The Carboxyl Terminus of the Granulocyte Colony-Stimulating Factor Receptor, Truncated in Patients with Severe Congenital Neutropenia/Acute Myeloid Leukemia, Is Required for SH2-Containing Phosphatase-1 Suppression of Stat Activation

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The Carboxyl Terminus of the Granulocyte Colony-Stimulating Factor Receptor, Truncated in Patients with Severe Congenital Neutropenia/Acute Myeloid Leukemia, Is Required for SH2-Containing Phosphatase-1 Suppression of Stat Activation

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The G-CSF receptor transduces signals that regulate the proliferation, differentiation, and survival of myeloid cells. A subgroup of patients with severe congenital neutropenia (SCN) has been shown to harbor mutations in the G-CSF receptor gene that resulted in the truncation of the receptor’s carboxyl-terminal region. SCN patients with mutations in the G-CSF receptor gene are predisposed to acute myeloid leukemia. The truncated receptors from SCN/acute myeloid leukemia patients mediate augmented and sustained activation of Stat transcription factors and are accordingly hyperactive in inducing cell proliferation and survival but are defective in inducing differentiation. Little is known about the molecular mechanisms underlying the negative role of the receptor’s carboxyl terminus in the regulation of Stat activation and cell proliferation/survival. In this study, we provide evidence that SH2-containing phosphatase-1 (SHP-1) plays a negative regulatory role in G-CSF-induced Stat activation. We also demonstrate that the carboxyl terminus of the G-CSF receptor is required for SHP-1 down-regulation of Stat activation induced by G-CSF. Our results indicate further that this regulation is highly specific since SHP-1 has no effect on the activation of Akt and extracellular signal-related kinase1/2 by G-CSF. The data together strongly suggest that SHP-1 may represent an important mechanism by which the carboxyl terminus of the G-CSF receptor down-regulates G-CSF-induced Stat activation and thereby inhibits cell proliferation and survival in response to G-CSF. The Journal of Immunology, 2001, 167: 6447–6452.

G ranulocyte colony-stimulating factor is the major hematopoietic growth factor involved in the regulation of granulopoiesis (1, 2). G-CSF regulates the proliferation, differentiation, and survival of myeloid progenitor cells. The biological activities of G-CSF are mediated by a specific cell surface receptor, a single transmembrane protein that is a member of the cytokine receptor superfamily. Similar to other members of the superfamily, the G-CSF receptor contains no intrinsic kinase activity in the cytoplasmic domain and transduces signals via interacting with cytoplasmic kinases. Stimulation of cells with G-CSF has been shown to activate multiple signal transduction pathways such as Janus kinase/Stat (3–7), mitogen-activated protein kinases including extracellular signal-regulated kinase (Erk)3 1/2, Erk5, c-Jun N-terminal kinase, and p38 (8–13), phosphatidylinositol 3-kinase/Akt (14, 15), and Src family kinases (16, 17). Mutations in the G-CSF receptor gene have been identified in a subgroup of patients with severe congenital neutropenia (SCN) (4, 18–20), a myeloid disorder characterized by profound selective neutropenia and a maturation arrest of bone marrow myeloid cells at early stages of development. These mutations introduce premature stop codons in the G-CSF receptor gene, leading to the carboxyl-terminal truncation of 82–98 amino acids. SCN patients with G-CSF receptor mutations are predisposed to acute myeloid leukemia (21). When expressed in murine myeloid cells, the truncated G-CSF receptors from patients with SCN/acute myeloid leukemia transduced stronger proliferation signals than the wild-type receptor but, unlike the wild-type receptor, failed to induce granulocytic differentiation (4, 18, 22). Mice carrying an equivalent G-CSF receptor mutation were neutropenic, although bone marrow cells from these mice were hyperproliferative in response to G-CSF (23, 24). Administration of G-CSF in vivo resulted in markedly increased levels of peripheral blood neutrophils as compared with normal mice.

