N-terminal Region of CCAAT/Enhancer-binding Protein ε Is Critical for Cell Cycle Arrest, Apoptosis, and Functional Maturation during Myeloid Differentiation*\(^{1,3}\)

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CCAT/enhancer-binding protein ε (C/EBPε) plays a critical role in terminal myeloid differentiation. Differentiation is an integrated process of cell cycle arrest, morphological change, functional maturation, and apoptosis. However, the molecular networks underlying these events in C/EBPε-induced differentiation remain poorly understood. To reveal these mechanisms, we performed a detailed molecular analysis of C/EBPε-induced differentiation using an inducible form of C/EBPε. The activation of C/EBPε induced growth arrest, morphological differentiation, the expression of CD11b and secondary granule proteins, and apoptosis in myeloid cell lines. Unlike C/EBPα, C/EBPε dramatically up-regulated p27 with a concomitant down-regulation of cdks/6 and cyclin D2/A/E. Moreover, the anti-apoptotic proteins Bcl-2 and Bcl-x were down-regulated, whereas pro-apoptotic protein Bax remained unchanged. Using a variety of mutants, we revealed that these events were all regulated by the N-terminal activation domain of C/EBPε. Interestingly, some of the differentiation processes such as the induction of secondary granule protein genes were clearly inhibited by c-Myc; however, inhibition of apoptosis by Bcl-x did not affect the entire differentiation processes. These data indicate the N terminus of C/EBPε to be solely responsible for most aspects of myeloid differentiation, and these events were differentially affected by c-Myc.

The differentiation of hematopoietic cells is an integrated process of morphological change, functional maturation, growth arrest, and apoptosis. We have previously shown that C/EBPε retarded cellular growth by some unknown mechanisms when it was expressed in cell lines (6). In addition, C/EBPε activates myeloid progenitors have an increased rate of proliferation (11). C/EBPα, another C/EBP family member that is essential for early granulopoiesis (12), induces growth arrest and differentiation in a manner similar to C/EBPε (13). C/EBPα inhibits the kinase activity of cyclin-dependent kinase (cdk) 4 and cdk6 by direct protein–protein interactions to inhibit cell cycle progression (14–18). The interaction occurs through the N terminus (amino acids 175–187) of C/EBPα, and this facilitates the posttranslational degradation of cdk4 and cdk6. The N-terminal region of C/EBPα also interacts with E2F to inhibit its function, and this region is critically required for granulocyte and adipocyte differentiation in vivo (19). As for C/EBPε, it has also been reported to repress the E2F activity by direct protein–protein interactions (20). However, the precise molecular mechanism regarding how C/EBPε regulates growth arrest remains largely unknown. Moreover, the mechanism regulating the induction of apoptosis and secondary granule protein genes by C/EBPε still remains unclear.

In this study, we established an inducible system of C/EBPε to reveal the molecular targets of C/EBPε during differentiation. In addition, we performed a detailed structure-function analysis of C/EBPε to map the responsible regions for various aspects of differentiation. We herein demonstrate that C/EBPε up-regulates p27 and down-regulates cyclins/cdks to induce growth arrest during differentiation. In addition, C/EBPε induces apoptosis by down-regulating anti-apoptotic proteins, Bcl-2 and Bcl-x. These effects are mediated by the N-terminal activation domain of C/EBPε, and this domain is also responsible for the induction of such as impaired chemotaxis and superoxide productions and the lack of secondary and tertiary granule proteins (i.e. lactoferrin, gelatinase B) (3–5). This phenotype can be explained by the fact that C/EBPε works downstream of granulocyte colony-stimulating factor (G-CSF), which plays a critical role in the development and function of neutrophils (6). An aberrant C/EBPε function is actually linked to a variety of disease processes in humans. A mutation of the C/EBPε gene is the cause of human secondary granule deficiency (7, 8). In human leukemia, C/EBPε is reported to be the critical target of PML-RARα, a leukemic fusion product of chromosomal translocation t(15;17) (9, 10). Taken together, C/EBPε is considered to play a critical role in normal granulocyte differentiation and leukemogenesis, and therefore, a full understanding of the molecular networks surrounding C/EBPε is essential to elucidate the mechanism of myeloid differentiation and leukemogenesis.
of secondary granule protein genes. An overexpression of c-Myc prevented the induction of secondary granule protein genes, whereas Bcl-x did not affect the differentiation processes. These results demonstrate that the molecular pathways of C/EBPε leading to growth arrest, apoptosis, and the functional maturation all emanate from the N-terminal activation domain, and these pathways were differentially affected by c-Myc.

