CCAAT/Enhancer-binding Protein δ Mediates Tumor Necrosis Factor α-induced Aurora Kinase C Transcription and Promotes Genomic Instability*§

Received for publication, June 14, 2011 Published, JBC Papers in Press, June 28, 2011, DOI 10.1074/jbc.M111.270710

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Epidemiologic and clinical research indicates that chronic inflammation increases the risk of certain cancers, possibly through chromosomal instability. However, the mechanism of inflammation-dependent chromosomal instability associated with tumorigenesis is not well characterized. The transcription factor CCAAT/enhancer-binding protein δ (C/EBPδ, CEBPD) is induced by tumor necrosis factor α (TNFα) and expressed in chronically inflamed tissue. In this study, we show that TNFα promotes aneuploidy. Loss of CEBPD attenuated TNFα-induced aneuploidy, and CEBPD caused centromere abnormality. Additionally, TNFα-induced CEBPD expression augmented anchorage-independent growth. We found that TNFα induced expression of aurora kinase C (AURKC) through CEBPD, and that AURKC also causes aneuploidy. Furthermore, high CEBPD expression correlated with AURKC expression in inflamed cervical tissue specimens. These data provide insight into a novel function for CEBPD in inducing genomic instability through the activation of AURKC expression in response to inflammatory signals.

Chronic inflammation increases the risk of normal cells to become tumorigenic (1, 2), and the immune system is thought to be the cause of chronic inflammation-associated tumorigenesis and cancer progression. Pro-inflammatory cytokines are thought to play a pathogenic role in age-related diseases, including cancer (3). The inflammatory cytokine TNFα promotes inflammation-associated carcinogenesis by activating nuclear factor-κB (NF-κB) (4) and signal transducers and activators of transcription (STATs) signaling (5). In addition, TNFα causes oxidative stress, enhances the population of >4N cells, and contributes to tumorigenesis (6). However, the activation of oncogenes such as NF-κB or STATs cannot fully explain why inflammation can kill tumor cells and yet promote tumor growth. This discrepancy further implies that there may be a molecule (or molecules) that plays a dual role in both killing and promoting tumor cells in inflammation.

The transcription factor CEBPD3 was suggested to be a tumor suppressor because it can induce growth arrest and apoptosis in cancer cells (7) and loss of Cebpd increases the tumor incidence in a mouse mammary tumor model (8). On the other hand, CEBPD can also support tumor progression, most likely through a role in hypoxia adaptation (8). In addition, opposite to CEBPD up-regulated proapoptotic and growth arrest genes (9), some intriguing downstream targets of CEBPD, including cyclooxygenase 2 (10), the Fox/Jun family (11), and superoxide dismutase 1 (12), suggest that the CEBPD biology is complex and requires further studies, particularly in the pathogenesis that links inflammation and tumorigenesis.

In mammals, the aurora kinase (AURK) family consists of three members: aurora kinase A (AURKA), aurora kinase B (AURKB), and aurora kinase C (AURKC). AURKs play important roles in the control of centrosome and spindle function, kinechores-microtubule interactions, cytokinesis, and cell division (13–16). Abnormal chromosome segregation can result in aneuploidy, which is associated with the majority of early embryonic loss in human, when it occurs in the germline (17). Aneuploidy is also characteristic of cancer cells. The dysregulation of AURKs is correlated with chromosomal instability and clinically aggressive cancer (18–21). AURKC is localized at chromocenters in diploptene spermatocytes and centromeres in metaphase I and II and persists to a lesser extent in round spermatids (22). Similar to AURKB, AURKC can phosphorylate histone H3 at Ser-10 (23, 24) and form a complex with inner centromere protein (INCENP) and survivin (24–26). The expression of AURKC is very low in normal somatic tissues and higher in testis (27). Recently, AURKC was found to be overexpressed in primary colorectal cancers and thyroid carcinoma as well as in several cancer cell lines (28, 29).