We recently showed that truncation of the carboxyl terminus of the G-CSF receptor resulted in dramatically enhanced and prolonged activation of Stat5 by G-CSF in hematopoietic cells, indicating that the carboxyl terminus of the G-CSF receptor is involved in the negative regulation of Stat5 activation (25). Pretreatment of cells expressing the wild-type G-CSF receptor with tyrosine phosphatase inhibitor vanadate also led to increased and prolonged activation of Stat5 by G-CSF. Interestingly, vanadate had no effect on G-CSF-induced Stat5 activation in cells expressing a truncated receptor lacking the carboxyl-terminal 98 amino acids (F. Dong, unpublished data). Together, these results suggest that a phosphatase or phosphatases, which may be regulated by the carboxyl terminus of the G-CSF receptor, play a role...
in the down-regulation of G-CSF-stimulated Stat5 activation in hematopoietic cells.

SH2-containing phosphatase-1 (SHP-1) is an SH2 domain-containing protein tyrosine phosphatase that is predominantly expressed in hematopoietic cells. SHP-1 has been identified as a negative regulator of signaling through a variety of receptors such as c-fms (26), c-kit (27), erythropoietin receptor (28), IL-3R (29), IFN-αβ receptor (30), B cell Ag receptor (31), T cell Ag receptor (32, 33), killer cell inhibitor receptor (34), and CD22 (35). Mothereaten and viable mothereaten mice, which express essentially no SHP-1 and mutant SHP-1 proteins with markedly compromised catalytic activity, respectively (36, 37), exhibit multiple defects in hematopoiesis. Notably, the most significant hematopoietic defect is the expansion of myeloid cells. Consistent with the role of SHP-1 as a negative regulator of signal transduction, hematopoietic cells from these mice displayed enhanced response to various growth factors and cytokines. In this study, we show that SHP-1 specifically down-regulates G-CSF-stimulated Stat activation but does not affect the activation of Erk1/2 and Akt that are stimulated by G-CSF. We also demonstrate that the carboxyl terminus of the G-CSF receptor, which is truncated in patients with SCN, interacts with SHP-1 and is required for SHP-1 down-regulation of G-CSF-stimulated Stat activation.

Materials and Methods

Cells

The chicken DT40 cells and the SHP-1-deficient cells, which were created from DT40 cells by gene targeting through homologous recombination, have been described (38) and were kindly provided by Dr. J. V. Ravetch (Rockefeller University, New York, NY). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 1% chicken serum, 50 μM 2-ME, and gentamicin.

Stable transfection

The cDNAs encoding the wild-type G-CSF receptor, the D715 mutant, and the mAb mutant were cloned into the pBabe-puro retroviral expression vector as described (18, 39). DT40 cells and SHP-1-deficient cells were transfected by electroporation and were selected in medium containing puromycin (1 μg/ml). 293T cells were maintained in DMEM containing 10% FCS and gentamicin.

Immunoprecipitation and Western blot analysis

Cells were starved in the absence of serum for 4 h and subsequently stimulated with G-CSF (20 ng/ml) for the times indicated. Cells (10⁶) were washed with ice-cold PBS and resuspended in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM NaF, 1% Triton X-100, 1 mM PMSF, and 1 mM vanadate). After incubation on ice for 20 min, lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Supernatants were collected and incubated with appropriate Abs for 2 h at 4°C. Immunocomplexes were recovered with protein G-Sepharose beads (Amersham Pharmacia Biotech) and washed three times with lysis buffer. Samples were heated at 95°C for 5 min and separated by SDS-PAGE before transfer to Immobilon membranes (Millipore, Bedford, MA). The membranes were incubated with the appropriate Abs. Western blots were developed using ECL-Plus kit.

EMSA

EMSA were performed as previously described using whole cell extracts (40). The IFN-γ response region (GRR) probe (5‘-AGCATTTCCAAG GATTTGAGATGTATTTCCCAGAAAAG-3’) was end-labeled using polynucleotide kinase and [γ-32P]ATP.

Luciferase reporter assay

Cells were transfected by electroporation with the luciferase reporter plasmids containing three repeats of an IFN-γ activated site element derived from IRF-1 gene promoter or the c-fos serum response element (SRE), together with the pCDNA3-β-gal plasmid that encodes the β-galactosidase. After 16 h, cells were starved in serum-free medium for 2 h before stimulation with G-CSF for 6 h. Cell extracts were prepared and luciferase activity was measured using a kit (luciferase assay system; Promega, Madison, WI). The β-galactosidase activity was also determined to normalize the luciferase activity.

