Signal Transducers and Activators of Transcription 3 Augments the Transcriptional Activity of CCAAT/Enhancer-binding Protein α in Granulocyte Colony-stimulating Factor Signaling Pathway*

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The Janus kinase (Jak)-Stat pathway plays an essential role in cytokine signaling. Granulocyte colony-stimulating factor (G-CSF) promotes granulopoiesis and granulocytic differentiation, and Stat3 is the principle Stat protein activated by G-CSF. Upon treatment with G-CSF, the interleukin-3-dependent cell line 32D clone 32Dcl3 differentiates into neutrophils, and 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in G-CSF without differentiation. Gene expression profile and quantitative PCR analysis of G-CSF-stimulated cell lines revealed that the expression of C/EBPα was up-regulated by the activation of Stat3. In addition, activated Stat3 bound to CCAAT/Enhancer-binding protein (C/EBP), leading to the enhancement of the transcription activity of C/EBPα. Conditional expression of C/EBPα in 32Dcl3/DNStat3 cells after G-CSF stimulation abolishes the G-CSF-dependent cell proliferation and induces granulocytic differentiation. Although granulocyte-specific genes, such as the G-CSF receptor, lysozyme M, and neutrophil gelatinase-associated lipocalin precursor (NGAL) are regulated by Stat3, only NGAL was induced by the restoration of C/EBPα after stimulation with G-CSF in 32Dcl3/DNStat3 cells. These results show that one of the major roles of Stat3 in the G-CSF signaling pathway is to augment the function of C/EBPα, which is essential for myeloid differentiation. Additionally, cooperation of C/EBPα with other Stat3-activated proteins are required for the induction of some G-CSF responsive genes including lysozyme M and the G-CSF receptor.

The proliferation and differentiation of hematopoietic progenitor cells are regulated by cytokines (1). Among these, granulocyte colony-stimulating factor (G-CSF) specifically stimulates cells that are committed to the myeloid lineage (2). The importance of G-CSF to the regulation of granulopoiesis has been confirmed by the observation of severe neutropenia in mice carrying homozygous deletions of their G-CSF or G-CSF receptor genes (3, 4). Cytokines activate several intracellular signaling pathways, and the Janus kinase (Jak) signal transducers and activators of transcription (Stat) pathway is essential for cytokine function (5, 6). The binding of G-CSF to cell surface G-CSF receptors activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7–9). Stat3 is the principle protein activated by G-CSF (8, 10). Phosphorylated Stats translocate from the cytoplasm into the nucleus and induce transcription of their target genes within a short period of time. 32Dcl3 cells differentiate to neutrophils following treatment with G-CSF. In contrast to their parental cells, 32Dcl3 cells expressing dominant-negative Stat3 (32Dcl3/DNStat3) proliferate in the presence of G-CSF, but they maintain immature morphologic characteristics without evidence of differentiation (11). Additionally, transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). To clarify the role of Stat3 in the G-CSF signaling pathway, we wished to identify target genes of Stat3.

We found that the levels of CCAAT/enhancer-binding protein (C/EBP) α mRNA were up-regulated following G-CSF stimulation in 32Dcl3 but were unchanged in 32Dcl3/DNStat3. In addition, the activation of Stat3 augmented the function of C/EBPα, which is the essential transcriptional factor for myeloid differentiation. G-CSF-induced granulocytic differentiation was restored in 32Dcl3/DNStat3 cells by the conditional expression of C/EBPα. These results show that one of the major

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¶ The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; C/EBPα, CCAAT/enhancer-binding protein; NGAL, neutrophil gelatinase-associated lipocalin precursor; Jak, Janus kinase; Stat, signal transducers and activators of transcription; DNStat3, dominant-negative Stat3; IRES, internal ribosome entry site; GFP, green fluorescent protein; ER, endoplasmic reticulum; IFN, interferon; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; LUC, luciferase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TK, thymidine kinase; 4-HT, 4-hydroxytamoxifen.
roles of Stat3 in the G-CSF signaling pathway is to enhance the function of C/EBPα.

**MATERIALS AND METHODS**

**Cell Culture, Expression Plasmid, and Cytokines**—32D cl3 and 32Dcl3/DNStat3 cells (DNStat3 deletes the transactivation domain of Stat3) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ICN, Osaka, Japan), penicillin/streptomycin (Invitrogen), recombinant murine interleukin-3 (IL-3) (Kirin Brewery, Takasaki, Japan), and recombinant human G-CSF (Chugai Pharmaceutical, Tokyo, Japan). 293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin/streptomycin, and l-glutamine.

