MgcRacGAP is involved in the control of growth and differentiation of hematopoietic cells

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In a search for key molecules that prevent murine M1 leukemia cells from undergoing interleukin (IL)-6–induced differentiation into macrophages, we isolated an antisense complementary DNA (cDNA) that encodes full-length mouse MgcRac-GTPase-activating protein (GAP) through functional cloning. Forced expression of this antisense cDNA profoundly inhibited IL-6–induced differentiation of M1 cells into macrophage lineages. We also isolated a full-length human MgcRacGAP cDNA, which encodes an additional N-terminal polypeptide of 105 amino acid residues compared with the previously published human MgcRacGAP. In human HL-60 leukemic cells, overexpression of the full-length form of human MgcRacGAP alone induced growth suppression and macrophage differentiation associated with hypertoculization and de novo expression of the myelomonocytic marker CD14. Analyses using a GAP-inactive mutant and 2 deletion mutants of MgcRacGAP indicated that the GAP activity was dispensable, but the myosin-like domain and the cysteine-rich domain were indispensable for growth suppression and macrophage differentiation. The present results indicated that MgcRacGAP plays key roles in controlling growth and differentiation of hematopoietic cells through mechanisms other than regulating Rac GTPase activity. (Blood. 2000;96:2116-2124)

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Materials and methods

Cell lines

The murine myeloid leukemia cell line M1 was grown in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Rockville, MD) containing 10% fetal calf serum (FCS). A human myeloid leukemia cell line HL-60 was grown in RPMI 1640 medium (GIBCO) containing 10% FCS. An ecotropic retrovirus packaging cell line BOSC23 12 was maintained in DMEM containing 10% FCS and GPT selection reagents (Specialty Media, Lavallette, NJ). Two days before transfection, the cells were transferred into DMEM-10% FCS without GPT selection reagents.

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Retrovirus vectors
A bicistronic retroviral vector pMX-IREs-EGFP was constructed as described,\textsuperscript{13} pMX-MgcRacGAP-IREs-EGFP was constructed by inserting a 1.9-kb EcoRI-NorI fragment of the Flag-tagged full-length form of MgcRacGAP gene into EcoRI and NorI sites of pMX-IREs-EGFP, upstream of the IRES (internal ribosomal entry site) sequence so that both MgcRacGAP and EGFP (enhanced green fluorescent protein) are expressed from a single messenger RNA (mRNA) in the same cells.

Production of retroviruses and infection with them
High-titer retroviruses carrying the hMgcRacGAP-IREs-EGFP were produced with a transient retrovirus packaging cell line BOSC23 \textsuperscript{12} as described previously.\textsuperscript{15} We first established a stable transfectant expressing the ecotropic virus receptor.\textsuperscript{14,15} Infection was performed as described.\textsuperscript{16} Briefly, cells were incubated with 10 mL of the retroviruses in the presence of 10-μg/mL hexamethrin bromide (Sigma, St Louis, MO). Twenty-four hours after infection, cells were washed, refed with growth medium, and left for one more day before cell sorting with EGFP.

Cell sorting and flow cytometry
EGFP \textsuperscript{+} cells were sorted using a modification of the technique described earlier.\textsuperscript{17} Briefly, 2 days after virus infection, the infected cells were washed twice with phosphate buffered saline (PBS), suspended in PBS containing 1% bovine serum albumin (BSA), and then sorted based on EGFP expression on a FACSStat (Becton Dickinson, San Jose, CA). The sorted cells (40 per well) were then expanded in growth medium. Flow cytometric analysis was carried out to quantify morphologic changes and to confirm the expression of EGFP in the HL-60 transfectants on a FACScalibur flow cytometer (Becton Dickinson). The cells were also stained with a phycoerythrin (PE)-conjugated mouse antihuman CD14 monoclonal antibody to confirm the expression of EGFP in the HL-60 transfectants on a FACScalibur flow cytometer (Becton Dickinson). The cells were also stained with a phycoerythrin (PE)-conjugated mouse antihuman CD14 monoclonal antibody (PharMingen, San Diego, CA) on ice for 30 minutes after blocking with 100-fold excess of mouse immunoglobulin (Ig) G, and analyzed on a FACScalibur flow cytometer. PE-conjugated mouse IgG\textsubscript{2a} was used as an isotype-matched negative control.

