THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 34, Issue of August 23, pp. 30508–30514, 2002

Printed in U.S.A.

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

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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/diacyglycerol-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)[1] 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 (1). 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 (2). 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 (3). 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 (4). 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 (5). 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 (6). 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]

* Our research was supported by Grant CA42978 from the National Institutes of Health (to C. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF448437.

‖ Supported by Training Grant T32-H07774 from NHLBI, National Institutes of Health.

‡‡ Supported by the Coleman Leukemia Research Fund.

§§ Supported by American Cancer Society Grant RPG-00-125-01TBE and the Walther Cancer Institute.

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[1] The abbreviations used are: AML, acute myeloid leukemia; GEF, guanine nucleotide exchange factor; PMA, phorbol 12-myristate 13-acetate; RBD, Ras-binding domain; RasGRP, Ras guanyl nucleotide-releasing protein; HA, hemagglutinin; FBS, fetal bovine serum; IL-3, interleukin 3; GST, glutathione *S*-transferase.

[2] Although our initial database search failed to identify sequences

EXPERIMENTAL PROCEDURES

Northern Blot Analysis—A human multiple tissue mRNA Northern blot (BD Biosciences/CLONTECH) was probed per 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 pBabepuro-HA, a retroviral vector designed to place a HA epitope tag coding sequence at the 5′-end of the cDNA, as PCR-amplified *Eco*RI fragments. These cDNAs were also cloned into pcDNA3HA, a modified version of pcDNA3 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 *Eco*RV/*Xba*I fragment of RasGRP3 was subcloned into pcDNA3. RasGRP2 was constructed from two EST clones (GenBank™ accession numbers aa035643 and t78563) as described (7). This construct creates the nonmyristoylated, Rap-specific form of RasGRP2 (8). A *Bam*HI site was incorporated immediately 5′ of the initiating methionine, and the cDNA was subcloned into the *Bam*HI and *Not*I 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—Ecotropic retrovirus was made in 293T cells using the pVPack retroviral production system (Stratagene). 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 medium as a source of IL-3 (10). Stable cell lines were generated by retroviral infection as previously described (5). Puromycin (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(61L) have been described previously (5). Soft agar assays were performed using single-cell suspensions in agar (5×10^3 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 Raf-1 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% Me_2SO for 30 min prior to harvesting. For experiments with 32D cells, 3×10^6 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 (Me_2SO) 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). S100/P100 subcellular fractionation was performed essentially as described except the protein in each fraction was resuspended in 2× sample buffer (20 mM $NaPO_4$ pH 7.0, 20% glycerol, 10% β-mercaptoethanol, 0.2 M dithiothreitol, and 0.02% bromphenol blue) following acetone precipitation (13). Equivalent proportions of S100 and P100 were analyzed by immunoblotting.

32D Cell Growth Analysis—32D cells were washed twice with RPMI to deplete the culture medium of IL-3 (WEHI3B conditioned medium). Cells were than plated at a concentration of 2×10^5/ml in RPMI supplemented with 10% FBS. 100 nM PMA or 0.1% Me_2SO was added, and cells were counted daily by trypan blue exclusion. Growth medium with PMA was replenished every 2–3 days.

Immunoblot Analyses—The following antibodies were used for immunoblot analyses: anti-HA (Covance), anti-pan Ras (Calbiochem), and anti-FLAG (Sigma).

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. 1*A*.

RasGRP4 exhibits a similar domain structure to the three human members of the RasGRP protein family (Fig. 1*B*) (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 Ral 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 Ral exchange factors is shown in Fig. 1*C*. 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 vivo* but not *in vitro*.

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 RasGRP subcellular location 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).

identical to our isolated transforming sequences, during the course of our study and the review of our manuscript, R. L. Stevens and colleagues 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 number XM056604).

