Hematopoietic Cell Regulation by Rac1 and Rac2 Guanosine Triphosphatases

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The Rho guanosine triphosphatases (GTPases) Rac1 and Rac2 are critical signaling regulators in mammalian cells. The deletion of both Rac1 and Rac2 murine alleles leads to a massive egress of hematopoietic stem/progenitor cells (HSC/Ps) into the blood from the marrow, whereas Rac1−/− but not Rac2−/− HSC/Ps fail to engraft in the bone marrow of irradiated recipient mice. In contrast, Rac2, but not Rac1, regulates superoxide production and directed migration in neutrophils, and in each cell type, the two GTPases play distinct roles in actin organization, cell survival, and proliferation. Thus, Rac1 and Rac2 regulate unique aspects of hematopoietic development and function.

Rho GTPases, members of the Ras superfamily, are critical regulators of cellular function and signal transduction pathways in eukaryotic cells. In mammalian cells, the best-studied members—Rho, Rac, and Cdc42—play distinct roles in regulating actin assembly and motility (1). However, the roles of Rho GTPases in hematopoietic cell development and function have only recently begun to be elucidated. There are three Rac GTPases—Rac1, Rac2, and Rac3—and their high conservation suggests potential overlapping functions (2, 3). Rac1 is ubiquitously expressed, whereas the expression of Rac2 is restricted to cells of hematopoietic origin (2, 3) and Rac3 is expressed primarily in the brain (4).

We have previously reported the critical roles of Rac2 in a wide variety of primary hematopoietic cells, including the regulation of adhesion, migration, oxidative activity, and gene expression (5–10). These same functions have been attributed to Rac1 in nonhematopoietic cells in which Rac2 is not expressed (11–14). The roles of Rac1 compared with the roles of Rac2 in blood cells, which express both GTPases, remain to be elucidated.

Because homozygous Rac1-deficient mice die at ~E8 (embryonic day 8) in utero (15), we generated mice with a conditional Rac1 (floxed) allele (Rac1flox/flox) (16) (fig. S1). These were compared with mice that were homozygous for both the Rac1 flox allele and a Rac2-null allele (Rac1flox/flox;Rac2−/−). Floxed Rac1 sequences were deleted by means of two methods, and the deletion of Rac1 sequences was confirmed with polymerase chain reactions (PCRs) and immunoblotts (figs. S2 and S3) (17).

Most prominently, the absence of Rac1 led to a significant reduction compared with that of the wild type in the ability of hematopoietic stem/progenitor cells (HSC/Ps) to reconstitute hematopoiesis in a non-obese diabetic/severe combined-immunodeficiency (NOD/SCID) engraftment model (18) (Fig. 1A). Engraftment is a multistep process requiring proliferation and differentiation of stem cells after the movement of these cells into the bone marrow from the blood and adhesion in the hematopoietic microenvironment (19). Rac2−/− cells demonstrated normal short-term engraftment, indicating that defective engraftment was specific for Rac1 deficiency. Although modest movement (mobilization) of HSC/Ps out of the marrow cavity into the circulating blood was observed in Rac2−/− mice, as previously reported (6), the absence of both Rac1 and Rac2 resulted in a massive mobilization of progenitor colony-forming unit cells (CFU-C) into the peripheral circulation (Fig. 1B). These phenotypes occurred despite the normal expression of β1 integrin adhesion molecules (20) and the normal expression of CXCR4, the receptor for the stromal-derived-factor-1 (SDF-1), both of which have previously been implicated in the engraftment and mobilization of stem cells (21) (Fig. 1C). However, whereas Rac1−/− HSC/Ps showed normal adhesion to fibronectin, Rac2−/− HSC/Ps and, more prominently, Rac1−/−;Rac2−/− cells displayed significantly decreased adhesion to fibronectin (Fig. 1D), strongly suggesting that Rac2 has a predominant but overlapping role with Rac1 in integrin-mediated stem cell adhesion. Moreover, mobilization of Rac1−/−;Rac2−/− HSC/Ps was associated with significantly increased expression of CXCR4 (Fig. 1C).

