Biochemical and Biological Characterization of a Human Rac2 GTPase Mutant Associated with Phagocytic Immunodeficiency*

Yi Gu‡§, Baoqing Jia¶, Feng-Chun Yang§§, Maria D’Souza‡, Chad E. Harris‡¶, Caroline W. Derrow¶, Yi Zheng¶, and David A. Williams‡¶¶

Received for publication, November 20, 2000, and in revised form, January 24, 2001
Published, JBC Papers in Press, February 22, 2001, DOI 10.1074/jbc.M010445200

The Rho GTPase family, including Rho, Cdc42, and Rac, is a growing subgroup of Ras proteins. A number of studies have shown that Rho GTPases are involved in multiple cellular processes such as actin polymerization and cytoskeleton reorganization, regulation of gene transcription, cell cycle progression, and cell survival. Rho GTPases were initially found to be required for the regulation of actin polymerization in eukaryotic cells. In these and subsequent studies it has been demonstrated that Rho activates the assembly of actin-myosin stress fibers and focal adhesions, Rac induces lamellipodial extension and membrane ruffling, and Cdc42 promotes filopodia or microspike formation (for review, see Refs. 3–6). To date, Rho GTPases have been implicated in cell adhesion, cell motility, cytokinesis, and membrane trafficking (7–9). When microinjected into fibroblasts, it has also been reported that Rho, Rac, and Cdc42 stimulate cell cycle progression through G1 and DNA synthesis (10); and expression of Rho, Rac, and Cdc42 activates gene transcription via serum response factor and nuclear factor κB (11, 12). Studies of downstream signaling pathways have shown that Rac and Cdc42 are distinguished from Rho by activating c-Jun kinase, also known as stress-activated protein kinase (10). In addition, cross-talk between Rho proteins has also been observed. Cdc42 acts as a stronger activator of Rac, and activated Rac can activate or inhibit Rho depending on the cells involved (3, 13). Very little is known about how Rho GTPases differentially orchestrate cellular processes and signaling pathways in different mammalian cell lineages.

The Rac subfamily of Rho GTPases has three highly homologous members, Rac1, Rac2, and Rac3. Unlike Rac1 and Rac3, which are widely expressed, Rac2 is found only in hematopoietic cells (14–17). In addition to its reported roles in actin remodeling, the Rac proteins have been implicated in the generation of O2·− via the phagocytic NADPH oxidase complex (for review, see Ref. 18). Rac1 was identified as an oxidase-related factor from guinea pig peritoneal macrophages (19), whereas Rac2 was demonstrated as an oxidase-related factor in human neutrophils (20). In a cell-free system, both Rac recombinant proteins can reconstitute a fully active NADPH oxidase complex that produces superoxide (21). In mice, gene targeting studies have demonstrated that the loss of Rac2 activity decreases superoxide production in unactivated bone marrow neutrophils in response to some but not all agonists (22). This finding is consistent with other previous results suggesting that Rac2 is a primary GTPase regulator in this cellular event (23, 24). Rac2 activity was also found to be required for neutrophil chemotaxis both in vivo and in vitro, in part because of the reduced F-actin formation in response to chemoattractants (22). In addition, Rac2-deficient mast cells display diminished cell migration in response to stem cell factor (25). Surprisingly,
Rac2—/— mast cells also demonstrate a significant decrease in growth factor-dependent survival. Studies of kinase signaling pathways indicate that Rac2 function is required for activation of Akt downstream of phosphatidylinositol 3-kinase (25). In addition, Rac2 appears to be critical for the appropriate expression of Bcl2 family members Bcl-X<sub>L</sub> and BAD in these cells. Although ectopic expression of activated Rac mutants in fibroblasts has been demonstrated to stimulate cell cycle progression through G<sub>1</sub> and subsequent DNA synthesis (10), no significant cell proliferation defect has been found in Rac2—/— neutrophils or mast cells (25).

Like all other GTPases, Rac2 functions as a molecular switch by cycling between an inactive GDP-bound form and an active GTP-bound form. A number of proteins, such as guanine exchange factors (GEFs)<sup>1</sup> and GTPase-activating proteins, regulate the ratio of GTP/GDP-bound forms which determines the activities of Rac GTPases (8). Mutations that disrupt the cycling of the two forms of GTPases have been identified in the guanine nucleotide binding domain or the effector domain regions. These mutations have been shown in Rac, Rho, and Cdc42, and in some cases are similar to mutations reported in Ras. Mutations at positions 12 (G12V) and 61 (Q61L) inhibit GTP hydrolysis to produce constitutively active mutants (26), and a mutation at position 17 (T17N) destabilizes the guanine nucleotide-binding site resulting in a dominant negative mutant (27).

Recently, Ambroso et al. (2) and our laboratory (1) identified a genetic mutation (D57N) in Rac2 GTPase which is associated with a human phagocyte immunodeficiency. The patient suffered severe recurrent infections and defective neutrophil cellular functions similar to those found in Rac2—/— mice. The patient’s genotype wild type (WT) Rac2/D57N Rac2 (2) was associated with normal Rac2 message levels (1) and normal cDNA sequences in 50% of cloned cDNAs (1). Neutrophils derived from normal human bone marrow progenitor cells transduced by the cloned D57N Rac2 cDNA mimic the patient’s neutrophil phenotypes of decreased cell migration and superoxide production (28). All of these data suggest that the D57N protein had a dominant negative effect on WT Rac2 protein. In addition, expression of the D57N mutant in NIH/3T3 cells, which express Rac1 but not Rac2 endogenously, resulted in diminished growth, abnormal cell shape, and reduced membrane ruffling in response to PDGF. These data imply that the D57N mutant may affect the function of other Rac GTPases and that the patient’s phenotype could be caused by dysfunction not only of Rac2, but also of Rac1, function.

