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A Novel In-Frame Deletion in the Leucine Zipper Domain of C/EBP ϵ Leads to Neutrophil-Specific Granule Deficiency

Taizo Wada,^{*,1} Tadayuki Akagi,^{†,1} Masahiro Muraoka,^{*} Tomoko Toma,^{*} Kenzo Kaji,[‡] Kazunaga Agematsu,[§] H. Phillip Koeffler,^{¶,||} Takashi Yokota,[†] and Akihiro Yachie^{*}

Neutrophil-specific granule deficiency (SGD) is a rare autosomal recessive primary immunodeficiency characterized by neutrophil dysfunction, bilobed neutrophil nuclei and lack of neutrophil-specific granules. Defects in a myeloid-specific transcription factor, CCAAT/enhancer binding protein- ϵ (C/EBP ϵ), have been identified in two cases in which homozygous frameshift mutations led to loss of the leucine zipper domain. In this study, we report a 55-y-old woman affected with SGD caused by a novel homozygous 2-aa deletion (Δ RS) in the leucine zipper domain of the C/EBP ϵ gene. The patient showed characteristic neutrophil abnormalities and recurrent skin infections; however, there was no history of deep organ infections. Biochemical analysis revealed that, in contrast to the two frameshift mutations, the Δ RS mutant maintained normal cellular localization, DNA-binding activity, and dimerization, and all three mutants exhibited marked reduction in transcriptional activity. The Δ RS mutant was defective in its association with Gata1 and PU.1, as well as aberrant cooperative transcriptional activation of eosinophil major basic protein. Thus, the Δ RS likely impairs protein-protein interaction with other transcription factors, resulting in a loss of transcriptional activation. These results further support the importance of the leucine zipper domain of C/EBP ϵ for its essential function, and indicate that multiple molecular mechanisms lead to SGD. *The Journal of Immunology*, 2015, 195: 000–000.

Neutrophil-specific granule deficiency (SGD) is a rare autosomal recessive primary immunodeficiency characterized by either profound reduction or absence of neutrophil-specific granules and bilobed neutrophil nuclei (pseudo-Pelger-Huët anomaly) (1). It was previously called “lactoferrin deficiency.” Patients with SGD exhibit increased susceptibility to bacterial infections, especially affecting the skin, ears, lungs, and lymph nodes. The gene responsible for SGD is the CCAAT/enhancer binding protein- ϵ (C/EBP ϵ) gene. To date, two patients have been reported who carry C/EBP ϵ frameshift mutations that result in abrogated protein expression (2, 3). In addition, another patient with SGD had a heterozygous missense mutation of the C/EBP ϵ gene, but this mutation was unlikely to have caused disease because it resulted

in elevated levels of C/EBP ϵ (4). In this patient, the growth factor independence 1 (Gfi-1) protein that represses transcription of C/EBP ϵ was decreased, although the patient had no mutation of the Gfi-1 gene. Taken together, these previous findings suggest that SGD is a genetically heterogeneous disease. Because of the extreme rarity of SGD, the full spectrum of clinical symptoms and cellular abnormalities of the disease is unclear.

C/EBP ϵ is a member of the C/EBP family of widely expressed transcription factors that regulate proliferation, differentiation, and apoptosis in a variety of cell types (5, 6). The C/EBP family consists of six members (C/EBP α , β , γ , δ , ϵ , and ζ), and cellular expression of each C/EBP is tightly regulated. They bind to DNA through the highly conserved basic leucine zipper (bZIP) domain. C/EBP ϵ is restricted to granulocytes and is essential for their terminal differentiation (6, 7). C/EBP ϵ transcription primarily occurs at the myelocyte–metamyelocyte stage of differentiation and decreases in polymorphonuclear neutrophils. The human C/EBP ϵ gene produces four isoforms of 32, 30, 27, and 14 kDa that are functionally different; only the 32-kDa C/EBP ϵ has full transactivating potential (8–10). C/EBP ϵ is indispensable for expression of genes encoding proteins that reside in specific granules of neutrophils, such as lactoferrin and defensins. Many features of SGD are manifested by C/EBP ϵ -deficient mice in which neutrophils are morphologically and functionally abnormal and eosinophil numbers are decreased (8).

In this study, we describe another case of SGD with a novel 2-aa deletion in the bZIP domain of C/EBP ϵ , and we report the mechanism that leads to SGD. We also completed clinical, cellular, and molecular comparisons for this patient and the previously reported two cases of SGD (2, 3), and we discuss the functional significance of the mutation.

Materials and Methods

Patients

We studied two Japanese patients with SGD. Patient P1 is a 55-y-old woman who has suffered since late infancy from recurrent skin infections that often required more than 2 mo to heal. After hospitalization because of severe

*Department of Pediatrics, Institute of Medical, Pharmaceutical and Health Sciences, School of Medicine, Kanazawa University, Kanazawa 920-8641, Japan;

†Department of Stem Cell Biology, Institute of Medical, Pharmaceutical and Health Sciences, School of Medicine, Kanazawa University, Kanazawa 920-8640, Japan;

‡Department of Dermatology, Komatsu Municipal Hospital, Komatsu 923-0961, Japan; §Department of Infection and Host Defense, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan; ¶Division of Hematology and Oncology, Cedars-Sinai Medical Center, University of California Los Angeles School of Medicine, Los Angeles, CA 90048; and ||Cancer Science Institute of Singapore, National University of Singapore, Singapore 117599, Singapore

1T.W. and T.A. contributed equally to this work.