Results

To investigate the involvement of SHP-1 in the regulation of G-CSF receptor signaling, we stably expressed the human wild-type G-CSF receptor in chicken DT40 cells and SHP-1-deficient cells that were derived from the DT40 cell line (38). It has been shown that expression of the human G-CSF receptor in DT40 cells reconstituted G-CSF signaling (17). The levels of expression of the G-CSF receptor in the two cell lines were comparable as determined by Western blotting (Fig. 1B). After starvation in serum-free medium for 4 h, cells were stimulated with G-CSF for different times before preparation of whole cell extracts. Activation of Stat transcription factors was examined by EMSA using the IFN-γ response region (GRR) probe. G-CSF stimulated the DNA binding activity of Stat transcription factors in both cell lines (Fig. 2A). Stat activation in SHP-1-deficient cells was ~5-fold stronger than that in parental DT40 cells, as determined by phospho imager analysis (data not shown). The rates at which the Stat DNA binding activity decayed were similar in the two cell lines, although Stat activation was prolonged in SHP-1-deficient cells because of the enhanced activation that took longer to diminish.

EMSA

B

FIGURE 1. Expression of the wild-type and the truncated G-CSF receptor proteins in DT40 and SHP-1-deficient cells. A, Diagram of the wild-type and truncated forms of the G-CSF receptor. Numbers 1, 2, and 3 denote regions conserved in members of the cytokine receptor superfamily. The four tyrosine residues present in the cytoplasmic domain of the G-CSF receptor are also indicated. TM, transmembrane domain. B, Whole cell extracts were prepared from DT40 (+/+ and SHP-1-deficient (−/−) cells stably transfected with the wild-type (WT) or the truncated receptor (D715) and subjected to immunoprecipitation with an Ab against the G-CSF receptor. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with the anti-G-CSF receptor Ab.
G-CSF has been shown to activate Stat3 (6–8). A polyclonal Ab was available that recognized the activated form of chicken Stat3 phosphorylated on tyrosine 705. We examined Stat3 phosphorylation in response to G-CSF in DT40 and SHP-1-deficient cells. Similar to Stat DNA binding activity, Stat3 phosphorylation was augmented and somewhat prolonged in SHP-1-deficient cells (Fig. 2B). SHP-1-deficient cells were also more sensitive to G-CSF and required lower concentrations of G-CSF for inducing Stat3 activation as compared with DT40 cells (Fig. 2C).

We then investigated whether re-expression of SHP-1 in SHP-1-deficient DT40 cells would attenuate Stat activation by G-CSF. SHP-1-deficient cells were transiently transfected with cDNA encoding SHP-1. Expression of SHP-1 in SHP-1-deficient cells significantly reduced the activation of Stat DNA binding activity that was stimulated by G-CSF treatment (Fig. 3).

In addition to Stat pathway, G-CSF also induces the activation of Erk1/2 and Akt (8, 10, 14). We asked whether activation of these kinases by G-CSF was regulated by SHP-1. Activation of Erk1/2 and Akt was examined by Western blotting using phosphospecific Abs that recognize only the active forms of these kinases. As shown in Fig. 4, treatment of DT40 and SHP-1-deficient cells with G-CSF resulted in the activation of Erk1/2 and Akt. However, the magnitude and the duration of activation of these kinases were comparable in the two cell lines, suggesting that SHP-1 may not play a major role in the regulation of Erk1/2 and Akt activation by G-CSF.

We further examined Stat transcriptional activation in response to G-CSF in DT40 and SHP-1-deficient cells using a Stat-dependent luciferase reporter containing three repeats of an IFN-γ activated site element derived from IRF-1 gene promoter. G-CSF stimulated Stat-dependent transcriptional activity in both parental and SHP-1-deficient cells (Fig. 5). However, Stat transcriptional activation in SHP-1-deficient cells was at least 2-fold stronger than that in DT40 cells, consistent with the enhanced Stat DNA binding activity. In contrast, activation of another luciferase reporter containing the c-fos SRE, which is dependent on Erk signaling pathway (41, 42), was comparable in the two cell lines. Together, these data indicated that SHP-1 deficiency resulted in enhanced Stat activation by G-CSF and that SHP-1 is specifically involved in the down-regulation of G-CSF-stimulated Stat activation.