The abbreviations used are: CEBPD, CCAAT/enhancer-binding protein δ; AURK, aurora kinase; MEF, mouse embryonic fibroblast; AURKA, aurora kinase A; AURKB, aurora kinase B; AURKC, aurora kinase C.
Therefore, the deregulation of AURKC expression during tumorigenesis appears to be highly relevant and important.

In this study, TNFα was found to stimulate CEBPD expression in non-hematopoietic cells. Evidence that links the increase of CEBPD and the induction of aneuploidy was established. Briefly, CEBPD induction resulted in increasing aneuploidy, and caused abnormal DNA segregation, and a prolonged mitotic phase. Moreover, we identified that CEBPD specifically induced expression of AURKC in response to TNFα. The clinical evidence that correlates CEBPD with AURKC expression in inflamed cervical tissue was also demonstrated.

RESULTS

CEBPΔ Mediates TNFα-induced Genomic Instability—As mentioned above, TNFα causes enhancement of the population of >4N cells and contributes to tumorigenesis. In hematopoietic cells, CEBPD is induced by inflammatory stimuli including TNFα (31). To assess the potential involvement of CEBPD in TNFα-induced aneuploidy of non-hematopoietic cells, we first determined if long-term TNFα treatment can promote aneuploidy in the HeLa cervical carcinoma cell line. As shown in Fig. 1A and supplemental Fig. S1, TNFα treatment indeed increased the >4N cell population in HeLa cells regardless of nocodazole treatment. We next examined if CEBPD induction contributed to TNFα-induced genomic instability. We found that the TNFα-induced >4N HeLa cell population was reduced when CEBPD expression was attenuated by two RNAi (Fig. 1B), suggesting that CEBPD plays a functional role in the TNFα-induced genomic instability. Next, we examined the effect of CEBPD on TNFα-induced transformation using an anchorage-independent growth assay in soft agar. We found that the insensitive induction of CEBPD upon TNFα treatment caused larger HeLa cell focus formation on soft agar (Fig. 1C, left panel), in agreement with the hypothesis that CEBPD acts as a tumor suppressor. Interestingly, when we examined the CEBPD expression in smaller foci, sensitive induction of CEBPD upon TNFα treatment (Fig. 1C, right panel, compare lane 10 with lane 11) showed a coincident effect on TNFα-induced focus formation (Fig. 1C, right panel, compare lanes 8 and 9 with lanes 11 and 12). This important discovery suggests that CEBPD, depending
on its expression level, could play a dual role in determining long-term TNFα-induced tumorigenic transformation.

CEBPΔ Activation Enhances Genomic Instability—As mentioned above, genomic instability is a hallmark of cancer cells and is suggested to play a critical role in shifting the cells from normal to the malignant stage. To further confirm whether increased CEBPΔ affects genomic integrity, a CEBPΔ-inducible system was introduced into HeLa cells. Consistent with the results presented above, the induction of CEBPΔ resulted in the increase of 4N cell population in synchronized cells (Fig. 2A). It is known that abnormal chromosomal segregation during mitosis impair genome integrity. We therefore assessed the role of CEBPΔ in chromosomal abnormality. We observed that CEBPΔ induction caused an increase in the number of tripolar nuclei, DNA bridge occurrence, and micronuclei formation (Fig. 2B). These results indicate that CEBPΔ can affect genomic integrity through induction of abnormalities in chromosomes and centrosome. Cancer cells are known to contain extra chromosomes. Therefore one would expect that their mitosis are prolonged. We examined the possibility of prolonged mitosis by CEBPΔ-induced genomic instability by phase-contrast time-lapse microscopy. The results in Fig. 2C show that CEBPΔ-activated cells take an average of 103.7 min (n = 91). The results show that CEBPΔ expression increased the duration of cell division. In addition, to assess the effect of CEBPΔ on genomic integrity, GFP-stabilized wild-type Cebpd and GFP-stabilized Cebpd-deficient MEF cells were individually generated. After culturing for several passages, GFP expression was lost more readily in wild-type Cebpd MEF cells upon long-term TNFα treatment (supplemental Fig. S2), which indicated the greater ability to induce genomic instability. Taken together, these results show that CEBPΔ contributes to genomic instability.