To examine carefully the biochemical and biological consequences of D57N Rac2 expression in hematopoietic cells further, we studied recombinant protein in vitro and introduced this mutant into murine hematopoietic stem and progenitor cells via retrovirus-mediated gene transfer. Biochemical analyses show that D57N Rac2 has significantly decreased binding affinity for GTP resulting from a very rapid dissociation rate of the mutant into murine hematopoietic stem and progenitor cells. Biochemical analysis and introduction of the cloned D57N Rac2 cDNA mimic the patient’s severe recurrent infections and defective neutrophil cell phenotypes of decreased cell migration and superoxide production, as well as increased apoptosis. Long-term engraftment in vivo after bone marrow transplantation was also impaired significantly. As expected, neutrophils derived from D57N-transduced myeloid progenitors displayed reduced chemotaxis and superoxide generation. On the other hand, overexpression of WT Rac2 or the constitutively activated mutant Q61L was associated with increased proliferation of normal cells and slightly reduced apoptosis. Thus, D57N Rac2 appears to affect the activity of both Rac2 and Rac1. Although normal Rac2 expression and function appear critical for the growth factor-induced survival, overexpression of Rac2 is associated with increased proliferation. The data imply that Rac proteins are critical regulators of both actin function and cell survival/proliferation in hematopoietic cells.

**Experimental Procedures**

**Protein Expression and Purification**—The human D57N Rac2 mutant cDNA was cloned into the bacterial expression plasmid pET-28a at EcoRI and XhoI sites; and WT human Rac2 cDNA was cloned into the pET-15b plasmid without the 11-amino acid T7 tag (Novagen, Milwaukee, WI). The Rac2 constructs were expressed in the Escherichia coli BL21 strain as (His)<sub>6</sub>-tagged fusion proteins. Recombinant proteins were purified through the His-binding-resin as described in the manufacturer’s protocol. The isolated proteins were >90% pure judged by Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (New England Biolabs, Beverly, MA).

**Guanine Nucleotide Binding and Exchange Assays**—GST and [H]<sub>3</sub>GTP and [H]<sub>3</sub>GDP (PerkinElmer Life Sciences) were preloaded onto 1–2 μg of recombinant Rac2 proteins (WT and D57N) in the presence of 100 μM NaCl, 20 μM Tris-HCl, pH 7.6, 2 mM EDTA, and 1 mM GTP for 20 min at room temperature. The labeled Rac2 protein complexes were stabilized by adding MgCl<sub>2</sub> to a final concentration of 5 mM. Binding of guanine nucleotides to Rac2 proteins was determined by counting radioactivity bound to filters as described previously (29, 30). Dissociation assays were performed by adding activation solution (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM DTT, 1 mM GTP, and 5 mM MgCl<sub>2</sub>) to the preloaded reaction mixture (26), and the remaining bound radioactivity examined at different time points by filtration (Midwest Scientific, Valley Park, MO).

To measure GEF-stimulated change in nucleotide binding, 1 μg of recombinant Rac2 proteins was preloaded with [H]<sub>3</sub>GDP and exchanged for cold GTP in a buffer of 100 mM NaCl, 20 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.5 mM GTP with or without the addition of GEF TrioN. The remaining bound [H]<sub>3</sub>GDP (Amersham Pharmacia Biotech) was determined at five different time points by filtration.

**Exchange Factor Binding Assay**—Complex formation of His-tagged WT Rac2 and D57N Rac2 with GST-TrioN was carried out as described for the Dbl-G protein interactions (31). Briefly, 1 μg of purified (His)<sub>6</sub>-WT Rac2 or (His)<sub>6</sub>-D57N Rac2 in a buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 0.5% Triton 100, and 1 mM DTT was incubated with 2 μg of GST or GST-TrioN immobilized on agarose beads for 30 min at 4 °C under constant agitation. The coprecipitates were washed three times with the incubation buffer and were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blot analysis using anti-His polyclonal antibody (Amersham Pharmacia Biotech). The immune complexes were visualized by chemiluminescence reagents.

---

<sup>1</sup> The abbreviations used are: GEF(s), guanine nucleotide exchange factor; WT, wild type; GST, glutathione S-transferase; PBD, p21 binding domain; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting; BM, bone marrow; LDBM, low density bone marrow; hG-CSF, human granulocyte colony-stimulating factor; MDG, megakaryocyte growth and development factor; SCF, stem cell factor; EGFP, enhanced green fluorescence protein-positive; GST-S, guanosine 5′-3′-O-[(thio)triphosphate]; PI, phosphatidylinositol; PDI, phosphatidylethanolamine; TUNEL, terminal nucleotidyl transferase; fMLP, formylmethionyl-leucyl-phenylalanine; NBT, nitro blue tetrazolium; FMA, phorbol 12-myristate 13-acetate.
Human Mutation of Rho GTPase, Rac2
15931