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Address correspondence to Dr Taizo Wada, Department of Pediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-8641, Japan. E-mail address: taizo@staff.kanazawa-u.ac.jp

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Abbreviations used in this article: bZIP, basic leucine zipper; C/EBP ϵ , CCAAT/enhancer binding protein- ϵ ; Gata1, GATA binding protein 1; MaBP, major basic protein; MBP, maltose-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; Prg2, proteoglycan 2; SGD, neutrophil-specific granule deficiency; WT, wild-type.

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otitis media at 54 y of age, she was referred to our hospital for suspected immunodeficiency. A history of parental consanguinity appeared likely. However, her father had already died of a heart attack, and her mother refused genetic analysis. Her elder brother had similar skin symptoms and died of enterocolitis at 10 y of age. Another brother also died early after birth from unspecified causes. Two children of patient P1 and her granddaughter were healthy, and they did not want genetic testing. Clinical and genetic data of patient P2 have already been published (3, 11). Patient P2 is now 40 y old. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

Flow cytometry and immunohistochemistry

For flow cytometric analysis, the following mAbs were used: FITC-conjugated anti-CD16, anti-CD32 or CD66b and PE-conjugated anti-CD11b, anti-CD14, anti-CD15 anti-CD16 or anti-CD64 (BD, San Diego, CA); and FITC-conjugated anti-CD16b and PE-conjugated CD11c (Beckman Coulter, Fullerton, CA). Analysis was performed on a FACSCalibur using CellQuest software (BD Biosciences, Tokyo, Japan) (12). Cytospin preparations were made using whole leukocytes. For lactoferrin staining, cytospin samples were stained with anti-lactoferrin mAb (Beckman Coulter), followed by universal immuno-alkaline-phosphatase polymer (Simple Stain AP; Nichirei Biosciences, Tokyo, Japan). The alkaline phosphatase activity was visualized using Fast Red TR salt and Naphtol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO). For analysis of internal alkaline phosphatase and peroxidase activity, alkaline-phosphatase substrate solution (BCIP/NBT Substrate System; DAKO, Glostrup, Denmark) and a peroxidase staining kit (Muto Pure Chemicals, Tokyo, Japan) were used, respectively.

Mutation analysis

Peripheral blood or buccal mucosa samples, or both, were obtained from patients, and DNA was extracted from the samples using a standard method. Direct sequencing of the C/EBP ϵ gene was performed as described previously (2).

Cell culture and RT-PCR

Human embryonic kidney (HEK) 293 and mouse NIH3T3 cells were cultured in DMEM containing either 10% FBS or 10% calf serum, respectively. Total RNA was extracted from transfected NIH3T3 cells with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and converted to cDNAs by ReverTraAce (Toyobo, Tokyo, Japan). Expression of human C/EBP ϵ and PU.1, and murine cathelicidin B9/neutrophil granule protein, neutrophil gelatinase-associated lipocalin (NGAL)/ lipocalin 2, lactoferrin, proteoglycan 2 (Prg2)/eosinophil granule eosinophil major basic protein (MaBP), GATA binding protein 1 (Gata1), and GAPDH was examined by RT-PCR. Primer sequences are reported in Supplemental Table I. The number of amplification cycles was 20 for GAPDH; 25 for C/EBP ϵ , PU.1, MaBP, and Gata1; 30 for B9 and NGAL; and 35 for lactoferrin. The PCR cycle consisted of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. For lactoferrin, the annealing temperature was 64°C. All PCR reactions were completed with Taq polymerase (Ampliqon, Odense, Denmark) except for C/EBP ϵ (PrimeSTAR Max DNA Polymerase; Takara Bio, Otsu, Japan).

Construction of plasmid, luciferase assay, and cellular localization

Construction of mammalian expression vectors, including pCMV5-Flag maltose-binding protein (MBP), pCAGIP-enhanced GFP, and pCAGIP-Myc, was described previously (13, 14). Coding region of wild-type (WT) human C/EBP ϵ and its mutants, Δ RS, del5bp, and insA, were amplified by PCR using specific primers (Supplemental Table I) and cloned into expression vectors. Murine Gata1 and human PU.1 expression vectors were described previously (15), and their coding regions were cloned into the pCAGIP-Myc vector. The luciferase reporter plasmid containing the G-CSF receptor promoter (pGCSFR-Luc) has been described previously (16). The reporter plasmid, C/EBP ϵ and C/EBP ϵ mutant expression vectors (Δ RS, del5bp, and insA), were transfected into HEK293 cells by Lipofectamine 2000 (Life Technologies, Grand Island, NY). To determine luciferase activity, cell extracts were prepared 48 h after transfection, and luciferase activity was measured with a luciferase assay kit (Promega, Madison, WI) using an AB-2200 luminometer (ATTO, Tokyo, Japan). Protein concentration of each sample was measured by Protein Assay CBB solution (Nacalai Tesque). Amounts of cell extracts used to obtain relative luciferase activities were normalized to 1 μ g total protein per sample. To observe cellular localization of C/EBP ϵ and its mutants, GFP-tagged fusion protein expression vectors were introduced into NIH3T3 cells. Nuclei were stained by Hoechst 33258 (Sigma). Signals were observed 48 h after transfection.