Rac1−/− HSC/Ps also displayed impaired growth factor–stimulated in vitro growth, as determined by progenitor colony formation (Fig. 2A and fig. S4) and expansion in liquid culture (fig. S5). Reduced Rac1−/− HSC/P growth was associated with significantly decreased thymidine incorporation (Fig. 2B). Rac1−/−;Rac2−/− cells had a more severe reduction in proliferation compared with that of the wild-type and Rac2−/− cells, and they formed profoundly abnormal colonies with no cellular halo, suggesting combined effects of impaired growth and migration. Indeed, this severe phenotype in Rac1−/−;Rac2−/− cells was associated with reduced proliferation (Fig. 2B), increased apoptosis associated with Rac2 deficiency (Fig. 2C and fig. S6), and profoundly decreased migration in response to SDF-1, as compared with that of wild-type cells (Fig. 2D).

To determine the mechanism of reduced HSC/P growth, we undertook additional analysis. Significantly fewer Rac1−/− HSC/Ps entered S and G2/M over 24 to 48 hours in response to stem cell factor (SCF), a growth factor for primitive hematopoietic cells, as compared with those in wild-type or Rac2−/− cells (Fig. 3A and fig. S7). We found that levels of cyclin D1, which is required...
for G1-S progression, were not detectable in Rac1−/− HSC/Ps after SCF stimulation (Fig. 3B). Rac1−/− but not Rac2−/− HSC/Ps also showed decreased extracellular signal-regulated kinase (ERK) (p42/p44) phosphorylation (Fig. 3C). We found these defects in cell cycle progression and signaling directly related to Rac1 deficiency, because expression of the Rac1 protein but not the Rac2 protein, induced with retrovirus-mediated gene transfer, restored cycle progression and ERK activation in Rac1−/− cells (Fig. 3A and fig. S8). Inhibitor studies in wild-type cells confirmed the
role of ERK in cell cycle progression and cyclin D1 induction (figs. S9 and S10). In addition, SCF-induced reduction in expression of cyclin-dependent kinase (Cdk) inhibitor p27kip1 was absent in Rac1−/− cells (Fig. 3B).

In contrast, increased apoptosis of Rac2−/− HSC/Ps was associated with reduced Akt activation compared with that of wild-type cells after SCF stimulation (Fig. 3D). Apoptosis and Akt activation in Rac1−/− HSC/Ps were similar to those of the wild-type cells. Expression of Rac2 but not Rac1 in Rac2−/− HSC/Ps by means of retrovirus-mediated gene transfer led to a complete reversal of the apoptotic phenotype (fig. S11). Inhibitor studies in wild-type cells confirmed the roles of phosphoinositide 3-kinase and Akt in mediating survival (fig. S12). Thus, although some overlap exists in these pathways, Rac1 predominantly regulates HSC/P cell cycle progression. Defective proliferation in these cells likely contributes to their lack of engraftment in vivo. Rac2 predominantly regulates apoptosis, which likely contributes to the profound defect in growth in vitro seen in Rac1−/−/Rac2−/− HSC/Ps.

To further examine the effects of Rac1 and Rac2 on cytoskeleton changes that are important in engraftment and mobilization, SDF-1–induced actin polymerization and cell shape changes were studied. Rac2−/− but not Rac1−/− HSC/Ps demonstrated markedly impaired cortical F-actin assembly (Fig. 3E) (22). In time-lapse video microscopy images, Rac1−/−/Rac2−/− cells demonstrated significantly reduced cell spreading and actin-based membrane protrusion and essentially no coordinated migration in response to SDF-1. Although Rac1−/− HSC/Ps showed relatively normal migration in this assay, in some cases, Rac1 deficiency was associated with an apparent delay or defect in the retraction of the uropod trailing the cell (movie S1). Thus, Rac1 and Rac2 appear to play both unique and overlapping roles in regulating the cytoskeleton, which affects adhesion and mobilization of HSC/Ps from the medullary cavity into the blood circulation.

We next studied whether Rac1 and Rac2 play unique roles in the regulation of cytoskeleton in neutrophils that are derived from HSC/Ps. Consistent with HSC/Ps, agonist-induced assembly of F-actin in neutrophils was distinctly different in Rac1−/− and Rac2−/− neutrophils (Fig. 4A). Rac2−/−, but not Rac1−/−, neutrophils demonstrated impaired cortical F-actin assembly (23). Rac1−/− and Rac2−/− neutrophils displayed normal adhesion, but Rac1−/− cells notably showed increased cell spreading by means of β2 integrins compared with that of wild-type cells (Fig. 4, B to D). Rac2−/− cells showed decreased cell spreading and Rac1−/−/Rac2−/− neutrophils were comparable in circumference to Rac2−/− cells but showed decreased adhesion by means of β2 integrins (Fig. 4D).

