MgcRacGAP, A New Human GTPase-activating Protein for Rac and Cdc42 Similar to Drosophila rotundRacGAP Gene Product, Is Expressed in Male Germ Cells*

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In a search for new partners of the activated form of Rac GTPase, we have isolated through a two-hybrid cloning procedure a human cDNA encoding a new GTPase-activating protein (GAP) for Rho family GTPases. A specific mRNA of 3.2 kilobases was detected in low abundance in many cell types and found highly expressed in testis. A protein of the predicted size 58 kDa, which we call MgcRacGAP, was detected in human testis as well as in germ cell tumor extracts by immunoblotting with antibodies specific to recombinant protein. In vitro, the GAP domain of MgcRacGAP strongly stimulates Rac1 and Cdc42 GTPase activity but is almost inactive on RhoA. N-terminal to its GAP domain, MgcRacGAP contains a cysteine-rich zinc finger-like motif characteristic of the Chimaerin family of RhoGAPs. The closest homolog of MgcRacGAP is RotundRacGAP, a product of the Drosophila rotund locus. In situ hybridization experiments in human testis demonstrate a specific expression of mgcRacGAP mRNA in spermatocytes similar to that of rotundRacGAP in Drosophila testis. Therefore, protein sequence similarity and analogous developmental and tissue specificities of gene expression support the hypothesis that RotundRacGAP and MgcRacGAP have equivalent functions in insect and mammalian germ cells. Since rotundRacGAP deletion leads to male sterility in the fruit fly, the mgcRacGAP gene may prove likewise to play a key role in mammalian male fertility.

Rho GTPases constitute a group of the Ras superfamily of small G proteins comprising three subtypes in mammals, namely Rho, Rac, and Cdc42 (1). Through microinjection or transfection experiments in cultured fibroblasts, Rho GTPases have been shown to control a variety of cellular processes, including organization of the actin cytoskeleton, cell cycle progression, and Rac-induced transformation (1–3). Along with the description of these pleiotropic functions, elucidation of the signaling pathways involving Rho GTPases is being actively pursued, and a number of Rho-interacting proteins have recently been described (4). GTPase-activating proteins (GAPs) for Rho GTPases constitute a class of regulatory proteins that can bind activated (or GTP-bound) forms of Rho GTPases and stimulate GTP hydrolysis (5, 6). Through this catalytic function, Rho GAPs negatively regulate Rho-mediated signals. However, it has long been speculated that GAPs may also serve as effector molecules and play a role in signaling downstream of Rho and other Ras-like GTPases (5, 6). This idea has been recently substantiated in the case of n-alpha, a Rho GAP mainly expressed in the central nervous system. More specifically, n-alpha is shown to cooperate with Rac1 and Cdc42 in inducing specific changes in cytoskeletal morphology i.e. the formation of lamellipodia and filopodia, respectively (7). Interestingly, this effect of n-alpha requires G protein binding capacity but not GAP activity; it also requires the non-GAP N-terminal portion of the protein (7), which includes a cysteine-rich region, shared by all chimaerins, and a specific N-terminal extension (5, 8, 9).

These data support the idea that members of the Chimaerin family may mediate Rho-dependent signals in various cell types, as most of them show tissue-specific expression (5, 9). Analysis of these Rho GAPs may therefore help in defining Rho-dependent functions in differentiated cells.

We report here the cloning and characterization of a new human chimaerin-like protein showing GAP activity toward Rac and Cdc42 and mainly expressed in male germ cells that we call MgcRacGAP (Male Germ Cell RacGAP). It shows a strong structural similarity with the Drosophila protein Rotund (Rn/RacGAP), the product of a spermatocyte-specific gene essential for male fertility in the fruit fly (10). We propose that MgcRacGAP operates in a Rac/Cdc42-dependent signaling pathway involved in the development and/or function of male germ cells in mammals.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The two-hybrid system used in this study has already been described (11, 12). The Jurkat cell cDNA library in plasmid pGAD was generously provided by Dr. J. Camonis (13). The bait in our screen was a fusion between LexA and a human Rac2L61 mutant previously described (14). This fusion protein was expressed in L40 yeast from plasmid pVJL10, a derivative from pBTM116 (12) with modified polylinker. LexA fusion protein expression was checked on Western blots with anti-LexA antibodies (a gift from P. Moreau, Gif/Yvette).

Yeast culture conditions and the two-hybrid procedures were carried out according to published methods (12, 15). Library plasmids from transformed yeast colonies were recovered using HB101 as a recipient

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1 The abbreviations used are: GAP, GTPase-activating protein; rn, rotund gene; Rn, Rotund protein; PKC, protein kinase C; SGP-2, sulfated glycoprotein 2; bp, base pair(s).

