Rac GTPase Isoform-specific Regulation of NADPH Oxidase and Chemotaxis in Murine Neutrophils in Vivo

ROLE OF THE C-TERMINAL POLYBASIC DOMAIN*

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The Rho family GTPase Rac acts as a molecular switch for signal transduction to regulate various cellular functions. Mice deficient in the hematopoietic-specific Rac2 isoform exhibit agonist-specific defects in neutrophil chemotaxis and superoxide production, despite expression of the highly homologous Rac1 isoform. To examine whether functional defects in rac2−/− neutrophils reflect effects of an overall decrease in total cellular Rac or an isoform-specific role for Rac2, retroviral vectors were used to express exogenous Rac1 or Rac2 at levels similar to endogenous. In rac2−/− neutrophils differentiated from transduced myeloid progenitors in vitro, increasing cellular Rac levels by expression of either exogenous Rac1 or Rac2 increased formylmethionylleucylphenylalanine-or phorbol ester-stimulated NADPH oxidase activity. Of note, placement of an epitope tag on the N terminus of Rac1 or Rac2 blunted reconstitution of responses in rac2−/− neutrophils. In rac2−/− neutrophils isolated from mice transplanted with Rac-transduced bone marrow cells, superoxide production and chemotaxis were fully reconstituted by expression of exogenous Rac2, but not Rac1. A chimeric Rac1 protein in which the Rac1 C-terminal polybasic domain, which contains six lysines or arginines, was replaced with that of the human Rac2 polybasic domain containing only three basic residues, also reconstituted superoxide production and chemotaxis, whereas expression of a Rac2 derivative in which the polybasic domain was replaced with that of Rac1 did not and resulted in disoriented cell motility. Thus, the composition of the polybasic domain is sufficient for determining Rac isoform specificity in the production of superoxide and chemotaxis in murine neutrophils in vivo.

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Neutrophils are important effector cells in the host response to bacterial and fungal pathogens and are endowed with chemotactic and microbicidal functions that are activated by signal transduction pathways downstream of receptors for inflammatory and microbial stimuli (1). Migration from blood vessels to inflamed sites is initiated by various chemoattractants, including chemokines secreted by host cells and by bacterial products such as fMLP.1 Superoxide produced by the neutrophil NADPH oxidase is the precursor to toxic reactive oxidants important for normal microbial killing.

Rac GTPases, members of the Rho family of small GTPases, play a central role in regulating neutrophil chemotaxis, superoxide production, and other neutrophil functions (2). Rho GTPases act as molecular switches in signaling pathways, alternating between activated GTP-bound and inactive GDP-bound states. In unstimulated cells, the GDP-bound form of Rac is bound to Rho guanine-nucleotide dissociation inhibitor, which inhibits nucleotide exchange and maintains Rac-GDP in the cytoplasm. Upon stimulation, Rac translocates to the membrane where agonist-activated guanine nucleotide exchange factors mediate the exchange of GDP for GTP, resulting in a conformational change that permits Rac binding to downstream target proteins (3). Rac has three isoforms, the ubiquitously expressed Rac1, a hematopoietic cell-specific isoform Rac2, and Rac3, which appears to be expressed in a variety of tissues but not phagocytes (4-8). The amino acid sequence of murine Rac1 is identical to human Rac1, and murine Rac2 differs by only two amino acids from human Rac2, with a conservative substitution of aspartate instead of glutamate at position 148 and a proline instead of an alanine at position 188 (5, 6). Rac1 and Rac2 have 92% identical amino acid sequences. These two isoforms have an identical effector domain (amino acids 26–45), which is a critical site of interaction with both guanine nucleotide exchange factors and downstream protein targets. However, long range effects exerted by distant isoform-divergent amino acids may alter the flexibility of the effector domain to produce different affinities of Rac1 and Rac2 for these proteins (9). The greatest sequence divergence between Rac1 and Rac2 is in the C-terminal polybasic domain (residues 183–188), which is adjacent to a prenylated cysteine residue that can insert into cellular membranes. Rac1 has six adjacent basic residues in the polybasic domain (KKRRKKR), whereas...
Rac2 has only three basic residues interspersed by neutral amino acids (RQQKR/AFP) (Fig. 1A). The polybasic domain mediates differential localization of overexpressed Rac1 and Rac2 to fibroblast and epithelial cell membranes (10) and contributes to differential interactions with at least one downstream target of activated Rac GTPases, the serine-threonine kinase PAK1 (11).²

Studies in murine Rac2-deficient neutrophils and neutrophils from a patient with a dominant-negative mutation in Rac2 suggest that this isoform is essential for normal neutrophil function (8, 12–15). Although murine neutrophils have similar amounts of Rac1 and Rac2 (8), genetic deletion of Rac2 results in severely impaired F-actin polymerization and chemotaxis in response to chemoattractants, decreased L-selectin-mediated adhesion, and reduced superoxide production in response to FMLP, phorbol ester, and IgG-opsonized sheep red blood cells (8, 12, 15). Heterozygous rac2+/− murine neutrophils have intermediate activity between rac2−/− and rac2+/− neutrophils in F-actin formation, chemotaxis, and superoxide production (8). In human neutrophils, which have predominantly Rac2 (16), expression of a dominant-negative Rac2 results in cellular defects similar to those seen in rac2−/− murine neutrophils (13, 14). Although Rac1-null murine neutrophils also exhibit decreased actin cytoskeleton assembly and a modest decrease in directed migration, superoxide production is normal in contrast to Rac2-null counterparts (17). These findings suggest that Rac1 and Rac2 play distinct roles in regulating neutrophil functions. Alternatively, the defects in Rac2-deficient neutrophils could reflect effects of an overall reduction in cellular Rac levels.