For the construction of pTag2A-G-CSF receptor human G-CSF receptor cDNA (13) (pRQI, kindly provided by S. Nagata and R. Fukunaga) was excised from the pBluescript vector and inserted into the FLAG-tagged mammalian expression plasmid pCMV-Tag2A (Clontech). pDNA3-rat C/EBPα was described before (14). Stat3 cDNA was amplified by PCR and inserted into pCMV-HA vector (Clontech). Stat3c cDNA was elicted from RCMV-Stat3c (15), kindly given from Dr. Darnell, and inserted into pDNA3.1 (Clontech). For the construction of pMY-IRES-GFP/C/EBPα full-length human C/EBPα cDNA was fused in-frame with ligand-binding domain (amino acids 281–599) of mouse estrogen receptor harboring a mutation (G525R) that confers selective responsiveness to 4-hydroxamoxifen (4-HT). A reporter construct of a minimal TK promoter with CEIP-binding sites (p/C/EBP-2TK) was described previously (14).

Murine recombinant leukemia inhibitory factor, natural IFN-α, and recombinant IFN-γ were purchased from Sigma, HyCult Biotechnology (Uden, The Netherlands), and Peprotech (Rocky Hill, NJ), respectively. For Western blotting, 32Dcl3 cells or 32Dcl3/DNStat3 cells were deprived of IL-3 for 12 h. Then cells were stimulated with G-CSF (10 ng/ml), IL-3 (10 ng/ml), leukemia inhibitory factor (10 ng/ml), IFN-α (1,000 units/ml), or IFN-γ (1,000 units/ml) for 30 min.

**Microarray Analysis**—32D cl3 and 32Dcl3/DNStat3 cells maintained in IL-3 were cultured with PBS and starved of cytokines in RPMI 1640 containing 10% fetal bovine serum for 8 h and then stimulated with 10 ng/ml G-CSF. Total RNA was extracted by the acid guanidinium method, from 32Dcl3 and 32Dcl3/DNStat3 cells before or after the stimulation for 2 h with G-CSF. Double-stranded cDNA synthesized from the total RNA (20 μg/sample) was then used to prepare biotin-labeled cRNA for the hybridization with GeneChip MGU74Av2 microarrays (Affymetrix, Santa Clara, CA) harboring oligonucleotides corresponding to ~60,000 known genes as well as ~60,000 expressed sequence tag sequences. Hybridization, washing, and detection of signals on the arrays were performed with the GeneChip system (Affymetrix).

**Quantitative Real-time Reverse Transcription-PCR Assay**—32Dcl3 and 32Dcl3/DNStat3 cells maintained in IL-3, were washed twice with PBS and starved of cytokines for 8 h, and then stimulated for 1 h with 10 ng/ml G-CSF. cDNA was synthesized from the indicated times, and then separated on a 2% agarose gel and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. p-ERK1/2, phosphorylated ERK1/2.

**Luciferase Assay**—293T cells were transfected by the calcium phosphate precipitation method in 6-well plates, and luciferase activity was measured using the indicated antibodies. p-C/EBPα-HA, phosphorylated C/EBPα.

**Transfection**—1 × 10⁵ cells were transfected with 20 μg of expression vector, and GFP-positive cells were sorted by FACs Vantage (BD Biosciences). Expression of C/EBPα was determined by Western blotting analysis (see below).

**Conditional C/EBPα Expression**—pMY-ires-GFP/C/EBPα-ER was transfected into 32Dcl3 and 32Dcl3/DNStat3 cells by electroporation. 5 × 10⁵ cells were transfected with 20 μg of expression vector, and GFP-positive cells were sorted by FACs Vantage (BD Biosciences). Expression of C/EBPα was determined by Western blotting analysis (see below).

**Flow Cytometry**—1 × 10⁵ cells were incubated with 5 μl of recombinant phycoerythrin-conjugated rabbit anti-murine Gr1 monoclonal antibody (BD Biosciences) for 30 min at 4 ⁰C, washed twice in PBS, and analyzed on a FACs Calibur (BD Biosciences).

**Immunoprecipitation and Immunoblotting**—Cells were lysed with lysis buffer, and lysates were immunoprecipitated with anti-C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (8). Total cell lysates or the immunoprecipitates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. p-ERK1/2, phosphorylated ERK1/2.