**EXPERIMENTAL PROCEDURES**

**Cells—**32D (6), LG, and LGM3 (kindly provided by Dr. T. Honjo) cells and their transfectants are cultured in an RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mM l-glutamine, and 2.5 units/ml of recombinant murine IL-3. All of the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO2. 32D/DN-STAT3 cells and 32D/e cells were described previously (6). 32D, LG, and LGM3 cells expressing C/EBPε-ER were generated by infecting C/EBPε-ER retrovirus. Briefly, cells in a logarithmic growth phase were suspended in medium containing retrovirus with 10 μg/ml of polybrene (Sigma) and 10 units/ml of murine IL-3. An equal amount of medium (RPMI 1640, 10% fetal bovine serum, 2.5 units/ml of murine IL-3) was supplemented after 5 h of infection, and GFP-positive cells were sorted by FACS vantage (Becton Dickinson) after several days. To avoid clonal variation, pools of sorted cells were used for all experiments. The intensities of GFP in established cell lines were checked periodically by FACS, showing that GFP expressions remained constant during experiments.

**Retroviral Constructs—**cDNAs for full-length C/EBPε and their mutants were fused in-frame to a mutated (G525R) ligand-binding domain of murine estrogen receptor and subcloned into MSCV-IRES-GFP retrovirus vector. cDNA for the ligand-binding domain (amino acids 281–599) of murine estrogen receptor was amplified by PCR using bone marrow cDNA as a template. cDNAs for Bcl-x and c-Myc were also amplified by PCR using cDNA as a template. cDNAs for Bcl-x and c-Myc were also amplified by PCR using bone marrow cDNA as a template and subcloned into MSCV-IRES-GFP retrovirus vector. All of the amplified sequences were verified by DNA sequencing.

**Transfection and Retrovirus Production—**Retroviral constructs were transfected into PlatE producer cell line (21) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Retroviral supernatants were harvested after 48 h of transfection, filtered, and stored at −80 °C until use.

**4-Hydroxytamoxifen—**4-Hydroxytamoxifen (4-HT; Sigma) was dissolved in EtOH (stock concentration at 5 mM) and was used at the final concentration of 1 μM in all experiments.

**Gel Shift Assay—**Nuclear extracts were made from LG/C/EBPε-ER cells stimulated with 1 μM of 4-HT for various times indicated as described previously (22). Cell extracts (10 μg of total protein) were incubated with 2 μg of poly(dl-dC) for 15 min and then with double-stranded C/EBP consensus oligonucleotide probes (5′-TGGCAGATTCGGCAACTCTGCA-3′) labeled with [γ-32P]ATP for 30 min on ice. For supershift, anti-C/EBPε (C-22; Santa Cruz) or anti-ER (MC-20; Santa Cruz) antibodies were added to the reactions. The resulting complexes were resolved on 4.5% polyacrylamide gel in 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer. The gels were dried and visualized by autoradiography.

**DNA Fragmentation Analysis—**The cells (1 × 10⁶) were washed twice with ice-cold phosphate-buffered saline and lysed in a lysis buffer (10 mM Tris-HCl, pH 8.0, 34 mMol/liter sodium citrate, and 0.1 mM EDTA, 50 μg/ml propidium iodide). The DNA content was analyzed by FACSCalibur (Becton Dickinson) using the Cell Quest software package.

**Cell Cycle Analysis—**1 × 10⁶ cells were suspended in hypotonic buffer containing propidium iodide (0.1% Triton X-100, 1 mM Tris-HCl, pH 8.0, 3.4 mMol/liter sodium citrate, and 0.1 mM EDTA, 50 μg/ml propidium iodide). The DNA content was analyzed by FACSCalibur (Becton Dickinson) using the Cell Quest software package.