Biotin-labeled DNA pull-down assay, MBP pull-down assay, and Western blot analysis

Biotin-labeled DNA pull-down assay was performed as described previously (13, 17). Briefly, biotin-labeled oligonucleotides containing the human lactoferrin gene C/EBP ϵ binding site (5'-GGGTGTCTATTGGGCAACA-GGGCGGG-3') were incubated with cell extracts from HEK293 cells transfected with either pCAGIP-Myc-C/EBP ϵ or its mutant counterparts (Δ RS, del5bp, and insA) in the presence of streptavidin-agarose (Novagen, Darmstadt, Germany). Twenty-five-fold nonlabeled oligonucleotides (either WT or mutant nonbinding control) were added for the competition assays. The beads were washed three times with a washing buffer, and the bound proteins were eluted by boiling in 2X SDS sample buffer. Samples were then examined by Western blot analysis as described below. MBP pull-down assay was performed as described previously (13, 17). HEK293 cells were cotransfected with pCAGIP-Myc-C/EBP ϵ (WT or Δ RS) and pCMV5-Flag-MBP-C/EBP ϵ (WT or Δ RS). HEK293 cells were also cotransfected with pCAGIP-Myc-Gata1 and pCMV5-Flag-MBP-C/EBP ϵ or its mutant counterparts, or with pCAGIP-Myc-PU.1 and pCMV5-Flag-MBP-C/EBP ϵ or its mutant counterparts. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis. For Western blot analysis, samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with either anti-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Flag (F3165; Sigma-Aldrich) Abs followed by HRP-conjugated anti-mouse Ab (Millipore, Billerica, MA). The blot was visualized using ECL reagents (PerkinElmer, Waltham, MA) with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

Results

Bilobed nucleus and lack of granules in neutrophils

Patient P1 exhibited an ulcerative skin abscess and numerous skin scars (Fig. 1A, 1B). Her peripheral blood smear showed unique bilobed nuclei and a lack of cytoplasmic granules in her neutrophils (Fig. 1C). Absence of eosinophils, increased basophils with normal cytoplasmic granularity, and monocytosis were also noted on smears of the peripheral blood (data not shown). The immunohistochemical analysis of her neutrophils clearly demonstrated the presence of peroxidase, a primary granule protein, and the absence

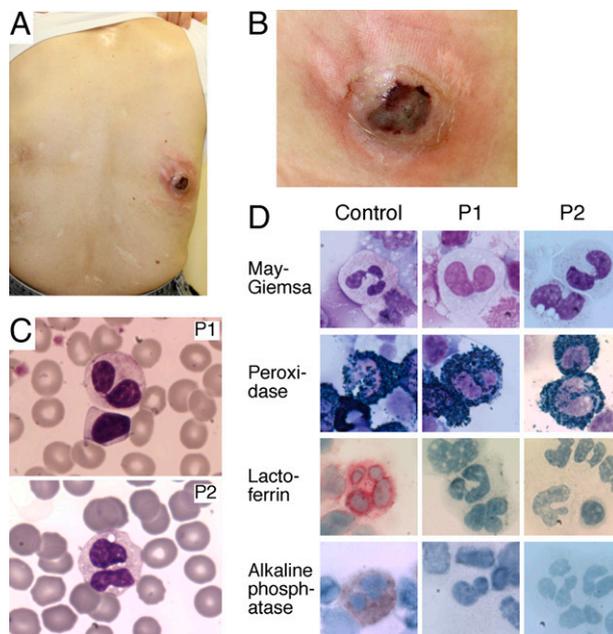


FIGURE 1. Patient characteristics. (A and B) Skin abscess with ulceration and scar. (C) Peripheral blood smears. Neutrophils from patients P1 and P2 have bilobed nuclei and lack cytoplasmic granules (May-Grünwald-Giemsa staining). Original magnification $\times 400$. (D) Immunohistochemical analysis. Cytospin preparations of leukocytes stained with anti-lactoferrin mAb. Cellular alkaline phosphatase and peroxidase activity were also analyzed. Original magnification $\times 400$.

of lactoferrin and alkaline phosphatase, both of which are normally expressed in specific granules of normal neutrophils (Fig. 1D). These characteristic features were similar to those of patient P2, the second case of genetically defined SGD, who carries a homozygous C/EBPε mutation, c.508_509insA (insA) (3).

Flow cytometric analysis of peripheral blood showed lower side scatter of P1 neutrophils compared with normal controls (Fig. 2) (18). More importantly, the majority of the patient's neutrophils that were defined on the basis of forward and side scatter expressed a monocyte marker CD14, indicating aberrant development toward the monocyte pathway. These cells did not express neutrophil markers such as CD15, CD16b (FcγRIIB), and CD66b. Although CD64 (FcγRI) was not detected on her neutrophils and monocytes, CD11b, CD11c, and CD32 (FcγRII) were detectable equally on both cells (data not shown). Again, all these characteristic features were similar to those of patient P2. Neutrophils from patient P2 showed more CD16 expression compared with P1.

2-aa deletion in bZIP and reduced transcriptional activity

Direct sequence analysis revealed that patient P1 had a homozygous 6-bp deletion in exon 2 of the C/EBPε gene (c.739_744delCGCAGC). This novel mutation leads to a 2-aa deletion, p.Arg247_Ser248del (ΔRS), which is located in the bZIP domain (Fig. 3). The mutation was present in DNA isolated from both the peripheral blood and buccal mucosa, indicating a germline mutation, not a somatic one. Analysis of 100 alleles of ethnically matched healthy controls demonstrated the absence of the mutation in the general population.

