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Forum Review

Rho GTPases in Hematopoietic Cells

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ABSTRACT

The ubiquitous Rho GTPases are instrumental in the organization of the actin cytoskeleton, but also for the control of gene expression. Here we review the role of the major members of this family, *i.e.,* RhoA, Rac1, Rac2, and Cdc42, and their intracellular signaling in hematopoietic cells. Although these proteins have been classically implicated in chemotaxis, there are now clear indications on how differential signaling toward other, more specific functions, such as phagocytosis or the production of reactive oxygen species, is regulated by relatively small differences in primary sequence. The identification of mutations in these GTPases or their regulators has provided novel insights in their function as well as their relevance for the development of hematological diseases. *Antioxid. Redox Signal.* 7, 1440–1455.

INTRODUCTION

The Rho guanosine trisphosphatases (GTPases) are members of the Ras superfamily and include the subfamilies Rho, Rac, and Cdc42. They function as molecular switches that cycle between the inactive state when bound to GDP and the active state when bound to GTP. In the GTP-bound state, GTPases recognize target proteins and generate a response until GTP hydrolysis brings the GTPase back to the inactive state. Spatial and temporal regulation of GTPase activity is carefully regulated by activating proteins, *i.e.,* guanine nucleotide exchange factors (GEFs) and GTPase activating factors (GAPs), and proteins that extract inactive GTPases from the membrane, *i.e.,* guanine nucleotide dissociation inhibitors. Active GTPases transduce signals from membrane receptors to the cytoskeleton, but are also involved in activating related signal transduction pathways, thereby regulating many different cellular processes, such as actin assembly (98, 99), adhesion (98, 99), motility (97), oxidase activity (1), cell-cycle progression (89, 90, 133), and gene expression (13, 34). Rho GTPases act through a myriad of effector proteins to activate these different processes. The outcome of Rho GTPase activation is, to a certain extent, dictated by cellular makeup. However, because most of the signaling of these GTPases drives basic cellular functions, the majority of effects of Rho GTPases can be observed in any type of cell. Detailed overviews on a discussion of Rho GTPase signaling mechanisms can be found in several excellent recent reviews (19, 29, 95, 100, 126). In this review, we discuss the role of the Rho GTPases in migration, proliferation, and differentiation of hematopoietic cells in physiological and pathophysiological conditions (for overview, see Fig. 1).

RHO GTPASES IN MIGRATION OF HEMATOPOIETIC CELLS

As in all other cells, polarization and directional migration are orchestrated by a large number of receptors and intracellular signaling proteins, of which the Rho GTPases are particular major players. The best characterized members of this family are RhoA, Rac1, Rac2, and Cdc42. These GTPases relay signals from receptors at the plasma membrane to the actin and microtubule cytoskeleton, as well as to kinase cascades that either affect adhesion or lead to nuclear signaling and the modulation of transcription. Although models describing the specific roles of different GTPases in cell polarity or migrational capacity may differ, it is generally accepted that these proteins are crucial for cytoskeletal dynamics and

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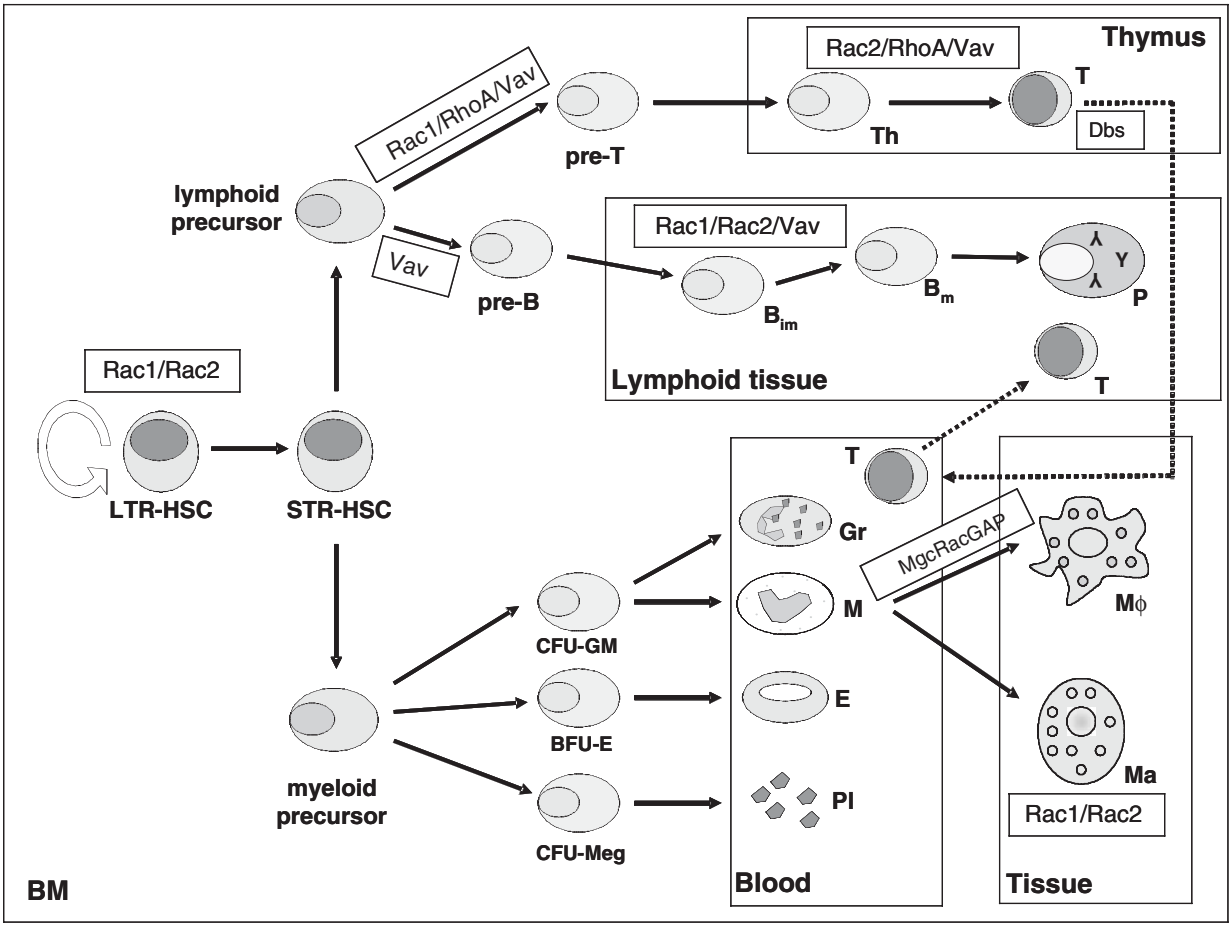

