The small GTP-binding proteins Rac1 and Rac2 are critically important in regulating multiple signal-transduction pathways in eukaryotic cells. Here we report the isolation of a novel third Rac family member, Rac3. Rac3 differs from Rac1/2 at its carboxyl-terminal end, a domain associated with subcellular localization and binding to specific cellular regulators. Rac3 mRNA expression patterns differ from those of Rac2, which is hematopoietic specific and also from those of Rac1. The Rac3 gene was mapped to chromosome 17q25–25, a region frequently deleted in breast cancer. Rac3 protein levels are not affected by organization of the actin cytoskeleton but remarkably, are serum-inducible. Rac3 is an active GTPase, and this activity is regulated by Bcr. When constitutively activated, Rac3 is able to stimulate efficiently the c-Jun amino-terminal kinase signaling pathway. These findings support a role for Rac3 in intracellular signaling.

The small G proteins Rac1 and Rac2 are highly related GTPases belonging to the Rho subfamily of Ras proteins (1–3). Rho family members regulate the organization of the actin cytoskeleton. Specifically, activation of Rac is associated with actin reorganization accompanying the appearance of lamellipodia and membrane ruffles in fibroblasts (4, 5). In neuroblastoma cells, Rac1 stimulates the formation of lamellipodia leading to neurite development (6). Activated Rac stimulates F-actin uncapping and morphological transformation of platelets but inhibits receptor-mediated endocytosis (7, 8). In neutrophils and macrophages, Rac is essential for activation of a multiprotein complex that produces superoxide in phagocytes, the NADPH oxidase (9–11). Moreover, Rac1 regulates intracellular reactive oxygen species production in fibroblasts and the activity of the redox-dependent transcription factor nuclear factor-κB (12, 13).

Rac participates in signal transduction from the membrane to the nucleus via two distinct mitogen-activated protein kinase cascades. Exposure of cells to endotoxins, proinflammatory cytokines, or hyperosmolarity effects the activation of Rac (14). These findings support a role for Rac in in vivo signaling.

Cloning and Sequencing of RAC3—A 350-base pair fragment from the coding region of mouse rac1 was used to screen a K562 agt10 cDNA library. Four cDNAs were isolated and partially sequenced. The 5'-untranslated and coding region of cDNA clone R10 of 1.0 kb was sequenced completely on both strands.

Cell Lines—The K562, DU4475, HL60, SCAβER, Swiss 3T3, and COS-1 cell lines were obtained from the ATCC, Rockville, MD. The cell line GM04155 was from NIGMS Human Genetic Mutant Cell Repository, Camden, NJ, and 5838 was kindly provided by Dr. T. Triche. Spodoptera frugiperda Sf9 insect cells were from Invitrogen, San Diego.

Expression of mRNA—RNAs were isolated using the LiCl-urea method (27). Total RNAs (15 μg) were run on guanidine thiocyanate-agarose gels (28), blotted to Hybond-N (Amersham Corp.), and hybridized as described (29). A multiple tissue Northern blot (CLONTECH) was used to analyze tissue distribution of RAC3 in human. For detecting RAC3, a 0.24-kb BamHI probe from the 3'-untranslated region was used and blots were washed to 0.01 × SSC at 65 °C. RAC1 was detected with a 0.21-kb NcoI-EcoRI probe and RAC2 with a 0.28-kb MscI-PstI probe, both of which included 3'-untranslated sequences.

Chromosomal Localization—Somatic cell hybrid and regional mapping panels for chromosome 17 were from the NIGMS Human Genetic Characterization of RAC3, a Novel Member of the Rho Family*

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The nucleotide sequence(s) reported in this paper has been submitted solely to indicate this fact.

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* This work was supported in part by funds provided by the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California Grant 1RB-0001 and by Public Health Service National Institutes of Health Grants CA47456 and CA 50248. 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 GenBankTM/EBI Data Bank with accession numbers AF008591.

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The abbreviations used are: JNK, c-Jun amino-terminal kinase; kb, kilobase(s); GST, glutathione S-transferase.
Mutant Cell Repository. DNAs were digested with EcoRI and hybridized with a 0.45-kb XbaI fragment from the 3'-untranslated region of RAC3.