**Flow Cytometry—**The cells were stained as described previously (6) with anti-Gr-1-fluorescein isothiocyanate or anti-Mac-1-fluorescein isothiocyanate antibodies (Pharmingen). A FACS analysis was performed using FACSCalibur flow cytometer (Becton Dickinson), and the data were analyzed using the Cell Quest software package.
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FIGURE 2. Effects of C/EBPε on growth, differentiation, and apoptosis of myeloid cells. LG cells expressing C/EBPε-ER were generated and examined for the outcome of C/EBPε activation. A, DNA binding activity of C/EBPε-ER protein induced by 4-HT. The cells were treated with IL-3 (10 ng/ml) or 4-HT for the times indicated, and a gel shift analysis was performed as described under “Experimental Procedures.” Supershifts were performed by adding anti-ER or anti-C/EBPε antibodies to the extract of 4-HT for 120 min. The
buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA) and separated on a 2% agarose gel. The fragmentation of DNA was visualized by ethidium bromide staining of the gel.

RESULTS

Expression of C/EBPα Correlates with Up-regulation of p27—C/EBPα, another C/EBP family member, induces cell cycle arrest through modulation of p21, cdk2, and cdk4 (14–18). Therefore, we hypothesized that C/EBPα induces withdrawal from the cell cycle similarly to C/EBPβ by modulating a family of cdk or cdk inhibitors. To get some insight into the molecular mechanism of cell cycle arrest induced by C/EBPα, we first examined the role of two major cdk inhibitors, p21 and p27 in 32D cells treated by

DNA-binding complex of C/EBPβ-ER and its supershifted complexes (s.s.) are indicated by arrows. The asterisk indicates endogenous C/EBP proteins. The schematic structures of C/EBPα and C/EBPβ-ER proteins are shown on the left. AD, activation domain; DBD, DNA-binding domain; LZ, leucine zipper motif; ER, ligand-binding domain of estrogen receptor. B, cell growth. LG cells transfected with vector alone are shown in red, and LG cells expressing C/EBPβ-ER are shown in blue. The cells were counted every day and diluted to maintain cell concentrations between 1 × 10^5/ml and 8 × 10^5/ml. The cumulative cell numbers are shown.

Cell cycle analysis. The cells were treated with vehicle (EtOH) or 4-HT for the times indicated, and the percentages of cells in G0/G1 or sub-G0/G1 were analyzed by FACS.

Apoptosis. The cells were treated with vehicle (EtOH) or 4-HT, and apoptotic cells were enumerated under a microscopy at the times indicated. A fragmentation of DNA was examined as described under “Experimental Procedures.” E, cell morphology. Cells treated with vehicle (EtOH) or 4-HT for 4 days were cytospun onto glass slides and stained by Wright-Giemsa staining. Original magnification was 1000× or 400×. The closed and open arrowheads indicate cells with granulocyte and macrophage morphology, respectively. F, expression of Mac-1α and Gr-1. The expressions of Mac-1α and Gr-1 were analyzed by FACS as described under “Experimental Procedures,” and their positivity was plotted in graphs.
G-CSF. C/EBPε is up-regulated by G-CSF in these cells where it plays a critical role in inducing differentiation to the granulocytes (6). Interestingly, p27 protein is markedly up-regulated in G-CSF treated 32D cells (Fig. 1), whereas the p21 protein level did not change substantially (data not shown). We speculated that C/EBPε could thus be involved in the up-regulation of p27 and therefore examined the p27 protein levels in 32D cells overexpressing C/EBPε (32D/C/EBPε). As expected, p27 protein was markedly up-regulated in these cells (Fig. 1). We also examined 32D cells expressing dominant negative signal transducers and activators of transcription (STAT) 3 (32D/DN-STAT3). These cells do not differentiate but rather proliferate in G-CSF at a slower rate compared with those in IL-3. Because G-CSF induces C/EBPε in these cells, we speculated that this lead to the induction of p27. As expected, p27 was clearly up-regulated in 32D/DN-STAT3 cells cultured in G-CSF, but not in IL-3. These data show a clear correlation between C/EBPε and p27 and suggest that p27 could be the critical target of C/EBPε during cell cycle arrest.