FIG. 1. Rho GTPases in hematopoietic cell proliferation and differentiation. See text for details. BM, bone marrow; LTR-HSC, long-term repopulating hematopioetic stem cell; STR-HSC, short-term repopulating hematopoietic stem cell; CFU-GM, colony-forming unit-granulocyte-macrophage; BFU-E, burst-forming unit-erythroid; CFU-Meg, colony-forming unit-megakaryocyte; Gr, granulocyte; M, monocyte; E, erythrocyte; Pl, platelet; Mϕ, macrophage; Ma, mast cells; Vav, GEF for Rac; pre-T, pre-T-cell; Th, thymocyte; T, T-cell; Dbs, GEF for Cdc42, RhoA, and RhoG; pre-B, pre-B-cell; B_{im}, immature B-cell; B_m, mature B-cell; P, plasma cell. Open arrow indicates self-renewal; solid arrow indicates proliferation and differentiation; dashed arrow indicates migration of mature cells.

integrin activation, and represent master regulators of (chemokine-driven) migration. Whereas RhoA, Rac1, and Cdc42 are ubiquitously expressed, Rac2 expression is confined to the hematopoietic compartment. Rac2, similar to the other Rho GTPases, is involved in cell migration, but plays also important roles in various physiological processes that are more relevant for leukocytes, such as phagocytosis and the generation of an oxidative burst.

Initial studies on the role of Rho GTPases in hematopoietic cell migration have relied mainly on expression of activated or dominant-negative mutants of RhoA, Rac1, or Cdc42 (22). These studies showed that both active and inactive mutants of RhoA, Rac, and Cdc42 impaired lymphocyte polarization and migration, underscoring their importance in these functions and also indicating that cycling is an essential aspect of GTPase signaling.

For hematopoietic stem and progenitor cells (HSPC), the best defined chemokine is stromal derived factor-1 (SDF-1). Although the role of Rho GTPases in cell migration, also in HSPC, is well accepted, there are surprisingly few articles that actually show that SDF-1 activates these proteins in hematopoietic cells. Nishita *et al.* showed that SDF-1 activates Rac1 and Cdc42 in Jurkat T cells in a pertussis toxin (PTX)-sensitive fashion, whereas RhoA activation was PTX-insensitive (88). This suggests specific signaling via $G\alpha_i$ proteins toward Rac and Cdc42, but not to RhoA. In another study, Whetton *et al.* showed that overall migratory potential is lower in primitive compared with more mature hematopoietic cells (138). They found that this migration can be boosted by stimulating the cells with lysophospholipids (lysophosphatidic acid, sphingosine-1-phosphate) in combination with SDF-1 and that Rho GTPases were implicated. The authors based this on the observed effects of *Clostridium difficile* toxin B, which inactivates all three major Rho GTPases (Rho, Rac, Cdc42), and on the use of Y27632, an inhibitor of one of the downstream targets of RhoA, ROCK (Rho kinase). In cells that lack the hematopoietic-specific activator of these Rho GTPases, Vav-1, migration was also severely impaired. However, the differential expression of Rho GTPases throughout hematopoiesis and its possible relation

to migratory capacity of the various cell types remain to be described in more detail.

Most emphasis over the past years has been on the differential role of the very homologous Rac1 and Rac2 GTPases in migration and host defense in hematopoietic cells. Their high level of homology and the resultant lack of discriminating tools, such as specific antibodies, have hampered the analysis of the differential role of these Rac proteins. Only recently, thanks to the use of elegant mouse models and transgenic/reconstitution models based on chimeric Rac proteins, specific functions of Rac1 and Rac2 in HSPC, as well as in more mature hematopoietic cells, have been addressed in more detail.

The Rac2 GTPase is specifically expressed in hematopoietic cells, in varying ratios compared with Rac1 depending on the lineage of differentiation and maturation stage. Its expression suggests that Rac2 plays an important role in those functions that may be relatively specific for hematopoietic cells. These functional differences were studied directly when Rac2-deficient mice were generated, allowing studies on hematopoiesis and leukocyte-specific functions (101). Indeed, this study showed that Rac2 deficiency particularly affected functions related to host defense, such as L-selectin-mediated tethering, chemotaxis and actin polymerization, oxidative burst, and activation of mitogen-activated protein kinases (MAPKs). The bias toward analysis of host defense in this study relates to the fact that this work was mainly based on $Rac2^{-/-}$ neutrophils, a cell type in which Rac2 is relatively abundant, *e.g.,* compared with monocytes. Although no gross alterations in hematopoiesis were found, Roberts *et al.* report that $Rac2^{-/-}$ mice have an increased number of granulocytes in the bone marrow, suggesting that Rac2 also plays a role in granulocyte differentiation (101).

Further *in vivo* evidence that Rac2 is specifically important for host defense was obtained upon mutational analysis of a patient with phagocytic immunodeficiency (recurrent infections, reduced or absent neutrophil function). This patient was found to carry a mutation in the Rac2 gene (D75N) resulting in expression of a dominant-negative version of the Rac2 protein (5, 139; see also below). From most studies, it has become clear that effects of Rac1 or Rac2 deficiency are due to specific defects, rather than to an overall reduction of Rac levels. For instance, in wild-type murine neutrophils, Rac2 was preferentially activated over Rac1, despite similar levels of expression (73). These authors further conclude that the preferential activation of Rac2 over Rac1 in neutrophils is one of the reasons that may explain the functional differences between these GTPases in these cells. To what extent there are differences in GEF selectivity toward Rac1 versus Rac2 is presently unknown.

Whereas the number of granulocytes in the bone marrow is increased in Rac2-deficient animals, the numbers of HSPC in the bone marrow of these animals remained equal compared with control. In contrast, the Rac2 deficiency results in increased numbers of HSPC in the peripheral blood, suggesting that Rac2 is required to retain cells in the bone marrow microenvironment. This notion is supported by the observation of a three- to fourfold increase in granulocyte colony stimulating factor-induced mobilization of HSPC in the $Rac2^{-/-}$ mice over control animals. Although VLA4 and VLA5 expression was unaltered in Rac2-deficient cells, the VLA4-, but not VLA5-mediated adhesion was significantly reduced, indicating that Rac2 apparently specifically signals to regulate VLA4 integrins. This finding correlates with the increased mobilization of HSPC *in vivo* in $Rac2^{-/-}$ mice, which was mimicked by injection of anti-α-4 antibodies. The effects on motility of $Rac2^{-/-}$ cells were found to be due to increased compensatory activation of Rac1 and Cdc42 and increased filamentous actin (F-actin) polymerization following SDF-1 (146).