The goal of the current study was to examine whether the impaired functions of Rac2-null neutrophils result from a relative cellular deficiency of total Rac or an isoform-specific role for Rac2 that is mediated by specific sequences. Retroviral vectors were used to express exogenous Rac1 or Rac2 in rac2−/− neutrophils at levels similar to endogenous ones. Increasing Rac levels by expression of either exogenous Rac1 or Rac2 enhanced NADPH oxidase activity in rac2−/− neutrophils differentiated in vitro with growth factors. However, we found that only expression of exogenous Rac2, but not Rac1, fully reconstituted defects in NADPH oxidase activity and chemotaxis in neutrophils harvested from rac2−/− mice transplanted with transduced rac2−/− marrow cells. This result could be recapitulated using a derivative of Rac1 in which the polybasic domain was substituted with the corresponding region from human Rac2, and conversely, expression of Rac2 chimeric for the polybasic Rac1 domain failed to rescue defects in rac2−/− neutrophils and resulted in disoriented motility. Thus, the polybasic domain is sufficient to confer isoform-specific Rac functions in regulating neutrophil chemotaxis and NADPH oxidase activity.

**MATERIALS AND METHODS**

**Reagents and Buffers**—A polyclonal Rac2 antibody was previously raised in rabbits (18), and a mouse monoclonal antibody against Rac1 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A rabbit polyclonal for p38 MAPK was obtained from Cell Signaling Inc. Phosphate-buffered saline (pH 7.2), ddH₂O, glycerol, a-MEM and Iscove’s modified Dulbecco’s medium, penicillin, streptomycin, and HEPES (125 mM, pH 7.5) were from Invitrogen. Fetal calf serum was from HyClone Laboratory (Logan, UT). Rat SCF, murine IL-6, and murine IL-3 were from PeproTech (Rocky Hill, NJ), human megakaryocyte-derives growth factor, and human granulocyte-colony stimulating factor were from Amgen. CH-296 was from Takara Bio (Osu, Japan). Other chemicals were purchased from Sigma unless otherwise stated. Buffers used in this study included phosphate-buffered saline with 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 7.5 mM glucose, Triton IPB lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, 20 µg/ml chymostain, 2 µm phenylmethylsulfonyl fluoride, 10 µM leupetin, and 1 µm 4-(2-aminoethylbenzenesulfonfyl) fluoride). All reagents are endotoxin-free grade. 96-well plates were from BD Biosciences (Franklin Lakes, NJ).

Mice—Mice were maintained under specific pathogen-free conditions. Wild type (WT) mice used in this study were 8–to 10-week-old male or female C57Bl/6J mice purchased from Jackson Laboratory Inc. Rac2−/− mice were previously generated (15) by targeted homologous recombination to disrupt the Rac2 gene. Rac2−/− mice used here were 8–to 10-week-old male or female mice that had been backcrossed onto the C57Bl/6J for more than 11 generations.

**Retroviral Vectors for Expression of Rac Proteins**—An MSCV retroviral backbone with a linked expression cassette for puromycin-N-acetyl-transferase (PAC) (19), MSCV-puc, was a kind gift from R. Hawley (American Red Cross, Rockville, MD). The MIEG3 retroviral vector was derived from the MIEG3 vectors described previously (11). N-terminal ribosome entry sequence linked to a cDNA for the enhanced green fluorescent protein (EGFP) instead of an antibiotic-resistance expression cassette (13). Murine Rac1 and Rac2 cDNAs flanked by BamHI and XhoI restriction sites were originally generated using reverse transcription-PCR from RNA prepared from the RAW 264.7 mouse macrophage cell line. The flanking restriction sites were changed to XhoI, along with engineered 5’ EcoRI and 3’ XhoI sites at the initiator ATG, using appropriate primers and PCR, and cloned into the XhoI site in MSCV-pac and MIEG3 (Fig. 1B). The murine Rac1 or Rac2 cDNAs with an N-terminal hemagglutinin (HA) or FLAG epitope tag (13, 20), respectively, were also cloned into MSCV-pac (Fig. 1B). The human Rac1 cDNA in which the Rac2 polybasic domain was replaced with that of Rac1 (Rac2-1) and the human Rac2 cDNA in which the polybasic domain was replaced with that of Rac2-1 (Rac2-2) were described previously (11). PCR was used to place EcoRI and XhoI linkers at the 5’ and 3’ ends, respectively, and each was subcloned into the corresponding sites in MIEG3 (Fig. 1). Details of oligonucleotide primers used for PCR amplifications are provided upon request. For all PCR-amplified cDNAs, DNA sequencing was performed to verify the fidelity of amplification. Vector plasmids were transfected using calcium phosphate into eectrophic Phoenix packaging cells provided by Gary Nolan, Stanford University; ATCC number SD3444, and retroviral vector supernatants were collected in alpha-MEM with 20% fetal calf serum.