C/EBPε Induces Morphological Differentiation, Growth Arrest, and Apoptosis in Early Myeloid Cell Line—To further explore the molecular mechanism of growth arrest induced by C/EBPε, we created an inducible form of C/EBPε that is a fusion of full-length C/EBPε and the mutated ligand-binding domain of estrogen receptor (C/EBPε-ER) and examined its effect in various hematopoietic cell lines. This fusion protein can be activated selectively by an estrogen antagonist, 4-HT. As shown in Fig. 2A, stimulation of cells expressing C/EBPε-ER with 4-HT clearly impaired the growth of these cell lines within 3 days of stimulation even in the presence of IL-3. Cell cycle analysis revealed that induction of C/EBPε activity led to a rapid accumulation of cells in G0/G1 (Fig. 2C). Notably, this was accompanied by enhanced cell death with apoptosis as revealed by the cell morphology, an increased percentage of cells in sub-G0/G1, and DNA ladder formation (Fig. 2, C and D). A significant fraction (up to 35% at day 6) of LG cells also arrested in sub-G0/G1.
4-HT stimulation; however, this was not as dramatic as that of LG/CEBP-ER cells and hence was regarded as a nonspecific, toxic effect of 4-HT. A morphological analysis revealed that treating LG/CEBP-ER and LGM3/CEBP-ER cells for 5 days with 4-HT induced a clear differentiation to the myeloid lineage, mainly mature granulocytes (Fig. 2E, closed arrowheads, and data not shown). We observed differentiation to the macrophage-like cells in a significant fraction of LG/CEBP-ER and LGM3/CEBP-ER cells (open arrowheads in Fig. 2E and data not shown), thus indicating that C/EBP could also be directing cells to a monocytic lineage in these cells. Differentiation to a myeloid lineage was also confirmed by a FACS analysis showing the 4-HT-treated LG/CEBP-ER cells to express increased levels of Mac-1 (CD11b) and Gr-1 (Fig. 2F). These data indicate that the induction of the C/EBP activity is sufficient to induce myeloid differentiation, growth arrest, and apoptosis in myeloid cell lines.

C/EBP Induces the Up-regulation of p27 and the Down-regulation of Cyclins and Cyclin-dependent Kinases—To reveal the mechanism of growth arrest and apoptosis induced by C/EBP, we analyzed the expression of various molecules involved in cell cycle regulation and apoptosis by Western blot (Fig. 3A). The most striking difference induced by C/EBP-ER induction is the robust up-regulation of p27. In sharp contrast, cyclin D2, cyclin A, cyclin E, cdk4, and cdk6 were clearly down-regulated after 4 days of 4-HT treatment. In addition, hypophosphorylation of Rb protein was specifically observed in LG/CEBP-ER cells treated with 4-HT. It should be mentioned that EtOH alone caused similar changes of some protein expressions seen in the 4-HT-treated LG/CEBP-ER cells (cyclin D2, cdk4/6 in Fig. 3 and Bcl-2 in Fig. 4). However, the extent of the changes were more dramatic in 4-HT-treated cells, and moreover, it was not observed in other experiments (see Fig. 6C). Therefore, we speculate this could be due to the specific experimental condition or to the background activity of the C/EBP-ER protein, which is commonly seen in inducible systems. Collectively, these data demonstrate that p27 is indeed the downstream target of C/EBP, as suggested by the previous experiment. Moreover, C/EBP not only up-regulates p27 but also down-regulates various cyclins and cdk5. This suggests that the concomitant modulation of positive and negative regulators for cell cycle may thus be the major underlying mechanism for C/EBP-induced growth arrest during myeloid differentiation.