**FIG. 1. The effect of dominant-negative Stat3 on G-CSF signaling pathway.** A, transfection in 293T cells with a reporter construct with -2-macroglobulin promoter (STAT3-LUC), dominant-negative Stat3, and G-CSF receptor. Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis is fold induction when compared with control. B, 32Dcl3 cells or 32Dcl3/DNStat3 cells were cultured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated with the G-CSF for 30 min and lysed. Lysate supernatants were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using the indicated antibodies. p-ERK1/2, phosphorylated ERK1/2.

mm MgCl₂, 200 μM each primer, 0.625 units of AmpliTaqGold, and 0.25 units of AmpliErase uracil N-glycosylase. Each amplification reaction also contained 100 μM appropriate detection probe. Each PCR amplification was performed in duplicate, using conditions of 50 ⁰C for 2 min preceding 95 ⁰C for 10 min followed by 40 cycles of amplification (95 ⁰C for 15 s, 60 ⁰C for 1 min). In each reaction, GAPDH was amplified as a housekeeping gene to calculate a standard curve and allow for the correction for variations in target sample quantities. Relative copy numbers were calculated for each sample from the standard curve after normalization to GAPDH by the instrument software.
were probed using the indicated antibodies followed by an IgG-horse- 
radish peroxidase-conjugated secondary antibody (Amersham Bio-
sciences) and visualized with the ECL detection system (Amersham
Biosciences). Anti-phospho-ERK1/2 antibodies were purchased from
Cell Signaling (Beverly, MA). Anti-phospho-Stat1 and -Stat5 antibodies
were obtained from New England Biolabs (Beverly, MA), and anti-
Stat1, -Stat3, and -C/EBPα antibodies were purchased from Santa Cruz
Biotechnology. Membranes were probed using and visualizes with the

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Proliferation Assay—

32D cl3 and 32Dcl3/DNStat3 cells maintained
in IL-3 were washed twice with PBS and starved of cytokine for 8 h
and then stimulated with 10 ng/ml G-CSF. The number of viable cells
was determined by trypan blue dye exclusion using a hemocytometer.


\[3 \text{H} \text{Thymidine incorporation assays were also performed. Briefly, cells (1 \times 10^5) in 100 \mu l of medium stimulated with murine IL-3 (1.0 ng/ml) or recombinant human G-CSF (10 ng/ml) were cultured for 48 h. During the final 4 h, }^{3} \text{H} \text{thymidine (1 \mu Ci/well) was added. Cells were then harvested by filtration, and radioactivity was counted by scintillation spectrophotometer.}

RESULTS

G-CSF-induced Intracellular Signal Response in 32Dcl3/
DNStat3 Cells—32Dcl3 cells differentiate into neutrophils follow-
ing treatment with G-CSF, but 32Dcl3 cells expressing a

TABLE 1

| Gene product name | Abbreviation | Accession number | Fold induction 32Dcl3 | Fold induction 32Dcl3/DNStat3 |
|-------------------|--------------|------------------|-----------------------|-------------------------------|
| B-cell leukemia/lymphoma α | Bcl2 | L31532 | 35.6 | 0.0629 |
| CyclinE1 | Ccne1 | NM007633 | 29.7 | 0.690 |
| Serotonin-gated ion channel | SHT3 | M74425 | 27.2 | 0.592 |
| KIF3B protein | kif3b | D26077 | 21.5 | 0.921 |
| Protein kinase, serine/arginine-specific 1 | Srpk1 | AB012290 | 18.7 | 0.321 |
| MAP kinase-interacting serine/threonine kinase 1 | Mnk1 | Y11091 | 15.7 | 0.845 |
| Protein tyrosine phosphatase | Ptpn13 | D63966 | 12.4 | 0.964 |
| Transferrin receptor | Tf | X57349 | 10.6 | 0.964 |
| Lymphocyte antigen 57 | Ly57 | AF068182 | 9.62 | 0.968 |
| Macrophage stimulating 1 receptor | Mst1r | X74736 | 8.83 | 0.762 |
| Mitogen-activated protein kinase kinase 7 | MKK7 | AB005654 | 8.14 | 0.980 |
| RAR-related orphan receptor alpha | Rora | U53228 | 7.94 | 0.861 |
| Hemoglobin Y, β-like embryonic chain | Hbb-β | V00726 | 7.28 | 0.375 |
| Runt related transcription factor 1 | Runx1 | NM009821 | 7.01 | 0.226 |
| Microtubule-associated protein 6 | Mtap6 | Y14754 | 5.06 | 0.885 |
| CCAAT/enhancer binding protein α | C/EBPα | M62362 | 2.05 | 0.840 |
| Ecotrophic viral integration site 1 | Evi1 | M21829 | 1.55 | 0.239 |
| Integrin alpha L | Itgal | M60775 | 1.55 | 0.567 |
| Ninjurin 1 | Ninj1 | U91513 | 1.34 | 0.783 |
| Interleukin 17 receptor | IL17R | U31993 | 1.24 | 0.449 |
| Mucosal addressin | MadCAM | D50434 | 1.14 | 0.527 |
| Carbon catabolite repression 4 homolog | Ccr4 | X18670 | 1.06 | 0.0768 |
| Friend leukemia integration 1 | Fit1 | X59421 | 1.01 | 0.905 |