**Transduction of Murine Bone Marrow Cells with Retroviral Vectors for Expression of Rac Proteins**—Retroviral transduction of WT or rac2−/− murine BM was generally described previously (21), with the following modifications. Three days following injection of 150 mg/kg 5-fluorouracil (5FU) into bone marrow donors, BM of donors, that were taken from femurs and tibias by flushing with α-MEM, was derived from the MSCV series, and contains an internal ribosome entry site (IREs) (American Red Cross, Rockville, MD). The MIEG3 retroviral vector was used to transduce cells in vitro differentiation in the presence of puromycin, and MIEG3 vectors were used to transduce cells that were sorted for EGFP fluorescence and transplanted into lethally irradiated recipient mice. There was no difference between the TD media A or B in either the transduction efficiency or subsequent in vitro differentiation and functional assays. Medium without virus was used for mock transduction. On day 5, the combined suspended and adherent cells were collected using Cell Dissociation Buffer (Invitrogen) and then were sorted for EGFP fluorescence and transplanted into lethally irradiated recipient mice. In Vivo Differentiation of Granulocytes following Retroviral Transduction of Murine Bone Marrow—Neutrophils were differentiated in vitro from mock transduced or transduced BM cells by culturing in α-MEM with 20% serum, 50 IU/ml murine IL-3, 0.3 ng/ml (= 30 IU/ml) human granulocyte-colony stimulating factor, and 2% penicillin/streptomycin in T75 flasks. Every other day (on days 6, 8, 10, 12, and 14 post harvest) cell number was counted, and cell density was adjusted to 5 × 10⁶ cells/ml.

² A. Yamauchi and M. Dinauer, unpublished data.
using fresh differentiation media. On days 8–12, cells were selected by adding 2 μg/ml puromycin, and by day 12, all mock-transduced cells exposed to puromycin had died. On day 14, cells were harvested for NADPH oxidase assays, using either transduced cells selected with puromycin or mock-transduced cells cultured in the absence of puromycin. Diff-Quik-stained slides prepared by Cytospin (Thermo Electron Corp., Waltham, MA) on day 14 showed ~80–90% mature neutrophils, with no differences seen between WT, Rac2-null, or Rac-transduced cells. The fraction of Gr-1-positive cells as analyzed by flow cytometry was also similar between different groups. Cell lysate was prepared on days 14–16 from WT, Rac2-null, or Rac-transduced cells. The mieg vector-transduced BM cells, EGFP-positive cells were selected on day 5 of the transduction protocol by sorting with a FACStar instrument (BD Biosciences), and 0.5–1.0 x 10^6 sorted, MIEG vector-transduced and motility. In some experiments, BM from mice transplanted with sorted, MIEG vector-transduced rac2^-/- cells were used for secondary transplants into additional 1100-cGy-irradiated rac2^-/- mice to generate additional in vivo differentiated neutrophils expressing exogenous Rac. For rac2^-/- controls, a group of rac2^-/- mice was transplanted in parallel with freshly isolated rac2^-/- BM and used as a source of rac2^-/- neutrophils in subsequent functional assays. However, NADPH oxidase activity and chemotaxis in neutrophils from these mice were different from neutrophils harvested from non-transplanted rac2^-/- mice.

**Immunoblotting**—Preparation of neutrophil lysates, SDS-PAGE, and immunoblotting were performed as described below (12, 15, 22). Briefly, neutrophils differentiated in vitro or purified from BM were treated with diisopropylphosphofluoridate prior to lysis with IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, 1 mM EGTA, pH 8.0) containing 20 μg/ml chymostatin, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride-HCl. Following SDS-PAGE and transfer to nitrocellulose membranes, blots were sequentially probed with either an anti-Rac monoclonal mouse antibody, an anti-Rac2 rabbit polyclonal antibody, and an anti-p38 MAPK rabbit polyclonal antibody, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG. Proteins were visualized using an ECL detection kit (Amersham Biosciences). Bands were scanned, and the densities were analyzed by National Institutes of Health image software (rsb.info.nih.gov/nih-image). Multiple exposures were analyzed to ensure that relative signal intensities measured were in the linear range.

**Measurement of NADPH Oxidase Activity**—For quantitative assays, either isoluminol chemiluminescence or a colorimetric assay based on reduction of cytochrome c was used to detect reactive oxygen species (8, 23). Under these conditions, superoxide dismutase inhibited the chemiluminescence signal by ~97.5% and cytochrome c reduction by ~100%. Chemiluminescence in fMLP-stimulated cells was detected as relative luminescence units (RLUs) by fast kinetic mode for 100 s using an Lmax microplate luminometer and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The rate of cytochrome c reduction was measured as Vmax using SpectraMax340 microplate reader and the SoftMax Pro software (Molecular Devices).