Immunoprecipitation and Western blotting
Immunoprecipitation, gel electrophoresis, and immunoblotting were performed as described\textsuperscript{10} but with minor modifications. Exponentially growing cells were washed with PBS, lysed in lysis buffer (50-mmol/L Tris-HCl, pH 7.5; 150-mmol/L NaCl; 1% Triton X-100; 1-mmol/L ethylenediaminetetraacetic acid [EDTA]; 0.2-mmol/L Na\textsubscript{2}VO\textsubscript{4}; 2-mmol/L phenylmethylsulfonyl fluoride; 2-μg/mL leupeptin; 10-μg/mL aprotinin) (5 \times 10\textsuperscript{5} cells/mL), and incubated on ice for 30 minutes. Cell lysates were clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C over night. The immunoprecipitates were washed 3 times with lysis buffer, subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and electrophoretically transferred onto Immobilon filters (Millipore, Bedford, MA). After blocking in a solution containing 3% BSA, the filter was probed with the anti-Flag M2 monoclonal antibody. The filter-bound antibody was detected using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

Northern blot analysis
Poly(A)\textsuperscript{+} mRNA was isolated from cells using FastTrack 2.0 kit (Invitrogen, San Diego, CA). Two micrograms of Poly(A)\textsuperscript{+} RNA was denatured and blotted to Hybond-N nylon filter (Amersham). The filter was incubated at 42°C in 50% formamide, 3× Denhardt’s reagent (0.06% polyvinylpyrrolidone, 0.06% BSA, 0.06% Ficoll), 5× standard saline citrate (SSC; 1× SSC = 0.15-mol/L NaCl, 0.015-mol/L trisodium citrate), 1× SDS, 200-μg/mL denatured salmon sperm DNA, and a 5′-labeled complementary DNA (cDNA) probe, prepared using Random Prime Kit (Stratagene, La Jolla, CA). After hybridization, the filter was washed in 0.1× SSC, 0.1% SDS at room temperature, and autoradiographed. Reprobing was carried out after washing the filter in a stripping buffer of 10-mmol/L Tris-HCl (pH 7.5), 1-μmol/L EDTA, 0.1% SDS, and 0.3× Denhardt’s reagent at 90°C for 20 minutes.

GTP hydrolysis assays
The [\textsuperscript{γ-32}P] GTP-bound form of small GTPases was prepared by incubating 10 pmol of protein with 74 kBq of [\textsuperscript{γ-32}P] GTP (1.11 TBq/mmol, NEN Life Science Products, Boston, MA) in a 50-L volume of 25-mmol/L Tris-HCl (pH 7.5), 5-mmol/L EDTA, 0.2-mmol/L MgCl\textsubscript{2}, 0.1-mg/ml BSA, 1-μmol/L dithiotreitol, and 4-μmol/L GTP for 15 minutes at room temperature. GTP hydrolysis was initiated by raising MgCl\textsubscript{2} and GTP to a final concentration of 20-mmol/L MgCl\textsubscript{2} and 200 μmol/L, respectively, and was stopped after 3 minutes by adding 2 mL of ice-cold 50-mmol/L Tris-HCl (pH 8), 35-mmol/L MgCl\textsubscript{2}, 1-μmol/L dithiotreitol, and 150-mmol/L NaCl. Amounts of [\textsuperscript{γ-32}P] GTP bound to GTPases were determined by radioactivity counting after rapid vacuum filtration of the samples on BA 85 nitrocellulose (Schleicher and Schuell, Dassel, Germany). Typically, 100% of [\textsuperscript{γ-32}P] GTP bound to GTPases is around 25 000 cpm in our hands. The GAP assay was conducted in the presence of the GAP domain (20 pmol) of hMgcRacGAP or a GAP inactive mutant of hMgcRacGAP (R385A\textsuperscript{+}MgcRacGAP) during the step of GTP hydrolysis. GTP exchange reactions during the GTP hydrolysis step were examined using [\textsuperscript{α-32}P] GTP-preloaded GTPases under similar conditions and were not significantly affected by the presence of hMgcRacGAP.