Retroviral Vectors and Stable Packaging Cell Lines—WT mouse Rac2 and mutant Rac2 (human D57N and Q61L) cDNAs were cloned into a modified murine stem cell virus-based bicistronic vector (MIEG3); (1) at unique EcoRI and XhoI sites. The resultant retroviral plasmids were introduced into a Phoenix-ampho packaging cell line (American Type Culture Collection, Rockville, MD) at 27°C in 5% CO2. After 48 h, the culture was centrifuged on Histopaque-1083 (Sigma) and the supernatant was collected. LDBM cells were isolated from fresh BM by centrifugation on Histopaque-1083 (Sigma) gradient for 30 min at 1,500 rpm at room temperature. Cells at the interface were collected and washed twice on a hemocytometer. 20 million LDBM cells were plated on a 10-cm non-tissue culture Petri dish and prestimulated in RPMI medium supplemented with 10% fetal calf serum, 2% penicillin and streptomycin, cytokine (100 ng/ml HGF, 100 ng/ml MGF, 100 ng/ml SCF, all from Amgen, Thousand Oaks, CA) and 0.5% dextran. Cells were cultured in RPMI medium supplemented with 100 ng/ml murine interleukin-3 (PeproTech, Rocky Hill, NJ) and 0.5% dextran. Cells were cultured in the complete RPMI medium for 5 days at 37°C and then washed twice with RPMI and then counted on the filter in six random 400 microscope fields.

Mouse Lysed Bone Marrow (LDBM) Isolation and Viral Transduction—Whole bone marrow was collected from WT and Rac2−/− mice 48 h after treatment with 5-fluorouracil (150 mg/kg of body weight; American Pharmaceutical Partners, Los Angeles, CA) and was resuspended in RPMI medium (Life Technologies, Inc.). LDBM cells were isolated from fresh BM by centrifugation on Histopaque-1083 (Sigma) gradient for 30 min at 1,500 rpm at room temperature. Cells at the interface were collected and washed twice on a hemocytometer. 20 million LDBM cells were plated on a 10-cm non-tissue culture Petri dish and prestimulated in RPMI medium supplemented with 10% fetal calf serum, 2% penicillin and streptomycin, cytokine (100 ng/ml HGF, 100 ng/ml MGF, 100 ng/ml SCF, all from Amgen, Thousand Oaks, CA) and 0.5% dextran. Cells were cultured in the complete RPMI medium supplemented with 100 ng/ml murine interleukin-3 (PeproTech, Rocky Hill, NJ) and 0.5% dextran. Cells were cultured in the complete RPMI medium for 5 days at 37°C and then washed twice with RPMI and then counted on the filter in six random 400 microscope fields.

Virus-mediated LDBM transduction was performed as described previously (32). Briefly, 2 × 106 cells were infected by viral supernatant overnight on CH296 (Tokara Shuzo Co., Japan)-coated six-well plates. The infected cells were kept in culture with complete RPMI medium for 48 h. Cells were subsequently analyzed and sorted for green fluorescence using a FACSort or FACStar Plus (Becton Dickinson).

Western Blot and Affinity Precipitation of Active (GTP-bound) GTases Using GST-PBD Fusion Protein—Transduced and untransduced green fluorescence protein positive (EGFP+) BM cells were immunoblotted using mouse antibodies for Rac1 (1:500, a gift from Dr. Gary Bokoch, Scripps Institute, La Jolla, CA), Rac1 (23A8, 1:2,000, Upstate Biotechnology, Lake Placid, NY), Cdc42 (sc575G, 1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA), and total p38 (1:2,000, New England Biolabs). The immunoblots were developed by chemiluminescence (ECL, Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified using densitometry analysis software (Quantity One). The loading was checked by staining the gel with Ponceau-S (Sigma).

RESULTS

Sequestration of GEF by the D57N Rac2 Mutant—The G169A (aspartic acid to asparagine, D57N) mutation in Rho GTTPase Rac2 was originally identified in blood cells from a human patient and was associated with severe phagocyte immunodeficiency (1, 2). Along with D57N, similar mutations in the Ras proto-oncogene. These Ras mutations have been shown to bind GDP normally, but it binds to GTP at only 10% of the normal level. Biochemically, the D57N Rac2[γ35S]GTP complex is much less stable compared with that of WT Rac2. As shown in Fig. 1, WT Rac2 has a comparable intrinsic dissociation rate for both [γ35S]GTP and [γ35S]GTP has been dissociated in 15 min. D57N Rac2, on the other hand, retained a
similar rate of GDP dissociation, but it dissociates GTP significantly faster than WT Rac2, reaching about 75% in 2 min.

In vivo, Rac2 GTPase functions as a molecular switch through its transition between inactive GDP-bound and active GTP-bound forms. The exchange of GDP for GTP is catalyzed by GEFs. We further examined the D57N Rac2 exchange rate of GDP for GTP within 15 min at room temperature, and D57N Rac2 exchanged at a similar rate. In the presence of the GEF TrioN (36), WT Rac2 significantly increased the rate of GDP/GTP exchange such that 90% became GTP-bound in 15 min (Fig. 2A). In contrast, D57N Rac2 did not respond to TrioN. By immunoblotting the His-tagged GTPases, GST-TrioN was found to coprecipitate equally well with both WT Rac2 and D57N Rac2 (Fig. 2B). Because D57N Rac2 can still physically bind to TrioN, the lack of responsiveness to GEF is most likely the result of its poor GTP binding activity with consequent impaired turnover rates. Thus, these biochemical data suggest that D57N Rac2 may act in a dominant negative fashion by sequestering endogenous GEFs from other Rho GTPases in cells.

