RGS16 is a negative regulator of SDF-1–CXCR4 signaling in megakaryocytes

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Regulators of G-protein signaling (RGS) constitute a family of proteins involved in the negative regulation of signaling through heterotrimeric G protein–coupled receptors (GPCRs). Several RGS proteins have been implicated in the down-regulation of chemokine signaling in hematopoietic cells. The chemokine stromal-cell–derived factor 1 (SDF-1) activates migration of hematopoietic progenitors but fails to activate mature megakaryocytes despite high levels of CXC chemokine receptor 4 (CXCR4) receptor expression in these cells. This prompted us to analyze RGS expression and function during megakaryocyte differentiation. We found that RGS16 and RGS18 mRNA expression was up-regulated during this process. Overexpressing RGS16 mRNA in the megakaryocytic MO7e cell line inhibited SDF-1–induced migration, mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) activation, whereas RGS18 overexpression had no effect on CXCR4 signaling. Knocking down RGS16 mRNA via lentiviral-mediated RNA interference increased CXCR4 signaling in MO7e cells and in primary megakaryocytes. Thus, our data reveal that RGS16 is a negative regulator of CXCR4 signaling in megakaryocytes. We postulate that RGS16 regulation is a mechanism that controls megakaryocyte maturation by regulating signals from the microenvironment. (Blood. 2005;106:2962-2968)

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Introduction

Chemokines and their receptor(s) are broadly expressed in different tissues and regulate cell migration as well as several other important biologic processes. The chemokine stromal-cell–derived factor 1 (SDF-1) is a stromal-cell–derived factor that interacts with a specific receptor CXC chemokine receptor 4 (CXCR4) and plays a role in B lymphopoiesis and bone marrow myelopoiesis. Studies using mutant mice with targeted gene disruption have revealed that SDF-1 and CXCR4 are essential for B-cell differentiation, for colonization of bone marrow by hematopoietic stem cells (HSCs) and myeloid lineage during ontogeny as well as for blood-vessel formation in gastrointestinal tract, cardiac ventricular septum formation, and cerebellar differentiation. SDF-1–CXCR4 signaling appears to be essential for the homing of hematopoietic stem/progenitor cells because treatment of immature human hematopoietic progenitor cells with anti-CXCR4 antibodies prevents their short-term engraftment into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. In addition, transplantation of CXCR4−/− fetal liver cells results in low numbers of B-lymphoid and myeloid lineage precursors in bone marrow but increased numbers in the peripheral blood compared with control animals. Proteolytic cleavage of the N-terminus of CXCR4 and of SDF-1 is one mechanism that has been identified for the regulation of CXCR4/SDF1 signaling in circulating and mobilized blood cells.

The process of megakaryopoiesis occurs within a complex bone marrow microenvironment where chemokines, cytokines, and adhesive interactions play a major role. At the end of their maturation, polyploid megakaryocytes (MKs) migrate through bone marrow endothelial cells and release platelets directly into the marrow-intravascular sinusoidal space or the lung capillaries. The interactions of immature MKs with a permissive, endothelial-enchriched microenvironment is promoted by chemokines, including SDF-1. Consistent with this process, CXCR4 is expressed all along the megakaryocyte differentiation from MK progenitors to platelets, However, during MK development, the outcome of CXCR4 signaling as measured by chemotaxis and extracellular signal regulated protein kinase (Erk) activation becomes markedly reduced, indicating the presence of possible negative regulators. Such mechanisms have remained elusive. Recent studies have shown that CXCR4 signaling is regulated by Regulators of G-protein signaling (RGS) proteins in mature B cells. Reminiscent of the MK differentiation, CXCR4 expression is maintained all along the B-cell maturation, but mature B cells fail to respond to SDF-1.

RGS proteins have emerged as major modulators of signaling for heterotrimeric guanine nucleotide binding proteins (G proteins). Heterotrimeric G proteins are the link between many receptors, including chemokines, and downstream effectors. Heterotrimeric G proteins are composed of α, β, and γ subunits, each having multiple isoforms; that is, 4 isoforms for the α subunit: αi, αo, αq, and α12. Ligand-bound activated receptors catalyze the exchange of guanosine diphosphate (GDP) by guanosine triphosphate (GTP) on the α subunit, leading to dissociation of the α form from the βγ dimer, which both transduce the signal to effectors.