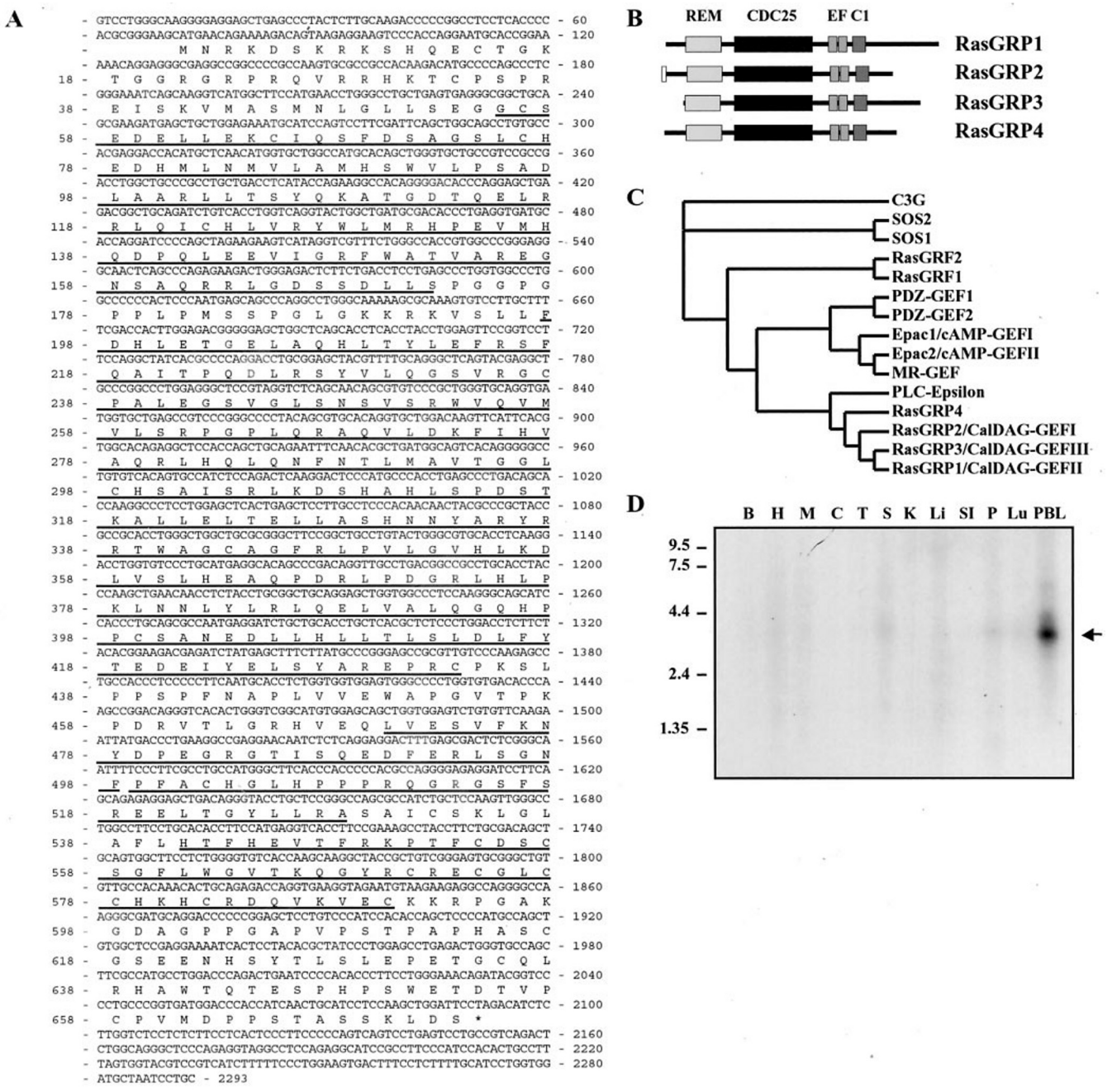

FIG. 1. **Sequence and expression of RasGRP4.** *A*, sequence of wild-type RasGRP4. The nucleotide sequence of wild-type *RasGRP4* cDNA is shown with the corresponding amino acid sequence *below* it. This sequence has been assigned the GenBank™ accession number AF448437. The following domains were identified in RasGRP4 using the Simple Modular Architecture Research Tool (smart.embl-heidelberg.de/): a Ras exchange motif (*REM*) (amino acids 55–172); a CDC25 homology domain (amino acids 197–433); potential EF hand domains (amino acids 470–498 and 499–528); and a C1 domain (amino acids 541–590). The amino acids of these domains are *underlined*. *B*, schematic representation of all RasGRP family members. *C*, dendrogram of the CDC25 domains of Ras exchange factors. A dendrogram was derived from a ClustalW alignment of the CDC25 homology domains of various human Ras, Rap, and Ral guanine nucleotide exchange factors. This was done using the ClustalW program at the EMBL European Bioinformatics Institute web site (www2.ebi.ac.uk/clustalw/). *D*, expression of RasGRP4. RasGRP4 cDNA was used as a probe on a human multiple tissue mRNA Northern blot (BD Biosciences/CLONTECH). The sizes of RNA markers are indicated in kilobases. Tissues include brain (*B*), heart (*H*), skeletal muscle (*M*), colon (*C*), thymus (*T*), spleen (*S*), kidney (*K*), liver (*Li*), small intestine (*SI*), placenta (*P*), lung (*Lu*), and peripheral blood leukocytes (*PBL*).

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. 1*D*). 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.[3] 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.

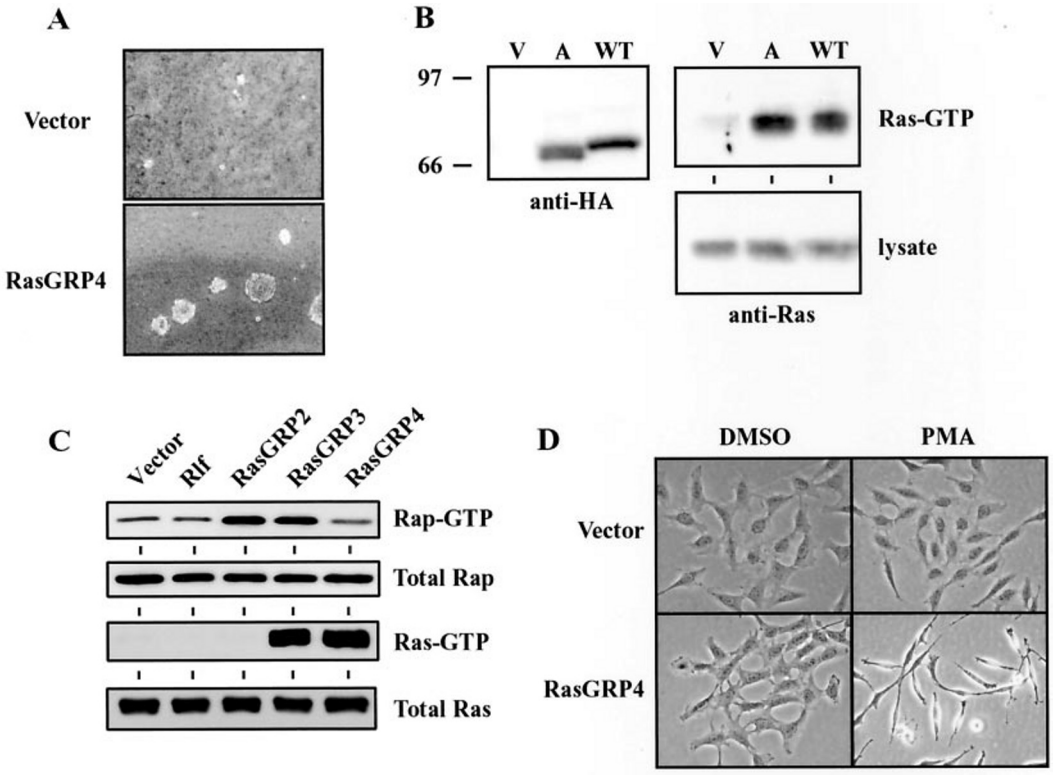

FIG. 2. **RasGRP4 transforms Rat1 fibroblasts and activates Ras in Rat1 fibroblasts and 293T cells.** *A*, RasGRP4 transforms Rat1 fibroblasts. Rat1 cells were generated to express RasGRP4 by retroviral infection. Cells stably expressing RasGRP4 readily formed colonies of proliferating cells in soft 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, Rlf, 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.