**Chemotaxis**—Neutrophil chemotaxis assays using 1 and 10 μM fMLP were performed using a modified Boyden chamber (48-well microchemotaxis chamber, Neuro Probes, Gaithersburg, MD) and 3-μm diameter polycarbonate filter membranes as described previously (8, 15). The number of migrated cells per high power view field (400×) were counted for a minimum of three fields per well, and an average estimation for individual sample was calculated from data of replicate wells. Values were compared relative to the number of migrated cells in the WT group.

**Time-lapse Video Microscopy and Motility Analysis**—Neutrophil motility in response to fMLP stimulation was recorded using a Dunn chamber (Pacific). BM neutrophils in Hanks’ balanced salt solution were allowed to adhere to clean glass coverslips for 15 min at 37 °C. The coverslips were mounted on the Dunn chamber with a gradient of 0–10 μM fMLP between the inner and outer wells of the chamber. The chamber was mounted on the microscope stage, and the temperature was maintained at 37 °C with a stage heater (Insect Instruments, Boulder, CO). The chamber was allowed to equilibrate for 25 min prior to image collection to allow a stable gradient to develop.

**Results**

**FIG. 1.** C terminus of Rac proteins and retroviral vectors for expression of Rac. A, sequence alignment of the C terminus of wild type Rac1, Rac2, and derivatives. Hyphens indicate sites where amino acid residues are identical to wild type murine Rac1, whereas differences are as indicated. The polybasic domain is indicated by a rectangle. Mu, murine; hu, human. Note that the entire murine Rac1 and human Rac amino acid sequence are identical. B, MSCV-pac and MIEG3 retroviral vectors. The genes between long terminal repeats (LTR) are shown. PGK, phosphoglycerate kinase; pac, puromycin-N-acetyltransferase; HA, epitope tag; FLAG, epitope tag; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein.

Images were recorded at 10-s intervals on a Nikon Diaphot 300 microscope with differential interference contrast optics using a SPOT cooled charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI). Cell positions were tracked using the particle tracking capabilities in Metamorph 6.1 software (Universal Imaging, Brandywine, PA). Cell trajectories were analyzed using the horizon method (24, 25), in which the position of the cell is marked once it reaches a “horizon” at 30 μm from the initial position, and used to define the trajectory of the cell relative to the orientation of the gradient. This methodology can only be applied to cell types that move a significant distance over the time period analyzed, and therefore directional data could not be obtained for rac2^-/- or Rac1-transduced rac2^-/- cells. Directionality was evaluated relative to the null hypothesis of uniformity using the Rayleigh test (25). In all cases over 350 cells of each type were analyzed.

**Statistical Analysis**—The two-tailed Student’s t test (either paired or unpaired, as indicated) was performed using Microsoft Excel software (Redmond, WA). Statistical comparisons of the distribution of rates of...
FIG. 2. Rac expression and NADPH oxidase activity in neutrophils differentiated in vitro. After transduction with MSCV-pac retrovirus vectors, or mock transduction, murine bone marrow cells were cultured with IL-3 and G-CSF for differentiation in vitro as described under "Materials and Methods." MSCV-pac retrovirus-transduced cells were selected in puromycin. Wild type, rac2+/−, or rac2+/− cells expressing exogenous Rac proteins are as indicated. Emp, empty (MSCV-pac lacking a Rac cDNA); R1, Rac1; HR1, HA-tagged Rac1; R2, Rac2; FR2, FLAG-tagged Rac2. A, immunoblot of extracts prepared from in vitro-differentiated neutrophils, using anti-Rac1, anti-Rac2, or anti-p38 MAPK as indicated. Note that epitope-tagged Rac proteins migrated slightly slower than untagged Rac proteins, and Rac1-transduced rac2+/− cells contain both endogenous and exogenous Rac1. Representative of four experiments. B and C, are representative time courses of ROS production by in vitro differentiated neutrophils detected by chemiluminescence (for fMLP-stimulated cells; B, or reduction of cytochrome c (PMA-stimulated superoxide production (C)). RLU, relative luminescence units. mOD, optical density (× 10−3). D and E, quantitative analysis of NADPH oxidase activity in in vitro differentiated neutrophils. Superoxide dismutase-inhibitable luminescence (integrated RLU’s over 100 s) is shown for
RESULTS

Expression of Recombinant Rac Proteins in Neutrophils Differentiated In Vitro—In initial studies, recombinant Rac proteins were expressed in rac2−/− neutrophils differentiated in vitro. Rac2−/− myeloid progenitor cells were transduced with MSCV-based retroviral vectors designed to express Rac1 or Rac2. In preliminary studies using MIEG-based retroviral vectors for transduction followed by in vitro differentiation, recombinant Rac proteins were expressed at levels that were 3- to 6-fold greater than endogenous levels in wild-type neutrophils. Therefore, subsequent in vitro studies utilized the MSCV-pac backbone, which contains a linked antibiotic resistance gene, puromycin-N-acetyl-transferase (pac) (Fig. 1B), where exogenous Rac proteins were not overexpressed (see below). Vectors included those in which an epitope tag was placed on the N terminus of Rac (an HA tag for Rac1 or a FLAG tag for Rac2) as well as those for expression of Rac1 or Rac2 without an epitope tag. Following transduction, bone marrow cells were differentiated in vitro with granulocyte-colony stimulating factor and IL-3 with or without puromycin, as described under “Materials and Methods,” with ~80–90% morphologically mature neutrophils present after 12–14 days. In the absence of selection, no differences in expansion or neutrophil differentiation were observed between WT or rac2−/− BM that was either mock transduced or transduced with a control vector containing only the puromycin cassette. There were also no significant differences in expansion or neutrophil differentiation between puromycin-selected WT and rac2−/− cells transduced with the control vector containing only the puromycin cassette, or in rac2−/− cells transduced with Rac-containing vectors.