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μg/mL hexadimethrine bromide (Sigma). Cells were stained with an anti-CD41–APC antibody and sorted to obtain CD41+/GFP+ and CD41+/GFP− populations for mRNA analysis and proplatelet formation. Cells were stained with an anti-CD34–PE antibody and sorted to obtain CD34+/GFP+ populations for MK colony-forming unit (CFU-MK) assay.

mRNA analysis using real-time RT-PCR

Total RNA was isolated from sorted megakaryocytes, platelets, MO7e cells, and EBNA cells using the SV total RNA isolation kit (Promega, Charbonnières, France). cDNA was generated by reverse transcription using Superscript II RNase H-reverse transcriptase (Invitrogen). The expression levels of RGSs and the internal reference β2-microglobulin (β2m) were measured by triplicate PCR which were performed using the ABI PRISM GeneAmp 5700 sequence Detection system and the TaqMan Universal PCR master Mix (PE Applied Biosystems, Courtaboeuf, France). The primers and probes were designed using Primer Express software and are listed in Table 2. A comparative threshold cycle (CT) method was used to determine gene expression. Indeed, the RGS Ct value was normalized to the β2m Ct value using the formula: 2−ΔΔCt (β2m − Ct). RNA samples were not used that exhibited fluorescence amidite (FAM)–labeled reverse transcription-quantitative polymerase chain reaction (RT-QPCR) products with Ct values greater than 37. This is because inaccurate and unreliable relative expression values might be obtained. All samples demonstrated Ct values less than 27 for FAM-labeled RT-QPCR products, indicating the samples contained undegraded cDNA that could be amplified.

Chemotactic assay

Cell migration was quantified through 5-μm pore filters for the MO7e cell line and 8-μm pore filters for MKs (Transwell, 24-well cell clusters; VWR, Strasbourg, France). Serum-free medium (200 μL) containing 2 × 105 cells was placed in the upper well of the transwell. In the lower chamber, 600 μL medium containing 300 ng/mL SDF-1α (Abcys, Paris, France) was added. After 4 hours at 37°C in 5% CO2, the cells of the lower chamber and the starting population were recovered in equal volumes, and the percentage of migration was calculated according to the following formula: number of cells in the lower well divided by the total cells put in the transwell at the beginning. The number of cells and their GFP levels were analyzed on a FACSort (Becton Dickinson). For megakaryocytes, cells in the lower chamber and the total cells were stained using anti-CD41–APC and anti-CD42–PE antibodies after migration and analyzed on a FACSort.

Analysis of p42/44 MAP kinase and AKT activation by Western blot

After 12 hours of serum and GM-CSF starvation, stably infected MO7e cells (RGS16, RGS18 or GFP alone) were stimulated or not with 300 ng/mL SDF-1 for 1 and 5 minutes. Equal amounts of proteins (30 μg) were transferred onto nitrocellulose. Endogenous mitogen-activated protein (MAP) kinase activity was detected with an antibody specific for the p42/44 phosphorylated forms of MAP kinase (Cell Signaling, Beverly MA). AKT phosphorylation was measured by triplicate PCR which were performed using the ABI PRISM GeneAmp 5700 sequence Detection system and the TaqMan Universal PCR master Mix (PE Applied Biosystems, Courtaboeuf, France). The primers and probes were designed using Primer Express software and are listed in Table 2. A comparative threshold cycle (CT) method was used to determine gene expression. Indeed, the RGS Ct value was normalized to the β2m Ct value using the formula: 2−ΔΔCt (β2m − Ct). RNA samples were not used that exhibited fluorescence amidite (FAM)–labeled reverse transcription-quantitative polymerase chain reaction (RT-QPCR) products with Ct values greater than 37. This is because inaccurate and unreliable relative expression values might be obtained. All samples demonstrated Ct values less than 27 for FAM-labeled RT-QPCR products, indicating the samples contained undegraded cDNA that could be amplified.

Quantification of proplatelet formation

pRRL-SCR or pRRL-24 CD41+/GFP+ sorted cells were cultured in 96-well plates at the concentration of 5000 cells/well. Petri dishes were cultured at 37°C for 12 days. Colonies were quantified by an indirect immunophosphatase alkaline labeling technique using an anti–glycoprotein III (GpIIIa) polyclonal antibody (Dako, Glostrup, Denmark). CFU-MK were counted under an inverted microscope.

Statistical analysis

Results of experimental points obtained from 3 repeated experiments are reported as the mean plus or minus standard deviation (SD). Statistical significance was determined using Student t test.