FIG. 2. Expression of C/EBPα mRNA in G-CSF-stimulated
32Dcl3 and 32Dcl3/DNStat3 cells. A and B, 32Dcl3 and 32Dcl3/
DNStat3 cells maintained in IL-3 were washed twice with PBS
and starved of cytokines for 8 h and stimulated with 10 ng/ml G-CSF (A) or
10 ng/ml G-CSF and 10 μg/ml cycloheximide (B). Total RNA was iso-
lated from both cell lines at the indicated times and transcribed to
cDNA, which was subjected to real-time PCR for murine C/EBPα. The
numbers given on the vertical axis represent the fold induction of the
ratios of GAPDH-normalized expression values when compared with
those before G-CSF stimulation. Results are expressed as mean fold of
two independent experiments.

Fig. 3. The effect of the abrogation of Stat3 on other cytokine
signaling pathway. 32Dcl3 cells or 32Dcl3/DNStat3 cells were cul-
tured with IL-3 and then deprived of IL-3 for 12 h. Cells were treated
with the indicated cytokines for 30 min and lysed. Post-nuclear super-
natants were resolved by 10% SDS-PAGE and transferred to nitrocel-
lulose membranes. Membranes were probed using the indicated anti-
bodies. LIF, leukemia inhibitory factor.

ECL detection system (Amersham Biosciences).

Proliferation Assay—32D cl3 and 32Dcl3/DNStat3 cells maintained
in IL-3 were washed twice with PBS and starved of cytokine for 8 h
and then stimulated with 10 ng/ml G-CSF. The number of viable cells
was determined by trypan blue dye exclusion using a hemocytometer.

[^3H]Thymidine incorporation assays were also performed. Briefly, cells (1 × 10^5) in 100 μl of medium stimulated with murine IL-3 (1.0 ng/ml) or recombinant human G-CSF (10 ng/ml) were cultured for 48 h. During the final 4 h, [^3H]thymidine (1 μCi/well) was added. Cells were then harvested by filtration, and radioactivity was counted by scintillation spectrophotometer.

RESULTS

G-CSF-induced Intracellular Signal Response in 32Dcl3/
DNStat3 Cells—32Dcl3 cells differentiate into neutrophils follow-
ing treatment with G-CSF, but 32Dcl3 cells expressing a
dominant-negative Stat3 (32Dcl3/DNStat3) proliferate following G-CSF treatment. These cells maintain immature morphologic characteristics without evidence of differentiation (11). First, we examined the effect of dominant-negative Stat3, carboxyl-truncated Stat3 that lacked 55 amino acids including the transactivation domain. We transfected reporter construct of G-CSF receptor, HA-Stat3, and C/EBP with 293T cells, together with empty vector (pcDNA3) or Stat3c, and control vectors. Promoter activity was measured as luciferase activity 24 h after transfection. The vertical axis number is the fold induction when compared with control. B and C, transient transfection in 293T cells with a reporter construct of a minimal TK promoter with CEBP-binding sites (p/CEBP/2TK), C/EBPα, and G-CSF receptor (G-CSFR). Twelve hours after transfection, cells were stimulated with 10 ng/ml G-CSF. Promoter activity was measured as luciferase activity 36 h after transfection. The vertical axis number is the fold induction when compared with control. D, transient transfection in 293T cells with a construct of G-CSF receptor, HA-Stat3, and C/EBPα and control vectors. After 24 h, cells were lysed and immunoprecipitated (IP) with anti C/EBPβ. Cells were stimulated with G-CSF during the final 9 h in the culture. The immunoprecipitates were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Stat3 was detected by immunoblotting.

Total RNA was isolated from 32Dc13 cells and 32Dc13/DNStat3 cells treated with G-CSF after 0 and 2 h, transcribed to biotinylated cRNA, and hybridized to GeneChip MGU74Av2 arrays to compare the expression profile of ~12,000 murine genes. The fold induction in the expression level of each gene was calculated as the ratio of GAPDH-normalized fluorescence intensity value of G-CSF-stimulated cells when compared with those before G-CSF stimulation. As shown in Table I, we could identify a set of candidate genes for Stat3 targets, expression of which was up-regulated in 32Dc13 cells but down-regulated or unchanged in 32Dc13/DNStat3 cells. Such Stat3-dependent expression profiles were confirmed in triplicate experiments.