Expression of Rac2 Mutant in Hematopoietic Cells—To examine more carefully the biological effects of mutant Rac2 GTPases on a variety of hematopoietic cell-related events, we introduced D57N, along with WT Rac2 and a dominant active Rac2 mutant, Q61L, into hematopoietic stem and progenitor cells. As shown in Fig. 3A, the WT Rac2, D57N Rac2, or Q61L Rac2 cDNA was ligated into retroviral vector MIEG3. An internal ribosome re-entry site is inserted between Rac2 and EGFP sequences to quantitate the expression of both genes in individual cells using the intensity of GFP staining assayed by flow cytometry. Stable GP+E86 retrovirus packaging cell lines expressing MIEG3 (vector only), WT Rac2, D57N Rac2, and Q61L Rac2 were established (for details, see "Experimental Procedures"). The integrity of integrated provirus in cells infected with virus from each producer line was demonstrated by Southern blot using probes for Rac2 cDNA and EGFP (data not shown).

High titer E86 viral supernatant generated from producer clones were used to transduce LDBM cells derived from either WT C57BL/6 or Rac2+/− mice (22) in the presence of cytokines (SCF, MGDF, and G-CSF) using a standard infection protocol (37). The transduced bone marrow cells were sorted for GFP− (Table I), and GFP+ cells were used for further biochemical and cellular analyses. We consistently saw only a low level of gene transfer in cells infected with Q61L. As shown in Fig. 3B, immunoblot of the transduced and GFP− Rac2−/− BM cells confirmed the ectopic expression of WT Rac2 and Rac2 mutants. WT BM cells transduced with the empty vector (MIEG3) were also used as a control of normal endogenous GTPase expression levels (lane 1). The Rac2 antibody has weak cross-reaction with another protein (likely Rac1 as shown in Ref. 22) in lysates from Rac2−/− BM cells (lane 2). In the transduced and GFP+ cells, the expression level of Q61L (lane 5) is lower than expression of either WT or D57N Rac2 (lanes 3 and 4). This may be because of the lower viral transduction rate (6–8%, Table 1) of Q61L Rac2 vector, resulting in lower proviral copy number cell in transduced cells. Southern blot analysis supports this interpretation (data not shown). As noted previously (1, 25), the WT Rac2 protein encoded by WT Rac2 is slightly larger (lane 3) because of the Flag tag. Expression of Q61L was associated with a decreased endogenous Rac1 protein level (lane 5 versus lane 2), but expression of dominant cellular analyses. We consistently saw only a low level of gene transfer in cells infected with Q61L. As shown in Fig. 3B, immunoblot of the transduced and GFP− Rac2−/− BM cells confirmed the ectopic expression of WT Rac2 and Rac2 mutants. WT BM cells transduced with the empty vector (MIEG3) were also used as a control of normal endogenous GTPase expression levels (lane 1). The Rac2 antibody has weak cross-reaction with another protein (likely Rac1 as shown in Ref. 22) in lysates from Rac2−/− BM cells (lane 2). In the transduced and GFP+ cells, the expression level of Q61L (lane 5) is lower than expression of either WT or D57N Rac2 (lanes 3 and 4). This may be because of the lower viral transduction rate (6–8%, Table 1) of Q61L Rac2 vector, resulting in lower proviral copy number cell in transduced cells. Southern blot analysis supports this interpretation (data not shown). As noted previously (1, 25), the WT Rac2 protein encoded by WT Rac2 is slightly larger (lane 3) because of the Flag tag. Expression of Q61L was associated with a decreased endogenous Rac1 protein level (lane 5 versus lane 2), but expression of dominant cellular analyses. We consistently saw only a low level of gene transfer in cells infected with Q61L. As shown in Fig. 3B, immunoblot of the transduced and GFP− Rac2−/− BM cells confirmed the ectopic expression of WT Rac2 and Rac2 mutants. WT BM cells transduced with the empty vector (MIEG3) were also used as a control of normal endogenous GTPase expression levels (lane 1). The Rac2 antibody has weak cross-reaction with another protein (likely Rac1 as shown in Ref. 22) in lysates from Rac2−/− BM cells (lane 2). In the transduced and GFP+ cells, the expression level of Q61L (lane 5) is lower than expression of either WT or D57N Rac2 (lanes 3 and 4). This may be because of the lower viral transduction rate (6–8%, Table 1) of Q61L Rac2 vector, resulting in lower proviral copy number cell in transduced cells. Southern blot analysis supports this interpretation (data not shown). As noted previously (1, 25), the WT Rac2 protein encoded by WT Rac2 is slightly larger (lane 3) because of the Flag tag. Expression of Q61L was associated with a decreased endogenous Rac1 protein level (lane 5 versus lane 2), but expression of dominant...
negative D57N or loss of Rac2 activity in Rac2−/− cells was associated with increased endogenous Rac1 (lanes 2 and 4 versus lane 1, and see below). Expression of WT Rac2 or D57N had little effect on the level of Cdc42 protein expression in the transduced bone marrow cells (lanes 1 and 3, 4 versus lane 2), whereas a very slight reduction of Cdc42 was seen in Rac2−/− cells expressing Q61L (lane 5 versus lane 2).

Ecotoxic Expression of Rac2 Alters Rac Activation in BM Cells—To determine whether Q61L and D57N Rac2 mutants affect endogenous GTPase activity in BM cells, we examined the level of active GTP-bound GTPases in transduced and GFP+ bone marrow cells using the PAK1 PBD GST effector pull-down assay. GTP-bound proteins were analyzed by immunoblot using antibodies for Rac2 (upper panel) and Rac1 (lower panel). Flag-tagged WT Rac2 protein bands were slightly higher (lanes 2 and 6, upper panel). The results shown are representative of four independent experiments.