Results

RGS16 and RGS18 mRNA expression is enhanced during megakaryocyte maturation

It has been previously reported that RGS16 and RGS18 mRNA are expressed by megakaryocytes.30,35 This prompted us to analyze whether these RGSs were regulated during the process of MK differentiation which is known to be associated with reduced SDF-1 response. MKs were derived from culture of cord-blood CD34+ cells, and cells at various stages of development were obtained from these cultures using flow cytometric cell sorting on the basis of the CD41, CD42, and CD42 markers (Figure 1A). CD34+ cells constitute progenitor cells, whereas CD41+CD42low cells are enriched in immature MKs and CD41+CD42+ cells in mature megakaryocytes. Total mRNA was isolated from each of these 3 populations, and the level of RGS16 and RGS18 was analyzed by quantitative RT-PCR. To control for differences in mRNA loading, the intensity of RGS16 and RGS18 signal was normalized to the housekeeping β2-microglobulin gene (Figure 1B). RGS16 transcripts were detected in all 3 populations. RGS18 expression increased 13-fold from immature progenitors (CD34+CD41−CD42+) to the more mature cells (CD41+CD42+). The amounts of RGS16 transcripts were markedly lower than RGS18 transcripts in all 3 populations. Similarly to RGS18, RGS16 mRNA expression increased 6-fold from immature progenitors (CD34+CD41−CD42+) to the more mature cells.
efficient transduction and high transgene expression in hematopoietic cells. Because of both the MK phenotype and efficient migratory response to SDF-1, the MO7e cell line was chosen for further study. Infection rates were evaluated to be about 80% as assessed by GFP expression in populations of stably transduced cells. GFP+ polyclonal populations (MO7e GFP, MO7e RGS16, and MO7e RGS18) were isolated by flow cytometry, and levels of expression of the various RGS constructs were evaluated by RT-PCR. As shown in Figure 2A, RGS16 mRNA level in MO7e RGS16 cells was 200-fold higher than RGS16 mRNA level in MO7e GFP cells. Similarly, RGS18 mRNA was 150-fold higher than endogenous RGS18 mRNA level obtained in MO7e GFP cells (Figure 2B). In MO7e RGS16 cells, overexpression of RGS16 did not alter the expression of RGS18 and vice versa.

Then we studied the effects of RGS16 and RGS18 overexpressions on SDF-1–induced chemotaxis in MO7e cells. RGS18 overexpression did not affect SDF-1–induced chemotaxis (Figure 2C). In contrast, RGS16 overexpression in MO7e cells was very effective in inhibiting SDF-1–mediated migration (75% inhibition compared with control cells transduced with GFP only). We subsequently tested the effect of these RGS overexpressions on the SDF-1–induced MAPK and AKT activation by Western blot. As shown in Figure 2D, MO7e GFP cells exhibited a strong Erk and AKT phosphorylation in response to SDF-1. Activation of CXCR4 by SDF-1 also induced a significant Erk and AKT activation in MO7e RGS18 cells. In contrast, MO7e RGS16 exhibited a lower Erk and AKT phosphorylation in response to SDF-1 as compared with MO7e GFP and MO7e RGS18. Thus, RGS16 protein overexpression led to the inhibition of SDF-1–mediated migration and intracellular signaling.

Inhibition of RGS16 expression using lentiviral-delivered short hairpin RNA

Results on the MO7e cell line prompted us to check whether RGS16 was implicated in SDF-1/CXCR4 signaling in primary megakaryocytes, using RNA interference. siRNA sequences specific for RGS16 mRNA were designed using the Ambion software available on the Internet and constructed as shRNA composed by forward and reverse sequences coding for siRNA linked by a short hairpin as described.25 The different cDNA shRNAs (shRGS16) were inserted into a pBlueScript vector containing the human H1 polymerase III promoter (Figure 3A). These different expression vectors were transfected into EBNA cells to assess endogenous RGS16 mRNA inhibition. EBNA cells were cotransfected with MIGR, a retrovirus vector containing the human H1 polymerase III promoter, using RNA interference. siRNA sequences specific for RGS16 mRNA were designed using the Ambion software available on the Internet and constructed as shRNA composed by forward and reverse sequences coding for siRNA linked by a short hairpin as described.25 These different cDNA shRNAs (shRGS16) were inserted into a pBlueScript vector containing the human H1 polymerase III promoter (Figure 3A). These different expression vectors were transfected into EBNA cells to assess endogenous RGS16 mRNA inhibition. EBNA cells were cotransfected with MIGR, a retrovirus vector containing the eGFP, which was used as a marker of transfection efficiency. GFP+
EBNA cells were sorted and analyzed by quantitative RT-PCR to assay the mRNA level of RGS16. Five of 6 shRGS16 sequences inhibited RGS16 mRNA levels by 2-fold or more (Figure 3 B). SEQ24 was the most effective (a 3-fold decrease) and was chosen, as well as the SEQ15 or a scramble control irrelevant siRNA, to construct lentiviral vectors to stably express anti-RGS16 siRNA into megakaryocytes.

