The α Subunit of the Granulocyte-Macrophage Colony-stimulating Factor Receptor Interacts with c-Kit and Inhibits c-Kit Signaling*

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The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates hematopoiesis and the function of mature host defense cells through the GM-CSF receptor (GMR), which is composed of α (αGMR) and β (βGMR) subunits. Stem cell factor is another important hematopoietic cytokine that signals through c-Kit, a receptor tyrosine kinase, and regulates hematopoietic stem cell maintenance and erythroid development. Like other cytokine receptors, GMR and c-Kit are generally deemed as independent adaptor molecules capable of transducing cytokine-specific signals. We found that the αGMR directly interacts with c-Kit and that the interaction is mediated by the cytoplasmic domains. Furthermore, αGMR inhibited c-Kit auto-phosphorylation induced by the ligand stem cell factor. Consistent with the inhibitory effect, the expression of αGMR was suppressed in cells whose viability was dependent on c-Kit signaling. In contrast, the alternatively spliced α2 isoform of the αGMR could not inhibit c-Kit signaling, providing a rationale for the existence of the α2 isoform. Our results suggest that in addition to having the commonly appreciated roles in cytokine signal transduction, the receptors αGMR and c-Kit could interact to coordinate their signal initiation.

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) is a key cytokine in host defense regulation, and the GM-CSF receptor (GMR) is expressed on hematopoietic precursors, in mature host defense cells, and in certain non-hematopoietic tissues (1–4). The high affinity receptor for GM-CSF is composed of an α subunit (αGMR) that binds ligand with low affinity and a β subunit (βGMR) that does not bind GM-CSF on its own (5–8). Although the βGMR initiates most GM-CSF-induced signaling pathways, the short intracellular domain (54 amino acids) of αGMR is required for GMR signaling (9–12). We and others have used genetic and biochemical approaches to find proteins interacting with αGMR, and several proteins were identified with different functional implications (13–15). There is a soluble isoform of αGMR as well as an α2 isoform (α2GMR) derived from alternative mRNA splicing (16, 17). The α2GMR has a different cytoplasmic domain but generally exhibits similar properties to αGMR in the cooperation with βGMR (17). Unlike the αGMR, the α2GMR is not up-regulated in granulocyte/macrophase (G/M) lineages, and the α2GMR appears to be more relevant in undifferentiated hematopoietic progenitor cells (18). Signaling of GM-CSF through αGMR/βGMR leads to more erythroid differentiation than through αGMR/βGMR, suggesting that the receptor isoforms could guide cell differentiation (18).

c-Kit is a transmembrane receptor tyrosine kinase that regulates the proliferation and differentiation of hematopoietic stem cells, erythropoiesis, melanogenesis, and gametogenesis (19–22). Stem cell factor (SCF, also known as kit ligand or Steel factor) is the ligand for c-Kit. The intracellular region of c-Kit has two kinase domains separated by a kinase insert (23, 24). SCF binding to c-Kit induces receptor dimerization and autophosphorylation, leading to activation of downstream signaling pathways (25). In mice, c-Kit is encoded at the white spotting (W) locus, its ligand SCF is encoded at the steel (Sl) locus, and mutations at the W or the Sl locus lead to sterility, pigment cell deficiency, and macrocytic anemia (26).

c-Kit and αGMR are well known markers of differentiation, especially in hematopoietic cells. Starting with a high level of c-Kit and negligible amount of αGMR, hematopoietic stem or progenitor cells down-regulate c-Kit and up-regulate αGMR when they enter into the G/M lineage (22, 27). The receptor subunits are generally deemed as passively regulated adaptor molecules, and a direct interaction between αGMR and c-Kit has not been reported. Using our preliminary computational method for predicting protein interactions, we identified c-Kit as a possible interacting protein for αGMR. Here we describe how the αGMR/c-Kit physical interaction regulates signal initiation of the receptors and how α2GMR is different from αGMR on modulating c-Kit signaling.

**EXPERIMENTAL PROCEDURES**

Vectors for 293T Cell Expression—Cells (293T) were maintained in high glucose Dulbecco’s modified Eagle’s medium.
supplemented with 10% bovine calf serum, 1% sodium pyruvate, 1% L-glutamine, and antibiotics. Human αGMR (in pmx) and mouse c-Kit (in pcDM8) were subcloned into HindIII and NotI sites of pcDNA4/His-V5c (Invitrogen). The 5′ primer for human αGMR is GCCGCAAGCTTACCGCGATGAGAGGCGCTCGCAG and 3′ primer GCCGCAAGCTTAGCACCATGCTTCTGGTGACA. The 3′ primer for full-length αGMR is GATGGTTAGCGCCGCCTGTTAATTTCTTCCAGG. Mouse c-Kit was subcloned using 3′ GATAGTTAGCGCCGCCGCTCGCAG and the 5′ primer for 256 amino acids at C terminus of c-Kit (kit256) is GCCGCAAGCTTCCCGTGATGAGAGGCGCTCGCAG, and the 5′ primer for 256 amino acids at C terminus of c-Kit (kit256) is GCCGCAAGCTTACCGCGATGAGAGGCGCTCGCAG.