Mutagenesis and production of retroviruses
The mutation R385A was introduced by overlap-extension polymerase chain reaction (PCR) mutagenesis,\textsuperscript{19} DNA encoding the conserved arginine residue was amplified by PCR using primer pairs as follows: Upstream primer: 5′-AGCTGCGGATTACTTGGATT-3′ Downstream primer: 5′-GCCACTGCTGTCAGTACAT-3′ Mutagenic primer, sense: 5′-AGCCTGATGCGATACAGGCT-3′. PCR products were joined and amplified by the upstream primer and the downstream primer and cloned into EcoRI-BstXI sites of pMX-MgcRacGAP-IREs-EGFP. Mutagenic sequence was confirmed by automated sequencing by an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Branchburg, NJ). To construct a mutant MgcRacGAP lacking the myosin-like domain (ΔMyo-MgcRacGAP), the deletion constructs were generated by PCR with a 5′ primer that contained an EcoRI site (5′-GAAAGATTTGCGAGAGATGCTAGTACATGCA-3′) and a 3′ primer that is located downstream of the BstXI site of hMgcRacGAP (5′-TCCTACAGACGCTTGCATCT-3′). The resulting PCR fragment was digested with EcoRI and BstXI and subcloned into the corresponding cloning sites of pMX-MgcRacGAP-IREs-EGFP. A mutant MgcRacGAP lacking the cysteine-rich domain (ΔCys-MgcRacGAP) was generated by overlapping extension using PCR. In brief, complementary oligonucleotide primers were used for PCR to generate 2 DNA fragments that have overlapping ends. To construct the N-terminal half of ΔCys-MgcRacGAP, primer pairs 5′-AGCTGCGGATATTCCGGAAAT-3′ (primer A) and 5′-GGTTCTCTATCAAGGTAGCGATCCTCCTCATTACTCTG-3′ (primer B) were used for PCR with the full-length hMgcRacGAP cDNA as a template. Primer pairs 5′-AACAGTAAATGAGGAGGATCCTACTATGAGAAACC-3′ (primer C) and 5′-TTCACCAACAGCTTGGTACAT-3′ (primer D) were used to generate the C-terminal half. These fragments were combined in a subsequent reaction in which the overlapping strands were annealed, allowing the 3′-overlap of each strand to serve as a primer for the extension of the complementary strand. The resulting fusion product was further amplified by PCR using primers A and D. The product was digested by EcoRI and BstXI, separated by agarose gel electrophoresis, purified, and cloned into pMX-MgcRacGAP-IREs-EGFP digested by EcoRI and BstXI. PCR was carried out using high-fidelity DNA polymerase Pyrobest (Takara, Ohtsu, Japan). The PCR condition was as follows: denaturation at 98°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes for 25 cycles.
Results

Expression of an antisense DNA encoding MgcRacGAP partially blocked IL-6–induced differentiation of M1 cells

To identify key molecules that regulate cell differentiation into macrophages, we generated a cDNA library in a retrovirus vector pMXneo\(^2\)\(^\text{a}\) from a subclone of mouse myeloid leukemia M1 cells (termed MD1 cells) expressing a constitutively active STAT5A mutant.\(^{2}2\) This particular mutant retained the potential to proliferate while differentiating into various stages along the monocytic differentiation pathway and giving rise to a heterogeneous population of blast, intermediate, and mature monocyctic cells through the production of interleukin-6 (IL-6) (T. Kawashima et al, unpublished data). We searched for a gene for this particular phenotype by retrovirus-mediated functional screening of the MD-1–derived cDNA library; M1 cells infected with the cDNA library were screened for clones resistant to IL-6–induced terminal differentiation in the medium containing IL-6 (100 ng/mL) and G418 (600 μg/mL) as described.\(^{16,22}\) After screening of \(1.5 \times 10^5\) independent cDNA clones, we happened to isolate an antisense cDNA encoding the complete coding sequence of mMgcRacGAP\(^2\)\(^\text{b}\) —from an M1 clone that became resistant to IL-6.