To evaluate the transcriptional activation by the ΔRS mutant, HEK293 cells were cotransfected with a G-CSF receptor promoter reporter construct and C/EBPε expression vectors. As shown in

Fig. 4A, WT C/EBPε presented robust reporter activity, whereas the ΔRS mutant exhibited a significant decrease in activity, similar to the previously reported mutants, insA and del5bp (c.249_253delTGACC) (2, 3), both of which were frameshift mutations with truncated proteins. HEK 293 cells transfected with the ΔRS mutant expressed levels of C/EBPε comparable to wild-type, as assessed with Western blot analysis, indicating ΔRS does not cause instability in the mutated C/EBPε protein (Fig. 4B). Increasing amounts of ΔRS had no negative effect on reporter activity of WT C/EBPε, indicating a lack of a dominant-negative effect of ΔRS on transcriptional activity (Fig. 4C). We also analyzed the ability of the C/EBPε mutant proteins to activate gene expression of secondary granule genes. As shown in Fig. 4D, WT C/EBPε was able to induce expression of endogenous B9, NGAL, and lactoferrin genes in transiently transfected NIH3T3 cells. In contrast, none of these genes was amplified from cells transfected with either the ΔRS mutant or the insA and del5bp mutants, consistent with loss of their capability to activate secondary granule genes.

Unaltered cellular localization, DNA-binding, and dimerization

To understand the mechanism by which the ΔRS mutation decreases transcriptional activity, we first investigated the cellular localization of WT and mutant C/EBPε proteins. GFP fluorescence was diffusely detected within the cytoplasm of NIH3T3 cells transfected with the control GFP vector, whereas WT and ΔRS mutant C/EBPε tagged with GFP was found in the nucleus (Fig. 5). Consistent with

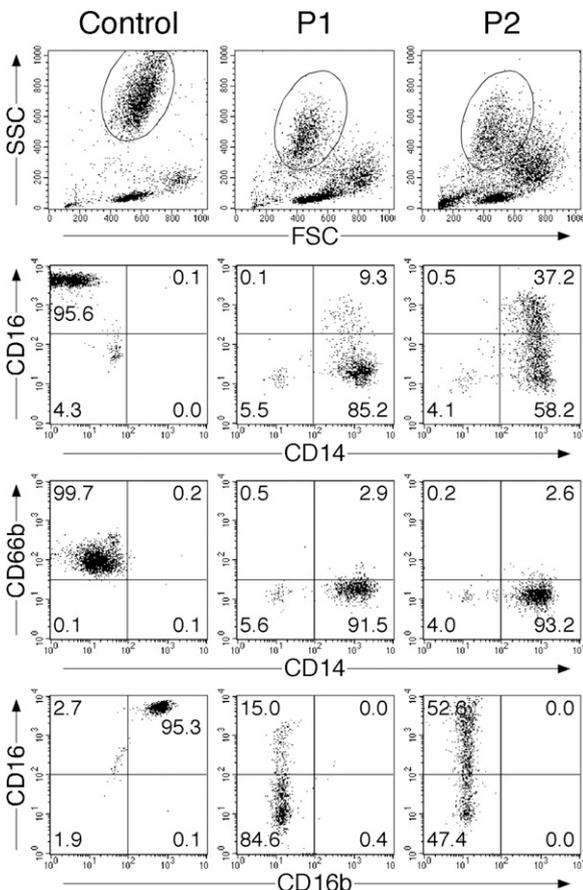


FIGURE 2. Characterization of granulocytes. Granulocytes were gated for fluorescence analysis. The percentage of cells gated in each region is shown.