Expression of Rac2 Mutants Affects BM Cell Growth—Rac2−/− mast cells display decreased cell growth and colony formation because of increased apoptosis even in the presence of growth factors (25). Because hematopoietic cell and colony growth studies were not reported in the patient from which the human D57N mutant was identified and cloned, we next examined the effect of D57N expression on transduced BM cell growth in vitro. Post-sort BM cells (both WT and Rac2−/− backgrounds) were confirmed by immunoblot using antibodies for Rac2 (upper panel) and Rac1 (lower panel). There is no significant reduction of active, GTP-bound Cdc42 in transduced WT, but we inconsistently saw small reductions in GTP-bound Cdc42 in Rac2−/− cells (data not shown). Thus, as predicted by the biochemical data presented above, D57N Rac2, when expressed via retrovirus vector, has a dominant negative effect not only on Rac2, but also on other Rac GTPases in BM cells.

As shown in Fig. 4, Q61L Rac2 expressed in both WT and Rac2−/− cells binds to GTP tightly (lanes 4 and 8, upper panel, Rac2 antibody) (26), and expression of this mutant is associated with a reduction of the endogenous active (GTP-bound) Rac1 (lane 4 versus lane 1 and lane 8 versus lane 5, lower panel, Rac1 antibody), but not GTP-bound Cdc42 (data not shown). Transgenic expression of WT Rac2 increases the amount of active Rac2 in transduced cells (lanes 2 and 6, upper panel), and this enhanced Rac2 activity results in a decrease in active Rac1 (lanes 2 and 6, lower panel). In bone marrow cells genetically deficient in Rac2, there is an apparent compensatory increase in GTP-bound Rac1 (lane 5 versus 1, lower panel, and see below). Results identical to those seen in Fig. 4 have been observed when cells transduced with each virus are stimulated with either PMA or fMLP (data not shown). Taken together, these results suggest that ectopic expression of either WT or mutant Rac2 can lead to changes in endogenous Rac GTPases activation.
were observed when clonogenic progenitor cells were examined in methylcellulose colony assays in the presence of the same cytokine combination (Fig. 5B). This reduction was even greater in Rac2−/− BM cells (Fig. 5B), showing a gene dosage effect of GTPases on cell growth and suggesting that both Rac2 and other Rho GTPases (at least Rac1) are required for BM cell expansion. Interestingly, overexpression of WT Rac2 or Q61L Rac2 in BM cells increased growth of WT cells significantly compared with cells transduced with the control vector, MIEG3 (−4-fold increase in WT cells, Fig. 5A; 10-fold increase in Rac2−/− cells, data not shown). Also, WT Rac2 and Q61L Rac2 in myeloid progenitor cells greatly increased colony numbers and colony size, suggesting that increased GTPase activity enhances expansion of normal cells.

To confirm these in vitro observations and to determine the physiological relevance of these observations in reconstituting hematopoietic stem cells, we transplanted transduced BM cells into lethally irradiated mice. Mice were subsequently bled monthly after the BM injection, and the level of GFP+ cells in the peripheral blood was determined. As shown in Fig. 6B, the percentage of D57N GFP+ cells dropped from more than 25% to less than 10% in 1 month and decreased to less than 5% by 3 months postinjection. In contrast to the increased expansion of cells seen in vitro, the engraftment of WT Rac2 (Fig. 6A) and Q61L Rac2 (data not shown) compared with empty vector MIEG3 (data not shown) was relatively stable during this same 3-month interval. These data suggest that the expression of dominant negative D57N mutant Rac2 in bone marrow cells is also associated with reduced reconstitution of stem/progenitor cells in vivo.

Rac2 Mutants Affect BM Cell Survival and/or Cell Proliferation—Rac proteins have been shown to be critical for cell proliferation by regulating G1 cell progression in Swiss 3T3 fibroblasts (39). In addition, Rac2 specifically has been shown to be critical in growth factor-induced survival in mast cells (25). To determine whether the D57N Rac2-induced expansion defect in bone marrow cells is caused by abnormal cell proliferation, we examined DNA synthesis of the cells using [3H]thymidine incorporation. As reported previously for Rac2-deficient mast cells, Rac2−/− bone marrow cells displayed normal DNA synthesis in response to SCF, MGDF, and G-CSF (data not shown). Expression of the dominant negative D57N mutant Rac2 had no demonstrable effect on DNA synthesis in transduced WT BM cells. In contrast, Q61L mutant Rac2 as well as WT Rac2 significantly increased DNA synthesis compared with untransduced cell or cells transduced with the empty vector (MIEG3) (Fig. 7A). Similar results were also found in the transduced Rac2−/− cells (data not shown). These observations suggest that Rac2 GTPase is not essential for cell proliferation in BM cells, although overexpression of Rac2 can stimulate DNA synthesis.

Because reduction of BM cell expansion and colony formation by D57N Rac2 is not associated with the abnormal cell cycle progression, we next examined the effect of Rac2 on programmed cell death in these cells. Cultured GFP−-transduced
BM cells stained for annexin V-PE or PI were analyzed by flow cytometry. After 10 days of culture, loss of Rac2 activity in BM cells was associated with increased cell death in the presence of SCF, MGDF, and G-CSF. 30% of Rac2−/− cells were annexin V+ compared with 15% of WT cells (p < 0.01), whereas the percentage of PI+ was 36% of Rac2−/− cells versus 17% of WT cells (p < 0.01, data not shown). Expression of WT Rac2 reversed this increased cell death in Rac2−/− BM cells (data not shown). In contrast, expression of D57N Rac2 dramatically increased cell apoptosis in both transduced WT and Rac2−/− cells, with up to 90% annexin V+ (Fig. 7B) and PI+ (data not shown) cells at 13 days after transduction. The Q61L mutant slightly, but not significantly, reduced the percent annexin V+ cells (Fig. 7B). In addition, D57N Rac2 significantly induced DNA fragmentation in transduced cells detected by TUNEL assay (14.8% for D57N versus 6.0% for MIEG3, p = 0.003, Fig. 7C). Interestingly, WT Rac2 and Q61L significantly reduced apoptosis as analyzed by TUNEL assay (Fig. 7C). Thus in BM cells, dominant negative D57N mutant Rac2 reduces cell survival but has no measurable effect on cell proliferation, whereas the dominant active Q61L mutant or overexpression of WT Rac2 increases cell proliferation and may reduce apoptosis slightly.