Preparation of GST-fusion Proteins—The entire BCR, RAC1, RAC2, or RAC3 coding region was inserted into the baculovirus transfer vector pAcG2T (PharMingen). Two μg of plasmid DNA was cotransfected to Sf9 cells with BaculoGold™ virus DNA according to the manufacturer’s instructions. Sf9 cells infected with two amplified viral stock of GST-Bcr were collected after 4 days and lysed in IP-lysis buffer (PharMingen). GST-Bcr fusion proteins were purified on glutathione-agarose (Sigma). After infection of Sf9 cells with GST-Rac3 viral stock, cells were sonicated in 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1% Triton X-100, 1 mM phenylmethanesulfonylfuoride, 10 μM leupeptin, 10 μg/ml aprotinin (buffer A). The supernatant was applied to glutathione-agarose column and protein eluted with 20 mM glutathione, 50 mM NaCl, pH 8, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol. Protein fractions were dialyzed against 50 mM Tris, pH 7.5, 2 mM dithiothreitol, 2 mM MgCl₂ and concentrated using Centri- con-10 cartridges (Amicon).

In addition, total cellular extracts were prepared from GST-Rac expressing Sf9 cells. RAC1 was also included into pGEX-2T (Pharmacia Biotech Inc.) and expressed as a GST-fusion protein in Escherichia coli. Bacteria were sonicated in buffer A without Triton X-100 and purified on glutathione-agarose (see above).

GST-pulse-activating protein assay—GST-pulse-activating protein activity was determined by measuring GAP hydrolysis as described (22). Briefly, Rac1 and Rac3 were loaded with [γ-32P]GTP by incubation of the GST-Rac fusion protein in 25 mM Tris, pH 7.5, 4.7 mM EDTA, 1 mM dithiothreitol, 0.1 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 20 μM unlabeled GTP, and 10 μM γ-[32P]GTP (5,000 Ci/mmol, Amersham) at 30 °C for 4 min. Equally loaded aliquots (as measured by cpm bound) of Rac1 and Rac3 were used for GST-pulse assays. 200 ng of GST-Bcr fusion protein was added to each of four samples. Hydrolysis was initiated by the addition of MgCl₂ and GTP to final concentrations of 17 mM and 170 μM, respectively, and [γ-32P]GTP remaining bound to protein was determined by filtration analysis (29).

Western Blotting and Immunodetection—Antibodies specific to Rac3 were raised in rabbit against amino acid residues 182–192 and were affinity purified (Zymed Laboratories). For immunoblotting, Rac3 antibody was used at a final concentration of 250 ng/ml. Antibodies against JNK1 (C-17) and GST were from Santa Cruz. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Hybond-ECL nitrocellulose (Amersham), probed with antibodies as described (30), and visualized using ECL (Amersham).

Rac3 Protein in Swiss 3T3 Cells—Swiss 3T3 cells were treated for 2 h with 10⁻³ or 10⁻⁴ M cytochalasin B (Sigma) dissolved in dimethyl sulfoxide (Sigma). The final concentration of dimethyl sulfoxide in the culture medium was 0.1%. Induction of Rac3 was studied by using serum-starved Swiss 3T3 cells for 24 h in Dulbecco’s modified Eagle’s medium plus 2% charcoal-stripped serum and stimulating with 10% fetal bovine serum for 1 h. Protein concentrations were determined using the BCA method (Pierce). 10 μg of total cellular protein was loaded per lane.

Transfection—For eukaryotic expression studies, Rac3 was cloned into an SV40-based mammalian vector. To generate a constitutively activated Rac3 protein (V12Rac3), the glycine at codon 12 was replaced into an SV40-based mammalian vector. To generate a constitutively active Rac3 protein (V12Rac3), the glycine at codon 12 was replaced by using lipofection. After 66 h, cells were lysed in 2x Laemmli sample buffer.

JNK assay—COS-1 cells were transfected with V12Rac3 or were mock transfected. 27 h after transfection, the percentage of fetal bovine serum was lowered from 10 to 0.5%. Lysates were prepared after 18 h. As positive control, COS-1 cells were stimulated with 0.4 μM sorbitol for 1 h to induce osmotic shock. Cells were lysed in Triton lysing buffer (30). Endogenous JNK was immunoprecipitated from cleared lysates by incubation with JNK1 antiserum for 2 h at 4°C. Immunocomplexes bound to protein A-agarose beads were washed four times with lysis buffer and once with kinase buffer (31). Immunoprecipitated JNK activity was determined using a method reported previously (15). GST-c-Jun (1–79) was kindly provided by Dr. G. M. Bokoch. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The relative amount of GST-c-Jun phosphorylation was quantitated by scintillation counting of excised bands. The presence of Rac3 and similar levels of JNK1 protein were confirmed by Western blot analysis.