Stat3 Augments C/EBPα Function in G-CSF Signaling Pathway—Among the identified genes, it was decided to focus further efforts on C/EBPα. C/EBPα has been shown to be critical for early granulocytic differentiation (17–19), and the factors regulating its activity are unclear. The expression of C/EBPα was examined by real-time quantitative reverse transcription-PCR. C/EBPα mRNA levels are rapidly up-regulated in 32Dc13 cells, being elevated 2.39-fold after 6 h and 4.20-fold after 48 h (Fig. 2A). In contrast to 32Dc13 cells, the C/EBPα mRNA levels were not changed in 32Dc13/DNStat3 cells after G-CSF stimulation (Fig. 2A). A similar expression pattern was seen in separate experiments with independently designed primers and probes (data not shown). Levels of C/EBPα mRNA were unaffected by cycloheximide treatment (Fig. 2B). The expression level of the sum of Stat3 plus dominant-negative Stat3 in 32Dc13/DNStat3 cells is a little larger than that of Stat3 in 32Dc13 cells (Fig. 2C).
Stat3 abrogation on the balance of intracellular signals in other cytokine pathways. Although Stat1 was not phosphorylated by leukemia inhibitory factor stimulation in neither 32Dcl3 cells nor 32Dcl3/DNStat3 cells, its activation in response to IFN-γ occurred at the same degree in both 32cl3 cells and 32Dcl3/DNStat3 cells (Fig. 3A). As for the Stat5 activation, the phosphorylation of Stat5 by IL-3 stimulation in 32Dcl3 cells was stronger than that in 32Dcl3/DNStat3 cells (Fig. 3B). These data indicated that there was the possibility that abrogation of Stat3 signaling can alter the balance of intracellular signals in other cytokine signaling pathways. The transcription of C/EBPα is regulated by C/EBPα itself (20, 21). Then we examined whether activated Stat3 in G-CSF signaling enhance C/EBPα activity or not.

We transfected a reporter construct of a minimal TK promoter with CEBP-binding sites (p(C/EBP)2TK), C/EBPα, and G-CSF receptor to 293T cells. After 12 h of transfection, cells were stimulated with 10 ng/ml G-CSF. Cells were cultured for more 24 h, and a luciferase assay was performed. C/EBPα up-regulated the C/EBPα-dependent gene expression, and the G-CSF stimulation enhanced this C/EBPα-dependent gene expression (Fig. 4A). Next we examined the effect of constitutive active Stat3 (Stat3C) on the augmentation of C/EBPα transcriptional activity instead of the G-CSF stimulation. We transfected reporter construct p(C/EBP)2TK, C/EBPα, and Stat3C to 293T cells. After 24 h of transfection, luciferase assay was performed. Stat3C augmented the C/EBPα-dependent gene expression, although Stat3C alone had no influence on the luciferase activity (Fig. 4, B and C).

As p(C/EBP)2TK contains only a C/EBPα-binding site and does not contain a Stat3-binding sequence, the possibility that Stat3C makes a complex with C/EBPα and augments the function of C/EBPα is raised. Then we transfected C/EBPα, Stat3, and G-CSF receptor to 293T cells and stimulated cells with G-CSF for 6 h. There is no detectable level of endogenous C/EBPα or C/EBPβ protein in 293T cells. Cells were lysed and immunoprecipitated with C/EBPβ antibody (this antibody cross-reacts with C/EBPα). As shown in Fig. 4D, immunoprecipitants with anti-C/EBPβ contain Stat3. In addition, the complex formation between C/EBPα and Stat3 is augmented by G-CSF stimulation, indicating that activated Stat3 makes the complex with C/EBPα.