To evaluate expression of Rac isoforms in neutrophils differentiated in vitro, we performed immunoblotting using antibodies specific for either Rac1 or Rac2 (Fig. 2A). Recombinant Rac proteins were expressed from the MSCV-pac vectors at endogenous levels, based on densitometric analysis of blots from four independent experiments (not shown). Placement of an epitope tag retarded the migration of the recombinant Rac proteins, as expected (Fig. 2A), but did not affect the relative level of expression. Endogenous Rac1 levels in the in vitro differentiated rac2−/− neutrophils were not significantly increased from wild-type levels (Fig. 2A).

NADPH Oxidase Activity in Wild Type and rac2−/− Neutrophils Differentiated in Vitro—Quantitative assessment of NADPH oxidase activity in neutrophils differentiated in vitro was performed using a chemiluminescence assay to detect fMLP-induced production of reactive oxygens and the cytochrome c reduction assay to measure superoxide production in cells stimulated with phorbol myristate acetate (PMA). Rac2−/− neutrophils had substantially reduced NADPH oxidase activity compared with WT neutrophils (Fig. 2), as previously reported for neutrophils harvested either from the bone marrow storage pool or from peritoneal exudate (8, 12, 15, 26). For both genotypes, there was no significant difference in oxidant production between neutrophils derived from mock transduced cells compared with puromycin-selected cells transduced with the control MSCV-pac vector (not shown). Expression of either exogenous Rac1 or Rac2 increased NADPH oxidase activity in Rac2-null cells stimulated with either fMLP or PMA. The time course of oxidant production in representative experiments is shown in Fig. 2 (B and C), and aggregate data are shown in Fig. 2 (D and E). Of note, Rac derivatives with an epitope tag had a reduced ability to rescue NADPH oxidase activity in rac2−/− neutrophils compared with exogenous Rac proteins lacking a tag, although both epitope- and non-epitope-tagged exogenous Rac proteins were expressed at similar levels (Fig. 2A).

Expression of Rac2 was consistently slightly more effective than exogenous Rac1 in enhancing NADPH oxidase activity in rac2−/− neutrophils (e.g. Fig. 2B), although the difference in activity between the Rac2- and Rac1-transduced groups did not reach statistical significance. Only partial reconstitution of NADPH oxidase activity was observed even in Rac2-transduced rac2−/− neutrophils, which may reflect the fact that exogenous Rac proteins were expressed at only ~60% of wild-type levels using the MSCV-pac vectors (see above). Neutrophils isolated from heterozygous rac2−/+ BM, which have approximately one half the level of Rac2 protein compared with rac2−/− neutrophils, also have reduced superoxide production compared with wild-type neutrophils (8). Similarly, NADPH oxidase activity in rac2−/− neutrophils differentiated in vitro as above is reduced by ~25% or ~50% following PMA or fMLP stimulation, respectively.

Retrovirus-mediated Expression of Exogenous Rac1 and Rac2 in Neutrophils Differentiated in Vivo—We next examined whether expression of exogenous Rac1 or Rac2 in rac2−/− neutrophils differentiated in vivo could reconstitute NADPH oxidase activity and motility. The above results using rac2−/− neutrophils differentiated in vitro with hematopoietic growth factors indicated that exogenous expression of either isoform improved NADPH oxidase activity, with expression of exogenous Rac2 only slightly more effective than Rac1. Granulocyte macrophage-colony stimulating factor or tumor necrosis factor-α treatment of neutrophils isolated from rac2−/− mice can partially correct defective NADPH oxidase activity and chemotactant-induced actin formation (15), raising the question that exposure to pharmacologic concentrations of growth factors during the in vitro differentiation process may have influenced these results. Thus, we transplanted Rac-transduced rac2−/− bone marrow cells into lethally irradiated rac2−/− mice to study function of neutrophils produced in vivo. Rac derivatives without epitope tags were utilized for these studies.