RESULTS

Isolation of a Rac-related cDNA Clone—The human genome contains many Rac-related sequences. To identify expressed sequences, a 0.35-kb murine rac1 cDNA fragment was used to screen a human K562 cell line cDNA library. Four clones were isolated, of which two represented RAC1 and one RAC2. The fourth, R10, with an insert of 1.0-kb, was sequenced (Fig. 1). Data base searches revealed that its nucleotide sequence was homologous to but clearly distinct from that of Rac1 (77% identity), Rac2 (83%), or RHOG (69%). Because of its high degree of homology to Rac1/2, we named our clone RAC3 (Fig. 2).

The R10 cDNA displayed an open reading frame, able to encode a protein of 192 amino acids. The clone also contained 70 base pairs of 5′-noncoding and 450 base pairs of 3′-untranslated regions. Interestingly, this 3′ region is well conserved between man and rodents (also see Fig. 3, lane 8), suggesting functional significance. Similar to mouse rac1, the 5′-untranslated region of RAC3 is exceptionally GC-rich (87%) albeit much shorter, with the potential to form hairpin loops (2).

Chromosomal Localization of RAC3—The 3′-untranslated region of RAC3 was chosen to prepare a highly specific probe. This probe detected a single band on Southern blots (see Fig. 3, lane 7) and did not cross-hybridize to the RAC1 or RAC2 cDNAs (not shown). A panel of somatic cell hybrid DNAs from hybrids that each retain one intact human chromosome was then used to localize RAC3 chromosomally. Only a single hybrid contained sequences hybridizing to the RAC3-specific probe, locating RAC3 on chromosome 17 (not shown). RAC1 and RAC2 were present on different chromosomes (not shown). To sublocalize RAC3 on chromosome 17, a regional mapping panel for human chromosome 17 was utilized. Human RAC3

2. L. Haataja, J. Groffen, and N. Heisterkamp, unpublished observations.
Rac3

Rac1

Rac2

-32P\]GTP, and the rate of hydrolysis was measured using a filter binding assay. As shown in Fig. 5, Bcr stimulated the GTPase activities of Rac3 and Rac1 in a similar fashion.

Rac3-specific Antiserum—To be able to detect Rac3 protein, antisera were raised against a carboxyl-terminal peptide of Rac3 containing a maximum degree of divergence with Rac1 and Rac2. Affinity-purified antibodies reacted only with GST-Rac3 (Fig. 6A, lane 2) but not with either GST-Rac1 or 2 (lanes 3 and 4), although all three extracts contained GST-Rac fusion proteins reacting with GST antibodies (Fig. 6A). Rac3 antibodies also clearly detected Rac3 overexpressed in COS-1 cells (Fig. 6B, lane 2) and endogenous Rac3 (lane 1).

Regulation of Rac3 Expression—Previous studies have shown that Rac1 expression levels can be modulated by the state of the actin network (2). To investigate this, endogenous Rac3 protein was analyzed in Swiss 3T3 cells. Treatment of these cells with 10^{-6} or 10^{-7} M cytochalasin B had no apparent effect on Rac3 levels (Fig. 6C, lanes 2–4), although this treatment caused a prominent morphological change in the cells caused by disruption of the actin cytoskeleton (not shown). Similarly, Rac3 levels were unaffected by detachment from the substratum (lane 5). However, deprivation of cells from serum for 24 h caused a decrease in Rac3 levels (Fig. 6C, compare lanes 6 and 7). Stimulation of the serum-starved cells with 10% fetal bovine serum for 1 h resulted in a return to normal Rac3 levels (lane 8).

V12Rac3 Activates Endogenous JNK—To investigate whether activation of Rac3 would affect signaling, it was mutated to a constitutively activated form by replacing glycine at position 12 with a valine. Overexpression of V12Rac3 in COS-1 cells and the activation of endogenous JNK was examined by phosphorilation of GST-c-Jun. As shown in Fig. 7 (bottom), mock-transfected, V12Rac3-transfected, and osmotically shocked COS-1 cells contained comparable levels of endogenous JNK. Exposure of cells to 0.4 M sorbitol resulted in a maximal stimulation of 4-fold of endogenous JNK activity. Overexpression of the constitutively active Rac3 resulted in the activation of JNK as measured by the 2-fold increased phosphorylation of the GST-c-Jun substrate (Fig. 7, top).