We recently showed that the carboxyl terminus of the G-CSF receptor down-regulated Stat activation by G-CSF in that truncation of this carboxyl terminus led to enhanced and prolonged activation of Stat5 and Stat3 in hematopoietic cells such as pro-B BAF3 and myeloid 32D cells (25, 39). To compare the effects of the receptor’s carboxyl-terminal truncation and SHP-1 deficiency...
on G-CSF-induced Stat activation, we stably transfected DT40 cells with a truncated G-CSF receptor (D715 mutant), derived from a patient with SCN (4), that lacked the carboxyl-terminal 98 amino acids. The D715 mutant was expressed at levels comparable from a patient with SCN (4), that lacked the carboxyl-terminal 98 amino acids. The D715 mutant was expressed at levels comparable to the wild-type receptor in DT40 cells, as confirmed by Western blotting (Fig. 1B). As compared with the wild-type receptor, the D715 receptor mediated significantly enhanced and prolonged Stat activation in DT40 cells (Fig. 6A). Notably, the magnitude of Stat activation by the D715 receptor in DT40 cells was comparable to that by the wild-type receptor in SHP-1-deficient cells. However, Stat activation by the D715 receptor was more sustained than that induced by the wild-type receptor in SHP-1-deficient cells. These results demonstrated that the receptor’s carboxyl-terminal truncation and SHP-1 deficiency had similar effects on the magnitude of Stat activation and that SHP-1 may not play a major role in the regulation of the duration of Stat activation.

We further explored the possibility that the negative effect of the receptor’s carboxyl terminus on the magnitude of Stat activation is mediated by SHP-1. If SHP-1 is involved in the down-regulation of Stat pathway by the receptor’s carboxyl terminus, it is conceivable that SHP-1 deficiency should not further potentiate Stat activation by the D715 mutant. SHP-1-deficient cells were stably transfected with the D715 receptor and Stat activation by G-CSF was examined by EMSA. As shown in Fig. 6A, Stat activation mediated by the D715 mutant was comparable in DT40 and SHP-1-deficient cells. Together, these results indicated that SHP-1 specifically interacts with the carboxyl-terminal region of the G-CSF receptor and that the carboxyl-terminal tyrosine residues are not required for such an interaction.

**Discussion**

Point mutations in the G-CSF receptor gene leading to the carboxyl-terminal truncation of the receptor have been identified in a...
subgroup of patients with SCN (4, 18–20). These mutations are associated with increased incident of leukemic transformation in SCN patients (21). Expression of the truncated receptors in murine hematopoietic cells results in increased cell proliferation and survival but impaired myeloid differentiation in response to G-CSF (18, 25). The truncated G-CSF receptors also mediate markedly augmented and prolonged activation of Stat3 and Stat5 in hematopoietic cells (Ref. 25 and F. Dong, unpublished data), which are critically involved in the regulation of cell proliferation, differentiation, and survival induced by G-CSF (25, 43–46). However, the molecular mechanisms underlying the negative effects exerted by the carboxyl terminus of the G-CSF receptor on Stat activation and cell proliferation/survival are still unknown.

Studies have indicated that SHP-1 may play a negative role in the regulation of G-CSF-stimulated signaling pathways. Tapley et al. (47) reported that bone marrow cells from viable motheaten mice, which exhibit substantially decreased SHP-1 activity, displayed dramatic increase in proliferative response to G-CSF. However, it was unclear whether the increased responsiveness to G-CSF was due to enhanced cellular response to G-CSF or to an increase in G-CSF-responsive granulocytic population. Ward et al. (48) recently showed that overexpression of a catalytically inactive SHP-1 mutant led to enhanced Stat5 activation and cell proliferation in response to G-CSF. The generation of the SHP-1-null cell line by gene targeting through homologous recombination provided a clean model system for studies addressing the role of SHP-1 in the regulation of intracellular signaling (38, 49). Using this cell model, we show that deficiency of SHP-1 is associated with augmented Stat activation by G-CSF, providing solid evidence that SHP-1 is involved in the down-regulation of G-CSF-induced Stat activation. We also demonstrate that this regulation is highly specific because SHP-1 has no effect on the activation of Akt and Erk1/2 by G-CSF.