CEBPΔ Mediates TNFα-induced AURKC, Which Increases Genomic Instability—Deregulation of AURKA, AURKB, and AURKC has been demonstrated in many cancers (32). However, it is unknown whether AURKs are responsive to TNFα treatment. We found that in HeLa cells, TNFα induced the expression of CEBPΔ and AURKC, slightly reduced AURKA expression, and had no effect on AURKB expression (Fig. 3A). Consistent with these data, the results of reporter assays suggest that the TNFα-induced AURKC expression was due to activation of the AURKC gene promoter (Fig. 3B). AURKC plays a functional role in mitosis and increased AURKC was observed in many cancers (27). However, the potential role of the increase in AURKC in disturbing genomic integrity is unknown. We found that induction of ectopic AURKC alone was sufficient to increase the 4N cell population (Fig. 3C, left panel), and the incidence of tripolar nuclei, DNA bridges, and...
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Micronuclei (Fig. 3C, right panel). To further assess the effect of AURKC on genomic integrity, stable HeLa-off AURKC#11-GFP and HeLa-off GFP cells were individually generated. Over time, GFP expression was lost more readily in AURKC-overexpressing cells (Fig. 3D). Taken together, these results show that AURKC contributes to genomic instability.

Both CEBPD and AURKC activation showed a concomitant increase in genomic instability and responsiveness to TNFα treatment. However, CEBPD is not directly located on the condensed DNA during mitosis (supplemental Fig. S3), suggesting that the CEBPD-induced chromosomal abnormalities may be caused by a transcription-dependent manner. These observations led us to test whether AURKC is the downstream target of CEBPD. Transcripts of AURKC, but not AURKA or AURKB, increase in response to CEBPD induction not only in HeLa cells but also in the U373MG astrocytoma cell line (Fig. 4A). Moreover, this CEBPD/AURKC axis is also observed in other cells responding to TNFα treatment (supplemental Fig. S4). On the other hand, loss of Cebpd attenuated TNFα-induced Aurkc transcription in MEF cells (Fig. 4B). To determine whether CEBPD and Cebpd activate AURKC and Aurkc gene expression through promoter activation, the AURKC and Cebpd reporters were cotransfected with human CEBPD and mouse Cebpd expression vectors, respectively. As shown in supplemental Fig. S5A, Cebpd can transactivate the Aurkc reporter, whereas CEBPD transactivates the AURKC reporter. Moreover, activation of the AURKC reporter is specifically regulated by CEBPD but not LAP1, a C/EBP family member (supplemental Fig. S5B).

Furthermore, the lentiviral-mediated expression of CEBPD shRNAs significantly attenuated the TNFα-induced AURKC reporter activity (Fig. 4C). Western blot analysis verified that CEBPD knockdown abolished TNFα-induced AURKC expression (Fig. 4D). Taken together, these results show that CEBPD plays an important role in TNFα-activated AURKC promoter activity.

CEBPα Directly Regulates AURKC Promoter Activation—There are five putative CEBPD binding motifs within 1000 base pairs of the 5′-flanking region of the AURKC gene (Fig. 5A) identified by the PCDBM program. We further shortened the AURKC promoter from −996/+84 bp (AC1P) to −593/+84 bp (AC2P) for the purpose of determining the CEBPD-responsive region. As shown in Fig. 5A, CEBPD-dependent transactivation of AC2P reporter activity is similar to AC1P reporter activity, suggesting that the AC2P reporter contains the CEBPD-responsive element(s). We next performed an in vivo DNA binding assay to determine whether CEBPD binds directly to the endogenous AURKC promoter. Both exogenous HA/CEBPα and TNFα-induced endogenous CEBPD were found to bind to the proximal 5′-flanking region of the AURKC promoter that contains the CEBPD-I and -II motifs (Fig. 5B). The same approach was conducted to assess whether Cebpd binds to the AURKC promoter region. This result showed that the binding of Cebpd is detectable on the AURKC promoter region upon TNFα treatment (supplemental Fig. S6). Furthermore, to specify the function of CEBPD-I and -II an in vitro DNA binding assay was conducted. The results show that the CEBPD-I motif exhibited a higher CEBPD binding activity than CEBPD-II in a gel shift assay (Fig. 5C). To determine the role of these elements in TNFα-induced AURKC promoter activity, the reporter assay was performed with AC2P, AC2PM1 (AC2P mutant on mutant CEBPD-I motif), or AC2PM2 (AC2P mutant on CEBPD-II motif). Mutation of the CEBPD-I site reduced the basal activity of the AURKC promoter (Fig. 5D, compare the first lane with the fourth and seventh lanes). However, both elements contribute to the response to TNFα in a CEBPD-dependent manner (Fig. 5D, compare the second lane with the
fifth and eighth lanes). These data suggested that the CEBP-I motif plays an important role in basal AURKC promoter activation and that both CEBP-I and -II are critical for TNFα-induced AURKC promoter activity.