To determine if the expression of this antisense cDNA (asMgcRacGAP) indeed made M1 cells resistant to IL-6–induced macrophage differentiation, we subcloned it into a bicistronic retrovirus vector pMX-ires-EGFP to construct pMX-asMgcRacGAP-ires-EGFP, and we reintroduced it into M1 cells via retrovirus infection. As a control, pMX-ires-EGFP was also introduced into M1 cells. After transduction of M1 cells with these vectors, GFP\(^\text{c}\) cells were sorted on a fluorescence-activated cell sorter (FACS) as described in “Materials and methods” and were cultured for 4 days in the presence or absence of 5-ng/mL IL-6. Flow cytometric analysis was performed to quantify morphologic changes after the culture. The increase in cell size and the granularity of cytoplasm were evaluated based on increases in forward scatter and side scatter, respectively, in M1 cells expressing either the antisense mMgcRacGAP or the control vector after IL-6 treatment (Figure 1A,C). Only 15.2% of M1 cells transduced with the antisense mMgcRacGAP showed a shift from region R1 to region R2, a hallmark of macrophage differentiation after treatment with IL-6, while 56.9% and 47.6% of the parental M1 cells and M1 cells transduced with the control vector showed similar shifts, respectively. Similarly, morphologically differentiated cells were evident in the control M1 cells but not in the antisense mMgcRacGAP-transduced M1 cells after treatment with IL-6 (Figure 1B), indicating that expression of antisense mMgcRacGAP profoundly inhibited the IL-6–induced differentiation of M1 cells. On the other hand, the growth rate of M1 cells was not affected by the expression of the antisense MgcRacGAP (data not shown).

Identification of the full-length form of hMgcRacGAP cDNA

To isolate cDNA clones encoding the complete coding sequences of both murine and human MgcRacGAP, we screened libraries derived from the murine M1 cell line and the human TF-1 erythroleukemic cell line. The isolated cDNAs for 2919 base pairs of the mMgcRacGAP and 3050 base pairs of the hMgcRacGAP were then sequenced. We found that the open reading frame of the hMgcRacGAP cDNA encoded a putative protein of 632 amino acids, which is longer than the previously reported sequence of 527 amino acids by N-terminal 105 amino acids.\(^{24}\) The corresponding cDNA and the protein sequence of the previously reported hMgcRacGAP were not found in the FASTA/BLAST program of the National Center for Biotechnology Information or the DNA Databank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence database. In addition, an analysis of other mRNA sources derived from Jurkat cells and phytohemagglutinin (PHA)-activated human T-cell blasts by reverse transcription PCR indicated that these cells expressed the full-length hMgcRacGAP cDNA, which was identical to the cDNA reported in the present paper (data not shown). Thus, it was shown that the hMgcRacGAP cDNA reported here is the full-length form of this RacGAP (accession No. AB030251). The surrounding sequence of the putative initiation codon ATG for the full-length form of
hMgcRacGAP well matched to the Kozak consensus sequence, and a calculated molecular mass of this full-length form is 71 000 kd. Murine MgcRacGAP and the full-length form of hMgcRacGAP are 84% identical and 97% similar to each other at the amino acid level (Figure 2A). Database searches revealed the similarity between the N-terminal region of the full-length hMgcRacGAP (amino acids 41-124) and myosins, mainly tropomyosins (Figure 2B). In this paper, we call the full-length form of this molecule hMgcRacGAP.

Expression of the hMgcRacGAP mRNA

Northern blot analysis revealed that a single hMgcRacGAP transcript of 3.3 kilobases (kb) was expressed in most tissues tested (Figure 3), with expression being high in thymus and placenta and low in spleen and peripheral blood leukocytes. It was noteworthy that hMgcRacGAP was highly expressed in tissues such as thymus and placenta containing immature hematopoietic cells but not in nonproliferating peripheral blood leukocytes. We also confirmed that mMgcRacGAP mRNA was expressed in murine fetal liver cells and in hematopoietic cell lines including Ba/F3 cells, CTLL-2 cells, and DA-1 cells (data not shown).