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strain, selected on M9 medium lacking leucine.

To obtain the full-length sequence of the mgcRacGAP cDNA, overlapping cDNA clones were isolated from a human placenta AExLox cDNA library (kindly provided by J. M. Garnier and P. Chambon) using a 450-bp EcoRI 5′ fragment of the two-hybrid clone as a probe.

**Sequence Analysis**—Clones of interest were sequenced using the Sanger dideoxy termination method adapted to ABI 373A automated sequencer. Sequence analysis was performed using computer facilities provided by the Centre Interuniversitaire de Traitement Informatique (CITII) and Groupement d’Intérêt Scientifique (GIS) Infobiogen.

Gene expression was analyzed on Multiple Tissue Northern blots (Clontech) using mgcRacGAP cDNA as a probe. A β-actin cDNA was used as a control.

**Recombinant Proteins**—GAP domains of MgcRacGAP (amino acids 238–513) and Bcr (amino acids 1050–1271) and Rac1 were expressed in Escherichia coli, purified as glutathione S-transferase fusion proteins, and cleaved with thrombin. Protein purity, as estimated by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis, was more than 95%. The purified Rac1, Cdc42, Rab6, and Rap2a proteins were kind gifts of G. M. Bokoch, P. Boquet, B. Goud, and J. de Gunzburg, respectively.

**Immunodetection of MgcRacGAP**—Antibodies to recombinant MgcRacGAP (amino acids 238–513) were raised in chicken, affinity-purified on a MgcRacGAP/Affi-Gel column (Bio-Rad), and used to detect MgcRacGAP (amino acids 238–513) of MgcRacGAP in Western blot. Proteins from human testis extracted on 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Filters were incubated sequentially with anti-MgcRacGAP-purified antibodies and peroxidase-labeled rabbit anti-chicken immunoglobulins (Dako). Blots were developed using the ECL detection system (Amersham Corp.).

**GTP Hydrolysis Assays**—The [γ-32P]GTP-bound form of small GTPases were prepared by incubating 10 pmol of protein with 2 μCi of [γ-32P]GTP (30 Ci/mmol, NEN Life Science Products) in a 50-μl volume of 25 mM Tris, pH 7.5, 5 mM EDTA, 0.2 mM MgCl2, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 4 μM GTP for 15 min at room temperature. GTP hydrolysis was initiated by raising MgCl2 and GTP to a final concentration of 20 mM and 200 μM, respectively, and was stopped after 3 min by the addition of 2 ml of ice-cold 50 mM Tris, pH 8, 35 mM NaCl, 1 mM dithiothreitol, 150 μM NaiCl. Amounts of 20 pmol of MgcRacGAP or Bcr GAP domains during the GTP hydrolysis step were determined by radioactivity counting after rapid vacuum filtration of the samples on BA 85 nitrocellulose (Schleicher and Schuell). Typically, 100% of [γ-32P]GTP bound to GTPases were detected by radioactivity counting after rapid vacuum filtration of the samples on BA 85 nitrocellulose (Schleicher and Schuell). Typically, 100% of [γ-32P]GTP bound to GTPases was around 20,000 cpm. GAP assays were conducted in the presence of 20 pmol of MgcRacGAP or Bcr GAP domains during the GTP hydrolysis step, and similar data were obtained at least three times. In an additional set of experiments, substrate specificity of MgcRacGAP toward Rho GTPases was determined as described previously (16), using decreasing amounts (20 pmol to 0.4 pmole) of GTP domain during GAP assays; measurements were done in duplicate after 5 min of GTP hydrolysis. GTP exchange reactions during the GTP hydrolysis step were checked using [α-32P]GTP-preloaded GTPases under similar conditions and were found not to be significantly affected by the presence of MgcRacGAP.

**In Situ Hybridization Analysis**—An 5′-UTP-labeled antisense mgcRacGAP cDNA probe was generated by linearizing with Kpnl a pExLox plasmid containing a 1837-bp insert of mgcRacGAP cDNA and transcribing with SP6 RNA polymerase. An SGP-2 antisense RNA probe used in control experiments was synthesized by SP6 transcription of a 1,197-bp subclone of rat SGP-2 cDNA in the pGEMblue vector, kindly donated by Dr. Michael W. Collard of Utah State University.

6-μm paraffin sections of formalin-fixed adult human testis were hybridized overnight at 55 °C, washed as described (17) and exposed to Ilford K5 emulsion for 6 days. After development of the emulsion, the sections were stained with toluidine blue.