Gu *et al.* showed that short-term engraftment of HSPC in NOD/SCID mice is impaired in cells lacking Rac1, whereas deficiency of Rac2 did not affect the engraftment (39). Interestingly, deletion of both Rac1 and Rac2 deprives the cells of the capacity to stay in the bone marrow, resulting in massive mobilization. This is likely due to strongly reduced β-1-mediated adhesion in the $Rac1^{-/-}$/$Rac2^{-/-}$ cells. Single deficiency of Rac1 or Rac2 leads to impaired *in vitro* migration toward SDF-1, a response that is further reduced when both GTPases are absent, indicating that for migration, these GTPases are partially redundant. Intriguingly, in particular Rac2 was found to be required for cortical actin reorganization, both in HSPC and HSPC-derived neutrophils. In these cells, also polarization was impaired when Rac2 was absent; tail retraction was shown to be dependent on Rac1 (39).

In bone marrow-derived macrophages, deletion of Rac1, using a tissue-specific knockout, does not impair migration or chemotaxis (132). In contrast, these $Rac1^{-/-}$ macrophages showed reduced membrane ruffling, which indicates that perhaps Rac1 in these cells is more important for membrane protrusion and phagocytosis. In hematopoietic cells, Rac1 and Rac2 show variable levels of redundancy as in some models (73, 146), but not in others (132), compensatory up-regulation and increased activation of other Rho GTPases have been observed. The idea that Rac1 would be the more relevant GTPase for murine macrophage phagocytosis (132) is contradicted by a study by Yamauchi *et al.,* who showed that, even though Rac1 is four times more abundant than Rac2, deficiency of Rac2 in these cells leads to impaired phagocytosis of IgG-opsonized sheep red blood cells, but not of serum-opsonized zymosan (144). This suggests that Rac2 has a specific role in Fcγ-R-mediated, but not β2-integrin-mediated phagocytosis and that Rac1 cannot efficiently compensate for this defect. Similarly, a reduced induction of the oxidative burst, triggered by either phorbol ester, FcγR, or zymosan-induced phagocytosis, was observed in $Rac2^{-/-}$ macrophages. In contrast, actin polymerization in these cells was normal, indicating that this is a Rac1-mediated response for which Rac2 is not required. Clearly, Rac1 and Rac2 have both overlapping as well as distinct roles in various functions of hematopoietic cells.

CONTROL OF DIFFERENTIAL SIGNALING BY RHO GTPASES

Rac2 is >95% homologous to Rac1, but the fact that cells that lack either of these GTPases show selective functional defects indicates that there is no or only limited redundancy. So how is this signaling specificity between two small proteins that are almost identical achieved? The explanation for

this relates to a small number of amino acid differences that are primarily concentrated in the C-termini of these GTPases. These C-termini, which also harbor the CAAX box that mediates lipid modification, have classically been assumed to mediate membrane association, based on the lipid anchor and via the negatively charged residues. More recently, it was shown that the C-terminal domain is important for subcellular (membrane) localization of a range of small GTPases (80). Based on a number of studies, including from our own lab, there is increasing evidence that these C-termini not only carry the lipid anchor for membrane localization, but also mediate protein–protein interactions that may be the crucial determinants for proper intracellular targeting of the different GTPases (124, 131). These interactions include, for Rac1, binding to phosphoinositide-5-phosphate kinase (PIP-5-kinase), which may be involved in polarized lipid metabolism and thus in directional migration, and to the Crk adapter protein, which may connect the Rac pathway to, *e.g.,* Rap signaling and the control of integrin-mediated adhesion (54). The association of Rac1 signaling with localized phosphoinositide formation is further supported by the finding that the Rac1 C-terminus, without the lipid anchor, by itself is sufficient to localize to membrane lipid rafts (124).

Using cell-permeable C-terminal peptides as selective inhibitors, our lab has shown that primary CD34+ cells rely primarily on Rac1 and Cdc42 for migration, whereas inhibition of Rac2 and RhoA did not impair migration significantly in these primary cells. This inhibitory effect of the Rac1 C-terminus on cell migration is related to its inhibition of actin polymerization (124) and of microvilli dynamics (87). Mechanistically, Rac1 functioning has been linked to its association with various proteins, such as the lipid kinase PIP-5-kinase and the adapter protein Crk (124), and to its inactivation of ezrin radixin moezin family proteins (87). The Rac-related GTPase Cdc42 has been shown in T-cells to be activated by SDF-1 and to activate Wiskott–Aldrich syndrome protein (WASp), which is required for cytoskeletal remodeling and cell migration (41). Additional proteins that were recently proposed to mediate SDF-1-induced migration in HSPC include adseverin and gelsolin. These are proteins related to the control of actin dynamics (28). The mode of regulation of these proteins by SDF-1 remains to be established.

Our own work has shown that the C-terminus of Rac1 can mediate Rac1 association with lipid rafts (124). In addition, CXCR4 associates with lipid rafts and is distributed in a polarized fashion in the leading edge of SDF-1-stimulated, migrating cells (36, 122). As Rac activation has been described to occur at the leading edge in neutrophils (33), this suggests that SDF-1-mediated signaling by CXCR4 occurs in rafts, and that Rac1 is targeted to these sites to control membrane protrusion and migration. This notion is also in line with recent data obtained with THP1 cells (142).

Two recent studies by the Williams and Dinauer groups provide further insight in the control of differential signaling by the Rac1 and Rac2 GTPases. These groups have reconstituted Rac1 or Rac2 expression in GTPase-deficient murine neutrophils or used chimeric Rac1 and Rac2 proteins that carry the C-terminus of Rac2 or Rac1, respectively (30, 144). They have shown that the C-termini of Rac2 and Rac1 mediate localization differences that are crucial for functionality. In $Rac2^{-/-}$ hematopoietic progenitor cell-derived neutrophils, reintroduction for Rac2, but not Rac1, restores the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced production of superoxide. Similarly, the defect in fMLP-induced chemotaxis was restored by Rac2, but not by Rac1, in line with the notion that Rac1 in neutrophils (30), as in macrophages (132), is not essential for migration.

Using green fluorescent protein-fusion proteins, Filippi *et al.* also showed that Rac1 and Rac2 have a different intracellular distribution following fMLP stimulation, with Rac1 more associated with F-actin, compared with Rac2. In addition, using chimeric Rac proteins that have the C-terminal tail of the other Rac protein, they showed that the Rac2 C-terminus in the background of Rac1 is sufficient for restoration of superoxide production, but not of fMLP-induced chemotaxis (30). This suggests that, for migration, additional sequences outside the C-terminus are also important. Detailed mutational analysis revealed that a negatively charged residue at position 150 (aspartic acid in Rac2) is required for cell migration. Rac1 has a glycine at this position, and mutating this to an aspartic acid, in conjunction with a Rac2 C-terminus, allowed the Rac1 chimera to fully restore migration as well as actin polymerization in $Rac2^{-/-}$ neutrophils. In addition, the C-terminus and residue 150 were shown to be critical determinants for the intracellular localization of Rac2 (30).