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 RasGRP4 cDNA readily grew in soft agar (Fig. 2*A*). Rat1 cells expressing the AML-derived point-mutated RasGRP4 promoted equivalent colony formation in soft agar, indicating that the mutation in the tumor-derived sequence did not alter the transforming signal of the protein, at least in the assays that we performed.[3] Previous studies have described a potent transforming activity for RasGRP1 and a weak activity for RasGRP3, whereas RasGRP2 did not exhibit transforming activity (19).

RasGRP4 Is a Ras-specific Guanine Nucleotide Exchange Factor—RasGRPs have been shown to function as GEFs for Ras and/or Rap proteins (6). Constitutively activated mutants of Ras, but not Rap1, are potent transforming proteins (23). Hence, we sought to determine whether RasGRP4 functions as an activator of Ras. For these analyses, we utilized a glutathione *S*-transferase (GST) fusion protein containing the GTP-dependent Ras-binding domain from the Ras effector, the Raf-1 serine/threonine kinase, in a pull-down assay (12) to determine whether the level of activated GTP-bound Ras was elevated in Rat1 cells stably overexpressing RasGRP4. Cells expressing the AML-mutated or wild type RasGRP4 contained an elevated level of GTP-bound Ras when compared with empty vector-infected control cells (Fig. 2*B*). As expected from their similar transforming activities, we found no significant difference in the ability of the AML-derived point mutated RasGRP4 or wild-type RasGRP4 to activate Ras. Thus, we conclude that transformation by RasGRP4 likely occurs by causing persistent activation of endogenous Ras proteins.

RasGRP2 (in this case the shorter unmyristylated form, CalDAG-GEFI) activates Rap1 but not Ras (8, 18), whereas RasGRP3 activates both Ras and Rap (17, 19). Therefore, we next compared the ability of RasGRP4 to activate Ras and Rap in 293T cells with that of other RasGRP proteins. These analyses involved pull-down assays using the GST-Raf-RBD for Ras

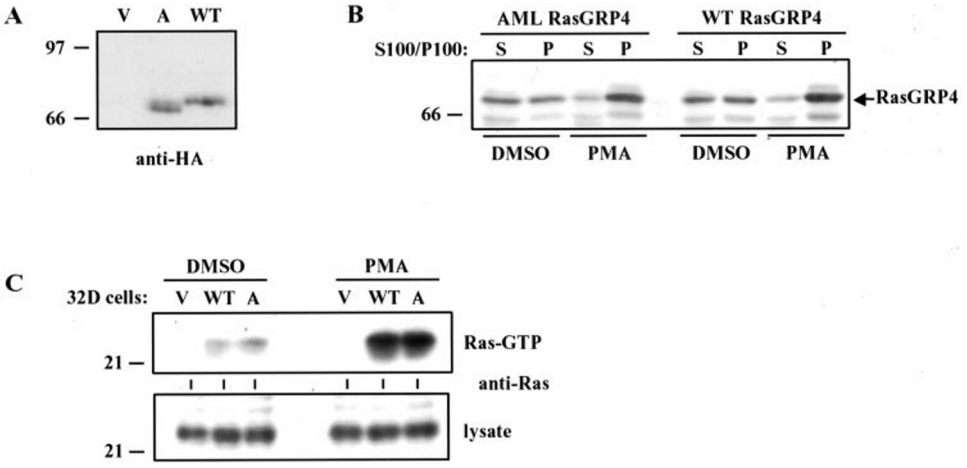

FIG. 3. **PMA promotes RasGRP4 membrane association and RasGRP4-mediated activation of Ras in 32D myeloid cells.** *A*, expression of the RasGRP4 proteins in 32D cells was determined by immunoblot analyses of total cell lysates with anti-HA antibodies. The molecular masses of protein markers are indicated in kilodaltons. *V*, control vector; *A*, AML-mutated form; *WT*, wild type. *B*, 32D cells stably expressing wild-type or AML-mutated forms of RasGRP4 were treated with PMA for 30 min and analyzed by S100/P100 fractionation. RasGRP4 present in each fraction was detected by immunoblot analyses with anti-HA antibodies. *S*, soluble; *P*, particulate; *DMSO*, dimethyl sulfoxide. *C*, 32D cells expressing RasGRP4 proteins were starved of FBS and IL-3 for 1 h and then stimulated with PMA for 30 min, and the relative amount of activated Ras-GTP was determined by utilizing a GST-RafRBD pull-down assay. The levels of active Ras-GTP in the pull-downs and of total Ras in lysates were determined by immunoblotting with anti-pan Ras antibodies.