**Immunoprecipitation**—The cDNAs encoding αGMR (pcDNA4/His-V5c, 5 μg) and c-Kit (in pcDNA4/His-V5c, 5 μg) were transfected into 293T cells by calcium phosphate precipitation (28). Antibodies M-14 and C-18 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for immunoprecipitation of c-Kit and αGMR, respectively, in lysis buffer (modified phosphate-buffered saline with 135 mM K⁺, 5 mM Na⁺, and 1.0% Triton X-100, protease, and phosphatase inhibitor cocktails from Sigma). Protein A (Sigma) was used to capture the antibodies. αGMR and c-Kit were detected with anti-V5-HRP (Invitrogen). The αGMR and kit256 (C terminus part of c-Kit) interaction experiments used αGMR in the pMX vector and kit256 in the pcDNA4/His-V5c vector. αGMR was immunoprecipitated and detected with C-18 antibody. The kit256 protein was detected with anti-V5-HRP.

**Detection of Phosphorylated c-Kit**—Cells (293T) were transfected with mouse c-Kit (0.7 μg in pcDM8 vector) alone or in combination with αGMR (4 μg in pmx vector) for 16 h. Cells were harvested and treated with 100 ng/ml mouse SCF (R&D Systems Inc., Minneapolis, MN) for 10 or 20 min at room temperature. The cells were then lysed in lysis buffer (0.1% Triton X-100), and c-Kit was immunoprecipitated with the M-14 antibody. Protein A was used for capturing the antibody. Phosphorylated c-Kit was detected with anti-phosphotyrosine antibody clone 4G10 (Upstate Biotechnology, Inc. Lake Placid, NY), and c-Kit was detected with the M-14 antibody.

**Retroviral Vector Expression of αGMR Isoforms and Mutants**—TF-1 and K562 cells were obtained from the American Type Culture Collection (Manassas, VA). TF-1 cells were maintained in RPMI 1640 medium with 2 mM l-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 10% fetal bovine serum, and 10 ng/ml human SCF (29). K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin). Recombinant human SCF and GM-CSF are from R&D Systems. αGMR, αGMR-375, αGMR-356, and αGMR-352 were subcloned by PCR into the HindIII site and NotI site of pmRETRO-GFP (kindly provided by Dr. Adrian Ting) (30) using the 5′ primer GCCGCAAGCTTACCGCGATGAGAGGCGCTCGCAG and 3′ primers AGTTAGCGCCGCCGCTACCTTGCTTCCACTCATG (αGMR), AGTTAGCGCCGCCGCTACCTTGCTTCCACTCATG (αGMR-375), AGTTAGCGCCGCCGCTACCTTGCTTCCACTCATG (αGMR-356), and AGTTAGCGGAGCGCGCTACAGAGATGACTCTGAGCCGCTCGCAGG.

**RESULTS**

**αGMR Interacts with c-Kit**—The short intracellular domain of αGMR is required for GM-CSF signaling, and the membrane-proximal proline-rich region is especially important (9–12). An 18-amino acid peptide of αGMR (KRFLRIQLRFPPVPIKD) from this region (347–364) was used in the application of our computational protein interaction prediction method. A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4 A peptide from caprine c-Kit (731–747, YVVPKAAKRRSARIG) in the kinase insert domain was used in the application of our computational protein interaction prediction method.4
found to be a likely interaction partner with the αGMR peptide in reverse orientation (Fig. 1A). Since the sequences of human, mouse, and caprine c-Kit have a very high degree of similarity (Fig. 1B), we tested the interaction between human αGMR and mouse c-Kit in co-immunoprecipitation experiments. When co-expressed in 293T cells, human αGMR was co-immunoprecipitated with mouse c-Kit (Fig. 1C). We also made a construct with 256 amino acids (kit256) from the C terminus of mouse c-Kit that contains the putative αGMR-interacting domain and found that kit256 was co-immunoprecipitated with αGMR when co-expressed in 293T cells (Fig. 1D). Therefore the αGMR and c-Kit appeared to interact through their intracellular domains.