DISCUSSION

We have identified a new member of the Rho family, Rac3, which has a high degree of amino acid identity with human Rac1 and Rac2 (92 and 89%, respectively). This raises the question of why three highly related proteins exist. Despite this close degree of relatedness, it is likely that each Rac species plays a distinct regulatory role in vivo, although there may be overlap both in vitro and in vivo. Differences among the three could be found in the regulation of their GTPase activity through interaction with other proteins or at the level of gene expression.

With respect to protein structure, experiments with mutant Rac proteins have delineated domains of functional importance for the interaction of both Rac1 and Rac2 with effectors and other regulatory molecules. Two effector sites are needed for in vitro activation of the NADPH oxidase component p67^phox_. Rac-induced actin polymerization in fibroblasts, and interaction with p65^PAK in vitro (33). The amino-terminal effector site is identical in all three Rac proteins, and the carboxyl-terminal effector site in Rac3 is 94 and 91% identical to the homologous region in Rac1 and Rac2, respectively (Fig. 2 and Ref. 33). This suggests that Rac3 is also capable of binding p67^phox and p65^PAK at least in vitro. In addition, the carboxyl-terminal residues have been identified as an important region for NADPH oxidase and JNK activation (34–36).
Our experiments on the functional activity of Rac3 show that it is a GTPase. Like Rac1, it interacts with Bcr, which enhances its GTPase activity in vitro. COS-1 cells overexpressing a constitutively activated Rac3 show a physiologically significant 2-fold activation of endogenous COS-1 JNK compared with its 4-fold activation by osmotic stress. This demonstrates that Rac3 is able to participate in the stress activation pathway.

The greatest divergence among Rac3, Rac1, and Rac2 occurs at residues 180–192, which are also hypervariable regions in the Ras proteins (37). Indeed, the only functional difference demonstrated to date between Rac1 and Rac2 using an in vivo assay is that p67phox interacts 6-fold better with Rac2 than with Rac1 (38). Therefore, this region may specify differences in in vivo binding activities among the three Rac proteins. All three Rac proteins contain a Cys-A-A-X-COOH sequence, in which A is an aliphatic residue and X is any amino acid. In vivo in Rac1, the cysteine residue becomes geranylgeranylated, followed by proteolytic removal of the A-A-X residues and carboxyl methylation of the isoprenylated cysteine (39). This posttranslational modification is important for specific intracellular localization and interaction with target proteins (19, 40, 41). Thus, the identified differences between Rac3 and Rac1/2 in their very carboxy-terminal end may define differences in subcellular localization and/or binding to specific regulatory molecules.
FIG. 7. Activation of endogenous JNK by activated RAC3. COS-1 cells were mock-transfected (lane 1) or transfected with V12RAC3 (lane 2). Treatment of cells with 0.4 M sorbitol for 1 h was used as a positive control for JNK activation (lane 3). Similar data were obtained in total cellular lysates from transfected COS-1 cells using a Western blot (bottom). Similar data were obtained in two independent experiments.

this treatment. Surprisingly, Rac3 expression was down-regulated in quiescent fibroblasts and was clearly induced by serum stimulation. This suggests the Rac3 promoter is serum-responsive. To date, of the extended Rho family, only RacB was shown to be serum-inducible; there have been no reports indicating that either Rac1 or Rac2 expression is modulated by serum. RacB is closely related to RacA and RacC on an amino acid level. In contrast to RacA and RacC, RacB does not have a clearly defined function in actin cytoskeletal reorganization, but rather it appears to be involved in cell proliferation (42, 43).

In view of the increasing evidence for critical roles of Rho family members in actin reorganization and cellular signaling including cancer, it will be of interest to define the specific role of Rac3 in these processes. This, in addition to our finding that RAC3 is located at chromosome 17q23–25, a region frequently deleted in breast cancer (44, 45), provides further impetus to investigate Rac3 expression and activity in this type of malignancy.

Acknowledgments—We thank Vesa Kaartinen for critical reading of the manuscript and Ron de Jong and Jacqueline Velmaat for introduction of the JNK assay.

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J. Biol. Chem. 1997, 272:20384-20388.
doi: 10.1074/jbc.272.33.20384

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