C/EBPα Restores G-CSF-induced Granulocytic Differentiation in 32Dcl3/DNStat3 Cells—To analyze the role of Stat3-
regulated C/EBPα function in the G-CSF signaling pathway, we transfected a C/EBPα-tamoxifen receptor fusion protein (C/EBPα-ER) into 32Dcl3 and 32Dcl3/DNStat3 cells (32Dcl3/C/EBPα cells, 32Dcl3/DNStat3/C/EBPα cells, respectively). The expression of C/EBPα-ER in these cells was verified by Western blotting (Fig. 5A). C/EBPα-ER localizes to the cytoplasm and is in an inactive form in the absence of tamoxifen. Upon treatment with tamoxifen, it translocates from cytoplasm to nucleus and becomes active. 32Dcl3, 32Dcl3/C/EBPα, 32Dcl3/DNStat3, and 32Dcl3/DNStat3/C/EBPα cells were cultured with G-CSF in the presence or absence of tamoxifen, and cell proliferation was examined by both counting viable cells and [3H]thymidine incorporation. 32Dcl3/DNStat3 proliferated in response to G-CSF, and proliferation was not affected by the presence of tamoxifen. Conversely, G-CSF-induced proliferation of 32Dcl3/DNStat3/C/EBPα cells in the presence of tamoxifen was dramatically reduced (Fig. 5, B and C).

32Dcl3/DNStat3 cells maintain morphologically immature characteristics and proliferate without granulocytic differentiation after G-CSF stimulation. We examined the morphological changes in 32Dcl3 and 32Dcl3/DNStat3 cells induced by G-CSF after translocation of C/EBPα from the cytoplasm to the nucleus. When tamoxifen was added to medium containing G-CSF, 32Dcl3/DNStat3/C/EBPα cells rapidly began to differentiate into granulocytes, and 5 days later, about 40% of the cells were morphologically similar to mature neutrophils. In contrast, 32Dcl3/DNStat3/C/EBPα cells cultured in G-CSF-containing medium without tamoxifen appeared immature with blast-like morphologic features (Fig. 6, Table II). To quantitatively analyze the difference in granulocyte maturation in 32Dcl3/DNStat3/C/EBPα cells stimulated by G-CSF in the presence of tamoxifen, the mature granulocyte marker Gr-1 was monitored by FACS analysis. 32Dcl3 cells differentiate into Gr-1-positive neutrophils in response to G-CSF (Fig. 7A). As shown in Fig. 7D, Gr-1-positive cells were increased by the addition of tamoxifen in 32Dcl3/DNStat3/C/EBPα cells treated with G-CSF, although low levels were detected in the absence of tamoxifen.

C/EBPα Up-regulates Genes That Are Related to Granulocytic Differentiation—In a conditional expression system, induction of C/EBPα leads to expression of granulocyte-specific genes, such as neutrophil primary granule genes (lysozyme M, NGAL) and the G-CSF receptor gene (17). In 32Dcl3/DNStat3 cells, the expression of these genes following G-CSF stimulation was inhibited (Fig. 8, A, C, and E). Interestingly, only NGAL was up-regulated by G-CSF in 32Dcl3/DNStat3/C/EBPα cells following the restoration of C/EBPα (Fig. 8B). Conversely, the expression of lysozyme M and the G-CSF receptor were not changed by the restoration of C/EBPα (Fig. 8, D and F). These data suggest that regulatory factors in addition to C/EBPα may be involved in the induction of expression of granulocyte-specific genes by G-CSF.

**DISCUSSION**

G-CSF plays a pivotal role in granulopoiesis and granulocytic differentiation. The binding of G-CSF to its receptor leads to the activation of the Jak-Stat pathway, phosphatidylinositol-3 kinase pathway, and Ras-MAP kinase cascade (22). In the Jak-Stat pathway, G-CSF activates Jak1, Jak2, and Tyk2 followed by the activation of Stat1, Stat3, and Stat5 (7, 8).

Dominant-negative Stat3 inhibits G-CSF-induced transcriptional activity of Stat3 (Fig. 1A), as does G-CSF-induced granulocytic differentiation in vitro (11). Also, more transgenic mice with a targeted mutation of their G-CSF receptor that abolishes G-CSF-dependent Stat3 activation show severe neutropenia with an accumulation of immature myeloid precursors in their bone marrows (12). Consequently, Stat3 is thought to play an essential role in G-CSF-induced granulocytic differentiation.

32Dcl3 cells differentiate into neutrophils after treatment with G-CSF, and 32Dcl3/DNStat3 cells (32Dcl3 cells expressing dominant-negative Stat3) proliferate in G-CSF without differentiation. The degree of the phosphorylation of ERK1/2 by
G-CSF stimulation in 32Dcl3/DNStat3 cells was stronger than that in 32Dcl3 cells (Fig. 1B). We reported that Stat3 null bone marrow cells displayed a significant activation of ERK1/2 after G-CSF stimulation than wild-type bone marrow cells did using Stat3 conditional deficient mice (23). Then the augmented phosphorylation of ERK1/2 in response to G-CSF in 32Dcl3/DNStat3 cells might be caused by the functional abrogation of Stat3 in 32Dcl3/DNStat3 cells.