**FIGURE 2. αGMR inhibits c-Kit auto-phosphorylation induced by SCF.** 293T cells transfected with mouse c-Kit (pcDM8) or co-transfected with c-Kit and αGMR (pMX) were treated with 100 ng/ml mouse SCF for 10 or 20 min, and phosphorilated c-Kit (Phospho-c-Kit, top panel) was detected by anti-phosphotyrosine antibody clone 4G10 after immunoprecipitation of c-Kit with antibody M-14. The lower panel shows the levels of c-Kit protein expression.

**FIGURE 3. SCF/c-Kit signaling and αGMR do not co-exist.** A, construction of retroviral expression vectors including wild type human αGMR, intracellular domain deletion mutants αGMR-375 and αGMR-356, and another isoform, α2GMR. B, flow cytometry analysis of αGMR, αGMR-375, αGMR-356, and α2GMR expression in K562 cells infected by retroviruses for 48 h without cytokine supplements. The four proteins were detected with FITC-conjugated anti-αGMR antibody clone S-20, and the percentages of positive cells in the polygonal region are indicated. The x axis is FITC fluorescence intensity, and the y axis is nonspecific fluorescence. Non-infected cells are included for control (first panel). C, TF-1 cells were cultured in 2 ng/ml human SCF and infected with retroviruses for 48 h for protein expression, αGMR and its mutants as well as its isoform were recognized by anti-αGMR-FITC. Control cells are not infected with retroviruses. The percentages of positive cells in the polygonal region are indicated and also normalized to the percentages in K562 cells (panel B) that received the same amounts of respective retroviruses shown at the bottom.

**αGMR Inhibits c-Kit Auto-phosphorylation Induced by SCF**—The physical interaction between c-Kit and αGMR suggested cooperation or cross-inhibition between SCF/c-Kit and GM-CSF/αβGMR signaling. Binding of αGMR to c-Kit, most likely to the kinase insert domain, implied that αGMR could modulate c-Kit kinase activity, and therefore we investigated whether αGMR influenced c-Kit kinase activity in 293T cells expressing mouse c-Kit and human αGMR. Mouse SCF stimulated time- and dose-dependent phosphorylation of c-Kit in 293T cells transfected only with c-Kit as detected by anti-phosphotyrosine antibody (Fig. 2). When cells were co-transfected with αGMR and c-Kit, the phosphorylation of c-Kit induced by SCF was clearly inhibited (Fig. 2). This result suggested an inhibitory function of the αGMR/c-Kit interaction.

**SCF/c-Kit-maintained Cells Restrict αGMR Expression**—We asked whether αGMR could inhibit c-Kit signaling in the human hematopoietic cell line TF-1 that responds to growth factors such as SCF, GM-CSF, interleukin-3, or erythropoietin (29). SCF stimulation through human c-Kit maintains the viability and proliferation of TF-1 cells, providing a good model of human c-Kit signaling for testing the αGMR inhibitory activity. We generated retroviral expression vectors for αGMR, α2GMR (the αGMR isoform with a variant cytoplasmic tail), αGMR-375 (the common portion of αGMR and α2GMR with 25 amino acids deleted from αGMR C terminus), and αGMR-356 (44 amino acids deleted from αGMR C terminus) (Fig. 3A). The four αGMR-related proteins have an identical extracellular domain that can be detected by flow cytometry using the same FITC-conjugated anti-αGMR antibody, conveniently allowing comparison of expression levels. The retroviruses produced in 293T cells were tested in the erythroleukemia cell line K562 that proliferates without requirement for growth factor supplements (31). The four αGMR-related proteins were expressed by retrovirus infection of K562 cells with variations in expression levels and infection percentages, possibly reflecting different amounts of retroviruses delivered (Fig. 3B). When a retroviral vector expresses an inhibitor for SCF/c-Kit signaling in TF-1 cells maintained by SCF, the viability and proliferation of the infected cells should be decreased when compared with cells expressing a non-inhibitory protein. The results showed that in TF-1 cells, αGMR-356 (the intracellular domain deletion mutant) and α2GMR were expressed at high levels, whereas αGMR expression level was low as measured by fluorescence intensity (Fig. 3C). αGMR-375 was expressed at an intermediate level.
GM-CSF Receptor α Subunit Inhibits c-Kit