We next examined the functional significance of these changes in neutrophil actin assembly. In contrast to Rac2−/− neutrophils, which showed impaired chemotaxis (Fig. 4E), no defect in migration was observed in Rac1-deficient neutrophils stimulated with N-formyl-Met-Leu-Phe (fMLP). Rac1−/−/Rac2−/− neutrophils demonstrated markedly reduced migration. Rac1−/−/Rac2−/− cells also displayed normal frequency of migration when observed by video microscopy (movie S11).

**Fig. 3.** Effect of Rac deficiency on cell cycle progression, signaling pathways, and actin polymerization in HSC/Ps. Wild-type, Rac2−/−, Rac1−/−, and Rac1−/−/Rac2−/− HSC/Ps (littermate controls) that were generated by in vivo Cre expression (as in Fig. 2) were used for these assays. Similar results were obtained with cells transduced in vitro with a Cre-expressing retrovirus vector. (A) Cell cycle progression. Cells were stained and stimulated with SCF (100 ng/ml). Cycle progression into S and G2/M phases was determined by bromodeoxyuridine (BrDU) incorporation 48 hours after SCF stimulation, and cell cycle distribution was analyzed by flow cytometry using the antibody to BrDU and 7-amino actinomycin D staining. Error bars show the mean ± SD, n = 3. For genetic restoration studies, Rac1−/− and Rac2−/− HSC/Ps were transduced with a retrovirus, MIEG3–hemagglutinin antigen (HA)–Rac2 (expressing wild-type Rac2) or MIEG3–HA–Rac1 (expressing wild-type Rac1). Two days after transduction, EGFP+ and c-Kit+ cells were isolated by a fluorescence-activated cell sorter and used for cell cycle analysis. (B to D) Immunoblot analyses. HSC/Ps of each genotype were starved as above and stimulated with SCF (100 ng/ml) at the time points indicated. Cell lysates were prepared for immunoblot analyses using antibodies specific for (B) cyclin D1, p27kip1, and p21cip1, (C) phospho-p42/p44, and (D) phospho-Akt. Membranes were then stripped and blotted with antibodies for (B) β-actin, (C) total p42/p44, and (D) total Akt. Membranes loading controls. HSC/Ps were serum-depleted in Hanks’ balanced salt solution and stimulated with the chemokine, SDF-1 (100 ng/ml), for 30 s before they were fixed with 2% paraformaldehyde. Cells were stained with rhodamine-labeled phalloidin on chamber slides. Fluorescence images were acquired on a Leica microscope equipped with a deconvolution system driven by OpenLab software (31). Images shown are representatives of more than 100 cells examined for each genotype. The results shown in each panel are representatives of three independent experiments.
S2). However, as seen in HSC/Ps, Rac1 deficiency was also accompanied by an abnormal retraction of the uropod in some migrating neutrophils, suggesting a subtle defect in F-actin polymerization (24). Rac2−/− neutrophils and, more prominently, Rac1−/−;Rac2−/− neutrophils showed defects in cell polarization and migration.

Both Rac1 and Rac2 have been demonstrated to be essential for oxidase activity of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) in cell-free assays (25, 26). However, after stimulation with fMLP, the percentage of NBT− cells, a measure of oxidase activity, was identical in Rac1−/− neutrophils and wild-type cells (Fig. 4F). Rac2−/− neutrophils displayed a 60% reduction in the number of NBT− cells compared with that in wild-type cells. Deficiency of both Rac1 and Rac2 was associated with a higher reduction in the percentage of NBT− cells compared with that of Rac2−/− cells (Fig. 4F).

We next determined whether, in a manner similar to HSC/Ps, Rac proteins regulated ERK activation in neutrophils. A reduction in fMLP-induced ERK phosphorylation was seen in both Rac1−/− and Rac2−/− cells, but this reduction was reproducibly more pronounced in Rac2−/− neutrophils (Fig. 4G). Rac1−/−;Rac2−/− neutrophils demonstrated severely reduced ERK phosphorylation. In wild-type neutrophils, cortical F-actin assembly, cell migration but not superoxide production was inhibited in the presence of the Mek (ERK kinase) inhibitor U0126 (figs. S13 to S16). Thus, Rac2 appears to be a physiologically critical Rac GTPase in neutrophil migration and NADPH oxidase function, whereas Rac1 plays a role in controlling cell spreading.