In conclusion, in neutrophils as well as in macrophages, Rac2 is the most important isoform for the generation of reactive oxygen species (ROS) as well as for chemotaxis. This is not due to altered relative expression levels in knockouts, but is due to specific sequences in the C-terminus, as well as at Rac2 residue 150, that control intracellular localization and, thereby, specific biological effects. The latter may relate to interactions with either targeting or docking proteins, with exchange factors or with downstream effectors, or a combination thereof.

RHO GTPASES IN PROLIFERATION AND DIFFERENTIATION OF HEMATOPOIETIC CELLS

Mature peripheral blood cells have specialized functions in combination with a limited life span (except for lymphocytes), have limited or no ability to proliferate, and are incapable of self-renewal. The replacement of these cells depends on the function of less differentiated hematopoietic progenitor cells with proliferative capabilities that respond to humoral regulators. The most primitive hematopoietic cells are the multipotent hematopoietic stem cells that possess extensive self-renewal capabilities and generate primitive progenitors that are programmed to differentiate (commitment).

The pluripotent hematopoietic stem cells reside in the bone marrow cavity during adult life (2) with only a few cell clones participating in the production of committed progenitors and eventually billions of mature blood cells (44–46, 57, 60). The largest part of the stem cell pool remains in a quiescent state (42, 53, 69, 70, 113). The availability of dominant-negative or dominant-active constructs of the respective GTPases and the possibility to make conditional knockouts have provided the

possibility to reveal the differential role of the GTPases in regulating the size and function of the hematopoietic stem cell compartment as well as differentiation into the various hematopoietic lineages.

As deletion of Rac1 results in embryonic death (114), a conditional knockout of the Rac1 gene (39) allowed the investigation of Rac1 and Rac2 function in hematopoietic stem cell proliferation and differentiation [for review, see Weston *et al.* (136)]. Gu *et al.* found that although Rac1$^{-/-}$ HSPC showed impaired *in vitro* growth factor-induced proliferation and abnormal colony formation compared with wild-type and Rac2 knockouts, Rac1/Rac2 double knockout-derived HSPC formed colonies with both impaired growth and migration (39). The proliferation defect was caused by defective cell-cycle progression. In addition, an increased rate of apoptosis was associated with the Rac2$^{-/-}$ phenotype, which revealed defective activation of the pro-survival protein Akt-1, in response to stem cell factor (SCF). Therefore, although there is some functional redundancy, Rac1 appears to predominantly regulate cell-cycle progression and therefore proliferation, whereas Rac2 predominantly controls apoptosis and thus growth of the HSPC compartment (50).

In addition to the defects in the HSPC compartment, Rac2$^{-/-}$ mice were shown to exhibit multiple defects in B-cell development. In addition to reduced numbers of peripheral blood B-cells and IgM-secreting plasma cells, a severe reduction in the number of marginal zone and peritoneal B1 cells was observed (20). Walmsley *et al.* generated a conditional B-cell lineage-specific Rac1 knockout, *i.e.,* Rac1B. Rac1B mice had a comparable number of B-cells in bone marrow, spleen, and lymph nodes to control mice (129). The possibility of functional redundancy with Rac2 was excluded as Rac1B;Rac2$^{-/-}$ mice also exhibited apparently normal immature B-cell development. However, these double knockout mice showed an enhanced phenotype of Rac2$^{-/-}$ mice with severely reduced numbers of splenic marginal zone B-cells and peritoneal B1-cells (20, 129). As B-cell receptor (BCR) signaling regulates B-cell differentiation, the developmental block at the T1 stage of B-cell development in the Rac1B; Rac2$^{-/-}$ suggests that Rac1 and Rac2 are critical for BCR signaling. Furthermore, counterselection against Rac1 deletion was observed only in mature B-cells, suggesting that both Rac GTPases are essential for B-cell differentiation past the T1 stage (129).

With regard to the role of Rac2 in T-cell differentiation, Li *et al.* have reported that Rac2 is a T-helper cell 1 (Th1)-specific gene that activates interferon-γ (IFNγ) production both *in vitro* and *in vivo* through simultaneous activation of both the nuclear factor-κB (NFκB) and p38 pathways (71). The finding that inactivation of Rac2 in primary T-cells, either by a dominant-negative transgene or by gene targeting of Rac2, inhibits IFNγ production, demonstrates that Rac2 activation is required for IFNγ production during normal T-cell activation and Th1-cell differentiation (71).

Henning *et al.* showed that Rho has a critical role in determining the size of the mature T-cell compartment (49). Using thymic targeting of a transgene encoding bacterial C3 transferase from the *Clostridium botulinum*, leading to tissue-specific Rho inhibition, severe impairment of the generation of normal numbers of thymocytes and mature T-cells was observed. This appeared to be due to a dramatic proliferative and cell-survival defect during T-cell development. The positive and negative selection of thymocytes in the thymus, as well as the differentiation of thymocytes to mature T-cells, appeared to be normal in the absence of functional Rho (49). Data showing that Rac1 activity in pre-T-cells requires RhoA, but that RhoA cannot replace Rac1 to induce the cytoskeletal rearrangements necessary for pre-T-cell development, indicate the mutual dependence of these GTPases in signaling regulating T-lymphocyte maturation (19).

Timokhina *et al.* have provided evidence for a role of Rac1 in the proliferation of mast cells (121). They showed that in bone marrow-derived mast cells (BMMC), phosphatidylinositol 3-kinase (PI3-kinase) and Src kinase are activated by SCF and that both pathways converge to activate Rac1 and Jun N-terminal kinase (JNK). The concomitant activation of both Rac1 and JNK appeared to be critical in SCF-induced proliferation of BMMC, but not for suppression of apoptosis (121). By using mast cells derived from Rac2-deficient mouse bone marrow, Yang *et al.* subsequently reported that Rac2, and indeed not Rac1, is critical in regulating the growth factor-induced survival, similar to the situation in HSPC (145). The underlying mechanism appeared to be the activation of Akt and a change in expression levels of the Bcl-2 family members BAD and Bcl-XL (145). Moreover, the importance of Rac2 expression in mast cell function was shown in a study in which gene expression profiles between wild-type and Rac2$^{-/-}$ BMMC after cytokine stimulation were compared (38). The authors concluded that Rac2 is involved in the regulation of transcription of genes relevant for the function of mature mast cells in response to SCF. This was shown to be regulated via the activation of JNK proteins and c-Jun-regulated transcription. The Rac1 and NFκB pathway appeared not to be involved, in spite of the increased cellular levels of these proteins in activated form (38).