**FIGURE 4. αGMR inhibits SCF/c-Kit signaling in TF-1 cells.** A, FSC/SSC plot of normal TF-1 cells cultured with 10 ng/ml SCF + 100 pm GM-CSF (left panel). FSC roughly measures cell size, and SSC is a cell morphology indicator. When the culture was switched from SCF + GM-CSF to 10 ng/ml SCF alone for 48 h, cells were mostly shifted to smaller size in the lower left quadrant (right panel). B, in 10 ng/ml SCF + 100 pm GM-CSF culture, TF-1 cells express all four αGMR-related proteins. Cells were then cultured in 10 ng/ml SCF for 48 h and analyzed for αGMR expression. The polygonal regions for αGMR, αGMR-375, αGMR-356, and α2GMR select positive cells subject to further analysis (panels C and D). For control, we selected a region of non-infected cells from the same data file of αGMR. C, FSC/SSC analysis of control and infected cells. SCF induced a cell size change that shifts the cell population to low FSC and low SSC region in control, αGMR-356-, and α2GMR-expressing cells. Cells expressing αGMR do not have such shift, and αGMR-375-expressing cells have a partial shift. D, ratios of LL/LR from panel C are summarized in the column graph. A higher ratio indicates a higher SCF response.

(Fig. 3C), suggesting that the two domains sequentially deleted from the intracellular portion of αGMR are both involved in the interaction with c-Kit. We also normalized the percentages of the TF-1 cells expressing the four αGMR-related proteins to those of K562 cells (Fig. 3B), in which we used the same doses of retroviruses. The normalized percentages also supported that αGMR inhibited c-Kit and αGMR-375 partially inhibited c-Kit (Fig. 3C).

**Exogenous αGMR Expression in TF-1 Cells Inhibits Endogenous SCF/c-Kit Signaling**—We further attempted to achieve the expression of αGMR and α2GMR in TF-1 cells and test whether exogenously expressed αGMR could inhibit endogenous c-Kit signaling. To allow expression of αGMR in TF-1 cells, another growth factor, GM-CSF, was included in addition to SCF, relieving the viability dependence on SCF/c-Kit signaling. We noticed under a microscope that the addition of GM-CSF to SCF caused a significant TF-1 cell size increase and that the bigger cells could be driven back to smaller size in culture with SCF alone, quantifiable in the flow cytometry forward scatter/side scatter (FSC/SSC) plot (Fig. 4A). If TF-1 cells express a protein inhibitor of SCF/c-Kit signaling, SCF should be unable to drive enlarged cells to smaller cells. Therefore we used the cell population ratio of the lower left quadrant (LL, smaller cells) to the lower right quadrant (LR, bigger cells) from the FSC/SSC plot of TF-1 cells treated first with GM-CSF and SCF and then with SCF alone to measure the SCF response. A bigger LL/LR ratio would indicate a higher response. We identified cells positive with αGMR, αGMR-375, αGMR-356, and α2GMR with an anti-αGMR antibody in flow cytometry (Fig. 4B) and analyzed their FSC/SSC plots (Fig. 4C). α2GMR- and αGMR-356-positive cells had an LL/LR ratio similar to non-infected cells (low fluorescence population) (Fig. 4, C and D), indicating that α2GMR and αGMR-356 did not inhibit SCF/c-Kit signaling. αGMR-375 partially inhibited SCF response, and αGMR had the strongest inhibition (Fig. 4, C and D). The results demonstrated that the expression of human αGMR but not α2GMR inhibited human c-Kit signaling and that the inhibition was dependent on the intracellular domain of αGMR. Our results supported that the membrane-proximal region of the αGMR intracellular domain (used in our protein-interaction prediction) was involved in the interaction with c-Kit. It appeared that the membrane-distal region of αGMR intracellular domain enhanced the interaction with c-Kit and that the membrane-distal cytoplasmic tail of α2GMR prevented the protein-protein interaction with c-Kit.

**DISCUSSION**

Our present study defines a molecular interaction between the αGMR and c-Kit, which is quite unique in that it involves a receptor tyrosine kinase and a non-kinase receptor subunit. The c-Kit kinase insert domain is known to bind phosphatidylinositol 3-kinase (24), suggesting that it is a domain accessible for interaction with other proteins, and therefore it is understandable that the αGMR/c-Kit interaction utilizes their intracellular domains.

Our findings suggest that the inhibition of c-Kit signaling by αGMR is a result of direct receptor-receptor modulation. Not only could we roughly confirm the predicted interaction domains, we also demonstrated that αGMR inhibited c-Kit auto-phosphorylation in 293T cells with protein overexpression. Auto-phosphorylation is an early step in c-Kit signaling pathways, indicating that the inhibition is at the receptor level. If the inhibition occurred indirectly, i.e. through a third party mediator, the would-be mediator also needs to reach overexpression level to overcome c-Kit. It is very unlikely that 293T cells would happen to have such a mediator at that high level.
induced by αGMR; therefore the αGMR and c-Kit interaction appears to be direct. Our data do not exclude the possibility that αGMR binding to c-Kit affects SCF binding. Direct receptor-receptor trans-modulations are probably not rare as there are other examples including the hepatocyte growth factor receptor Met inhibition of Fas signaling and the 67-kDa laminin receptor inhibition of GM-CSF receptor signaling (15, 32).