**Expressions of CEBP and AURKC Are Coincident in Inflamed Cervical Tissue**—The evidence presented above demonstrated that CEBP is an important mediator of TNFα-induced AURKC transcription. To assess the clinical relevance of the correlation between CEBP and AURKC expressions in inflamed tissue, immunohistochemical staining was conducted on a clinical cervicitis specimen compared with normal cervical tissue. The results showed a simultaneous increase in expression of CEBP and AURKC, but not AURKA, in inflamed cervical tissues (Fig. 6). These data strongly support clinical relevance for CEBP-induced AURKC expression.

**DISCUSSION**

Although it is clear that cell proliferation alone does not cause cancer, sustained cell proliferation in an inflammatory environment combined with accumulation of genetic abnormalities contributes to initiation and promotion of cancer (33). Evidence is accumulating that demonstrates the link between inflammation and cancer (34). However, the precise mechanism remains uncertain. Two inflammation-responsive transcription factors, NF-κB and STATs, have been suggested to initiate tumor growth by interfering with genomic integrity (6). In this report, we provide evidence that an additional inflammation responsive factor, CEBP, affects genomic integrity at least in part through the activation of AURKC transcription.
Due to a tight feedback control between proinflammatory and anti-inflammatory cytokines, normal inflammation, but not chronic inflammation, is a self-limiting process to prevent the persistence of immune responses (35). This raises the intriguing scenario of inflammation serving as a two-edged sword to both suppress and promote tumor formation and cancer progression. Previously, CEBPD was reported to act as an innate inflammation-responsive factor (36), which persists in high concentration in chronically inflamed tissues (9). However, unlike nuclear factor-κB (NF-κB) and signal transducers and activators of transcription (STATs), the level of CEBPD is low during tumorigenesis, as confirmed in multiple cancers (7, 9). We recently demonstrated that an epigenetic regulator, the polycomb group/DNA methyltransferases pathway, increases CEBPD gene hypermethylation, which results in CEBPD being resistant to external stimulation (7). Whereas these reports are in line with a tumor suppressor function of CEBPD, the results of this study suggest that TNFα-induced CEBPD mediates in part the transforming activity of TNFα.

Genomic instability is observed in most cancers, and although tumorigenesis has been attributed to the dysregulation of mitotic checkpoint proteins (37), the molecular mechanisms underlying inflammation-related chromosomal instability associated with tumorigenesis are poorly understood. For example, the mitotic regulators that control genomic stability in chronic inflammation-induced tumors remain relatively unknown. Increased cyclooxygenase 2 is known to induce genomic instability, and we previously reported that cyclooxygenase 2 is a CEBPD-responsive gene (38), which suggests that a sustained CEBPD expression may play a role in oncogenesis. Moreover, based on the current study, the association between aneuploidy and chronic inflammation can be linked to CEBPD-mediated AURKC expression. This study not only highlights the role of chronic CEBPD expression promoting genomic...
FIGURE 5. CEBPD binds to the AURKC promoter in vivo, and the identification of CEBPD-responsive motifs. A, the AURKC promoter −593/+84 is important for activation by CEBPD. Following the description under “Experimental Procedures,” a luciferase assay was performed using the AURKC promoter −996/+84 reporter (AC1P) or −593/+84 reporter (AC2P) and cotransfected with HA/CEBPD. The reporter activities of the transfectants expressing the empty vector (pCDNA3-HA) were set to 100 as the standard. The data represent three independent experiments.