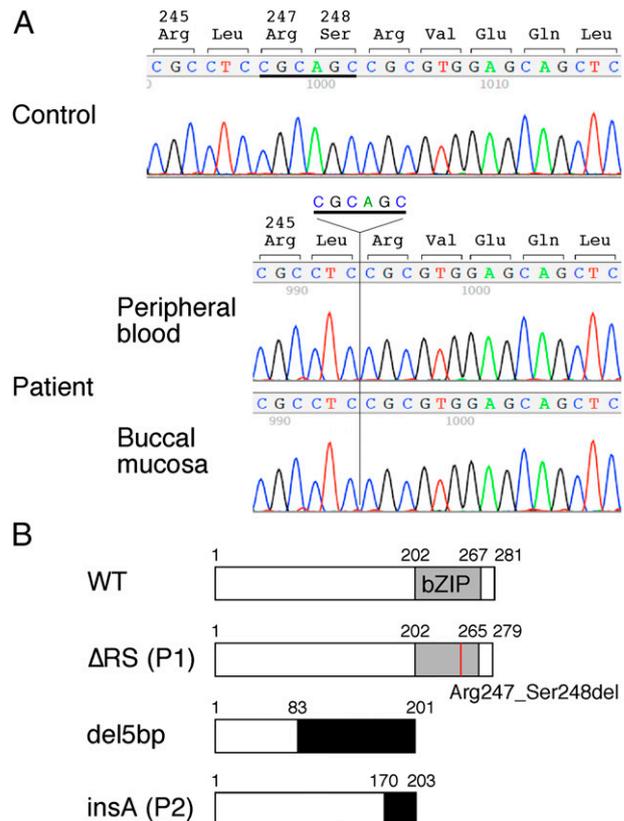
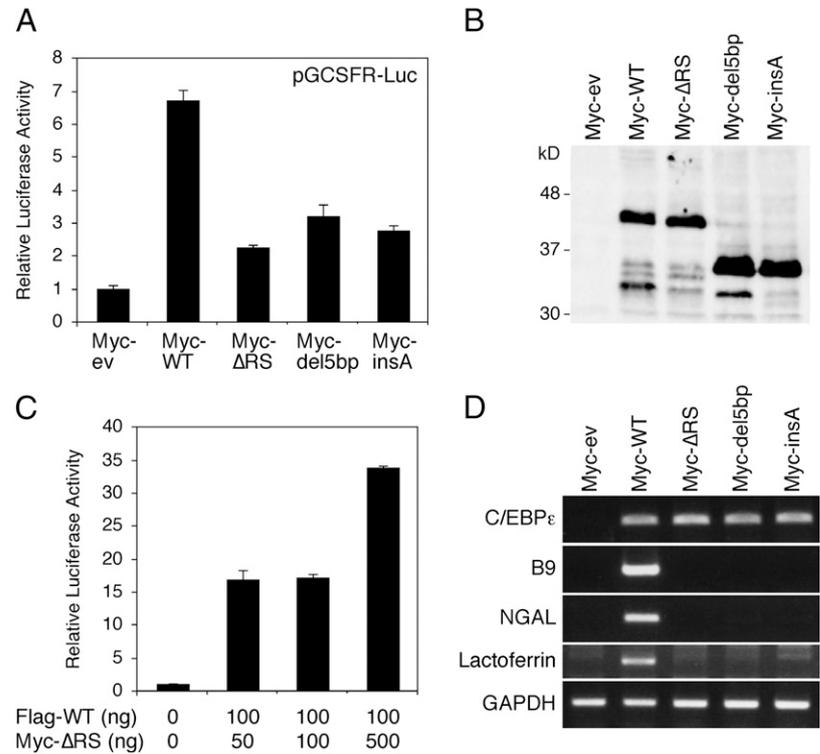


FIGURE 3. Mutation analysis of C/EBPε gene. (A) C/EBPε gene exon 2 was amplified from DNA extracted from normal control, as well as peripheral blood and buccal mucosa of patient P1. Direct sequencing was performed using an automated sequencer. A thick bar highlights the position of the 6-bp deletion (ΔRS). (B) Predicted structures of mutated C/EBPε molecules. The previously reported frameshift mutations, del5bp and insA, produced frameshifts that result in incorrect amino acid sequence subsequent to the mutations (shaded area) and premature termination.

FIGURE 4. Transcriptional activity and induction of endogenous expression of granule genes. **(A)** Luciferase reporter plasmid containing the G-CSF receptor promoter (pGCSFR-Luc) was transfected into HEK293 cells with either control empty (ev), WT, Δ RS, del5bp, or insA C/EBP ϵ expression vector. Luciferase activity was measured 48 h after transfection. Bars represent the means and SDs of triplicate assay. **(B)** Western blot analysis of C/EBP ϵ was performed using lysates of HEK293 cells transfected with ev, WT, Δ RS, del5bp or insA C/EBP ϵ vector. **(C)** WT C/EBP ϵ vector was mixed with increasing amounts of Δ RS C/EBP ϵ vector, and luciferase reporter assay was performed. **(D)** Expression vectors of ev, WT, Δ RS, del5bp, or insA C/EBP ϵ were transfected into NIH3T3 cells, and expression of endogenous B9, NGAL, and lactoferrin was examined by RT-PCR. GAPDH was used as a loading control. The numbers of PCR cycles were 20 for GAPDH; 30 for C/EBP ϵ , B9, and NGAL; and 35 for lactoferrin.



the previous report (3), the insA mutant was localized in the cytoplasm and the nucleus; and a similar abnormal localization was detected in the del5bp.

We next assessed the ability of the WT and mutant C/EBP ϵ to bind to C/EBP ϵ binding site at the 5'UTR of human lactoferrin gene (19). The oligonucleotides containing the C/EBP ϵ binding site bound *in vitro* to C/EBP ϵ from lysates of HEK293 cells transfected with WT Myc-C/EBP ϵ vector (Fig. 6A, *top left blot*). Importantly, the Δ RS mutant showed DNA-binding ability comparable to the WT C/EBP ϵ (Fig. 6A, *top right blot*). Nonlabeled oligonucleotides of the same sequence, but not those with a mutated sequence, were able to compete with the biotinylated oligonucleotides for sequence-specific binding of both the WT and Δ RS mutant C/EBP ϵ . In contrast, no product was precipitated from lysates of cells transfected with the del5bp and the insA mutants, indicating that no oligonucleotide binding took place (Fig. 6A, *bottom panels*).

To assess further the functionality of the Δ RS mutant, we examined dimer formation in lysates of HEK293 cells cotransfected with Myc-C/EBP ϵ and Flag-MBP-C/EBP ϵ vectors. The Δ RS mutant was able to homodimerize or heterodimerize with WT C/EBP ϵ at levels comparable to WT C/EBP ϵ (Fig. 6B and data not shown). These results were consistent with the ability of the Δ RS mutant to bind to DNA.

