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Rac GTPases in erythroid biology

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Abstract

Rac1 and Rac2 GTPases, members of the Rho GTPases family, control actin organization and play distinct and overlapping roles in hematopoietic and mature blood cells of all lineages. Here we review our findings on the role of Rac GTPases in erythroid cells, by using conditional gene-targeting in mice. Rac1 and Rac2 deficiency causes anemia with reticulocytosis, indicating decreased red blood (RBC) survival, altered actin assembly in the erythrocyte membrane skeleton and decreased RBC deformability. On the other hand, Rac1^{-/-};Rac2^{-/-} megakaryocyte-erythrocyte progenitors demonstrate decreased proliferation in the bone marrow, but increased survival and proliferation in the spleen, indicating that stress erythropoiesis circumvents Rac GTPases deficiency. Further elucidation of the signaling pathways controlled by Rac GTPases in erythroid cells may reveal potential therapeutic targets for diseases characterized by hemolytic anemia and erythropoiesis disorders.

Keywords

Rac GTPases; red blood cell membrane skeleton; erythropoiesis; actin; adducin

INTRODUCTION

Rac GTPases consist a subfamily of the Rho family of small GTPases, itself a subgroup of the Ras superfamily of small G proteins. The Rho family of small GTPases includes at least 22 members which are categorized into 6 subfamilies depending on their primary sequence, domain structure, and function [1]. Rho GTPases regulate a wide spectrum of cellular functions, many of them via control of actin polymerization. They control actin cytoskeleton, cell polarity and motility, microtubule dynamics, and vesicular transport pathways, as well as cell adhesion and migration, proliferation, and survival [2–5]. As GTPases, they cycle between an active GTP-bound form and an inactive GDP-bound form. Their activation is mediated by GEFs (guanine nucleotide exchange factors), which exchange GDP for GTP. GEFs are antagonized by GAPs (GTPase-activating proteins) which increase intrinsic GTPase activity resulting in GTP hydrolysis and the return of the GTPase to the inactive GDP-bound state [6]. Further regulation of GTPase activity is

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achieved through GDIs (guanosine nucleotide dissociation inhibitors), which inhibit nucleotide exchange and sequester small GTPases away from the membrane.

The members of the Rac GTPase subfamily are Rac1, Rac2, Rac3 and RhoG. Homologues of Rac GTPases have been found in all eukaryotic cells studied so far but not in prokaryotes. Rac1 is ubiquitously expressed, while Rac2 is expressed only in hematopoietic cells [7, 8]. Rac3 was initially considered confined to the nervous tissue but has now been identified in a wide variety of tissues [7–9]. RhoG is also widely expressed [10]. Rac1 and Rac2 GTPases have been shown to play distinct and overlapping roles in hematopoietic and mature blood cells. In neutrophils, Rac1 affects cell spreading and adhesion, while Rac2 regulates directed migration and superoxide production [11, 12]. Both Rac1 and Rac2 GTPases regulate homing, engraftment, actin cytoskeleton, cell survival, and proliferation in hematopoietic stem cells [11, 13]. We explored the role of Rac GTPases in erythropoiesis and mature RBCs in gene targeted mice where Rac1 and Rac2 GTPases have been deleted from the hematopoietic cells [14, 15]. Since Rac1 is indispensable for embryonic development [16], we used mice with germline deletion of Rac2 gene, conditional Rac1 (flox) allele and a transgene for Cre recombinase expressed via an IFN- γ -inducible Mx1 promoter (Cre^{Tg/+}; Rac1^{flox/flox}; Rac2^{-/-}). Cre-mediated recombination was carried out by intraperitoneal injections of polyinosinic-polycytidylic acid (pI-pC). Here we review our team's and other researchers' findings on the role of Rac GTPases in erythroid biology.

Rac GTPases and the erythrocyte membrane skeleton

Rac1^{-/-};Rac2^{-/-} mice develop microcytic anemia with hemoglobin decreased by an average of 20%, significant anisocytosis and poikilocytosis in erythrocyte morphology, and approximately doubling of the reticulocyte count indicating hemolysis. The decreased survival of Rac1^{-/-};Rac2^{-/-} erythrocytes is likely due to decreased cellular deformability (down to ~80% of WT control cells), leading to higher rates of RBC rupture while journeying through the microvasculature [14]. This decreased erythrocyte deformability appears to be secondary to impaired membrane skeleton mechanical stability.