**Myeloid Cell Function Is Affected by Expression of Rac2 Mutants**—Rac2 has previously been shown to be required for normal neutrophil chemotaxis, rolling via L-selectin and superoxide production both in vitro and in vivo in mice (22). In addition, the D57N mutant Rac2 was associated with abnormal neutrophil migration, rolling, and superoxide generation in a human patient with recurrent infections (1). The effect of Q61L Rac2 expression on normal neutrophil function has not been studied previously. To understand better the physiological effects of expression of these two different Rac2 mutants in myeloid cell function, we induced myeloid differentiation of transduced and GFP−-sorted BM cells in vitro. After 10 days of culture (7 days after transduction) in the presence of SCF, MGDF, and G-CSF cytokines, greater than 90% of the cells were Mac1+ and Gr-1+. These cells were subsequently analyzed for cell migration using a modified Boyden chamber assay. Expression of D57N Rac2 completely inhibited cell migration, particularly in Rac2−/− cells (Fig. 8), again suggesting a gene dosage effect. Because, as shown above, D57N Rac2 biochemically blocks Rac1 GTPase activity (Fig. 4), these data support a compensatory role for Rac1 in cultured BM cells (details discussed below). Expression of WT Rac2 or the dominant active Q61L mutant slightly increases chemotaxis. Interestingly in contrast with freshly isolated cells, we observed less of a difference in cell migration between the in vitro differentiated WT and Rac2−/− BM neutrophils, although this difference was still significant (p < 0.05) (Fig. 8). This is possibly due to increased Rac1 activity noted after extensive in vitro culture in stimulating growth factors (see below).

Rac GTPases play an essential role in generating superoxide via NADPH oxidase in a cell-free system in vitro. Both Rac1 and Rac2 have been demonstrated to be involved in assembly and activation of the NADPH-dependent respiratory burst oxidase (40, 41). In cultured and in vitro differentiated BM neutrophils, D57N mutant Rac2 completely abolishes superoxide production, particularly in Rac2−/− cells, using either fMLP (Fig. 9A) or PMA (Fig. 9B) as an agonist. However, ectopic expression of WT Rac2 and Q61L Rac2 in in vitro derived BM neutrophils had no significant affect on superoxide production (Fig. 9). Thus, in contrast to the effects on cell proliferation, increased expression of functional Rac protein does not appear to enhance oxidase activity.

**Compensatory Function of Rac Proteins**—All three members of the Rac subfamily are highly conserved in their primary sequences. Rac1 and Rac2 have overlapping expression in the hematopoietic cell lineages. Unlike Rac1, which is ubiquitously

![Fig. 7. Effect of Rac2 mutants on DNA synthesis and cell apoptosis.](http://www.jbc.org/)
expressed, Rac2 is expressed primarily in the hematopoietic cells (42). Previous gene-targeting studies demonstrated defective migration and F-actin generation by freshly explanted BM neutrophils from Rac2−/− mice (22), suggesting that Rac2 has essential roles in these cellular events. In contrast, as noted above, after 10 days of in vitro culture and differentiation in the presence of SCF, MGDF, and GM-CSF, Rac2−/− BM-derived neutrophils showed a reduced phenotype with respect to chemotaxis compared with WT cells after stimulation with 1 μM fMLP (Fig. 8). To determine if this change in cell migration phenotype was related to Rac activity, we quantified the active (GTP-bound) Rac1 in fresh and in vitro cultured Rac2−/− cells. Both total Rac1 protein and active (GTP-bound) Rac1 are up-regulated in cultured Rac2−/− cells (lanes 4, Fig. 10) but not in freshly prepared Rac2−/− cells (lanes 2, Fig. 10). As shown in Fig. 4 (lower panel, lane 5), Rac1 activity as measured using the PBD GST pull-down assay (38) in cultured Rac2−/− cells increased more than 5-fold compared with in vitro cultured WT cells (lane 1). The excess activity of Rac1 was also associated with restoration of defective superoxide production (measured by the number of NBT+ cells) in Rac2-deficient neutrophils in response to PMA or fMLP after in vitro culture (Fig. 9). Taken together, these data suggest that up-regulation of Rac1 occurs with in vitro culture of Rac2−/− progenitor cells but not in vivo, and this increase in Rac1 activity can partially rescue Rac2-deficient phenotypes.

**DISCUSSION**

Rho GTPase members of the Ras superfamily have been shown to control actin cytoskeleton organization in eukaryotic cells (9, 43). Similar to Ras, by cycling between inactive GDP-bound and active GTP-bound states, Rho GTPases are key regulators of a wide spectrum of cellular functions in eukaryotic cells, including actin polymerization, membrane trafficking, gene transcription, and cell cycle progression (3). The regulation of Rho GTPase activity is achieved through protein–protein interactions with GEFs, GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (44). In general, GEFs function to stimulate GDP/GTP exchange thus activating the Rho GTPase, whereas GTPase-activating proteins stimulate intrinsic GTPase activity, leading to decreased signaling. Guanine nucleotide dissociation inhibitors interfere with both late intrinsic GTPase activity, leading to decreased signaling. Guanine nucleotide dissociation inhibitors interfere with both GTP hydrolysis and GDP/GTP exchange and may function additionally in modulating subcellular localization patterns of the GTPases (8). The mammalian Rho-like GTPases consist of several distinct proteins, including the Rac subfamily (45). Among three identified Rac proteins, all of which share very high sequence homology (Rac1 and Rac2 are 92% homologous, and Rac2 and Rac3 are 91% homologous), Rac2 is expressed only in hematopoietic cells (42).