Aberrant association with Gata1 and PU.1

To determine whether the Δ RS mutant properly interacts with other proteins, cooperative transcriptional activation of MaBP was analyzed in NIH3T3 cells. Consistent with the previous report (15), MaBP gene expression was observed in cells transfected with Gata1 and PU.1 in addition to WT C/EBP ϵ , and no products were obtained from those without WT C/EBP ϵ (Fig. 7A). Interestingly, the Δ RS mutant, as well as the del5bp and the insA mutants, failed to induce MaBP gene expression.

To assess further the ability of the C/EBP ϵ mutants to bind to Gata1 as well as PU.1, the MBP pull-down assay was performed. As expected, both Gata1 and PU.1 were able to bind *in vitro* to

C/EBP ϵ in lysates of HEK293 cells transfected with the WT Flag-MBP-C/EBP ϵ vector, and no binding was observed in lysates of cells transfected with the del5bp and the insA mutants (Fig. 7B, 7C). Some binding of the Δ RS mutant to Gata1 or PU.1 was indicated by the results, but the amounts of precipitated Gata1 and PU.1 were extremely low in lysates from HEK293 cells transfected with the Δ RS mutant.

Discussion

C/EBP ϵ is essential for terminal differentiation of granulocytes. Frameshift mutations of the C/EBP ϵ gene have been identified in two patients with SGD. In this study, we report on a 55-y-old woman (P1) affected with SGD caused by a novel 2-aa deletion mutation of the C/EBP ϵ gene. This case represents a third case of genetically defined SGD. The availability of blood samples from the previous case (P2) offered us the unique opportunity to evaluate and compare phenotype of peripheral neutrophils in these patients. In addition to the morphologic abnormalities typical for SGD, we found characteristic surface phenotype in their neutrophils,

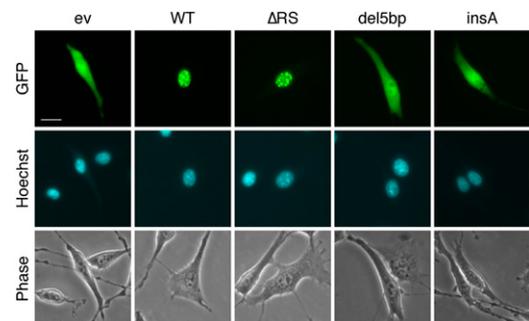


FIGURE 5. Cellular localization. GFP-tagged to either control empty (ev), WT (WT), Δ RS, del5bp or insA C/EBP ϵ expression vectors were transfected into NIH3T3 cells, and cells were analyzed by microscopy and fluorescent microscopy 2 d after transfection. Nuclei were stained with Hoechst stain. Scale bar, 20 μ m.

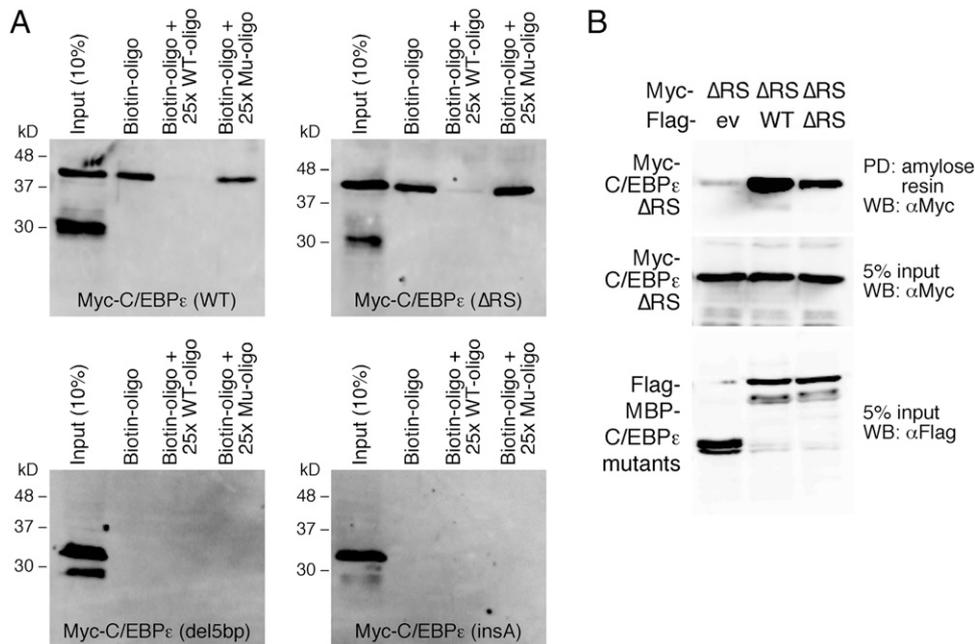
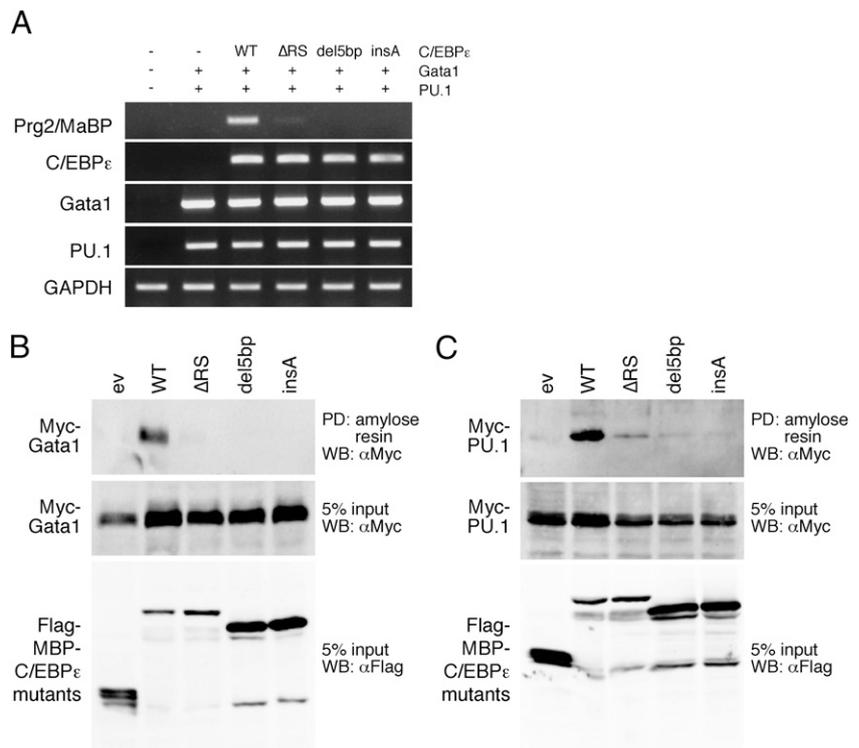