Enforced expression of hMgcRacGAP induced macrophage differentiation in the human myeloid leukemia HL-60 cells

We attempted to elucidate the role of MgcRacGAP in macrophage differentiation using murine myeloid leukemia M1 cells and human myeloid leukemia HL-60 cells. The mMgcRacGAP cDNA and the Flag-tagged hMgcRacGAP cDNA were transduced into M1 cells and HL-60 cells, respectively, using the retrovirus vector pMX-IRES-EGFP. GFP M1 and HL-60 cells transduced with these viruses were sorted on FACS 2 days after infection. GFP M1 and HL-60 cells transduced with a control pMX-IRES-EGFP vector were similarly sorted and served as negative controls.

Overexpression of either mMgcRacGAP or hMgcRacGAP in M1 cells induced no significant differentiation but did moderately inhibit the growth of M1 cells (data not shown). Overexpression of mMgcRacGAP or hMgcRacGAP also retarded proliferation of a murine proB cell line Ba/F3. On the other hand, enforced expression of hMgcRacGAP in HL-60 cells profoundly suppressed growth (Figure 4A) and induced differentiation (Figure 4B-D) of the cells. Differentiation of HL-60 cells into macrophages was evaluated by FACS, expression of a monocyte marker CD14, and the morphology. First, the increase in cell size and granularity of the cytoplasm were evaluated by increases in forward scatter and side scatter, respectively. As shown in Figure 4B, expression of hMgcRacGAP induced a shift from region R1 to region R2 in 65.8% of the transduced HL-60 cells, while expression of the control vector pMX-IRES-EGFP did not induce a significant shift. Second, expression of a myelomonocytic marker CD14 was induced in HL-60 cells expressing hMgcRacGAP but not in control HL-60 cells (Figure 4C). Morphologic changes were also noticed in HL-60 cells transduced with hMgcRacGAP; most HL-60 cells became larger with morphologic changes of monocytic differentiation and showed reduced nuclear/cytoplasmic ratios and notable hypervacuolation in the cytoplasm after transduction of hMgcRacGAP (Figure 4D). These results clearly demonstrated that overexpression of hMgcRacGAP alone induced differentiation of HL-60 cells into macrophages.

Stable expression of hMgcRacGAP transgene in HL-60 and M1 transfectants was confirmed by the Northern blot and the Western blot analysis (Figure 5A,B). In Northern blot analysis of HL-60 cells carrying the hMgcRacGAP transgene (Figure 5A, lane 3), we detected retroviral transcripts migrating at approximately 4.7 kb and 4.0 kb, which were presumably genomic and subgenomic forms of the transcripts, respectively. The retrovirus vector pMX harboring splicing donor and acceptor sites as well as...
the endogenous hMgcRacGAP mRNA at approximately 3.3 kb. Western blot analysis demonstrated that M1 cells transduced with the Flag-tagged hMgcRacGAP transgene expressed the recombinant protein of approximately 80 kd (Figure 5B).

Expression of endogenous MgcRacGAP was down-regulated during IL-6– and TPA-induced macrophage differentiation of M1 and HL-60 cells, respectively

To determine whether the expression level of endogenous MgcRacGAP was altered during macrophage differentiation, M1 and HL-60 cells were stimulated with IL-6 (50 ng/ml) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (16 nmol/L), respectively, and at various time points we isolated poly(A)

1 RNA. Intriguingly, Northern blot analysis of these samples showed that the expression of endogenous MgcRacGAP mRNA dramatically decreased along with macrophage differentiation of both M1 and HL-60 cells (Figure 6A,B).

The mutant MgcRacGAP in which the conserved arginine was replaced with an alanine exhibited no GTPase activity

A conserved arginine residue found in the first homology box of GAP domains of Rho-GAP family was also conserved in MgcRacGAP (Figure 7A), and structural studies suggested that this arginine residue was a key catalytic residue.27,28 Indeed, it was reported that
mutation (or deletion) of this conserved arginine residue disrupted GAP activities of n-chimaerin and Cdc42-GAP without affecting their binding activities to GTP-bound Rho GTPase. Therefore, to generate a GAP-inactive mutant of hMgcRacGAP, we replaced the conserved arginine (Arg385) of hMgcRacGAP with an alanine (R385A*MgcRacGAP). We expressed the mutant GAP domain of R385A*MgcRacGAP as GST fusion products in vitro and confirmed that the GAP activity of hMgcRacGAP toward Rac1 and Cdc42 was inactivated as a result of this mutation (Figure 7B).