We have generated Rac2-deficient mice by homologous recombination (22). Mast cells and neutrophils from Rac2−/− mice have a distinct phenotype, characterized by abnormal actin-based functions, such as adhesion, migration, L-selectin function, phagocytosis and degranulation, and abnormal cell survival, with increased apoptosis after growth factor stimulation (22, 25). Rac2 also appears to be critical for T helper 1 lymphocyte differentiation (46). In addition, our laboratory (1) and Ambroso et al. (2) have recently identified a mutation of human Rac2 (G169A; aspartic acid to asparagine at position 57 (D57N)), associated with a phagocytic immunodeficiency, demonstrating that Rac2 also plays a critical role in human blood cells. The abnormalities in Rac2−/− hematopoietic cells occur despite continued expression of the highly homologous Rac1 in these cells. Differences in Rac1 and Rac2 proteins are not...
The cellular phenotypes associated with Rac2 deficiency in mice and dominant negative D57N Rac2 in man have been characterized mainly in phagocytic cells, and they include defective migration, defective capture, and rolling on the L-selectin ligand, glycamin-1, and reduced superoxide generation in response to some agonist of the phagocytic oxidase pathway. As shown here, expression of D57N Rac2 in primary mouse hematopoietic cells was associated with markedly impaired migration and superoxide generation. However, recent data derived from other lineages deficient in Rac2, including lymphocytes (46) and mast cells (25), taken together with the biochemical data reported here would suggest the potential for multilineage abnormalities as a result of expression of D57N Rac2. As shown here, expression of D57N Rac2 compared with either WT Rac2 or the activated Q61L Rac2 was associated with markedly reduced expansion of transduced cells in vitro and a significant lack of engraftment in vivo. Impaired expansion in vitro in large part was caused by increased apoptosis even in the presence of growth factors that act as survival factors for primitive hematopoietic cells. In Rac2-deficient mast cells, increased apoptosis has been demonstrated to be associated with defective activation of Akt by phosphatidylinositol 3-kinase as well as reduced expression of the antiapoptotic protein Bel-XI, and enhanced expression of the proapoptotic protein BAD (25). Interestingly, there are no cell cycle progression changes as assayed by thymidine incorporation demonstrated in the absence of WT Rac2 or the presence of D57N Rac2, whereas increased proliferation is apparent when WT Rac2 or Q61L Rac2 is overexpressed. Thus previous data implicating Rac function in cell cycle progression using transduction of activated or dominant negative mutants of Rac in fibroblast cells may not accurately reflect the role of this GTPase in normal cell cycle events, and the D57N Rac2 phenotype in humans requires more careful analysis in the future to determine the full extent of the effect in all blood cells.

A surprising but consistent finding in the studies reported here is the loss of phenotypic abnormalities of migration and superoxide generation in Rac2-deficient neutrophils after extensive in vitro growth and differentiation in growth factors. These same cells demonstrated a significant induction of Rac1 expression and activity compared with cells freshly isolated from Rac2-deficient mice. These data suggest that factors regulating expression of Rac1 in vivo are overcome after stimulation in vitro and that Rac1, when highly overexpressed, can subserve at least some functions in blood cells normally performed by Rac2. The nature of the regulatory control of Rac1 (and Rac2) expression and its protein activity is currently unknown. In addition, the basis of the specificity of Rac2 function in humans requires more careful analysis in the future to determine the full extent of this effect.

Acknowledgments—We thank Eva Meunier and Sharon Smoot for excellent administrative assistance. We thank Dr. Mary Dinauer and members of our laboratory for helpful discussions.

REFERENCES

1. Williams, D. A., Tao, W., Yang, F. C., Kim, C., Gu, Y., Mansfield, P., Levine, J. E., Petryniak, B., Derrrow, C. W., Harris, C., Jia, B., Zheng, Y., Ambruso, D. R., Lowe, J. B., Atkinson, S. J., Dinauer, M. C., and Boxer, L. (2000) Blood 96, 1646–1654
2. Ambruso, D. R., Knall, C., Abell, A. N., Panepinto, J., Kurkchubasche, A., Thurman, G., Gonzalez-Aller, C., Hiester, A., deBoer, M., Harbeck, R. J., Oyer, R., Johnson, G. L., and Roos, D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4654–4659
3. MacKay, D. J., and Hall, A. (1998) J. Biol. Chem. 273, 20685–20688
4. Allen, W. E., Jones, G. E., Pollard, J. W., and Ridley, A. J. (1997) J. Cell Sci. 110, 707–720
Human Mutation of Rho GTPase, Rac2

5. Bengtsson, T., Sarndahl, E., Stendahl, O., and Andersson, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2921–2925

6. Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., and Greenberg, S. (1997) J. Exp. Med. 186, 1487–1494

7. Narumiya, S., Ishizaki, T., and Watanabe, N. (1997) FEBS Lett. 410, 68–72

8. Van Aelst, L., and D'Souza-Schorey, C. (1997) Genes Dev. 11, 2295–2322

9. Hall, A. (1998) Science 279, 549–554

10. Olson, M. F., Ashworth, A., and Hall, A. (1995) Science 269, 1270–1272

11. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170

12. Sulciner, D. J., Iriani, K., Yu, Z. X., Ferrans, V. J., Goldschmidt-Clermont, P., and Finkel, T. (1996) Mol. Cell. Biol. 16, 7115–7121

