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

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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/EBPε), 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-γ-old woman affected with SGD caused by a novel homozygous 2-aa deletion (ΔRS) in the leucine zipper domain of the C/EBPε 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 ΔRS mutant maintained normal cellular localization, DNA-binding activity, and dimerization, and all three mutants exhibited marked reduction in transcriptional activity. The ΔRS 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 ΔRS 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/EBPε for its essential function, and indicate that multiple molecular mechanisms lead to SGD. The Journal of Immunology, 2015, 195: 000–000.

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

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Materials and Methods

Patients

We studied two Japanese patients with SGD. Patient P1 is a 55-γ-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
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. 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.

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 of lactoferrin mAb. Cellular alkaline phosphatase and peroxidase activity was visualized using Fast Red TR salt and Naphtol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO). For 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.

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/EBPε binding site (5′-GGGCTGTTTATGTTGCAACA-GGGCGGG-3′) were incubated with cell extracts from HEK293 cells transfected with either pCAGIP-Myc-C/EBPε or its mutant counterparts (ΔRS, deLbp, 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/EBPε (WT or ΔRS) and pCMV5-Flag-MBP-C/EBPε (WT or ΔRS). HEK293 cells were also cotransfected with pCAGIP-Myc-Gata1 and pCMV5-Flag-MBP-C/EBPε or its mutant counterparts, or with pCAGIP-Myc-PU.1 and pCMV5-Flag-MBP-C/EBPε 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-MyHC20 (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).

FIGURE 1. Patient characteristics. (A and B) Skin abscess with ulceration and scar. (C) Peripheral blood smear. Neutrophils from patients P1 and P2 have bilobed nuclei and lack cytoplasmic granules (May–Grünewald–Giemsa staining). Original magnification ×400. (D) Immunohistochemical analysis. Cytosin preparations of leukocytes stained with anti-lactoferrin mAb. Cellular alkaline phosphatase and peroxidase activity were also analyzed. Original magnification ×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 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
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/EBPε to bind to C/EBPε binding site at the 5′UTR of human lactoferrin gene (19). The oligonucleotides containing the C/EBPε binding site bound in vitro to C/EBPε from lysates of HEK293 cells transfected with WT Myc-C/EBPε vector (Fig. 6A, top left blot). Importantly, the ΔRS mutant showed DNA-binding ability comparable to the WT C/EBPε (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 ΔRS mutant C/EBPε. 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 ΔRS mutant, we examined dimer formation in lysates of HEK293 cells cotransfected with Myc-C/EBPε and Flag-MBP-C/EBPε vectors. The ΔRS mutant was able to homodimerize or heterodimerize with WT C/EBPε at levels comparable to WT C/EBPε (Fig. 6B and data not shown). These results were consistent with the ability of the ΔRS mutant to bind to DNA.

Aberrant association with Gata1 and PU.1

To determine whether the ΔRS 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/EBPε; and no products were obtained from those without WT C/EBPε (Fig. 7A). Interestingly, the ΔRS mutant, as well as the del5bp and the insA mutants, failed to induce MaBP gene expression.

To assess further the ability of the C/EBPε 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/EBPε in lysates of HEK293 cells transfected with the WT Flag-MBP-C/EBPε 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 ΔRS 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 of HEK293 cells transfected with the ΔRS mutant.

Discussion

C/EBPε is essential for terminal differentiation of granulocytes. Frameshift mutations of the C/EBPε 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/EBPε 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,
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 (FcγRIIIA) 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 6. DNA binding activity and dimerization.](image)

**FIGURE 6.** DNA binding activity and dimerization. (A) DNA binding activity of WT, ∆RS, del5bp, and insA C/EBPε. Biotin-labeled oligonucleotide containing the C/EBPε-binding site of the lactoferrin gene was incubated with Myc-C/EBPε (WT, ∆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 ∆RS. HEK293 cells were transfected with Myc-C/EBPε-∆RS together with either empty control vector (MBP-ev), Flag-MBP-C/EBPε-WT or -∆RS. MBP-fused proteins were pulled down by amylase 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.

![Figure 7. Cooperative transcriptional activation of eosinophil major basic protein (MaBP).](image)

**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/EBPε expression vectors (WT, ∆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/EBPε, Gata1, PU.1 and MaBP. (B and C) Protein interactions between C/EBPε 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/EBPε-WT, -∆RS, -del5bp, or -insA. MBP-fused proteins were pulled down by amylase 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 ΔRS mutant as well as the two frameshift mutants. All three mutants exhibited marked reduction in transcriptional activity. The ΔRS 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 ΔRS 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 ΔRS mutant may suggest that the single normal C/EBPε 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 ΔRS mutation, remain in good health. The association of C/EBPε 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 ΔRS 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 ΔRS 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/EBPε on the G-CSF receptor promoter (24). Like the ΔRS 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 ΔRS mutant also exhibits defective interaction with other transcription factors such as c-Myb, PML, p500, E2F1, and Rb (9, 16, 25, 26). Because certain isoforms of C/EBPε have been reported to inhibit the synergistic activities of GATA1 and PU.1 (25), we also need to evaluate the isoforms of the C/EBPε mutants other than the full-length, 32-kDa C/EBPε.

These characteristics of the ΔRS 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 abscesses 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/EBPε-deficient mice exhibited impaired maturation and altered cytokine expression, such as increased levels of TNF-α and LTB4, in response to inflammation (28, 29). In addition, monocyte counts in C/EBPε-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 deletion mutation in the bZIP domain of C/EBPε and demonstrated its molecular pathogenesis leading to SGD. Comparative analysis of the C/EBPε mutations, including the previous frameshift mutations, also clarifies the functional significance of these mutants. Characterization of C/EBPε genetic defects and functional abnormalities will help to define the role of C/EBPε in human myelopoiesis and innate immunity.

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Disclosures

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