**RESULTS AND DISCUSSION**

In a search for new targets of Rac GTPase, we have used a two hybrid procedure in yeast (11, 12) to screen a Jurkat cDNA library (13) with a constitutively activated form of Rac1. Rac1 variant rather than Rac1V12 was chosen as a bait, since Mad 2 was shown previously to interact with a higher affinity with target and GAP molecules (14, 18). A 1,837-bp cDNA potentially encoding a new Rho GAP protein was isolated. Screening of a λ cDNA library from human placenta with the 5′ end of this cDNA as a probe allowed the isolation of an overlapping 1,837-bp cDNA insert. Combination of the two sequences yielded a 2,840-bp sequence, showing an open reading frame of 1,581 bp. This open reading frame starts with an ATG codon in a Kozak consensus sequence (19, 20) and encodes a putative protein of 527 amino acids with a molecular mass of 58,277 kDa. Stop codons are found in all three reading frames upstream of the start codon. In addition to the three blocks conserved among Rho Gaps (Fig. 1), the predicted protein exhibits a large N-terminal region including a cysteine-rich sequence with a C6H2 structure resembling a zinc finger and previously described in several isoforms of protein kinase C (PKC) (21). Therefore, this protein, which we call MgcRacGAP, appears clearly related to chimaerins that consist of a Rho GAP domain coupled to a PKC-like cysteine-rich motif with variable N-terminal sequences (8, 21).

FASTA and BLAST programs were used to search for homologies in protein data banks. By both methods, the best scores were obtained with Dro sophila RnRacGAP and human and rat n- and β-cha iner ions. Despite the phylogenetic distance between human and Dro sophila, the closest homolog to human MgcRacGAP is the Dro sophila RnRacGAP protein encoded by the rotund locus (10). The two proteins show 40% identity (60% similarity) in the region encompassing the cysteine-rich and GAP domains (Fig. 2), whereas identity scores of MgcRacGAP or RnRacGAP with other mammalian members of the Chi a merin family in this region do not exceed 30%.

In PKC and n-cha iner ions, cysteine-rich domains have been shown to bind phorbol esters and to mediate lipid-dependent regulation of enzymatic activity (21, 22). Comparative analysis of cysteine-rich domains in those proteins have indicated a 14-amino acid consensus sequence for phorbol ester binding (23, 24). However, low matching scores for both MgcRacGAP (10/14) and RnRacGAP (9/14) make them unlikely as phorbol ester receptors. Moreover, in a putative loop (amino acids 198–210 in MgcRacGAP) of zinc-finger-like domains, MgcRacGAP and RnRacGAP exhibit very similar basic sequences not found in other mammalian chimaerins (see Fig. 2B). This may indicate a conserved and specific function for this region in both proteins. Analysis of N-terminal sequences of MgcRacGAP and RnRacGAP did not reveal an SH2 domain as was found in α2 and β2 forms of mammalian chimaerin 2s (25, 26).

To confirm that the protein encoded by m gcRacGAP cDNA actually displays a GAP activity, we expressed the GAP domain (amino acids 238–513) of MgcRacGAP in E. coli and examined its ability to stimulate Rho GTPases. The GAP domain of MgcRacGAP exhibited strong GAP activities toward Cdc42 and Rac1 (or Rac2) but appeared much less active on RhoA (Fig. 3).

In another set of experiments, under rate-limiting conditions of GAP concentration, MgcRacGAP was estimated to be equally active on Rac1 (or Rac2) but appeared much less active on RhoA (Fig. 3). Therefore, this protein, which we call MgcRacGAP, appears clearly related to chimaerins that consist of a Rho GAP domain coupled to a PKC-like cysteine-rich motif with variable N-terminal sequences (8, 21).

**Amino acid sequence of MgcRacGAP**—The amino acid sequence of MgcRacGAP was deduced from the open reading frame detected in cDNA. In the cysteine-rich region, delimited by brackets, Cys and His residues conserved in the similar regions of chimaerins and PKCs are in bold characters; boxed sequences correspond to the three blocks conserved in RhoGaps.
active on Rac1 and Cdc42, although being 30 times less active on RhoA (Table I). In addition, MgcRacGAP did not significantly stimulate GTPase activity in Rap2a nor in Rab6 (Fig. 3). Non-chimaerin mammalian Rho GAPs such as Bcr and Abr proteins have been previously reported to have the same specificity as MgcRacGAP toward Cdc42 and Rac (5, 27); on the contrary, MgcRacGAP appears functionally distinct from other mammalian chimaerins, which are active primarily on Rac (8, 9, 28). The substrate specificity of RnRacGAP toward either mammalian or Drosophila Rho GTPases has not yet been reported.