Adhesion and migration of cells within the hematopoietic microenvironment are critical for blood formation and blood cell function (27). We show here that the Rho GTPases Rac1 and Rac2 are key regulators of these functions in hematopoietic cells. Despite the high degree of sequence identity, each GTPase plays unique physiological roles, particularly with respect to cell growth, survival signaling pathways, and distinct actin structures that mediate different cytoskeleton functions. Given that each Rac protein contains identical sequences mediating known effector interactions, the basis for the specificity of these functions is still to be determined. We propose that these differences likely reside in the subcellular localization of each protein (28).

References and Notes
7. F. C. Yang et al., Immunity 12, 557 (2000).
A Bayesian Networks Approach for Predicting Protein-Protein Interactions from Genomic Data

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We have developed an approach using Bayesian networks to predict protein-protein interactions genome-wide in yeast. Our method naturally weights and combines into reliable predictions genomic features only weakly associated with interaction (e.g., messenger RNA coexpression, coessentiality, and colocalization). In addition to de novo predictions, it can integrate often noisy, experimental interaction data sets. We observe that at given levels of sensitivity, our predictions are more accurate than the existing high-throughput experimental data sets. We validate our predictions with TAP (tandem affinity purification) tagging experiments. Our analysis, which gives a comprehensive view of yeast interactions, is available at genecensus.org/intact.

Many fundamental biological processes involve protein-protein interactions, and comprehensively identifying them is important to systematically defining their cellular role. New experimental and computational methods have vastly increased the number of known or putative interactions, cataloged in databases (1–7). Much genomic information also relates to interactions indirectly: Interacting proteins are often significantly coexpressed (as shown by microarrays) and colocalized to the same subcellular compartment (8, 9).

Unfortunately, interaction data sets are often incomplete and contradictory (10–12). In the context of genome-wide analyses, these inaccuracies are greatly magnified because the protein pairs that do not interact (negatives) far outnumber those that do (positives). For instance, in yeast, the ~6000 proteins allow for ~18 million potential interactions, but the estimated number of actual interactions is <100,000 (10, 13, 14). Thus, even reliable techniques can generate many false positives when applied genome-wide. This is similar to a diagnostic with a 1% false-positive rate for a rare disease occurring in 0.1% of the population, which would roughly produce one true positive for every 10 false ones. Further information is necessary. Consequently, when evaluating protein-protein interactions, one needs to integrate evidence from many different sources (15–17).

Here, we propose a Bayesian approach for integrating interaction information that allows for the probabilistic combination of multiple data sets and demonstrate its application to yeast (18). Our approach can be used for combining noisy interaction data sets and for predicting interactions de novo, from other genomic information. The basic idea is to assess each source of evidence for interactions by comparing it against samples of known positives and negatives (“gold-standards”), yielding a statistical reliability. Then, extrapolating genome-wide, we predict the chance of possible interactions for every protein pair by combining each independent evidence source according to its reliability. We verified our predictions by comparing them against existing experimental interaction data (not in the gold-standard) as well as new TAP (tandem affinity purification) tagging experiments.

Among the many possible machine-learning approaches that could be applied to predicting interactions (ranging from simple unions and intersections of data sets to neural networks, decision trees, and support-vector machines), Bayesian networks have several advantages (19): They allow for combining highly dissimilar types of data (i.e., numerical and categorical), converting them to a common probabilistic framework, without unnecessary simplification; they readily accommodate missing data; and they naturally weight each information source according to its reliability. In contrast to “black-box” predictors, Bayesian networks are readily interpretable as they represent conditional probability relationships among information sources.

The gold-standard data set on which we train (“parameterize”) the Bayesian network should ideally be (i) independent from the data sources serving as evidence, (ii) sufficiently large for reliable statistics, and (iii) free of systematic bias. We used the MIPS (Munich Information Center for Protein Sequences) complexes catalog as the gold-standard for positives (6). This hand-curated list of protein complexes is based on the literature [8250 pairs

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