Increased Half-life of p27 in C/EBP-expressing Cells—To gain insight into the mechanism of apoptosis induced by C/EBP-ER, we examined the levels of various pro- and anti-apoptotic proteins in LG/CEBP-ER cells by Western blot (Fig. 4). Interestingly, the anti-apoptotic proteins Bcl-2 and Bcl-x were clearly down-regulated after induction of C/EBP activity in LG/CEBP-ER cells even though the
cells were being cultured in IL-3. In contrast, the level of pro-apoptotic protein Bax did not change substantially. These data suggest that the apoptosis induced by C/EBP-H9280-ER is mainly triggered by the down-regulation of anti-apoptotic molecules, Bcl-2 and Bcl-x. The fact that this occurs in the presence of IL-3 strongly suggests that C/EBP-H9280-ER disrupts or overrides anti-apoptotic signals to induce Bcl-2 and Bcl-x expression generated by IL-3.

C/EBP-H9280 Alone Is Sufficient to Induce Expression of Secondary Granule Proteins—The next question we asked was whether C/EBP could induce functional differentiation by itself. To answer the question, we first checked the induction of secondary granule protein genes in LG/CEBP-H9280-ER cells. However, we could not observe any induction upon 4-HT stimulation by Northern blot (A) or for CD11b (Mac-1α) expression by FACS (B). Lanes C, unstimulated cells. In the FACS picture, the blue and red lines represent vehicle- and 4-HT-treated cells, respectively. The data of pretreated cells were almost identical to those of vehicle-treated cells, and hence they were omitted from the picture for simplicity.

FIGURE 7. Effects of c-Myc and Bcl-x on C/EBP-induced differentiation. 32D cells expressing C/EBP-ER were transfected with c-Myc or Bcl-x and examined for their effects on differentiation. The cells were stimulated with vehicle (EtOH) or 4-HT for 3 days and analyzed for the expression of secondary granule protein genes by Northern blot (A) or for CD11b (Mac-1α) expression by FACS (B). Lanes C, unstimulated cells. In the FACS picture, the blue and red lines represent vehicle- and 4-HT-treated cells, respectively. The data of pretreated cells were almost identical to those of vehicle-treated cells, and hence they were omitted from the picture for simplicity.

N-terminal Region of C/EBP Is Essential for Growth Arrest and Functional Differentiation—We next tried to dissect the region of C/EBP required for growth arrest, apoptosis, and functional differentiation. To do this, we generated a variety of C/EBP mutants fused to the ER ligand-binding domain (Fig. 6A) and assessed their effects in LG or 32D cells. The expressions of mutant proteins in the cell lines were confirmed by Western blot (Fig. 6A). As shown in Fig. 6B, only a small (32 amino acids) deletion of the N-terminal portion of C/EBP (which corresponds to the N0 mutant) abolished its growth suppressing activity, thus indicating that the N-terminal 32 amino acids play a critical role in growth suppression. In all of the mutants tested, only the A2 mutant that retained an intact N-terminal activation domain but lacked an internal repressor domain showed significant growth suppression by 4-HT treatment. To reveal the molecular elements working downstream of these mutants, various molecules related to cell cycle and apoptosis were analyzed by Western blot (Fig. 6C). We examined p27, cdk4, cyclin D2, and Bcl-2 in control and 4-HT-treated cells and did not see any changes of their protein levels in the N1–N5 mutants (data not shown). To our surprise, p27 was induced by 4-HT not only in cells expressing full length (F) or the A2 mutant of C/EBP but also in cells expressing the N0 and A1 mutants, albeit to a lesser extent (Fig. 6C).
contrast, down-regulations of cyclin D2, cdk4, and Bcl-2 were seen only in full length and the ΔA2 mutant, but not in the N0 and ΔA1 mutants, paralleling their capability of growth suppression. Moreover, analysis of secondary granule protein genes revealed that only full length and ΔA2 mutant were able to induce lactoferin and other secondary granule protein genes (Fig. 6D and the data not shown).

Collectively, these data revealed that the growth suppressive capacity of C/EBPζ mutants is not specified by the regulation of p27 but by the regulation of positive regulators for cellular growth and anti-apoptosis, such as cyclin D2, cdk4, and Bcl-2. In addition, growth suppression, down-regulation of cyclin D2, cdk4, Bcl-2, and induction of secondary granule protein genes are all mediated by the N-terminal 102 amino acids containing AD1 and AD2 activation domains, whereas only AD1 domain is sufficient for p27 up-regulation (summarized in Fig. 6A).