The normal erythrocyte membrane skeleton is organized as a hexagonal lattice and underlies the cell membrane. Each side of a hexagon consists of a long, flexible spectrin tetramer, constructed by two α - and β -spectrin heterodimers connected head to head, with their tails ending to junctional complexes, each centered around a short actin filament [17, 18]. The actin filament (F-actin) has an average length of 12–18 monomers (G-actin) [19]. The capping proteins adducin and tropomodulin, when bound at the rapidly assembling (barbed or leading) end and the slowly assembling (pointed) end, prevent further actin monomer association or dissociation [20–22]. Tropomodulin, protein 4.1R, and dematin also participate in the structure of the junctional complex [17]. The RBC membrane skeleton is anchored to the lipid bilayer at points along the spectrin heterodimers through multi-protein complexes containing ankyrin, band 3, and protein 4.2, as well as at the junctional complexes through transmembrane proteins such as band 3 and Glut1 (glucose and L-dehydroascorbic acid transporter) [17].

The actin oligomers of the junctional complexes have been traditionally considered as structural blocks that remain stable through the RBC lifetime, steadily capped by adducin and tropomodulin [23]. However, our studies in $Rac1^{-/-}; Rac2^{-/-}$ mice demonstrate that erythrocytes deficient of Rac1 and Rac2 GTPases have a disorganized membrane skeleton with meshwork gaps, F-actin aggregates, and irregular clamping of band 3, as seen by confocal microscopy [14]. Electron microscopy studies show junctional aggregates and irregularity of the hexagonal spectrin scaffold. The actin-to-spectrin ratio in membrane (ghost) preparations of these cells is increased, while adducin and dematin content is decreased compared to wild type cells. $Rac1^{-/-}; Rac2^{-/-}$ erythrocytes exhibit increased phosphorylation of adducin at Ser724, which is a protein kinase C (PKC) target. Phosphorylation of adducin at Ser726 in human platelets (which corresponds to Ser724 in murine adducin) leads to actin-spectrin dissociation [24–26]. Indeed, actin and phosphorylated adducin of $Rac1^{-/-}; Rac2^{-/-}$ erythrocytes are more easily extractable by Triton X-100, indicating weaker association to the membrane skeleton. Phosphorylation of adducin in platelets is expected upon platelet activation when actin filaments are formed and the cytoskeleton is remodeled. The fact that RBC adducin is also phosphorylated in $Rac1^{-/-}; Rac2^{-/-}$ erythrocytes implicates a dynamically-maintained balance in the erythrocyte membrane skeleton that is disrupted by the deficiency of Rac GTPases (Figure 1).

Rac GTPases in erythropoiesis

The production of mature blood cells of the different lineages requires the differentiation of HSCs through a successive series of increasingly lineage-restricted intermediate progenitors. The first step of commitment occurs at the levels of the common lymphoid progenitors (CLPs), which will differentiate into T, B, and natural killer (NK) cells [27], and of the common myeloid progenitors (CMPs) which will give rise to the granulocyte-macrophage progenitors (GMPs) and the megakaryocyte-erythroid progenitors (MEPs) [28]. Under the influence of cytokines, such as stem cell factor (SCF), interleukin-3 (IL3), and erythropoietin, erythroid progenitors and precursors proliferate and differentiate through a variety of stages until reaching maturity as enucleated reticulocytes that enter the circulation [28]. The whole process of hematopoiesis, including erythropoiesis, is tightly regulated by the interplay of mutually-dependent transcription factors with controls adjusted for both dosage and timing [29–31].

Studies performed in hematopoietic stem cell/progenitor cells (HSC/Ps), purified by FACS sorting as c-Kit⁺ (SCF receptor positive) cells depleted of lineage markers, demonstrated that Rac1 and Rac2 GTPases regulate unique aspects of hematopoietic development and function [11] (Figure 2). $Rac1^{-/-}; Rac2^{-/-}$ HSC/Ps demonstrate reduced proliferation in response to SCF which is also seen in the $Rac1^{-/-}$ HSC/Ps, associated with non-detectable cyclin D1 levels and with decreased extracellular signal-regulated kinase (ERK) (p42/p44) phosphorylation. Increased apoptosis is noted in $Rac1^{-/-}; Rac2^{-/-}$ and $Rac2^{-/-}$ HSC/Ps, associated with reduced AKT activation compared with that of wild-type cells after SCF stimulation. $Rac1^{-/-}; Rac2^{-/-}$ HSC/Ps display decreased adhesion to fibronectin, despite the normal expression of CD49d (α4β1) and CD49e (α5β1) integrin adhesion molecules on these cells [11]. Since $Rac1^{-/-}$ HSC/Ps showed normal adhesion to fibronectin and $Rac2^{-/-}$

HSC/Ps showed an intermediate decrease of adhesion, it appears that Rac2 has a predominant but overlapping role with Rac1 in integrin-mediated stem cell and progenitor adhesion. In addition, $Rac1^{-/-}; Rac2^{-/-}$ HSC/Ps show decreased migration in response to SDF-1, as compared with that of wild-type cells although they have a significantly increased expression of CXCR4, the receptor for the stromal-derived factor-1 (SDF-1) [11]. Since Rac GTPases are key components of the signaling pathways downstream of the SCF-ligand c-kit [32], the chemokine receptor CXCR4 [33], and the $\beta 1$ integrin-receptors for fibronectin [34, 35], all significant mechanisms of interaction of HSC/Ps with the bone marrow microenvironment, it is not surprising that combined Rac1 and Rac2 deficiency results in massive mobilization of progenitor colony-forming unit cells (CFU-C) into the peripheral circulation and results in increased homing of CFU-C in the spleen [13] (Figure 2).