We compared gene profiles between two cell lines, 32Dcl3 and 32Dcl3/DNStat3 cells, to identify target genes of Stat3 in G-CSF signaling. We found that C/EBPα mRNA levels are rapidly up-regulated in 32Dcl3 cells following G-CSF treatment; these levels are increased 2.39-fold after 6 h and 4.20-fold after 48 h of treatment. In contrast to 32Dcl3 cells, C/EBPα mRNA levels are not changed in 32Dcl3/DNStat3 cells after G-CSF stimulation (Fig. 2A). The observation that cycloheximide does not inhibit G-CSF-induced increases in C/EBPα transcript levels (Fig. 2B) suggests that C/EBPα is induced by G-CSF directly downstream of Stat3. Dahlin et al. (24) also reported that G-CSF induced the expression of C/EBPα in IL-3-dependent progenitors. SOCS3 is one of the major target genes of Stat3. We previously reported that the expression level of SOCS3 protein in Stat3-deficient bone marrow cells is a trace, and it is not augmented by G-CSF stimulation (23). Contrary to this suppression of SOCS3 in Stat3-deficient cells, the induction of SOCS3 by G-CSF is not abolished in 32Dcl3/DNStat3 cells (data not shown).

The phosphorylation of ERK1/2 by G-CSF is stronger and the phosphorylation of Stat5 by IL-3 is weaker in 32Dcl3/DNStat3 cells when compared with those in 32Dcl3 cells, although Stat1 phosphorylation by IFN-γ was not changed between these two cells (Figs. 1B and 2A). Then there is the possibility that the transfection of dominant-negative Stat3 affects other signaling pathways in 32Dcl3/DNStat3 cells, resulting in the change of C/EBPα regulation. To clarify whether Stat3 directly up-regulates C/EBPα in the G-CSF signaling pathway in 32Dcl3 cells or not, we examined the effect of Stat3C on the transcription of C/EBPα. C/EBPα up-regulated the C/EBPα-dependent gene expression, and the G-CSF stimulation enhanced this C/EBPα-dependent gene expression (Fig. 4A). Strikingly, Stat3C augmented the C/EBPα-dependent gene expression as G-CSF stimulation did (Fig. 4, B and C). This means that G-CSF-induced up-regulation of C/EBPα-dependent gene expression is, at least partly, due to the activation of Stat3.

Two possibilities arise for the mechanism of the induction of C/EBPα transcription by activated Stat3 in the G-CSF signaling pathway. One is that activated Stat3 binds to the promoter region of C/EBPα and induces the transcription of C/EBPα. Analysis of the reported murine C/EBPα promoter sequence (20) identified no Stat-responsive elements (TTN5AA) (25, 26), but we found six Stat-responsive elements between 6 and 4 kb upstream of the C/EBPα transcription initiation site. Activated Stat3 might bind these Stat-responsive elements between 6 and 4 kb upstream of the C/EBPα transcription initiation site. The other possibility is that activated Stat3 might form the complex with C/EBPα and augment the transcriptional activity of C/EBPα because C/EBPα itself is the only protein reported to activate the murine C/EBPα promoter (20, 21). When a minimal TK promoter with CEBP-binding sites (pC/EBP2TK) together with C/EBPα was transfected to 293T cells, C/EBPα up-regulated C/EBPα-dependent gene expression. Activated Stat3 (Stat3C) enhanced this C/EBPα-dependent gene expression in collaboration with C/EBPα, although only Stat3C has no transcriptional activity on C/EBPα because C/EBPα itself is the only protein reported to activate the murine C/EBPα promoter (20, 21). In addition, the stimulation of G-CSF allows Stat3 to make the complex with C/EBPα (Fig. 4D). Then activated Stat3 by G-CSF makes the complex with C/EBPα and augments the transcriptional activity of C/EBPα. This is one of the reasons why induction of C/EBPα transcript through Stat3 activation by G-CSF occurred in 32Dcl3 cells. Several reports have described factors that repress C/EBPα promoter activity, such as SP1 (27), AP2α (28), or MYC (29). We show here for the first time that Stat3 augments the C/EBPα promoter activity.