Effect of Bcl-x and c-Myc on C/EBPζ-induced Myeloid Differentiation—
The coordinated regulation of cell cycle arrest, apoptosis, change in morphology, and functional differentiation is thought to be essential for the integrity of entire differentiation processes, and therefore, dysregulation of one component might lead to an aberrant output. We tested this possibility by expressing the anti-apoptotic protein, Bcl-x, or a critical cell cycle regulator, c-Myc, in 32D/CEBPζ-ER cells and by evaluating their effects on various differentiation processes. Expression of c-Myc or Bcl-x did not grossly affect the proliferation of 32D/CEBPζ-ER cells in IL-3, and induction of C/EBPζ activity equally suppressed the growth of mock transfected cells and cells expressing Bcl-x and, to a lesser extent, cells expressing c-Myc (data not shown). Interestingly, c-Myc completely suppressed the induction of secondary granule protein genes by C/EBPζ, whereas Bcl-x did not (Fig. 7A). Surprisingly, however, the induction of CD11b by C/EBPζ was not apparently affected by c-Myc or Bcl-x (Fig. 7B).

These data suggest that 1) the suppression of apoptosis by Bcl-x does not affect other differentiation processes, such as growth arrest, secondary granule protein expression, and induction of CD11b and 2) c-Myc differentially affects a selective aspect of differentiation, such as the induction of secondary granule protein genes.

DISCUSSION

C/EBPζ is a member of basic leucine zipper (bZIP) family of transcription factors. It contains a transactivation domain at its N terminus and a repression domain in the middle, just before the basic DNA-binding domain (Fig. 6A) (26). It was first reported that N-terminal amino acids 33–102 retained transactivating capacity (26), but a later study revealed that the first 32 amino acids had more potent activities (27). As a consequence, the full-length 32-kDa C/EBPζ, most widely expressed isoform in the bone marrow and peripheral neutrophils (28), demonstrates higher transactivating capabilities than the truncated 30-kDa isoform, which lacks the first 32 amino acids.

In this study, we showed that the N-terminal transactivation domain (amino acids 1–102) of C/EBPζ is responsible for cell cycle arrest during myeloid differentiation. A molecular analysis revealed that this process involved the up-regulation of p27 through protein stabilization and the down-regulation of cyclins and cdkls. Furthermore, the same region plays a critical role in the induction of apoptosis. After inducing C/EBPζ activity, anti-apoptotic proteins Bcl-2 and Bcl-x were down-regulated, whereas pro-apoptotic protein Bax remained unchanged, suggesting that the reduction of anti-apoptotic protein levels triggers an apoptotic response. In addition to growth arrest and apoptosis, morphological differentiation and expression of secondary granule protein genes were also mediated by the same N-terminal region. Taken together, these data indicate that C/EBPζ alone is sufficient to execute the myeloid differentiation program including cell cycle arrest, apoptosis, morphological differentiation, and the induction of secondary granule protein genes, and all of these activities depend on the N-terminal activation domain of C/EBPζ. DNA binding activity and a protein dimerization of C/EBPζ are required for these activities, because a deletion of the basic region and the leucine zipper motif rendered this protein inactive for growth suppression and the secondary granule protein induction (supplemental Fig. S1). In addition, our data showed that the N0 mutant (Fig. 6A), which corresponds to the 30-kDa isoform of C/EBPζ, was compromised in its differentiation-inducing capacity, indicating that a strong transactivation capacity of the first 32 amino acids is critically important for myeloid differentiation. These observations highly suggest that the 30-kDa isoform of C/EBPζ is not playing an active role in the induction of myeloid differentiation in vivo. This idea is actually compatible with the fact that the 30-kDa isoform is only found in cell lines but not in primary bone marrow and neutrophils (28).