Inhibition of RGS16 expression in megakaryocytes up-regulates SDF-1/CXCR4 signaling

MO7e cells were infected with different lentiviral vectors encoding shRNASeq15, shRNASeq24, or SCR control shRNA, together with a reporter GFP gene. Transduced GFP+ cells were purified and analyzed by quantitative RT-PCR. A 3-fold decrease in the expression of RGS16 mRNA was observed with the pRRL-SEQ24 in comparison to pRRL-SCR, whereas no inhibition was observed with pRRL-SEQ15 (Figure 4 A). The lack of efficacy of the SEQ15 constructed in the lentiviral vector was surprising compared with the inhibition obtained by transfection of SEQ15 plasmid in EBNA cells. This difference may be due to a lower number of SEQ15 siRNA obtained after transduction compared with transfection. Nevertheless, the lentiviral vector encoding SEQ24 was efficient and allowed the study of SDF-1–induced migration in MO7e cells (Figure 4B). Cell migration was increased by 68% in cells with pRRL-SEQ24, confirming that RGS16 modulates the SDF-1/CXCR4 signaling in MO7e cell line.

We have shown that RGS16 expression increased during MK differentiation. This correlates with the SDF-1 responsiveness of the mature MKs. Thus, the effect of an inhibition of RGS16 expression on SDF-1–induced migration of primary MKs was evaluated. Cord-blood CD34+ cells were stimulated 2 days with TPO and infected twice with the RGS16 siRNA. After 7 days of culture, pRRL-SCR GFP+/CD41+, pRRL-SEQ24 GFP+/CD41+, and GFP-/CD41+ were obtained by flow cytometry cell sorting. Analysis of RGS16 mRNA expression (Figure 5A) revealed a 2-fold decrease in pRRL-SEQ24 GFP+/CD41+ in comparison to the 2 control populations, which exhibited similar RGS16 mRNA levels. Moreover, the SDF-1–induced MK migration was investigated. Figure 5B illustrates the percentage of CD41+/CD42+ cells that migrated in response to SDF-1. SDF-1–induced migration of MK expressing RGS16 siRNA was increased by 57%, suggesting that RGS16 modulates negatively CXCR4 signaling in primary MKs.

Adhesion assays of pRRL-SCR GFP+/CD41+ and pRRL-SEQ24 GFP+/CD41+ cells on fibronectin and collagen I were performed as described in “Materials and methods.” As shown in Figure 5C, a significant fraction of megakaryocytes were shown to adhere to fibronectin and collagen I. However, we did not find any change in the adhesion to these extracellular matrix after RGS16 knocking down either in the presence or absence of SDF-1, suggesting that the level of RGS16 expression did not modify basal or SDF-1–induced adhesion to either fibronectin or collagen I.

Effects of RGS16 knocking down on megakaryopoiesis and platelet production

We studied whether inhibition of RGS16 expression affects CFU-MK formation by performing CFU-MK assays. pRRL-SCR GFP+/CD34+, pRRL-SEQ24 GFP+/CD34+ cells were obtained by flow cytometry cell sorting and grown in plasma clot in the presence of TPO and SCF to reveal their MK potential. Results of 3 experiments performed from cord-blood proved that cells are summarized in Figure 6A. Cloning efficiency of CFU-MK was quite similar in pRRL-SCR GFP+/CD41+ and pRRL-SEQ24 GFP+/CD41+.

We also analyzed the proplatelet formation from pRRL-SCR GFP+/CD41+ and pRRL-SEQ24 GFP+/CD41+ cells (Figure 6B). For this purpose, sorted cells were stimulated by SCF and TPO for 7 days, and proplatelet production was assessed. These analyses revealed comparable numbers of proplatelets between pRRL-SCR GFP+/CD41+, pRRL-SEQ24 GFP+/CD41+ cells, suggesting that RGS16 knocking down did not modify platelet formation in these culture conditions.