GAP activity was dispensable, but the myosin-like domain and the cysteine-rich domain were indispensable for hMgcRacGAP-induced growth suppression and differentiation.

To determine if the GAP activity of hMgcRacGAP is crucial and which domain of this RacGAP is important for the growth suppression and macrophage differentiation in HL-60 cells, we used the GAP activity-negative mutant (R385A*MgcRacGAP) and 2 deletion mutants, lacking the N-terminal myosin-like domain or the cysteine-rich domain (ΔMyo-MgcRacGAP or ΔCys-MgcRacGAP, respectively) (Figure 8).

Each mutant was introduced into HL-60 cells via retrovirus infection using the retrovirus vector pMX-IRES-EGFP. GFP+ cells with comparable fluorescence intensities were then sorted. As shown in Figure 9A, growth of HL-60 cells was suppressed after transduction of the GAP activity-negative mutant of hMgcRacGAP to an extent similar to that seen with HL-60 cells transduced with the wild-type hMgcRacGAP, indicating that the GAP activity of hMgcRacGAP is dispensable for the hMgcRacGAP-induced growth suppression of HL-60 cells. On the other hand, ΔMyo-MgcRacGAP showed decreased activities of growth suppression of HL-60 cells, and ΔCys-MgcRacGAP did not significantly suppress proliferation of HL-60 cells. The mutant as well as the wild-type hMgcRacGAP proteins were expressed at similar levels (Figure 9B). These results suggested that the protein kinase C–like cysteine-rich domain played a crucial role in inhibiting the proliferation of HL-60 cells.

To determine effects of these mutations in inducing macrophage differentiation, we performed May-Grunwald-Giemsa staining of cytospin preparations of the sorted cells (Figure 9C). Large cells, which differentiated along the monocytic differentiation pathway with distinctive hypervacuolation in the cytoplasm, were observed in HL-60 cells transduced with mutants.
R385A*MgcRacGAP as well as those transduced with the wild-type hMgcRacGAP, thereby demonstrating that the GAP activity of hMgcRacGAP was not required for its activity to induce macrophage differentiation of HL-60 cells. However, well-differentiated cells were not observed in the population of HL-60 cells expressing ΔCys-MgcRacGAP. When transduced with the ΔMyo-MgcRacGAP, HL-60 cells gave rise to large cells that were frequently multinuclei but did not exhibit hypervacuolation. These results suggested that the myosin-like domain of hMgcRacGAP played some roles in cell division as well as induction of macrophage differentiation, including vacuolation.

Discussion

In this paper, we functionally cloned an antisense cDNA for mMgcRacGAP, which profoundly inhibited IL-6–induced differentiation of M1 cells. Although overexpression of sense cDNA for MgcRacGAP induced no detectable differentiation of M1 cells, it induced macrophage differentiation of human acute leukemic HL-60 cells. Moreover, overexpression of MgcRacGAP led to growth suppression of both M1 and HL-60 cells. These results indicated that MgcRacGAP was involved in control of growth and differentiation in both M1 and HL-60 cells. We also found that the GAP activity of hMgcRacGAP was dispensable for hMgcRacGAP-induced growth suppression and differentiation into macrophages.

Rac/Cdc42 small G proteins were implicated in cytoskeletal organization; membrane ruffling; production of superoxide, phagocytosis, and chemotaxis; as well as regulation of cell cycle. Rac/Cdc42 GAPs include Bcr, n-, b2-, a-chimaerin, IQGAP, Cdc42-GAP, Ral-BP1/RLIP1, and myosin-IXb, and they were also implicated in controlling a variety of cellular functions. However, to our knowledge, the present study is the first report demonstrating that enforced expression of a GAP protein alone is able to alter cell fate.