FIGURE 6. DNA binding activity and dimerization. **(A)** DNA binding activity of WT, Δ RS, del5bp, and insA C/EBP ϵ . Biotin-labeled oligonucleotide containing the C/EBP ϵ -binding site of the lactoferrin gene was incubated with Myc-C/EBP ϵ (WT, Δ RS, del5bp, or insA)-transfected HEK293 cell extracts either with or without 25-fold nonlabeled WT or mutated nonbinding (μ) oligonucleotide. Biotin-labeled oligonucleotides were pulled down by streptavidin-agarose. The precipitates and cell lysates were analyzed by Western blot analysis with anti-Myc Ab. **(B)** Dimer formation of Δ RS. HEK293 cells were transfected with Myc-C/EBP ϵ - Δ RS together with either empty control vector (MBP-ev), Flag-MBP-C/EBP ϵ -WT or - Δ RS. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc Ab. Expression of each protein was confirmed with anti-Myc and anti-Flag Abs, respectively.

including the presence of monocyte markers such as CD14 and the absence of neutrophil markers such as CD15, CD16b, and CD66b. It is therefore difficult to distinguish neutrophils from monocytes by surface markers in the patients. CD16 includes two isoforms, CD16a (Fc γ RIIA) and CD16b. CD16a is a transmembrane receptor expressed by monocytes, natural killer cells and natural killer T cells,

whereas CD16b is a GPI-anchored receptor that is thought to be expressed exclusively by neutrophils. Because pan-CD16 but not CD16b was detected in a subset of the patient's neutrophils, they likely expressed CD16a. Neutrophils from patient P2 showed higher levels of CD16 expression than those of patient P1 did, indicating a larger subpopulation of CD16a⁺ neutrophils. Human monocytes

FIGURE 7. Cooperative transcriptional activation of eosinophil major basic protein (MaBP). **(A)** Induction of Prg2/MaBP expression in NIH3T3 cell. Expression vectors Gata1 and PU.1 were transfected into NIH3T3 cells either with or without C/EBP ϵ expression vectors (WT, Δ RS, del5bp, or insA). Expression of endogenous Prg2/MaBP mRNA was examined by RT-PCR. GAPDH was used as a loading control. The numbers of PCR cycles were 20 for GAPDH, and 25 for C/EBP ϵ , Gata1, PU.1 and MaBP. **(B and C)** Protein interactions between C/EBP ϵ WT or mutants with either Gata1 or PU.1. HEK293 cells were transfected with either Myc-Gata1 or Myc-PU.1 together with an empty control vector (MBP-ev), Flag-MBP-C/EBP ϵ -WT, - Δ RS, -del5bp, or -insA. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc Ab. Expression of each protein was confirmed with anti-Myc and anti-Flag Abs, respectively.



are divided into two major subsets: CD14⁺⁺CD16a⁻ and CD14⁺CD16a⁺ cells. Various inflammatory conditions including infections lead to an increased subpopulation of CD16a⁺ monocytes (20, 21). CD16 expression of neutrophils of patient P1 was associated with infections (data not shown); therefore, CD16a expression on SGD neutrophils may depend on inflammatory immune stimuli.

The reason why the patient's neutrophils expressed monocyte markers, including CD14 and CD16a, is presently unclear. Studies of granulocytes from healthy volunteers who were given G-CSF and from human embryonic stem cells treated with multiple growth factors have demonstrated aberrant expression of CD14 on mature granulocytes (22, 23). Although we did not measure any soluble factors related to granulocytic differentiation in our patients, defective myeloid differentiation in SGD could lead to dysregulated secretion of growth factors resulting in aberrant surface expression of neutrophil proteins. In vitro modeling of neutrophil development in SGD using induced pluripotent stem cells will be required to address these issues.

