D cells to IL-3 independence.5 Cells expressing these forms of Ras behaved similarly to 32D cells expressing GTPase-deficient H-Ras(61L) in the absence of IL-3 (Fig. 4D). We confirmed the H-Ras(117E) and H-Ras(119H) DNA constructs used in these experiments were highly transforming in NIH3T3 cells.3 This finding suggests that the effect of PMA-activated RasGRP4 on 32D cell growth may be mediated by concurrent activation of multiple Ras isoforms or other Ras-like GTPases (e.g. R-Ras) by RasGRP4.

During the course of our studies, Stevens and colleagues2 deposited a human cDNA sequence encoding a protein nearly identical to RasGRP4 (GenBank™ accession number AJ048119). The sequence of this protein is identical to what we found for wild-type RasGRP4 except for three amino acid positions. Amino acid positions 120, 261, and 671 are Gln, Arg, and Leu, respectively, in our sequence, and Leu, Cys, and Pro, respectively, in their sequence. The reason for these discrepancies is unclear, but the sequences at these positions were identical in the three different cDNA sources we utilized. Identified

5 G. W. Reuther and C. J. Der, unpublished observations.
as a mast cell-restricted signaling molecule in an asthma patient, the Stevens’ group also designated their protein RasGRP4, and they further identified human sequences that encoded defective variants of RasGRP4 (GenBank™ accession numbers AY048120, AY048121, and NM052949). Whether these defective variants are simply inactive proteins or function as dominant negative proteins that can block the function of other RasGRP proteins, will be interesting to determine. In addition, after the submission of our study, the Stevens’ group also identified mouse (GenBank™ accession numbers AF331457 and AY040628) and rat (GenBank™ accession numbers AF465263, AF465264, and NM130824) RasGRP4 cDNAs and submitted an additional human sequence (GenBank™ accession numbers XM056604).

In summary, we have identified a new member (RasGRP4) of the RasGRP family of Ras family guanine nucleotide exchange factors through a screen for transforming genes expressed in AML patients. Although the patient-derived cDNA encodes for a mutated protein, our analyses did not determine a functional difference from wild-type RasGRP4. Perhaps the mutated form will exhibit altered functions in other biological assays or, alternatively, simply represents a polymorphism. Is the aberrant activation of RasGRP4 important for AML development? The chromosomal location of RasGRP3 is involved in chromosomal rearrangements in AML, and proviral integration-mediated activation of RasGRP2 has been observed in a mouse model of AML (17, 20). Interestingly, sequence analysis using the NCBI Genome database indicates RasGRP4 is located at chromosome 19q13, a region identified as undergoing rearrangement in human leukemias and other cancers (38). Thus, aberrant regulation of RasGRP4 may be a common undiscovered event in AML development. Is Ras activation important for AML oncogenesis? N-Ras is frequently mutated in AML patients (39, 40), although it has been proposed that at least two oncogenic events are required to induce AML (41). Also, activation of the ERK (extracellular signal-regulated kinase) pathway, a major downstream component of Ras signaling, is seen in about half of all AML cases (42). This suggests that direct (e.g. point mutation of N-Ras) or indirect (possibly through RasGRP4 activation) stimulation of Ras signaling plays an important role in AML. Our future studies will evaluate whether RasGRP4 is aberrantly overexpressed or activated in human AML and whether Ras-mediated signaling pathways are important for AML development.