Discussion

SDF-1, also called pre-B-cell growth-stimulating factor-1 (PBSF), is a CXC chemokine constitutively produced by bone marrow stromal cells and is the major chemoattractant of HSCs and committed progenitors.50 This chemokine may be involved also in the retention of hematopoietic precursor cells in the bone marrow.51 The SDF-1 receptor CXCR4 is expressed all along the MK differentiation pathway from CFU-MK to platelets. However, the chemotaxis mediated by SDF-1 markedly decreases during MK differentiation.10-14 The negative regulation of CXCR4 signaling at the postreceptor level has also been described for mature B cells.15-17 In this cell lineage, it has been suggested that RGS proteins may be involved in the negative regulation of chemotaxis mediated by SDF-1 and other chemokines.
In this study we show that expression levels of RGS16 and RGS18 mRNA are up-regulated during MK differentiation. The observations reported here are consistent with the notion that dynamic control of specific RGS protein expression acts as a general mechanism for controlling the duration of G protein–coupled receptor signaling. Results of RGS16 and RGS18 protein overexpression in MO7e cells demonstrated that CXCR4 signaling was significantly inhibited by RGS16 but not by RGS18. This effect was further documented by reduced Erk and AKT signaling. Note that MAP kinase activation is constitutively (without agonist) higher in MO7e cells overexpressing RGS16 or RGS18 as compared with MO7e GFP cells, suggesting that RGS proteins may affect signaling events leading to activation of MAP kinase. The mechanisms leading to this high MAPK activation are presently unclear. In addition to this effect on basal MAPK signaling, MAPK activation by SDF-1 was also markedly inhibited by RGS16 overexpression but not by RGS18. MAPK kinase activation is mediated by free Gβγ subunits. Thus, the decrease in CXCR4 signaling induced by RGS proteins is probably due to the GAP activity of RGS proteins themselves, increasing the rate of reassociation of G protein with βγ complexes and decreasing the amount of free βγ dimers. This is consistent with the most accepted model for RGS proteins, which bind directly to activated G proteins, stimulating GTP hydrolysis and promoting G-protein deactivation.

Few studies have addressed the issue of RGS protein effects in CXCR4 signaling. RGS1 has been reported to be an effective inhibitor of chemotaxis toward SDF-1 in human or murine B lymphocytes. A short form of RGS3 was shown to impair CXCR4 signaling in a mature B-cell line. In another study, transiently transfected cells with constructs encoding RGS1, RGS3, and RGS4 but not RGS2 exhibited inhibition of chemoattractant-induced migration. Neill et al found that receptor-induced increases in inositol triphosphate levels were strongly reduced only in cells transfected with RGS3. In the present study, RGS16 and RGS18 proteins differentially affected CXCR4 signaling, suggesting that functional specificity of RGS proteins may depend on receptor and/or G-protein selectivity. Indeed, we found that RGS18 is highly expressed in mature megakaryocytes and platelets. A report showed that RGS18 exhibits GAP activity toward Gαi. Binding assays of RGS18 with megakaryocytic-cell lysates demonstrated that RGS18 specifically binds Gαi, suggesting that the dynamic control of this RGS acts as a mechanism for controlling the duration of one or more G-protein–coupled receptors expressed in megakaryocytes and platelets. Indeed, a recent work shows that RGS18 is phosphorylated in thrombin receptor-activating peptide (TRAP)–activated platelets, suggesting that RGS18 may be involved essentially in the protease-activated receptor 1 (PAR-1)/Gαi or Gqα signaling in megakaryocytes. RGS knock-out mice may help to precisely understand the role of this RGS on MK differentiation and platelet production.

To further demonstrate that RGS16 plays a key role in CXCR4 signaling, a lentiviral-delivered short hairpin RNA was used to inhibit RGS16 expression in MKs. A 3-fold decrease of RGS16 mRNA was achieved and led to an increase in CXCR4 signaling. Indeed, the SDF-1–induced migration of MKs was augmented in the presence of lentiviral vectors expressing anti-RGS16 siRNA. RGS16 has been already described to be important for lymphocyte activation and trafficking by attenuating the signaling of chemokine receptors such as CXCR4. Our experiment describes that RGS16 is also important in the MK lineage. Indeed, this may be part of the mechanism by which CXCR4 signaling is down-regulated during MK differentiation, leading to the release of mature MKs from the bone marrow microenvironment and to their interaction with the vascular endothelium for efficient platelet production. The up-regulation of RGS16 and RGS18 during MK differentiation suggests that RGS16 and RGS18 proteins are important targets for the biologic responses of MKs, and this is particularly true for RGS16 in CXCR4 signaling in these cells. Further experiments performed on RGS knock-out mice may help to precisely understand the role of RGS on MK differentiation and platelet production.

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