13. Nobes, C. D., and Hall, A. (1995) Cell 81, 53–62

14. Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T., and Synderman, R. (1989) J. Biol. Chem. 264, 1638–16392

15. Shirsat, N. V., Pignolo, R. J., Kreider, B. L., and Rovera, G. (1990) Oncogene S, 769–772

16. Moll, J., Sansig, G., Fattori, E., and van der Putten, H. (1991) Oncogene 6, 863–866

17. Haataja, L., Groffen, J., and Heisterkamp, N. (1997) J. Biol. Chem. 272, 20384–20388

18. Segal, A. W., and Abo, A. (1993) Trends Biochem. Sci. 18, 43–47

19. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991) Nature 353, 668–670

20. Knaus, U. G., Heyworth, P. G., Evans, T., Curnutte, J. T., and Bokoch, G. M. (1991) Science 254, 1512–1515

21. Abo, A., Boyhan, A., West, I., Thrasher, A. J., and Segal, A. W. (1992) J. Biol. Chem. 267, 16767–16770

22. Roberts, A. W., Kim, C. Zhen, L., Lowe, J. B., Kapur, R., Petryniak, B., Spaetti, A., Pollock, J. D., Boreo, J. B., Bradford, G. B., Atkinson, S. J., and Wells, J. P. M. (1992) J. Biol. Chem. 267, 183–186

23. Heyworth, P. G., Bohl, B. P., Bokoch, G. M., and Curnutte, J. T. (1994) J. Biol. Chem. 269, 30749–30752

24. Dorsseuil, O., Reibel, L., Bokoch, G. M., Camonis, J., and Gacon, G. (1996) J. Biol. Chem. 271, 83–88

25. Yang, F. C., Kapur, R., King, A. J., Tao, W., Kim, C., Boreo, J. B., Brestle, R., Marshall, M., Dinauer, M. C., and Williams, D. A. (2000) Immunity 12, 57–68

26. Xu, X., Wang, Y., Barry, D. C., Chanock, S. J., and Bokoch, G. M. (1997) Biochemistry 36, 626–632

27. Menard, L., Tomhaye, E., Casey, P. J., Uhing, R. J., Snyderman, R., and Didsbury, J. R. (1992) Eur. J. Biochem. 206, 537–546

28. Williams, D. A., and Smith, F. O. (2000) Ham. Gene Ther. 11, 2059–2066

29. Chuang, T. H., Xu, Q., Quilliam, L. A., and Bokoch, G. M. (1994) Biochem. J. 303, 761–767

30. Knaus, U. G., Heyworth, P. G., Kinsella, B. T., Curnutte, J. T., and Bokoch, G. M. (1992) J. Biol. Chem. 267, 23575–23582

31. Zhu, K., Debreceni, B., Li, R., and Zheng, Y. (2000) J. Biol. Chem. 275, 25993–26001

32. Hanenberg, H., Hashino, K., Konishi, H., Hock, R. A., Kato, I., and Williams, D. A. (1997) Hum. Gene Ther. 183–196

33. Yee, N. S., Paek, I., and Besmer, P. (1994) J. Exp. Med. 179, 1777–1787

34. Bunde, S., Dill, D., Senn, R., Gifford, M., and Dinauer, M. C. (1997) J. Biol. Chem. 272, 41–48

35. Jung, V., Wei, W., Ballester, R., Camonis, J., Mi, S., Van Aelst, L., Wigler, M., and Broek, D. (1994) Mol. Cell. Biol. 14, 3707–3718

36. Seipel, K., Medley, Q. G., Kedersha, N. L., Zhang, X. A., O'Brien, S. P., Serra-Pages, C., Henler, M. E., and Strelui, M. (1999) J. Cell Sci. 112, 1825–1834

37. Hanenberg, H., Xiao, X. L., Dill, D., Hashino, K., Kato, I., and Williams, D. A. (1996) Nat. Med. 11, 876–882

38. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) J. Biol. Chem. 274, 15196–15204

39. Lamarche, N., Tappon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) Cell 87, 519–529

40. Kreck, M. L., Uhlinger, D. J., Tyagi, S. R., Inge, K. L., and Lambeth, J. D. (1994) J. Biol. Chem. 269, 4161–4168

41. Xu, X., Barry, D. C., Ballester, R., Camonis, J., Mi, S., Van Aelst, L., Pigler, M., and Broek, D. (1994) Mol. Cell. Biol. 14, 3707–3718

42. Reibel, L., Dorseuil, O., Stancou, R., Bertoglio, J., and Gacon, G. (1991) Biochem. Biophys. Res. Commun. 175, 451–458

43. Scita, G., Tenca, P., Fritto, E., Chicchetti, A., Innocenti, M., Giardina, G., and Di Fiore, P. P. (2000) J. Biol. Chem. 275, 750–759

44. Bokoch, G. M., and Der, C. J. (1993) Trends Biochem. Sci. 18, 288–293

45. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127

46. Li, B., Yu, H., Zheng, W., Vell, R., Na, S., Roberta, A. W., Williams, D. A., Davia, B. J., Ghosh, S., and Flavell, R. A. (2000) Science 288, 2219–2222

47. Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) Nature 341, 209–214

48. Felts, K., Bauer, J. C., and Vainclzouquet, P. (1999) J. Biol. Chem. 112, 74–77