REFERENCES

1. Miller, K. B. (1995) in Hematology: Basic Principles and Practice (Hoffman, R., Benz, E. J., Shattil, S. J., Furie, B., Cohen, H. J., and Silberstein, L. E., eds) pp. 983–1014, Churchill Livingstone, Inc., Edinburgh.
2. Look, A. T. (1997) Science 278, 1059–1064.
3. Mrozek, K., Heinonen, K., de la Chapelle, A., and Bloomfield, C. D. (1997) Semin. Oncol. 24, 17–31.
4. Whitehead, I., Kirk, H., and Kay, R. (1995) Mol. Cell. Biol. 15, 704–710.
5. Reuth, G. W., Lambert, Q. T., Caligiuri, M. A., and Der, C. J. (2000) Mol. Cell. Biol. 20, 8655–8666.
6. Quilliam, L. A., Rebhun, J. F., and Castro, A. F. (2002) Prog. Nucleic Acids Res. Mol. Biol. 71, 391–444.
7. Kopey, G., Seroussi, E., Franssens, I., Trifunovic, J., Clark, M., Lagercrantz, J., Blennow, E., Mihlin, H., and Damanski, J. (1997) Hum. Genet. 100, 611–619.
8. Clyde-Smith, J., Silins, G., Gartsides, M., Grimmond, S., Etheridge, M., Aplonni, A., Hayward, N., and Hancock, J. F. (2000) J. Biol. Chem. 275, 32620–32267.
9. Rebhun, J. F., Chen, H., and Quilliam, L. A. (2000) J. Biol. Chem. 275, 32646–32140.
10. Lee, J. C., Hapel, A. J., and Ile, J. N. (1982) J. Immunol. 128, 2393–2398.
11. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) Methods Enzymol. 255, 185–220.
12. Taylor, S. J., and Shalloway, D. (1996) Curr. Biol. 6, 1621–1627.
13. Cox, A. D., Solaki, P. A., Jordan, D. J., and Der, C. J. (1995) Methods Enzymol. 255, 185–220.
14. Kozui, M., G. (2000) Mol. Carcinog. 28, 5–11.
15. 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.
16. Ebini, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998) Science 280, 1082–1096.
17. Lorenzo, P. S., Jung, W., Bottorff, D. A., Garfield, S. H., Stone, J. C., and Blumberg, P. M. (2001) Cancer Res. 61, 943–949.
18. Williams, H. L., Springer, G. B. (1998) J. Clin. Invest. 102, 13278–13283.
19. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K., and Matsuda, S. (2000) J. Biol. Chem. 275, 25488–25493.
20. Depuy, A. J., Morgan, K., von Linigiz, F. C., Shen, H., Achar, H., Hase, D. E., Jenkins, N. A., Copeland, N. G., Boss, G. R., and Largaespada, D. A. (2001) J. Biol. Chem. 276, 11804–11811.
21. Ebini, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Huton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L., and Stone, J. C. (2000) Blood 95, 3199–3203.
22. Dower, N. A., Stang, S. L., Bottorff, D. A., Ebini, J. O., Dickie, P., Ostergaard, H. L., and Stone, J. C. (2000) Nat. Immunol. 1, 317–321.
23. Reuther, G. W., and Der, C. J. (2000) Curr. Opin. Cell Biol. 12, 157–165.
24. van Triest, M., and de Ruij, J., and Bos, J. L. (1991) Methods Enzymol. 193, 334–348.
25. Volkman, M., Bauer, V. van Leer, L. J., de Vries-Smits, A. M., Cool, H. B., Spaargaren, M., Wittinghofer, A., Burgering, B. M., and Bos, J. L. (1996) Oncogene 13, 353–362.
26. Qiu, R. G., Abo, M., McCormick, F., and Simons, M. (1997) Mol. Cell. Biol. 17, 3449–3458.
27. Prendergast, G. C., Davide, J. P., deSelm, S. J., Giuliani, E. A., Graham, S. L., Gibbs, J. B., Oliff, A., and Kohl, N. E. (1994) Mol. Cell. Biol. 14, 4183–4202.
28. Metcalf, D. (1985) Blood 65, 357–362.
29. Greenberger, J. S., Sakakeeny, M. A., Humphries, R. K., Eaves, C. J., and Eckner, R. J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2931–2935.
30. Mitelman, D. (1980) Cancer Res. 40, 1360–1366.
31. Mizuki, M., Fenski, R., Hafler, H., Matsusawa, I., Schmidt, R., Moller, C., Gruning, W., Kratz-Albers, K., Serve, S., Steur, C., Buchner, T., Kienast, J., Kanakura, Y., Bergel, W. E., and Serve, H. (2000) Blood 96, 3907–3914.
32. Mavilio, F., Kreider, R. L., Valleti, M., Nase, G., Shirai, N., Venturrelli, D., Reddy, E. P., and Rovera, G. (1989) Oncogene 4, 301–308.
33. Kinoshita, T., Yokota, T., Ariai, K., and Miyajima, A. (1995) Oncogene 10, 2207–2212.
34. Lin, R., Bagrodia, S., Cerione, R., and Moran, D. (1997) Curr. Biol. 7, 794–797.
35. Der, C. J., Weissman, B., and MacDonald, M. J. (1988) Oncogene 3, 105–112.
36. Mitelman, F., Johannsson, B., and Mertens, F., eds (2002) Mitelman Database of Chromosome Aberrations in Cancer, cgap.nci.nih.gov/Chromosomes/Mitelman.
37. Bos, J. L., Verlaan-de Vries, V. M., van der Eer, A. J., Janssen, J. W., Delwel, R. (1995) Nucleic Acids Res. 33, 1227–1221.
38. Needleman, S. W., Kraus, M. H., Srivastava, S. K., Levine, P. H., and Aronson, S. A. (1986) Blood 68, 753–757.