Further supplementary information on the SCF signaling route in mast cells was provided by a recent report by Sivalenka and Jessberger showing that SWAP-70, a PI3-kinase-dependent protein that interacts with Rac and that is highly expressed in mast cells, is upstream of the GTPases in SCF-stimulated mast cells. BMMC from SWAP-70-deficient mice were used to show that SWAP-70 is necessary for the translocation to the membrane of both Rac1 and Rac2 after exposure to SCF. In addition to a disturbed (de)polymerization of actin, an aberrant distribution of polymerized actin in stimulated cells, and a retarded but sustained translocation to the membrane of Akt in SWAP-70-deficient cells, this led to a defective mast cell activation, adhesion, and migration (*in vitro* and *in vivo*) (110).

Besides the direct effect of GTPase expression levels on the differentiation and proliferation of hematopoietic cells, an additional regulatory mechanism is provided by GAP and GEF protein activity. This is illustrated by the data obtained with Vav-deficient mice independently generated and described by three research groups (31, 119, 148). Extensive characterization of these mice revealed that the absence of Vav, an exchange factor for Rac, leads to reduced proliferation, differentiation, and activation of T- and B-cells, result-

ing in low numbers of mature B- and T-cells in the periphery. Only part of this immunological phenotype can be overcome if activated Rac1 is expressed in Vav-deficient cells. This indicates that the GTP exchange capacity of Vav is not the only relevant function of this protein in lymphocyte development and maturation, but that Vav also functions as an adapter molecule to assemble signaling complexes (35). The relative contribution of the Vav isoforms to the development of a functional compartment of mature B- and T-cells was provided by publications of Tedford *et al.* (120) and Fuijkawa *et al.* (32).

The T-cell receptor (TCR) repertoire, determining the ability of T-cells to recognize a multitude of MHC-presented antigens, is changed during thymocyte selection ensuring lack of reactivity toward self-antigens. Mice transgenic for constitutive active Dbs, an exchange factor for Cdc42, RhoA, and RhoG, revealed an increased sensitivity of certain subsets of thymocytes for deletion, suggesting that Dbs is involved in restriction or alteration of the TCR repertoire that is generated by thymocyte selection (62).

Another layer of complexity to the function of GAPs in the proliferation and differentiation of hematopoietic cells was added by the work on a Rac/Cdc42 GAP, MgcRacGAP. Overexpression of this protein induced differentiation of human leukemic HL60 cells into macrophages, retarded proliferation, and induced formation of multinucleated cells in HL60 and M1 cells (59). These data, and the observation that the highest expression level of MgcRacGAP mRNA was observed in the G2/M phase, led to the hypothesis that MgcRacGAP would be involved in the regulation of cytokinesis. Further evidence for this was provided by immunohistochemical studies showing that MgcRacGAP colocalized with the mitotic spindle in metaphase, was transferred to the midzone in anaphase and telophase, and moved to the midbody in cytokinesis (52). Recently, Minoshima *et al.* showed that AuroraB functionally converts MgcRacGAP from a Rac/Cdc42 GAP to a RhoA GAP during cytokinesis through serine/threonine phosphorylation (81).

ROS IN HSPC

One of the major and best studied consequences of Rac activation in leukocytes is the generation of an oxidative burst in phagocytic cells, such as neutrophils and monocytes/macrophages. The molecular details of this response, as well as its role in the killing of pathogens, are well known [for review, see Babior *et al.* (7)]. Since less than a decade, it has become clear that intracellular ROS are produced, not just in phagocytic cells, but rather in most, if not all, types of cell, and that ROS serve a role as signaling molecules, mediating inflammatory responses, cell growth, apoptosis, and migration.

The major reason that ROS in nonphagocytic cells have escaped detection for so long is likely due to the low levels of ROS in these cells, compared with the massive amounts produced by neutrophils. It is now more apparent that, whereas high levels of ROS are damaging to cells, low levels of these signaling intermediates are required for a wide range of cellular functions. In addition, since 1999 it is clear that, next to the classical neutrophil NADPH oxidase, a family of related enzymes exists, the NOXs, of which the members show partially overlapping tissue distribution in both hematopoietic and nonhematopoietic cells [for review, see Lambeth *et al.* (67)]. The regulation and physiological roles of these NOX proteins is currently under intensive investigation by many laboratories.

Literature on the role of ROS in HSPC is scarce. From some of these studies it is clear that ROS, through the induction of apoptosis, block growth and development of HSPC. This phenomenon was already described in 1988 (79) in a culture system wherein primary murine HSPC, in the presence of increasing numbers of neutrophils and macrophages, showed a ROS-mediated decline in number. Furthermore, there are several proteins that have been recently identified as regulators of ROS production and thereby of hematopoiesis (56, 84). Nakata *et al.* showed that inhibition of NFκB in embryonic stem cells using a dominant interfering Rel/NFκB (IκBSR) protein impairs development of primitive hematopoietic precursor cells. This protein also blocked terminal differentiation toward granulocytes and erythrocytes (84). These data are in line with the known function of NFkB as an antiapoptotic regulatory protein, in particular through the induction of expression of ROS scavenger enzymes, such as superoxide dismutase, which converts reactive oxygen to hydrogen peroxide, which is then metabolized to water by catalase. Moreover, there are indications that stem cells in general might express elevated levels of ROS scavenger enzymes, rendering them less sensitive to oxidative stress. This was shown for endothelial progenitors, for which it is very relevant to be resistant to oxidative stress (23), because these cells are recruited to sites of, *e.g.,* ischemia, where levels of ROS are significantly elevated due to the action of inflammatory cytokines.

Another gene that was recently implicated in reducing ROS levels in HSPC is "ataxia telangiectasia mutated" (ATM) (56). Deficiency of this protein leads to severe disease, including immunodeficiency, premature aging, and lymphoma. This study showed that ATM-deficient mice show defective hematopoiesis resulting in a multilineage bone-marrow failure. Cells from $ATM^{-/-}$ animals showed elevated levels of intracellular ROS, which could be reduced by oxygen scavengers, such as *N*-acetyl-L-cysteine (NAC). The defects in *in vitro* and *in vivo* outgrowth of $ATM^{-/-}$ HSPC could be restored by treatment of the cells and/or the recipient mice with NAC, especially when the treatment was continued after the transplantation. The ROS-dependent up-regulation of the p16 INK4-retinoblastoma gene was found to cause the defects in reconstitution of hematopoiesis by the $ATM^{-/-}$ cells. Whether it is a general feature of stem cells to keep their ROS levels low, through expressing scavenger enzymes, to preserve their repopulating abilities, remains to be firmly established.