Regarding p27 up-regulation, we were able to further narrow down the responsible region in C/EBPζ to amino acids 33–50 in the activation domain. This region should be critical for protein turnover of p27, because the induction of C/EBPζ activity led to the increased half-life of the protein. Although the precise molecular mechanism for p27 stabilization by C/EBPζ is not clear, we observed an inducible interaction between C/EBPζ and p27, thus leading to the hypothesis that this region controls the degradation of p27 by direct protein-protein interactions.

Another C/EBPζ family member, C/EBPα, induces cell cycle arrest by a mechanism similar to that of C/EBPζ. C/EBPα triggers the proteasome-dependent degradation of cdk4 (14) and inhibits the kinase activity of cdk2 and cdk4 through direct interaction (16). In this study, we demonstrated that C/EBPζ also down-regulated the levels of cdk4 and cdk6 proteins and induced the resultant hypophosphorylation of Rb. It was also reported that C/EBPζ repressed E2F-dependent transcription independently of the Rb-E2F pathway (19). Similarly, C/EBPζ was shown to interact with E2F1 to repress its transcriptional activity (20). On the other hand, we observed a clear difference in the regulation of cdk inhibitors by C/EBPα and C/EBPζ. C/EBPζ robustly up-regulates p27 during differentiation, whereas C/EBPα induces p21 instead of p27 (18, 29). This differential regulation of cdk inhibitors by C/EBPα and C/EBPζ might confer some functional specificity to each molecule. It should be noted that p21 is not absolutely required for C/EBPζ-induced growth arrest (16, 30). Likewise, the up-regulation of p27 is not sufficient for cell cycle arrest by C/EBPζ, because the N1 and ΔA1 mutants, which are defective for the arresting cycle, still induced p27 accumulation. This observation is also compatible with the phenotype of p27-deficient mice, whose myeloid differentiation remains basically intact (31–33). Further studies will be required to uncover the implications of this differential regulation of cdk inhibitors in C/EBPα- and C/EBPζ-induced differentiation.

It is well known that G-CSF induces p27 during myeloid differentiation, and STAT3 has been reported to be responsible for this induction (34). However, we propose that C/EBPζ, rather than STAT3, plays a critical role in this process for the following reasons. 1) We have previously shown that C/EBPζ is a critical downstream target of G-CSF (6), and our present data show that the activation of C/EBPζ alone can induce p27 in a time course consistent with that induced by G-CSF. 2) In our hands, 32D cells expressing dominant negative STAT3 (32D/DN-STAT3) proliferating in G-CSF express relatively high levels of p27 in a comparison to those cultured in IL-3 (Fig. 1). It should be noted that 32D/DN-STAT3 expresses high levels of C/EBPζ when cultured in

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G-CSF (6), thus suggesting that C/EBPε but not STAT3 is responsible for p27 up-regulation. 3) STAT3 is activated within 15 min of G-CSF stimulation. However, the induction of p27 protein by G-CSF takes at least a couple of days. Collectively, C/EBPε thus seems to be a more suitable mediator for p27 induction during myeloid differentiation.

We have previously shown that an overexpression of C/EBPε reverses c-Myc-induced differentiation block in 32D cells (6). In contrast, we herein demonstrate that the expression of c-Myc blocks some aspects of differentiation induced by C/EBPε. These results show that c-Myc and C/EBPε induce an opposite effect on myeloid differentiation, suggesting that the balance of their expression actually determines the final outcome whether the cells differentiate or proliferate. On the other hand, the suppression of apoptosis by expressing Bcl-x did not affect C/EBPε-induced differentiation processes, such as cell growth, the expression of secondary granule protein genes, and the up-regulation of CD11b. This suggests that the suppression of cellular growth by C/EBPε is not due to increased apoptosis but to the suppression of cell cycle progression.

In summary, we demonstrated that the induction of C/EBPε activity alone is sufficient to induce myeloid differentiation, and these processes are all mediated by the N-terminal activation domain of C/EBPε. The molecular mechanism of C/EBPε-induced differentiation is similar to but distinct from that of C/EBPα, such as regarding the regulation of cdk inhibitors. Our data provide a molecular basis for the functional differences between C/EBPα and C/EBPε that may pave a way to differentially regulate the function of these two related molecules.

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