Intracellular transcript levels of several genes were changed following G-CSF treatment downstream of Stat3 activation (Table 1). To better identify the role of C/EBPα in Stat3-mediated signaling in G-CSF-induced granulocyte differentiation, C/EBPα-ER (C/EBPα-tamoxifen receptor fusion protein) was stably expressed in 32Dcl3 and 32Dcl3/DNStat3 cells. C/EBPα-ER translocates from the cytoplasm to the nucleus and becomes activated upon treatment with tamoxifen. Strikingly, transfection of C/EBPα-ER into 32Dcl3/DNStat3 cells abolished proliferation and induced myeloid differentiation by G-CSF without Stat3 activation (Figs. 5, B and C). These data indicate that C/EBPα activation induced by G-CSF through Stat3 plays an essential role in stopping the cell proliferation and inducing the differentiation to the myeloid lineage.
The CEBP family of transcription factor is expressed in multiple cell types, including hepatocytes, adipocytes, keratinocytes, enterocytes, and cells of the lung (30, 31). C/EBPα transactivates the promoters of hepatocyte- and adipocyte-specific genes, which are important for energy homeostasis (32, 33), and C/EBPα-deficient mice lack hepatic glycogen stores and die from hypoglycemia within 8 h of birth (34). In the hematopoietic system, C/EBPα is exclusively expressed in myelomonocytic cells (35, 36). C/EBPα expression is prominent in mature myeloid cells, and previous investigations found that C/EBPα is critical for early granulocytic differentiation. Mice with a targeted disruption of the C/EBPα gene demonstrate an early block in granulocytic differentiation, but they develop normal monocytes (19). Conditional expression of C/EBPα is sufficient to induce granulocytic differentiation (17). In contrast to the essential role of C/EBPα in granulocytic differentiation, the role of Stat in granulopoiesis is controversial. Stat3 is the principle Stat protein activated by G-CSF, with Stat5 and Stat1 also activated to a lesser degree (8, 10). In mice lacking Stat5α and Stat5β, the number of colonies produced in response to G-CSF was reduced 2-fold despite normal circulating numbers of neutrophils (9). Myeloid cell lines expressing dominant-negative forms of Stat3 (11, 37, 38) and transgenic mice with a targeted mutation of the G-CSF receptor that abolishes G-CSF-dependent Stat3 activation (12) demonstrate that Stat3-activation is required for G-CSF-dependent granulocytic proliferation and differentiation.

In the present study, we clearly demonstrate that the expression of C/EBPα mRNA is up-regulated through the activation of Stat3 in response to G-CSF, and the Stat3-C/EBPα signaling cascade plays an important role in G-CSF-induced differentiation. Contrary to these data, however, we and others showed that mice conditionally lacking Stat3 in their hematopoietic progenitors developed neutrophilia, and bone marrow cells were hyper-responsive to G-CSF stimulation (23, 39). Additionally, mice with tissue-specific disruption of Stat3 in bone marrow cells die within 4–6 weeks after birth with Crohn’s disease-like pathogenesis (40). These mice exhibit phenotypes with dramatic expansion of myeloid cells, leading to massive infiltration of the intestine with neutrophils, macrophages, and eosinophils. Cells of the myeloid lineage also demonstrate autonomous proliferation. These apparently disparate results may be explained by the need for molecules in addition to Stat3 to regulate C/EBPα expression in vivo, the in vivo functional redundancy among C/EBPα regulators, or the absence of the abrogation of SOCS3 induction by G-CSF in 32Dcl3/DNStat3 cells. In 32Dc13 cells, the Stat3-C/EBPα pathway might be favored, and other pathways may contribute little to granulocytic differentiation in response to G-CSF.

Among C/EBP family, C/EBPε is important for late phase of granulocytic differentiation, and its expression is up-regulated by G-CSF independent of Stat3 (11). A previous report showed that C/EBPε is a transcriptional target of C/EBPα in 32Dcl3 cells (41). From these reports and our results, we speculated that a small amount of C/EBPα is enough for the induction of the transcription of C/EBPε by G-CSF or that there are multiple signaling steps except for Stat3-C/EBPα to induce the transcription of C/EBPε by G-CSF.
Induction of C/EBPα led to not only morphologic differentiation but also expression of granulocyte-specific genes (17). In 32Dcl3/DNStat3 cells, the induction of the G-CSF receptor, lysozyme M, and NGAL in response to G-CSF was abrogated (Fig. 8). Restoration of C/EBPα in these cells led to expression of only the NGAL gene, and thus, 32Dcl3/DNStat3 cells differentiated by the induction of C/EBPα may not be functional as mature neutrophils. In these cells, therefore, activation of C/EBPα is not sufficient for the induction of lysozyme M or G-CSF receptor genes, and the presence of other molecules appears to be required for their expression.

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