In contrast to the studies mentioned above, it appears that, in transformed cells, ROS may have a potentiating effect on cell growth. This was initially shown in fibroblasts (55), but was also shown for cells expressing the Bcr-Abl tyrosine kinase. Bcr/Abl induces ROS production in hematopoietic cells, and Sattler *et al.* showed that these ROS are required for efficient intracellular signaling by, as well as activation of, Bcr/Abl in the hematopoietic Mo7e cell line (104). The same

group has also shown that the Mo7e cells required intracellular ROS for efficient cell growth (103) and for proper signaling by growth factor receptors, *e.g.,* for granulocyte-macrophage colony stimulating factor. Thus, it might be that cellular transformation redirects the cells' responses to intracellular ROS, or that the levels of ROS differ in normal versus transformed cells, either by increased activity of ROS-generating enzymes or by altered expression of ROS scavenger enzymes such as superoxide dismutase.

GAPS, GEFS, SMALL GTPASES, AND THEIR EFFECTORS IN HEMATOLOGICAL DISEASE

GTPases are indispensable for the maintenance of homeostasis in hematopoiesis and the function of mature hematopoietic cells. Therefore, it is not surprising that mutations in GAPs, GEFs, GTPase proteins, or effector proteins have been described as causal for various diseases involving cells of the hematopoietic system (for overview of proteins discussed here, see Fig. 2).

Defects in GTPase activity

Rac2 and immunodeficiency. A well-known effect of a lack of Rac2 activity is immunodeficiency. Ambruso *et al.* described a single patient who was identified with a primary immunodeficiency syndrome resulting from a heterozygous mutation in the Rac2 gene (5, 139). In the first 5 months after birth, the patient presented with severe bacterial infection, poor wound healing, and absence of pus in the wounds, indicative of a phagocyte defect. The patient's neutrophils had decreased chemotactic motility, polarization, and secretion of azurophilic granules. Surface expression of the β1-integrin, CD11b, was similar to that in control neutrophils in both resting and activated cells, ruling out leukocyte adhesion deficiency. The pattern of NAPDH oxidase activity was shown to be distinct from that seen in chronic granulomatous disease patients. Accordingly, the Phox proteins were all found to be normal by immunoblot and functional in a cell-free system, but Rac2 levels were reduced, suggesting a defect in this small GTPase. The molecular defect was identified as a missense mutation on one allele of the Rac2 gene that causes the change Asp57Asn. Aspartic acid is invariant at this position within GTP-binding proteins, and its replacement by asparagine resulted in greatly reduced GTP binding (139). The role of the mutation was further confirmed by ectopic expression of this D57N Rac2 mutant (37). The protein induced a phenotype in fibroblasts, similar to that induced by dominant-negative Rac1, lending support to the notion that the patient's immunodeficiency was due to expression of a Rac2 mutant that also interferes with Rac1 activity. In addition, it was shown that the growth defect in $Rac2^{-/-}$ bone-marrow cells could be rescued by wild-type Rac2, but not by the D57N mutant and that this mutant actually induced a similar phenotype in normal cells and a more severe defect in $Rac2^{-/-}$ cells. Interestingly, expression of the D57N mutant in HSPC was found to result in defective long-term engraftment following transduction (37), which could be explained by increased apoptosis of bone-marrow cells. Finally, also the fMLP-induced chemotaxis and the fMLP- or phorbol 12-myristate 13-acetate-induced oxidative burst were blocked by this D57N Rac2.

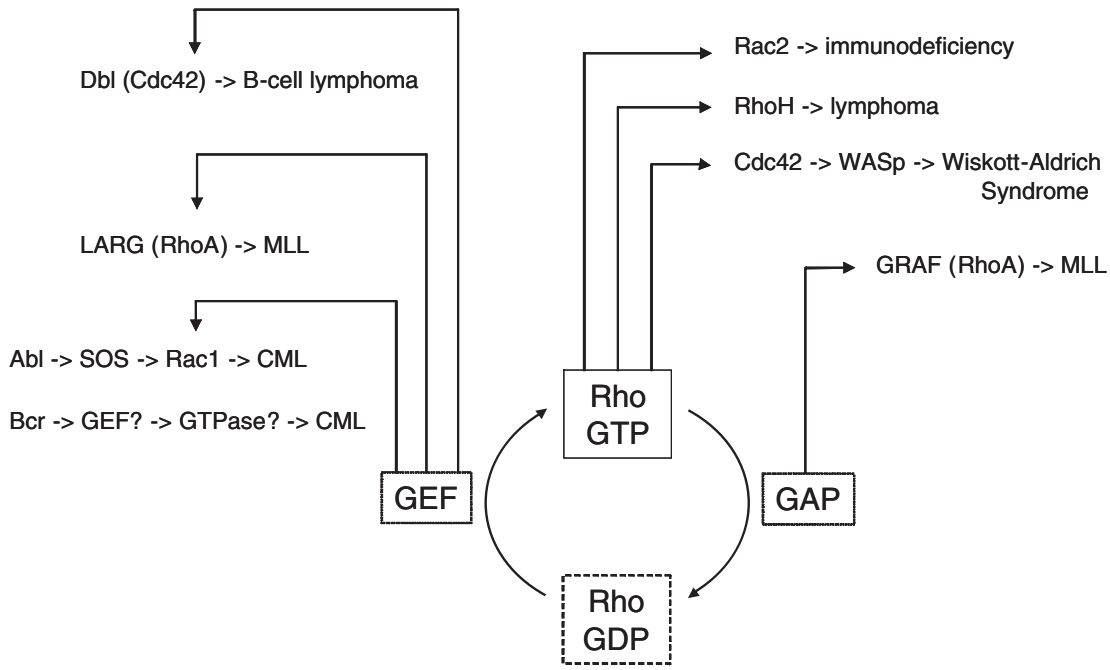

FIG. 2. GAPs, GEFs, small GTPases, and their effectors in hematological disease. See text for details. MLL, mixed lineage leukemia; CML, chronic myeloid leukemia.

RhoH and lymphoma. Recently, a series of studies have been published on a relatively unknown GTPase, the protooncogene RhoH/TTF (91, 93). RhoH is expressed primarily in hematopoietic tissues such as the thymus (40, 74) and in hematopoietic progenitor cells, as well as more differentiated cell types, like T- and B-cells. Preudhomme *et al.* were the first to report the recurrent chromosomal alteration of a GTP-binding protein-encoding gene in patients with hematopoietic malignancies (93). They showed in a set of three patients diagnosed with non-Hodgkin's lymphoma (NHL) that a t(3;4)(q27;p11–13) translocation resulted in a genetic fusion of a gene encoding the RhoH/TTF GTPase with the LAZ3/BCL6 gene. They also showed a rearrangement of the RhoH/TTF gene in a patient with multiple myeloma and t(4;14)(p13;q32) translocation. In addition, in extracerebral diffuse large B-cell lymphomas, the RhoH locus, together with loci of other protooncogenes, was affected by somatic hypermutation. The RhoH locus was hypermutated in 48% of the tumors analyzed with the mutations appearing in the 5′ untranslated or coding sequences (91). It was subsequently noted that the genes affected by the hypermutation, such as which RhoH, are also susceptible to chromosomal translocation in NHL.