Initial transplant studies using the MSCV-pac vectors (Fig. 1B) showed that recombinant Rac proteins were expressed at only ~5% of wild-type levels in neutrophils produced in vivo (not shown). Expression vectors for Rac1 and Rac2 based on the MIEG3 retrovirus (Fig. 1B) were next evaluated. Flow cytometry for EGFP expression was used to select transduced cells by fluorescence-activated cell sorting prior to transplantation into lethally irradiated rac2−/− recipients. Following hematopoietic recovery, between 60 and 92% of neutrophils were EGFP-positive in all but one mouse (a recipient of MIEG3-Rac1-transduced cells), with most animals having at least 80% EGFP-positive neutrophils. That fewer than 100% neutrophils were EGFP-positive despite sorting for EGFP fluorescence prior to

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fMLP-elicited ROS production (D). The maximal rate of superoxide dismutase-inhibitable cytochrome c reduction (Vmax) is shown for PMA-elicited superoxide production. Means ± S.E. are shown; n = 9 for WT and rac2−/− controls; n = 3–6 for Rac-transduced rac2−/− cells. †, p < 0.02 (versus rac2−/− emp); ‡, p < 0.005 (versus rac2−/− emp); ‡‡, p < 0.01 (versus Rac2, paired test, n ≥ 3). **, p = 0.057 (versus rac2−/− emp). Values except R2 versus FR2 are based on unpaired Student’s t test.
FIG. 3. Rac expression and NADPH oxidase activation of neutrophils isolated from WT, rac2<sup>−/−</sup>, and rac2<sup>−/−</sup> mice transplanted with Rac1- or Rac2-transduced BM. Rac2<sup>−/−</sup> BM was transduced with MIEG3 retroviral vectors for expression of either Rac1 or Rac2, sorted for EGFP-positive cells, and transplanted into rac2<sup>−/−</sup> mice as described under “Materials and Methods.” Immunoblots and NADPH oxidase assays were performed on freshly isolated neutrophils from transplanted mice. Rac2<sup>−/−</sup> control mice were transplanted with untransduced rac2<sup>−/−</sup> BM. Wild type, rac2<sup>−/−</sup>, or rac2<sup>−/−</sup> cells expressing exogenous Rac proteins are as indicated, and abbreviations are as in Fig. 2. A, immunoblot of extracts prepared from BM neutrophils, using anti-Rac1, anti-Rac2, or anti-p38MAPK, as indicated. Representative of three experiments. Note that the antibody for Rac2 has some cross-reactivity with Rac1 (15), which accounts for the faint band in Rac1-transduced rac2<sup>−/−</sup> extracts probed for Rac2.

B and C, representative time course of neutrophil ROS production detected by chemiluminescence (for fMLP-stimulated cells, B) or reduction of cytochrome c (PMA-stimulated superoxide production, C). RLU, relative luminescence units. mOD, optical density (× 10<sup>3</sup>). The fraction of EGFP-positive neutrophils for Rac1- and Rac2-transduced rac2<sup>−/−</sup> neutrophils was 84 and 92%, respectively. D and E, quantitative analysis of...
neutrophil NADPH oxidase activity. Superoxide dismutase-inhibitable luminescence (integrated RLUs over 100 s) is shown for fMLP-elicited ROS production (D). The maximal rate of superoxide dismutase-inhibitable cytochrome c reduction (V_max) is shown for PMN-elicited superoxide production. Means ± S.E. are shown. n = 14 and 8 for WT and rac2−/− controls, respectively. n = 3 for Rac-transduced rac2−/− neutrophils. * p < 0.05 (versus rac2−/−); **, p < 0.00005 (versus rac2−/−); #, p < 0.02 (versus WT); ##, p < 0.00005 (versus WT). p values are based on unpaired Student’s t test.
FIG. 4. Rac expression and NADPH oxidase activation of neutrophils isolated from WT, rac2−/−, and rac2−/− mice transplanted with Rac1-2- or Rac2-1-transduced BM. Experimental design and abbreviations are as described in Fig. 3. A, immunoblot of extracts prepared from freshly isolated neutrophils, using anti-Rac1, anti-Rac2, or anti-p38MAPK, as indicated. Representative of four experiments. B and C, representative time course of neutrophil ROS production detected by chemiluminescence (for fMLP-stimulated cells, B) or reduction of cytochrome c (PMA-stimulated superoxide production, C). RLU, relative luminescence units. mOD, optical density (×10−3). The fraction of EGFP-positive neutrophils for Rac1-2- and Rac2-1-transduced rac2−/− neutrophils was 83 and 80%, respectively. D and E, quantitative analysis of neutrophil NADPH oxidase activity. Superoxide dismutase-inhibitable luminescence (integrated RLU over 100 s) is shown for fMLP-elicited ROS production (D). The maximal rate of superoxide dismutase-inhibitable cytochrome c reduction (Vmax) is shown for PMA-elicited superoxide production. Means ± S.E. are shown. n = 14 and 8 for WT and rac2−/− controls, respectively. n = 3–5 for Rac-transduced rac2−/− cells. *, p < 0.0002 (versus rac2−/−); **, p < 0.00001 (versus rac2−/−). p values are based on unpaired Student’s t test.
Wild type murine neutrophils have similar amounts of Rac1 and Rac2, and total cellular levels of Rac are diminished by ~2-fold in rac2−/− neutrophils. We found that increasing cellular Rac levels by retrovirus-mediated expression of Rac2 in rac2−/− neutrophils in vivo restored NADPH activity and chemotaxis, whereas expression of additional Rac1 only partially corrected these defects. Moreover, isoform specificity could be determined solely by sequences derived from the C-terminal polybasic domain that is adjacent to the prenylation site. A determined solely by sequences derived from the C-terminal polybasic domain, whereas expression of additional Rac1 only partially downstream targets of Rac. For example, Rac1 binds to and interacts with proteins that regulate Rac-GTP levels or are interaction partners (35). Hence, the non-redundant role of also been shown to determine specifically in localization and interaction partners (35). Hence, the non-redundant role of Rac to subcellular membrane compartments or micro domains, the relative affinities with proteins that regulate Rac activation or serve as downstream targets, or a combination of these factors.