We carried out a comprehensive in vitro study to evaluate transcriptional activity, cellular localization, DNA-binding activity, dimerization and protein-interaction of the Δ RS mutant as well as the two frameshift mutants. All three mutants exhibited marked reduction in transcriptional activity. The Δ RS mutation is located in the bZIP domain, which is highly conserved among the C/EBP family members and has an important role in DNA binding and dimerization (5). However, the Δ RS mutant maintained normal cellular localization, DNA-binding activity, and dimerization, in contrast to the frameshift mutations del5bp and insA, which destroy the bZIP domain and thus are predicted to interfere with dimerization and binding to DNA. No dominant-negative effect of the Δ RS mutant may suggest that the single normal C/EBP ϵ allele is sufficient to maintain transcriptional activity, which is consistent with the fact that the mother and two children of patient P1, who are assumed to be heterozygous for the Δ RS mutation, remain in good health. The association of C/EBP ϵ with other transcription factors has been demonstrated to be important for the regulation of secondary granule gene expression in both neutrophils and eosinophils (15). In fact, the Δ RS mutant was found to be defective in association with Gata1 and PU.1, as well as aberrant cooperative transcriptional activation of eosinophil MaBP. Gata1 is primarily associated with erythroid and megakaryocyte differentiation, whereas PU.1 is more important for neutrophil differentiation. These results are in line with the fact that eosinophils were not detectable in patients P1 and P2. Taken together, our findings suggest that the Δ RS mutation impairs protein-protein interaction with Gata1 and with PU.1, resulting in loss of cooperative transcriptional activation.

A similar mutation has been described in a patient with acute myeloid leukemia, in which an in-frame 3-bp deletion within the leucine zipper domain of C/EBP α abrogated the transcriptional activation function of C/EBP α on the G-CSF receptor promoter (24). Like the Δ RS mutant, this mutant lacked a dominant-negative effect, although its protein-protein interaction with other transcription factors remained unexamined. Further investigation will be necessary to assess whether the Δ RS mutant also exhibits defective interaction with other transcription factors such as c-Myb, PML, p300, E2F1, and Rb (9, 16, 25, 26). Because certain isoforms of C/EBP ϵ have been reported to inhibit the synergistic activities of GATA1 and PU.1 (25), we also need to evaluate the isoforms of the C/EBP ϵ mutants other than the full-length, 32-kDa C/EBP ϵ .

These characteristics of the Δ RS mutant, wherein modest association with Gata1 and PU.1 is retained and nuclear localization remains intact, may be associated with less severe clinical symptoms of patient P1. To date, patient P1 has shown no deep organ

infection, whereas the other patients exhibited more severe presentation of the disease. The first reported patient with the del5bp mutation died of complications of pneumonia, and patient P2 suffered from recurrent pneumonia, as well as lung abscess (2, 3). On the other hand, recurrent bacterial skin abscess that persisted a few months was observed in all patients with SGD, including patient P1 (27). Skin abscess smears from patient P1 showed that most infiltrating cells were monocytes and macrophages, some of which phagocytosed bacteria (data not shown). Monocytes from C/EBP ϵ -deficient mice exhibited impaired maturation and altered cytokine expression, such as increased levels of TNF- α and LT β , in response to inflammation (28, 29). In addition, monocyte counts in C/EBP ϵ -deficient mice were higher than those of WT mice (30). Thus, impaired inflammatory response and killing of bacteria by SGD patients' monocytes can hinder the healing process, resulting in unique skin abscesses. Understanding which factors evoke an abnormal microenvironment at infectious sites will be necessary to develop more effective therapeutic approaches for patients with SGD.

In summary, our studies identified a novel in-frame deletional mutation in the bZIP domain of C/EBP ϵ and demonstrated its molecular pathogenesis leading to SGD. Comparative analysis of the C/EBP ϵ mutations, including the previous frameshift mutations, also clarifies the functional significance of these mutants. Characterization of C/EBP ϵ genetic defects and functional abnormalities will help to define the role of C/EBP ϵ in human myelopoiesis and innate immunity.

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Disclosures

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Table S1. Primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
For RT-PCR		
C/EBP ϵ	ATCCCCTGCAGTACCAAGTG	AATGCGCCTCTTGGCCTTG
B9/NGP	GAAGACCCCTCTTCCGCCTG	GGTATCCTCTCGACTGCAATC
NGAL	ATGTCACCTCCATCCTGGTC	TCCTTCAGTTCAGGGGACAG
Lactoferrin	CGAAGCACGAATGACAAAGA	ATCACACTTGCGCTTCTCCT
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGAGGCCATGTAGGCCAT
MBP/PRG2	CCCTCTACTTCTGGCTCTTC	GTCAAAGCATTGAGCCCTCC
Gata1	TTGTGAGGCCAGAGAGTGTG	TTCCTCGTCTGGATTCCATC
PU.1	AGATGCACGTCCTCGATACC	CAGGTCCAACAGGAACTGGT
For cloning		
C/EBP ϵ WT	GAATTCATGTCCCACGGGACCTAC	GCGGCCGCTCAGCTGCAACCCCCAC
C/EBP ϵ Δ RS	GAGCGCCTCCGCGTGGAG	CTCCACGCGGAGGCGCTC
C/EBP ϵ del5bp	ACTTGCCGCCCTCGGCCCTTT	AAAGGGCCGAGGGCGGCAAGT
C/EBP ϵ insA	TGCGCGTTCTCaAAGGCCCC	GGGGCCTTtGAGAACGCGCA