Compared with other Rho GTPases, RhoH has several amino acid differences in domains, involved in nucleotide binding and is constitutively GTP-bound. RhoH is proposed to be important for the balance in intracellular signaling as it blocks Rac- and Rho-mediated signal transduction, leading to activation of, *e.g.,* p38 MAPK or NFκB (74). In hematopoietic progenitor cells, RhoH controls survival as well as migration and engraftment, as was shown by overexpression and by siRNA-based studies. Part of this effect of RhoH may be related to its specific role in keeping lymphocyte function-associated antigen-1 on lymphocytes in a nonadhesive state (17). In line with this, RhoH-deficient cells have constitutive adhesive α4β1 integrins (17), leading to the suggestion that RhoH may interfere with the function of the Rap1 GTPase, which is positively implicated in adhesion through both β1 and β2 integrins (14).

Like Boettner and van Aelst stated, other mutations that may inactivate a Rho GTPase or lead to a constitutive active version of the protein either have escaped detection or simply are lethal (10). This latter notion is supported by the observation that Rac1 or Cdc42 knockout mice die early in development (16, 114). It may also reflect the multifunctional nature of Rho GTPases, and loss-of-function or constitutive gain-of-function mutations in many Rho GTPases may simultaneously interfere with various cellular processes (10). Finally, there is also continued speculation that Rho GTPases need to cycle between their active and inactive states in order to be fully functional (116).

Defects in GEF or GAP function

The diffuse B-cell lymphoma (Dbl) oncogene was first identified in screens designed to isolate transforming factors from a human diffuse B-cell lymphoma (27). Oncogenic activation of Dbl occurs through an N-terminal truncation of the 115-kDa proto-Dbl product. Later it was demonstrated that oncogenic Dbl acts as a GEF toward Cdc42 (47). In addition to identifying Dbl as an upstream activator of Cdc42, these findings provided important clues that deregulation of Cdc42-mediated signaling events is involved in oncogenic transformation. These initial observations led to the identification of a large number of proteins sharing a tandem arrangement of DH and PH domains, a feature of candidate GEFs for Rho family-binding proteins. Many of these proteins have been identified on the basis of their transforming activity or are known to be involved in cell growth regulation, making the Dbl family one of the largest groups of protooncogenes.

Leukemia-associated Rho guanine nucleotide exchange factor (LARG) was originally identified as a fusion protein with mixed-lineage leukemia (MLL) in a patient with acute myeloid leukemia (AML) (65) and is highly expressed in hematopoietic stem cell fractions (149). Reuther *et al.* have shown that LARG is a RhoA-specific GEF and postulated that sustained expression of the MLL–LARG fusion protein results in aberrant activation of LARG and that subsequent RhoA activation plays a mechanistic signaling role in the development of the leukemia (96). This notion is supported by the observation of the inactivation of a GAP for RhoA in leukemia. Borkhardt *et al.* (11) described the formation of an MLL fusion protein with GRAF, which acts as a GAP for RhoA (51). This fusion results in the deletion of the GAP domain of GRAF. Inactivation of a Rho GAP, which functions to convert active GTP-bound Rho proteins to the inactive GDP-bound form, would potentially have a RhoA-activating effect similar to the activation of a RhoA GEF such as LARG. That there is indeed a possible causal relationship between high RhoA activity and leukemia is also suggested by data of Olson *et al.* as they found that active RhoA suppresses p21, a known inhibitor of the cell cycle (90).

The p210(Bcr-Abl) and p190(Bcr-Abl) fusion proteins, responsible for chronic myelogenous leukemia (CML) (9, 48) and acute lymphoblastic leukemia (ALL) (18), respectively, show increased tyrosine kinase activity that causes the leukemia (76, 128). Interestingly, there is strong evidence that p210(Bcr-Abl) affects cytoskeletal structure as cells transformed by p210(Bcr-Abl) display a Rac-dependent increase in motility (111). A dominant-negative Rac mutant inhibits the Bcr-Abl-induced leukemic transformation (111). Thus, Rac appears to act downstream of Abl. Furthermore, the p210(Bcr-Abl) protein binds F-actin through a C-terminus actin-binding domain of the Abl protein (77, 78, 123, 137), and its distribution changes in migrating cells (112). Salgia *et al.* show that p210(Bcr-Abl)-transduced BaF3 cells exhibited an increased staining for F-actin and an enhanced rate of formation and retraction of actin-containing protrusions, such as pseudopodia and filopodia (102). As was observed previously, the cells showed a higher intrinsic motility that required the Bcr-Abl tyrosine kinase activity and was found in both Bcr-Abl transformed cell lines and primary CML progenitors. Recently, Sini *et al.* showed that Abl phosphorylates the Ras GEF, Sos-1, leading to activation of Rac, independent of the canonical Ras pathway, thus promoting Rac-dependent phenotypes (109).

In addition to the transforming effect of Abl in CML and ALL, also the Bcr protein might be involved in the aberrant regulation of the cytoskeleton as observed in Bcr-Abl-positive leukemias. The wild-type Bcr product has several

recognizable structural and functional motifs, including a domain that has GEF activity for Rho family GTPases (DH/PH domain). This domain is retained within p210(Bcr-Abl). Harnois *et al.* have shown that a stable complex exists between Rho GTPases and p210(Bcr-Abl) and that Rac, Rho, and Cdc42 were activated by p210(Bcr-Abl) through the Dbl homology domain of Bcr that is present in p210(Bcr-Abl), but absent in p190(Bcr-Abl) (43). Nevertheless, Rac and Cdc42, but not RhoA, were activated by p190(Bcr-Abl) *in vivo* and *in vitro*. Part of this GEF activity of p190(Bcr-Abl) is probably attributable to Vav (exchange factor for Rac), which is complexed to both p190(Bcr-Abl) and p210(Bcr-Abl).

Defects in the regulation of the actin cytoskeleton downstream of Cdc42

The Wiskott–Aldrich Syndrome (WAS) is a rare inherited X-linked recessive disease characterized by immune dysregulation and microthrombocytopenia (4, 140). The clinical phenotype of the immune disorder includes susceptibility to pyogenic, viral, and opportunistic infections and eczema (115). In its mildest form, known as X-linked thrombocytopenia, mutations in the same gene produce the characteristic platelet abnormality, but minimal immunological disturbance (127).