Although expression of Rac2 or Rac1-2 was required for full reconstitution of superoxide production in rac2−/− neutrophils in vivo, expression of exogenous Rac1 did produce small improvements. In rac2−/− neutrophils, Rac1 is already activated to a ~3-fold greater extent compared with wild-type neutrophils (8), and the current results suggest that increased expression of this isoform can improve phorbol ester- or fMLP-stimulated NADPH oxidase activity in rac2−/− neutrophils. Rac1 was also recently shown to be the predominant form of Rac in human monocytes, where it was suggested to function as the main isoform for superoxide production elicited by PMA and other agonists (36). Rac1 translocates to monocyte membranes in response to PMA, fMLP, or opsonized zymosan, and co-immunoprecipitates with the p47phox and p67phox NADPH oxidase subunits upon opsonized zymosan stimulation (36). Of note, murine neutrophils with a combined deficiency of both Rac1 and Rac2 have extremely low superoxide production (28).

Also noteworthy is that expression of the Rac2-1 chimera containing the Rac1 polybasic domain did not increase NADPH oxidase activity in rac2−/− neutrophils, in contrast to the small improvements seen with expression of exogenous Rac1. In addition, rac2−/− neutrophils expressing Rac2-1 exhibited disoriented cell motility. The underlying basis for these observations remains to be determined. However, the different responses when expressing exogenous Rac1 compared with Rac2-1 are not easily understood in terms of a mechanism for isoform specificity that is based solely on polybasic domain-directed localization of activated Rac.

Placement of an epitope tag on proteins is a useful strategy for localizing proteins and studying their functions, but this approach can have limitations. Epitope tags may affect protein stability, localization, or function, and they may also interfere with protein-protein interactions. Moreover, tagging proteins can alter their subcellular distribution, which can affect their interactions with other proteins and their ability to perform their physiological functions. Therefore, careful consideration should be given to the choice of epitope tag and its potential effects on protein function.

**Fig. 5.** Chemotaxis of neutrophils isolated from WT, rac2−/−, and Rac2−/− transplanted with Rac-transduced BM. Experimental design and abbreviations are as described in Fig. 3 and 4. Chemotaxis assays performed on freshly isolated neutrophils, using a modified Boyden chamber in response to 1 or 10 μM FMLP as described under “Materials and Methods.” The ligand was loaded in the lower wells and the cells (2 × 10⁶) were loaded in upper wells separated by a 3-μm pore size filter membrane, followed by incubation at 37 °C for 45 min. The number of migrated cells was subsequently counted by microscopic examination. Results are expressed as the percentage of migrated cells per field compared with WT control neutrophils. Means ± S.E. are shown. *, p < 0.002 (versus rac2−/−); **, p < 0.0005 (versus rac2−/−). p values are based on unpaired Student’s t tests (n = 3).
for distinguishing an exogenous protein from an endogenous one. Although not the focus of this report, it is of interest that placement of either an HA or FLAG tag on the N terminus of Rac1 and Rac2, respectively, blunted their ability to enhance NADPH oxidase activity in rac2⁻/⁻ neutrophils. This suggests that these tags impaired Rac activation and/or downstream signaling. To our knowledge, this is the first time epitope tagging of a small GTPase has been reported to interfere with

**Fig. 6. Videomicroscopy analysis of fMLP-induced motility in neutrophils isolated from WT, rac2⁻/⁻, and rac2⁻/⁻ transplanted with Rac-transduced BM.** Freshly isolated neutrophils were exposed to a linear gradient of 0–10 μM fMLP in a Dunn chemotaxis chamber, and the positions of cells were recorded at 10-s intervals over a 15-min period. See also accompanying movies S2–S6 and supplementary Fig. S1. A, histogram of the mean speed distribution of WT, rac2⁻/⁻, and Rac1- and Rac2-transduced rac2⁻/⁻ cells. Data were derived from three independent experiments. B, histogram of the mean speed distribution of WT, rac2⁻/⁻, and Rac1-2- and Rac2-1-transduced rac2⁻/⁻ cells. Data were derived from three independent experiments. C, circular histograms showing the distributions of cell trajectories of WT cells or rac2⁻/⁻ cells transduced with Rac2, Rac1-2, or Rac2-1 relative to the orientation of the gradient (0°). Plots show the mean direction and 95% confidence limit for the trajectories where there was a significant non-uniform distribution. Each plot shows a representative experiment from a total of three independent experiments with similar results.
flavocytochrome with roles in shared signaling pathways. We previously showed that 12, 15). The parallel effects of exogenous Rac1, Rac2, and that are associated with reduced NADPH oxidase activity (8, 12, 15). The agonist-selective defects in NADPH oxidase activity in rac2<sup>−/−</sup> neutrophils suggest that Rac2 regulates upstream events and/or is more efficiently incorporated into the oxidase complex compared with Rac1 in response to these agonists. The first scenario is supported by the observation that Rac2-null neutrophils have other functional defects in response to agonists that are associated with reduced NADPH oxidase activity (8, 12, 15). The parallel effects of exogenous Rac1, Rac2, and chimeric Rac proteins on chemotaxis and superoxide production by rac2<sup>−/−</sup> neutrophils in this study are also consistent with roles in shared signaling pathways.