and a GST fusion to the RBD of RalGDS to pull down activated Rap (9, 24) (Fig. 2*C*). In agreement with previous observations, we found that RasGRP3 activated both Ras and Rap1, whereas the Rap-specific short form of RasGRP2 (*i.e.* CalDAG-GEFI) activated Rap1 and not Ras. In contrast, transient expression of RasGRP4 activated Ras but did not activate Rap1 (Fig. 2*C*). Expression of Rlf, a guanine nucleotide exchange factor for the Ral GTPase (25), did not activate Ras or Rap1. Thus, RasGRP4 acts as a Ras-specific GEF and, like other RasGRPs, is not an activator of Ral.[4]

PMA Promotes RasGRP4 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 RasGRP proteins (8, 15–17). To determine whether RasGRP4 function is modulated by an increase in diacylglycerol, we determined whether treatment with the diacylglycerol analog, PMA, would enhance the transforming activity of RasGRP4 when expressed in Rat1 cells. PMA treatment induced morphological transformation of Rat1 cells expressing RasGRP4 but not vector control cells (Fig. 2*D*), suggesting that PMA further stimulated RasGRP4 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 RasGRP4 to grow in soft agar.[3] 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 RasGRP function by promoting enhanced plasma membrane association (8, 15–17). Because RasGRP4 mRNA is expressed highly in peripheral blood leukocytes and was isolated from a myeloid leukemia, we evaluated the ability of PMA to regulate RasGRP4 subcellular location and activity in 32D mouse myeloid progenitor cells (28, 29). For these analyses, we established 32D cells stably expressing HA epitope-tagged RasGRP4 and then determined whether PMA treatment also promoted membrane association of this exchange factor (Fig. 3*A*). We treated 32D cells expressing RasGRP4 with PMA for 30 min and performed subcellular fractionation. PMA treatment induced a translocation of RasGRP4 from the S100 soluble fraction to the P100 membrane-containing fraction (Fig. 3*B*). 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 RasGRP4. 32D cells expressing RasGRP4 exhibited elevated basal Ras-GTP levels when compared with control empty vector-infected cells (Fig 3*C*). This level of activated Ras was greatly enhanced by PMA treatment, whereas PMA alone did not further activate Ras in control cells (Fig. 3*C*). Rap1 was not activated under these conditions,[3] consistent with the inability of RasGRP4 to activate Rap1 in 293T cells (Fig. 2*C*). The elevated basal levels of Ras activation in cells expressing RasGRP4 is likely due to the significant amount of RasGRP4 that is already associated with membranes (Fig. 3*B*). Identical results were obtained with the wild-type and AML-mutated forms of RasGRP4, suggesting that the AML-derived mutation does not alter the ability of RasGRP4 to translocate to membranes and activate Ras in response to PMA treatment (Fig. 3, *B* and *C*). These data indicate that RasGRP4, like other RasGRP family members, has a functional C1 diacylglycerol-binding domain that regulates the activity of the CDC25 domain. Thus, RasGRP4 likely plays a role downstream of extracellular stimuli that elicit elevated diacylglycerol levels, such as those that stimulate G protein-coupled receptor or receptor tyrosine kinase activation of phospholipase C β or γ, respectively (30).

RasGRP4 Activation by PMA Promotes Cytokine-independent Growth of 32D Myeloid Cells—Finally, because had we identified RasGRP4 as a transforming protein, we determined whether RasGRP4 was capable of altering the growth properties of myeloid cells. To evaluate this possibility, we analyzed the ability of RasGRP4 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.