As a measurement for SCF-induced cellular responses, we utilized an observation that SCF-supplemented TF-1 cells were smaller than GM-CSF-supplemented cells. The reduction of TF-1 cell size with SCF/c-Kit signaling is consistent with previous reports that c-Kit signaling-deficient mice had severe macrocytic anemia, in which the average size of erythrocytes was larger than normal (21, 26).

The expressions of αGMR and α2GMR at the mRNA level were studied during CD34⁺ hematopoietic progenitor cell differentiation, and it was found that the ratio of α2GMR to αGMR was about 30–60-fold higher in undifferentiated hematopoietic cells (c-Kit positive) than in cells of G/M lineages (c-Kit negative) (18). This implies that the biological function of the alternatively spliced α2 isoform is to transduce GM-CSF signal in the presence of c-Kit. Both αGMR and α2GMR can cooperate with βGMR to transduce GM-CSF signals, and thus, their differential effects on c-Kit signaling might be an important biological reason for the existence of the receptor isoforms.

As an inhibitor for c-Kit signaling, αGMR would be expected to interfere with biological processes in which c-Kit is known to be important, such as erythropoiesis. It was found that expression of human αGMR, but not βGMR or interleukin-3 receptor α subunit, in avian primary erythroblasts actively inhibited their outgrowth and erythroid differentiation, in which erythroid progenitor receptor and c-Kit are involved (33). αGMR in those erythroblasts appeared to have induced changes reflective of the anemic phenotype in c-Kit-deficient mice, substantiating that αGMR is a c-Kit inhibitor. Another finding indicated that the signaling of GM-CSF through α2GMR/βGMR leads to more erythroid differentiation and less G/M differentiation than through αGMR/βGMR in FDCP-Mix cells (18). Our theory could potentially account for the observed differences in that α2GMR allows c-Kit signaling and permits both erythroid and G/M differentiations, whereas αGMR inhibits c-Kit signal and permits predominantly G/M differentiation. Using αGMR in the G/M lineage and c-Kit in the erythroid lineage, hematopoietic cells could ensure lineage divisions because the αGMR and c-Kit signaling pathways are intrinsically non-co-existent. The regulation of hematopoiesis has been extensively studied, and there are other known hematopoietic lineage regulators such as transcription factors PU.1 and CCAAT/enhancer binding protein α (C/EBPα) (34, 35). αGMR and c-Kit are quite special because these receptors were not previously known to coordinate their own signaling patterns.

We attempted to use the retroviral vectors to express αGMR and its variants in purified human bone marrow CD34⁺ cells and investigate whether αGMR could influence lineage differentiation in colony formation assays. The CD34⁺ cells supplemented with SCF, GM-CSF, interleukin-3, and erythropoietin could express α2GMR, αGMR-375, and αGMR-356 but not αGMR in 24 h, similar to SCF-supplemented TF-1 cells (data not shown). The incompatibility of αGMR expression in CD34⁺ cells probably reflects the importance of c-Kit signaling for the maintenance of CD34⁺ cells (22). The suppression of αGMR expression in c-Kit signaling-dependent cells suggested that some differentiation markers can be naturally incompatible in hematopoietic stem cells, providing the stem cells a possible maintenance mechanism.

Although the αGMR-expressing G/M lineage cells come from the c-Kit-expressing hematopoietic stem or progenitor cells, intermediate cells with both c-Kit and αGMR are rare (27), suggesting that the intermediate cells only exist for a short duration during differentiation. Therefore a natural cell system with both αGMR and c-Kit was mechanistically not readily available for us to demonstrate the endogenous αGMR/c-Kit association. Nevertheless, it can be inferred from our results that overexpression of αGMR is not required for inhibiting endogenous c-Kit signaling since SCF-supplemented TF-1 cells could only allow very little αGMR expression, suggesting that the amount of αGMR needed for inhibiting TF-1 endogenous c-Kit signaling is very low.

In summary, our studies showed that the αGMR could interact with c-Kit and inhibit c-Kit signaling, but α2GMR could not inhibit c-Kit signaling. The receptors αGMR and c-Kit are more than regulated adaptor molecules for transducing cytokine signals; rather, they are signal regulators in their own right.

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