Our observations also support a critical role for Rac2 in cell orientation in chemotaxis. We previously showed that rac2<sup>−/−</sup>-cells, in addition to their impaired ability to move, are not polarized in the direction of the chemotactic gradient (15). We show here that Rac1-2, as well as Rac2 itself, fully rescued the ability of cells to move and, moreover, to properly orient their motility in a chemotactic gradient. Rac2-1 cells displayed a greater defect in chemotaxis in Boyden chamber assays than the frequency and speed of migrating Rac2-1 cells might predict, but the discrepancy can easily be explained by the remarkably disoriented nature of their motility. This is consistent with the accepted critical role for Rac in chemotactic signaling pathways (39–41) and illuminates possible isoform-specific roles in this process that have not previously been recognized. The Rac GTPases are proposed to be a component of a positive feedback loop involving the GTPase and phosphatidylinositol 3-kinase that is important for establishment of cell polarity and the maintenance of a “front-ness” signal at the leading edge (39, 40). Although we were unable to definitively determine whether Rac1-expressing cells showed directed migration (due to their poor motility and consequent failure to reach the 30-μm horizon required for the analysis), the disoriented nature of the motile response displayed by Rac2-1-expressing cells is highly suggestive of a critical role for factors that specifically interact with the Rac2 polybasic domain in establishing cell polarity and orientation. Our results suggest the need for more work to determine interactions that specify distinct roles for Rac1 and Rac2 in regulating the cytoskeleton in hematopoietic cells.

While this manuscript was in preparation, a study by Filippi and colleagues (42) reported findings generally consistent with those described here, with a notable difference with respect to reconstitution of neutrophil motility. Similar to our results, migration of fMLP-stimulated rac2<sup>−/−</sup>-neutrophils in Boyden chambers was reconstituted by expression of Rac2. However, rescue by a Rac1-2 chimera additionally required replacement of a glycine at position 150 with aspartic acid, the residue at the corresponding position in Rac2 (42). This contrasts with our findings, where expression of a Rac1-2 chimera was sufficient to reconstitute fMLP-stimulated chemotaxis in rac2<sup>−/−</sup>-neutrophils (Figs. 5 and 6). However, the study by Filippi et al. used the murine Rac2 polybasic domain, which differs from the human Rac2 sequence used here, in having a proline instead of an alanine at position 188 (Fig. 1A). Other factors that may have contributed to the different results are that Filippi et al. used neutrophils differentiated in vitro and exogenous Rac proteins that were HA-tagged and relatively overexpressed (42).

Filippi and coworkers (42) also reported differences in localization of EGFP-tagged Rac proteins in murine neutrophils, which may contribute to their different functions. In resting cells, EGFP-Rac1 was dispersed compared with a more central cytoplasmic and perinuclear localization for EGFP-Rac2. Upon fMLP stimulation both forms were also detected at more peripheral locations, with Rac1 co-localizing with cortical actin and Rac2 being somewhat more interior. Moreover, in fMLP-stimulated rac2<sup>−/−</sup>-neutrophils, EGFP-Rac1 was poorly organized, suggesting that there may be cross-talk between the two Rac isoforms. Because deficient F-actin formation is a notable feature of fMLP-stimulated rac2<sup>−/−</sup>-neutrophils, Filippi et al. proposed that Rac1 distribution may be correlated to F-actin organization and that Rac1 dysfunction in rac2<sup>−/−</sup>-neutrophils might contribute to their abnormal chemotaxis. We are currently undertaking studies to examine Rac1 and Rac2 localization in neutrophils isolated from bone marrow or peripheral blood. In preliminary studies on unstimulated murine neutrophils stained with Rac1 and Rac2 antibodies or expressing small amounts of exogenous EGFP-Rac1 or EGFP-Rac2, both isoforms were distributed diffusely in the cytosol. These results are consistent with studies on resting human neutrophils, where Rac2 is cytosolic and in a complex with Rho guanine-nucleotide dissociation inhibitor (43) but differ from the localization described by Filippi and coworkers (see above) (42). However, Rac proteins were relatively overexpressed in the latter studies, possibly exceeding Rho-guanine-nucleotide dissociation inhibitor binding capacity (10) and also studied in neutrophils differentiated in vitro using relatively high concentrations of growth factors. Neither we nor Fillipi et al. (42) detected a significant pool of nuclear Rac1 in neutrophils.

In conclusion, these results establish that Rac2 plays a non-overlapping role with Rac1 to regulate neutrophil chemotaxis and NADPH oxidase activity and demonstrate that the C-terminal polybasic domain is sufficient for conferring this specificity. Future studies will address underlying mechanisms that discriminate between Rac1 and Rac2, which may include differences in subcellular targeting as well as binding to interacting regulatory or downstream target proteins.

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