The negative regulatory mechanisms controlled by the carboxyl terminus of the G-CSF receptor are obviously operational in DT40 cells, as evident from the fact that Stat activation mediated by the D715 receptor is augmented and prolonged in DT40 cells, analogous to that in murine pro-B BAF3 and myeloid 32D cells (25). Several lines of evidence implicate SHP-1 as a critical molecule involved in the negative regulation of the Stat signaling pathway by the carboxyl terminus of the G-CSF receptor. For instance, the magnitude of G-CSF-induced Stat activation is augmented to a comparable level by either the receptor’s carboxyl-terminal truncation or SHP-1 deficiency, suggesting that down-regulation of the magnitude of Stat activation requires the coexpression of the receptor’s carboxyl terminus and SHP-1. Notably, although SHP-1 deficiency enhances Stat activation by the wild-type G-CSF receptor, it does not augment Stat activation mediated by the D715 receptor, consistent with a model in which the carboxyl terminus and SHP-1 act in tandem to down-regulate Stat activation. Furthermore, we show that SHP-1 specifically interacts with the carboxyl terminus but not the other regions of the G-CSF receptor in 293T cells. Together, these data reveal an important molecular mechanism by which the carboxyl terminus of the G-CSF receptor down-modulates Stat activation by G-CSF.

It should be noted that the interaction between the G-CSF receptor and SHP-1 is weak. Although the G-CSF receptor and SHP-1 were abundantly expressed in 293T cells, it appears that only a small fraction of the two proteins associated with each other (see Fig. 6B). Such a weak interaction might explain why others have failed to detect an association between the G-CSF receptor and SHP-1 (47, 48). We were also unable to demonstrate SHP-1 association with the G-CSF receptor in DT40 cells (data not shown). In addition to the G-CSF receptor, SHP-1 has been shown to interact with a number of other cytokine receptors. Interestingly, the interactions of SHP-1 with the receptors for IL-3 and erythropoietin are induced by ligand stimulation (28, 29), whereas SHP-1 interactions with the IFN receptor and gp130 of the IL-6 receptor are constitutive (30, 50). We show in this work that the G-CSF receptor coassociate with SHP-1 in 293T cells and this association was not significantly altered by G-CSF stimulation. Furthermore, we demonstrate that the carboxyl-terminal tyrosine residues of the G-CSF receptor are not required for interaction with SHP-1, indicating that the interaction between the G-CSF receptor and SHP-1 is mediated via a phosphorylation-independent mechanism. Consistent with this, Ward et al. (48) recently showed that overexpression of SHP-1 suppressed G-CSF-dependent proliferation of BAF3 cells transfected with a G-CSF receptor mutant lacking all of the cytoplasmic tyrosine residues, suggesting that the cytoplasmic tyrosine residues of the G-CSF receptor are dispensable for the growth inhibitory effects of SHP-1.

Despite its significant role in G-CSF-induced Stat activation, SHP-1 is unlikely to be the only mechanism responsible for the inhibition of G-CSF signaling by the receptor’s carboxyl terminus. For instance, while receptor truncation leads to enhanced and prolonged activation of Stats in response to G-CSF, SHP-1 deficiency magnifies Stat activation but only marginally extends the duration of Stat activation by G-CSF. Likewise, Akt activation by G-CSF is significantly prolonged as a result of receptor truncation in hematopoietic cells including DT40 cells (Ref. 14 and F. Dong, unpublished data) but was not affected by SHP-1 deficiency (see Fig. 4). SHP-1 also does not seem to play a central role in the regulation of Erk1/2 activation by G-CSF. These results implicate SHP-1 as a specific regulator that primarily controls the magnitude of G-CSF-stimulated Stat activation. The prolonged activation of Stats and Akt associated with receptor carboxyl-terminal truncation appears to be caused by different mechanisms. It has been shown recently that truncation of the carboxyl terminus of the G-CSF receptor severely impairs receptor internalization triggered by G-CSF binding (39). Whether or not other mechanisms involved in terminating G-CSF signaling are also affected by receptor carboxyl-terminal truncation remains to be determined. However, the results presented in this work point to an important molecular mechanism that may explain, at least in part, why truncation of the carboxyl-terminal region of the G-CSF receptor leads to hyper-sensitivity of hematopoietic cells to G-CSF and thereby may contribute to leukemogenesis.

Acknowledgments

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References