$Rac1^{-/-}$ or $Rac2^{-/-}$ mice have normal erythropoiesis indicating a redundant role for these two GTPases in the erythroid lineage. However, with combined deficiency of Rac1 and Rac2 GTPases, significant defects of erythropoiesis are noted [15]. Burst forming units-erythroid (BFU-E) in the bone marrow of $Rac1^{-/-}; Rac2^{-/-}$ mice are slightly decreased in number but the morphology of the colonies produced in colony assays is significantly altered: small, round, and condensed indicating likely a defect in migration. The colony forming units-erythroid (CFU-E) and ensuing erythroblasts are significantly decreased in number in $Rac1^{-/-}; Rac2^{-/-}$ bone marrow. Correspondingly, MEPs (that include BFU-E and CFU-E along with megakaryocyte progenitors) are significantly decreased in the bone marrow due to decreased proliferation [15]. In contrast, MEPs (particularly the subset population of CFU-E) are significantly increased in the spleen, mounting a stress erythropoiesis in response to the hemolytic anemia due to the cytoskeleton defect described above [14]. The increase of MEPs in the spleen can likely be attributed, in part, to increased homing to the spleen (Figure 2), as well as to increased proliferation and decreased apoptosis as demonstrated by flow cytometry studies [15]. It is likely that erythropoietic cytokine signaling is impaired in the bone marrow-residing erythroid progenitors deficient of Rac1 and Rac2, due to mechanisms similar to the ones identified in $Rac1^{-/-}; Rac2^{-/-}$ HSC/Ps (Figure 2). However, within the splenic microenvironment or under the influence of unique cytokines acting in the spleen during stress erythropoiesis, like BMP4 (bone morphogenetic protein 4) [36] the deficiency of Rac GTPases in erythroid progenitors is successfully by-passed [37].

By using constitutively active or dominant negative mutants of Rac1 and Rac2 in cultured mouse fetal liver erythroblasts, Ji et al. showed that either excessive activation or inhibition of Rac GTPases inhibit enucleation [38]. Pharmacologic inhibition of Rac GTPases by NSC23766, a specific Rac inhibitor [39], was also shown to inhibit enucleation [38, 40]. However, enucleation is not compromised in $Rac1^{-/-}; Rac2^{-/-}$ erythroblasts, where Rac3 is upregulated which may compensate for Rac1 and Rac2 deficiency [40].

In conclusion, Rac GTPases play significant role in various stages of erythropoiesis and are vital for the stability, plasticity, and maintenance of the RBC membrane skeleton. Further elucidation of the signaling networks involving Rac GTPases in erythroid biology may offer therapeutic targets for the pharmacologic management of diseases characterized by defective erythropoiesis and/or hemolysis.

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Figure 1.

A. In erythrocytes deficient of Rac1 and Rac2 GTPases, there is increased phosphorylation of adducin by PKC, leading to decreased F-actin capping at the barbed ends, dissociation of spectrin from actin, and increased fragility of the RBC cytoskeleton. **B.** 3-dimensional reconstitution (using Volocity software, Improvision, Lexington, MA) of Z-stack images obtained by confocal microscopy of wild-type (WT) and $Rac1^{-/-}; Rac2^{-/-}$ erythrocytes, stained with rhodamine-phalloidin for F-actin. Arrowheads show aggregates of F-actin in $Rac1^{-/-}; Rac2^{-/-}$ erythrocytes.

Figure 2.

Rac1 and Rac2 GTPases mediate proliferation, survival, adhesion, and migration of hematopoietic stem cells and early myeloid and erythroid progenitors in the bone marrow microenvironment. Their deficiency results in massive mobilization of progenitors and increased homing in the spleen. While Rac1 and Rac2 are necessary for homeostatic erythropoiesis in the bone marrow, they are dispensable for stress-erythropoiesis in the spleen. (The bizarre morphology of $\text{Rac1}^{-/-};\text{Rac2}^{-/-}$ erythrocytes as visualized by confocal microscopy of fixed cells is depicted within the sketched vessel).