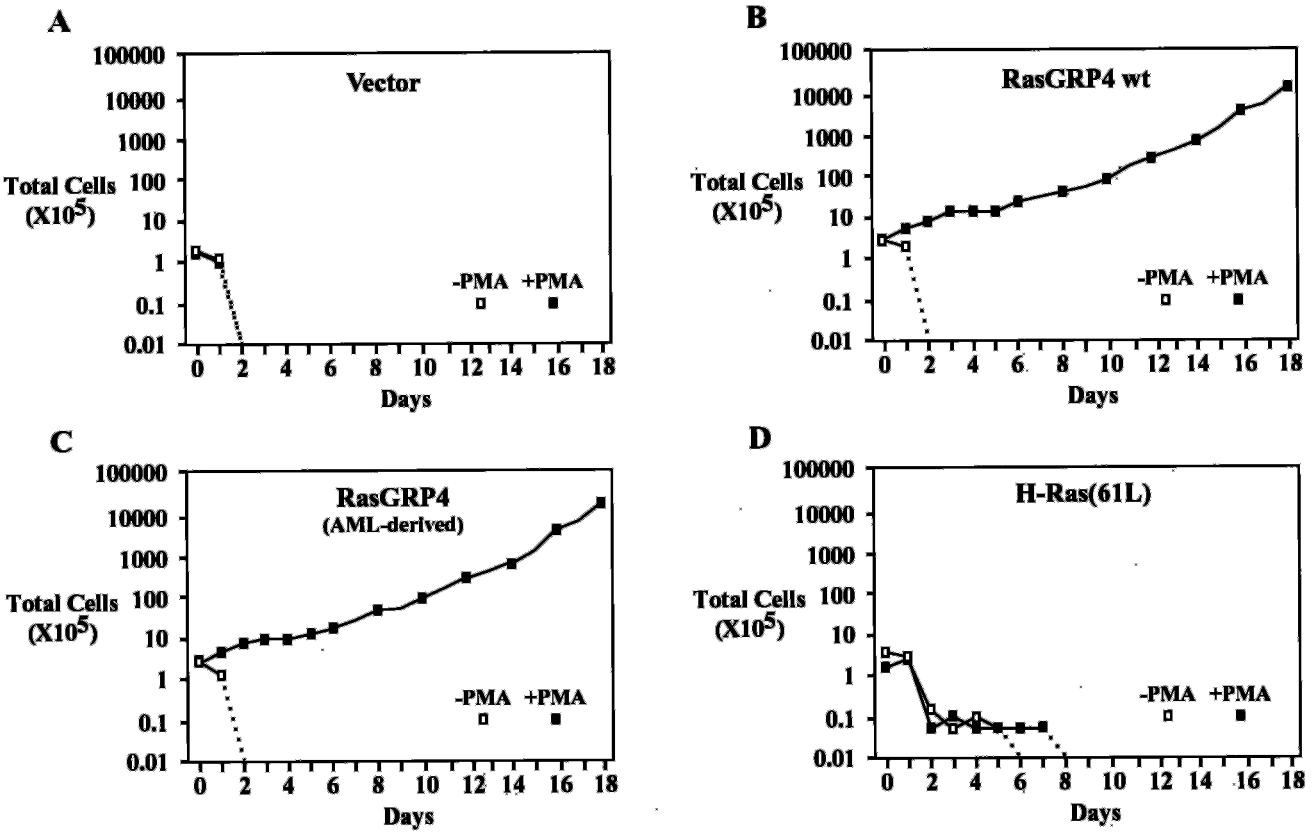

FIG. 4. **PMA promotes RasGRP4-mediated cytokine-independent growth of 32D myeloid cells.** 32D cells expressing control vector (*A*), wild-type RasGRP4 (*B*), AML-derived RasGRP4 (*C*), or H-Ras(61L) (*D*) were deprived of IL-3 on day zero and cultured in the presence (*closed symbols*) or absence (*open symbols*) of 100 nM PMA. The total number of viable cells was determined by trypan blue exclusion.

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 viability[5] 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. 4*A*). 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 affect of PMA on RasGRP4 cells is not merely a cooperation between Ras activation and other signals activated by PMA (Fig. 4*D*). 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. 4*D*). 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 colleagues[2] deposited a human cDNA sequence encoding a protein nearly identical to RasGRP4 (GenBank™ accession number AY048119). 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 protein in an asthma patient, the Stevens' group[2] 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[2] 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 data base 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.

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