Northern blot analysis revealed that an ~3.2-kilobase mgcRacGAP transcript is present in low abundance in most tissues and is highly expressed in testis (Fig. 4A). Antibodies to the GAP domain (amino acids 238–513) of MgcRacGAP were raised in chicken, affinity purified, and found to recognize recombinant GAP domain (Fig. 4B) without showing cross-reaction with two other recombinant mammalian RhoGAPs, Bcr and RLIP (29) (not shown). Western blot experiments using these antibodies allowed the detection of a major protein of ~60 kDa in testis extracts (Fig. 4B). This protein was not present in detectable amounts in several other tissues tested (except for a

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**Fig. 2. Sequence homology with RnRacGAP.** A, amino acid sequence homology of human MgcRacGAP and Drosophila RnRacGAP. Identical amino acids are represented by double dashes. Single dashes indicate conservative changes. B, alignment of cysteine-rich domains of MgcRacGAP and other chimaerins. Asterisks indicate conserved Cys and His residues characteristic of the C6H2 motif. Homologous residues in a putative loop of MgcRacGAP and RnRacGAP cysteine-rich domains are indicated in **bold** characters. MgcGAP, MgcRacGAP; RnGAP, RnRacGAP; NCHIh, human n-chimaerin; NCHIr, rat n-chimaerin; BChIh, human β-chimaerin.

**Fig. 3. GTPase-stimulating activity of MgcRacGAP.** GTP-hydrolyzing activity of various recombinant GTPases was measured in the presence or absence of recombinant GAP domain of MgcRacGAP or Bcr. Amounts of [γ-32P]GTP remaining bound to GTPases after 3 min were determined by a filter-binding assay and expressed as a percentage of the initial amount of GTP bound to each protein. Data shown are representative of at least three independent experiments.
TABLE I
Relative GAP activities of MgcRacGAP on Rho GTPases

| Rho GTPases | GAP activity |
|-------------|--------------|
| Rac         | 100 (12 nM)  |
| Cdc42       | 86 (14 nM)   |
| RhoA        | 3 (400 nM)   |

trace amount in fetal muscle) (not shown), thus confirming at the protein level that MgcRacGAP is preferentially expressed in testis. MgcRacGAP was also detected at a high level in human germ cell tumors (Fig. 4B). Equal amounts of protein extracts from a human germ cell tumor and from normal testis tissue of the same patient were run parallel to a liver extract (negative control) and recombinant MgcRacGAP (amino acids 234–512) (positive control). MgcRacGAP was detected using affinity-purified chicken antibodies.

Fig. 4. Tissue distribution of mgcRacGAP mRNA and protein. A, mgcRacGAP cDNA was used to probe multiple tissue Northern blots. Each lane contains two micrograms of poly(A)+ RNA from the indicated tissues. B, Western analysis of MgcRacGAP. Equal amounts of protein extracts from a human germ cell tumor and from normal testis tissue of the same patient were run parallel to a liver extract (negative control) and recombinant MgcRacGAP (amino acids 234–512) (positive control). MgcRacGAP was detected using affinity-purified chicken antibodies.

spermatogonia and mature spermatozoa were not above background. The developmental- and tissue-specific expression of the mgcRacGAP gene further supports the view of a close similarity with the rnRacGAP gene that was found by in situ hybridization to Drosophila testis specifically expressed in primary spermatocytes (10).

The sequence data reported here indicate that the mRacGAP gene encodes a Rho GAP belonging to the Chimaerin family of proteins, one most similar to Drosophila RnRacGAP. Although mgcRacGAP mRNA can be detected in many tissues, it is preferentially expressed in germ cells, specifically in spermatocytes. Taken together, these features distinguish MgcRacGAP from other known mammalian chimaerins and conversely, show a striking resemblance to Drosophila RnRacGAP, thus suggesting that MgcRacGAP is a mammalian homolog of rnRacGAP gene product.

In Drosophila, RnRacGAP is encoded by one of two transcripts of the rotund locus (10, 30). Genetic analyses of the rn locus have defined the rn null mutant phenotype as male and female sterility and an absence of structures in the subdistal part of the appendages (31). It was recently shown that a RnRacGAP transgene can rescue male sterility but not appendage morphogenetic function nor female sterility in flies deficient for the rn locus (32), implying that RnRacGAP is essential to development and/or function of male germ cells. Assuming that MgcRacGAP and RnRacGAP operate in equivalent pathways in mammalian and insect spermatocytes, respectively, the mgcRacGAP gene may prove to play a key role in spermatogenesis and male fertility in mammals as well.

Recent studies with n-chimaerin suggest that Rho GAPs of this family act as effectors for Rho GTPases and that this function requires the N-terminal non-GAP portion of the protein (7). Therefore, isolation of partners of the MgcRacGAP
N-terminal region in germ cells should provide new insights into Rac/Cdc42 signaling pathways in those cells.

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