The phenotype is caused by mutation in the WASp gene mapped on Xp11;22 that leads to a loss of function (117). In contrast, gain-of-function mutations may be responsible for neutropenia (25). The WAS gene encodes a 502-amino acid proline-rich intracellular protein expressed exclusively in hematopoietic cells (24), which belongs to a recently defined family of more widely expressed proteins involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton. WASp binds to the GTP-bound form of the Rho GTPase Cdc42 *in vitro*, less well to GTP-bound Rac, but not to Rho, and clusters physically with polymerized actin (6, 64, 117). The WASp–GTPase interaction is mediated by a CRIB (Cdc42 Rac-interactive binding) domain. The interaction between the GTP-bound Cdc42 and the CRIB domain of WASp induces conformational changes that allow the C-terminus verprolin cofilin acid (VCA) domain of WASp to bind the actin-related protein (Arp) complex 2/3 (61). The VCA–Arp2/3 interaction initiates branching structures by promoting addition of actin monomers to the barbed end of actin filament (50). Moreover, WASp contains a proline-rich sequence that binds to profilin, vasodilator-stimulated phosphoprotein, Src homology 3 domains of signaling proteins, and cytoplasmic tyrosine kinases (24). The result of the interaction between incoming signals and specific domains of the protein is the induction of actin polarization required for directed motility and phagocytosis (118).

Studies of hematopoietic cells from human patients have strongly suggested that the biological mechanisms responsible for the pathophysiology of WAS is due to deregulation of the actin cytoskeleton in response to stimuli [for recent review, see Burns *et al.* (12)]. Furthermore, it has been shown that a great majority of hematopoietic cells from the blood of heterozygous females with a nonsense WASp mutation normally express WASp (94). DNA extracted from CD34+ cells of WAS carriers exhibited a pattern of nonrandom X inactivation (134), demonstrating that the selective advantage of cells expressing the normal X chromosome occurs early during hematopoiesis. Studies using WASp knockout mice have shown that hematopoiesis is normal in spleen and marrow in WASp-deficient mice; however, hematopoietic stem cells from these mice have a defect in their ability to migrate into the bone-marrow hematopoietic microenvironment, possibly explaining the nonrandom X-inactivation pattern in the blood cells of female carriers (66).

STATINS AND HSPC

3-Hydroxymethylglutaryl-coenzyme A (HmG-CoA) reductase inhibitors or statins inhibit prenylation of many different proteins in the cell, among which are the members of the Ras and Rho family of GTPases. The absence of the hydrophobic farnesyl or geranyl tail will alter the subcellular localization of proteins and thereby disturb signaling. One of the major effects, by which the statins have become well-known in the past few years, is their ability to influence cholesterol metabolism and thereby change beneficially the risk profile of patients who are susceptible to ischemic vascular disease. Therefore, the use of these pharmacological inhibitors in lowering of low-density lipoprotein (LDL) cholesterol has been adopted by clinicians as part of a standard treatment regimen for patients with coronary disease or with an increased risk thereof (63, 92, 108).

However, statins do not just lower LDL cholesterol; they also have extensive pleiotropic effects, which include inhibiting monocyte adhesion to the endothelium (58, 147), inhibiting smooth muscle proliferation (68), reducing platelet aggregation (107), mediating antiinflammatory effects (3, 83, 105), mobilizing endothelial progenitor cells (75, 125) and enhancing reendothelialization (130, 135). Also the generation of ROS by the NADPH complex in monocytes (21) and in neutrophils (8) has been shown to be reduced after exposure of these cells to statins.

Analysis of possible effects of HmG-CoA reductase inhibitors on HSPC showed that short-term exposure selectively inhibits the growth of AML cells, both from primary material and from a cell line (85). These results were confirmed by data obtained by Scheffold *et al.* (106). The fact that the sensitivity of the leukemic bone marrow to statins is lost at remission (86) implies that not the bone-marrow environment, but the leukemic transformation of the hematopoietic stem cell renders its progeny more sensitive to the statins. This led to the hypothesis that the statins would be suitable to use as a *in vitro* purging agent in autologous transplantations for AML (86).

The working mechanism underlying the observed inhibition of cell growth by statins appeared to be the induction of apoptosis in myeloid leukemic cells (26), for which the blocking of protein geranylation appeared to be essential (143) and activation of mitochondrial caspase 9 was reported (15). For additional information on the experimental data concerning statins in the treatment of cancer, see the review of Wong *et al.* (141). Further research showed that combining HmG-CoA reductase inhibitors with conventional cytotoxic substances in the case of AML samples (72) or interferon when CML

material is concerned (82) made the cells more susceptible to apoptosis than either one of the treatments alone. These data together seem to warrant a clinical trial to establish the contribution of statins in the treatment of leukemia.

CONCLUSIONS

In this review, we have discussed the role of the Rho GTPases and their signaling in migration, proliferation, and differentiation of hematopoietic cells in physiological and pathophysiological conditions. Despite their high levels of homology, there is clear functional differentiation between the various members of the Rho GTPase family. The recent studies using chimeric Rac1 and Rac2 proteins have provided important information on the domains that determine the functional specificity of these proteins. A challenge for the field will be to determine which interactions control the intracellular targeting and thus the specificity of activation and signaling by Rho GTPases.

Given the complexity of Rho GTPase signaling and the multiple cellular and developmental aspects in which these GTPases are involved, there is a strong possibility that additional disease-causing mutations in genes encoding Rho-related signaling molecules will be uncovered in the future. Therefore, the studies on potential inhibitors, like the statins, will become more important in the near future, because these provide insight into the relevance of Rho GTPase signaling, as well as their potential as efficient drug targets in hematological disease.

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ABBREVIATIONS

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; Arp, actin-related protein; ATM, ataxia telangiectasia mutated; BCR, B-cell receptor; BMMC, bone marrow-derived mast cells; CML, chronic myelogenous leukemia; CRIB domain, Cdc42 Rac-interactive binding domain; Dbl , diffuse B-cell lymphoma; F-actin, filamentous actin; fMLP, formyl-methionyl-leucyl-phenylalanine; GAP, GTPase-activating factor; GEF, guanine nucleotide exchange factor; GRAF, GAP for RhoA associated with FAK; GTPase, Rho guanosine trisphosphatase; HmG-CoA, 3-hydroxymethylglutaryl-coenzyme A; HSPC, hematopoietic stem and progenitor cells; IFNγ, interferon-γ JNK, Jun N-terminal kinase; LARG, leukemia-associated Rho guanine nucleotide exchange factor; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MLL, mixed-lineage leukemia; NAC, *N*-acetyl-L-cysteine; NFκB, nuclear factor-κB; NHL, non-Hodgkin's lymphoma; PI3-kinase, phosphatidylinositol 3-kinase; PIP-5-kinase, phosphoinosititde-5-phosphate kinase; PTX, pertussis toxin; ROS, reactive oxygen species; SCF, stem cell factor; SDF-1, stromal-derived factor-1; TCR, T-cell receptor; Th1, T-helper cell 1; VCA, verprolin cofilin acid; WAS, Wiskott–Aldrich syndrome; WASp, Wiskott–Aldrich syndrome protein.

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