Interestingly, this potential of hMgcRacGAP to induce differentiation does not require its GAP activity but does require the N-terminal myosin-like domain and the cysteine-rich domain, as demonstrated by the experiments using a series of mutants. This result is reminiscent of that reported for n-chimaerin, which cooperates with Rac1 and Cdc42 in stimulating the formation of lamellipodia and filopodia, respectively; these functions of n-chimaerin require the G protein binding capacity and the non-GAP N-terminal extension but not GAP activity. Together, it is suggested that MgcRacGAP not only negatively regulates Rac-mediated signals through their catalytic functions, which stimulate GTP hydrolysis after binding to activated (or GTP-bound) forms of Rac GTPases, but also functions as downstream effectors of Rac proteins as a Rac-binding protein. The myosin-like domain of myosin-IXb and the cysteine-rich domain of n-chimaerin are required for interaction with actin filaments and phospholipids, respectively. Taken together, it seems likely that MgcRacGAP suppresses growth and differentiation through its multiple domains, which would interact with multiple signaling pathways but not through its GAP activity.

An N-terminus–truncated molecule of the hMgcRacGAP was previously isolated as a Rac-binding protein in a 2-hybrid experiment. Human MgcRacGAP was reported to be highly expressed in male germ cell and was implicated in spermatogenesis. However, the reported sequence corresponded to the deletion mutant R385A*MgcRacGAP.
ΔMyo-MgcRacGAP in the present paper and lacked the N-terminal 105 amino acid sequence when compared with the full-length form of the hMgcRacGAP described here. We further confirmed that the previously reported hMgcRacGAP was a truncated version by detecting the full-length cDNAs in Jurkat cells and PHA-activated human T-cell blasts (unpublished results), which was identical to the hMgcRacGAP cDNA that we isolated from erythroid TF-1 cells and used in this study.

Another group has recently cloned a cDNA for mMgcRacGAP under the name of band25.60 This was cloned using the differential display techniques as a cDNA whose expression well correlated with cell growth. In addition, it was shown that expression of the band25 decreased along with terminal differentiation into myocytes of a murine myogenic cell line C2C12. Thus, the expression level of MgcRacGAP was apparently parallel to the rate of cell proliferation.

In a similar context, we found that the expression level of hMgcRacGAP was high in thymus and placenta, which contained a number of proliferating cells, but was extremely low in peripheral blood leukocytes, most of which were terminally differentiated and lost proliferative activities. In addition, expression of endogenous MgcRacGAP dramatically decreased in HL-60 and M1 cells when induced to terminally differentiate into macrophages by TPA and IL-6, respectively (Figure 6), again is correlated with cell proliferation.

Then, the important question is why overexpression of MgcRacGAP suppressed cell growth and induced differentiation of HL-60 cells. Molecular mechanisms for this phenomenon remain to be clarified, but there are 2 possibilities. The first possibility is that MgcRacGAP is a protein primarily involved in cell proliferation and that its overexpression negatively regulates or disrupts the normal control of cell growth, thereby inducing differentiation of the cells as a secondary event. In fact, our results as well as those of others indicated that MgcRacGAP was rather associated with proliferation. This hypothesis also explains why expression of endogenous MgcRacGAP became undetectable along with terminal differentiation of M1 or HL-60 cells. What, then, is the role of MgcRacGAP in cell proliferation? Of particular interest, expression of a deletion mutant ΔMyo-MgcRacGAP suppressed cell growth and induced multinucleated cells in HL-60, suggesting that MgcRacGAP played critical roles in cell division through its myosin-like domain. However, this hypothesis could not explain why expression of the antisense MgcRacGAP profoundly inhibited IL-6–induced macrophage differentiation of M1 cells while leaving cell growth unaffected, strongly indicating that MgcRacGAP was directly involved in cell differentiation in M1 cells. Therefore, we prefer an alternative possibility that MgcRacGAP plays critical roles in both cell growth and differentiation. This possibility is consistent with most results so far presented by us and others concerning MgcRacGAP. However, how MgcRacGAP is involved in both cell growth and differentiation is currently unknown. To clarify the detailed mechanism of MgcRacGAP function, we are now raising antibodies against it.

Finally, the fact that the expression of MgcRacGAP is associated with proliferation and inversely correlates with differentiation in 3 different cell types, including hematopoietic cells, preadipocytes, and myogenic cells, suggests a rather common mechanism by which MgcRacGAP controls cellular proliferation and differentiation.

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References


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