B, CEBPD binds to the AURKC promoter in vivo. ChIP assays were performed with HeLa cells by using the indicated antibody-immunoprecipitated products from the experimental cells expressing HA/CEBPD (left panel) or treated with or without TNFα. The locations of the specific primers on the AURKC promoter region are shown in the upper panel. C, CEBPD binds to the C/EBPα Mediates TNFα-induced Genomic Instability.

C/EBPD-II C/EBPD-I
Comp. − + + − + + − I.V.T. C D D C D D

D, C/EBPD-I and -II motifs are important for TNFα-induced AURKC reporter activity. HeLa cells transfected with various luciferase reporters, as indicated, were stimulated with or without TNFα. The experiment was performed as described in the legend to Fig. 4C. Similar results were obtained from three independent experiments, and the data are from one experiment performed in triplicate. The average fold induction is shown as the mean ± S.E. The mutated sequence in each individual construct is shown by a black oval.
instability but also suggests that CEBPD could account in part for the connection between inflammation and tumorigenesis. The accurate expression of mitotic proteins, such as AURKs, plays a critical role in the maintenance of genomic integrity (32). Our results demonstrate that AURKA and AURKC are inactivated and activated, respectively, in response to TNFα, and that AURKC is specifically up-regulated by CEBPD. Although the mechanism of AURKA reduction during inflammation remains unknown, TNFα-induced CEBPD indeed plays a functional role in transcriptional activation of the AURKC gene, which subsequently causes aneuploidy and genomic instability (Figs. 3 and 4). Induction of AURKC was reported in the G2/M phase, which is coincident with or followed by, an increase in AURKB expression (25). However, following cell cycle progression we found that the expression of AURKC is comparable in asynchronized and synchronized HeLa cells (supplemental Fig. S7A). Furthermore, CEBPD showed a slightly shifted pattern, but not an increase, during the synchronized condition (supplemental Fig. S7A). In addition, CEBPD induction not only induces AURKC expression but also promotes the phosphorylation of histone H3 (supplemental Fig. S7B), which is known to associate with chromosome condensation and segregation (39). These observations suggest that the promotion of genomic instability regulated by the CEBPD-mediated TNFα-dependent induction of AURKC transcription uncouples mitotic control of cell cycle progression.

Cervical human papillomavirus infection is not believed to be inflammatory in nature (40). Nevertheless, there is some epide-miological evidence, albeit weak, to suggest that inflammation might be linked to cervical cancer, perhaps as an human papillomavirus cofactor (41, 42). Moreover, populations with high rates of cervical inflammation are associated with high incidence rates of cervical neoplasia (43). In this report, we demonstrated that long-term TNFα treatment increased aneuploidy and anchorage-independent growth of the HeLa cervical carcinoma cell line, which was attenuated by blocking CEBPD expression (Fig. 1, B and C). Therefore, we suggest that inflammation-induced CEBPD expression may promote human papillomavirus-mediated tumorigenesis in cervical cells through inflammation-disrupted genome stability.

Accurate mitotic protein levels play a critical role in maintaining genomic integrity. Primary Cebpd-deficient MEFs also exhibit genomic instability and centrosome abnormalities (44). Thus, it may be important to precisely regulate the levels of CEBPD protein to maintain genomic integrity. In addition, cells that survive TNFα-induced CEBPD-mediated genomic instability may be similar to cells that escape mitotic failure-induced apoptosis and contribute to the risk of tumorigenesis.

In summary, this study shows that CEBPD can cause genomic instability, which provides a new mechanism by which it may promote tumorigenesis. The dual role of CEBPD may in part underlie the dual functions of TNFα in both suppression and promotion of cancer progression and the complex role of inflammation in cancer.

Acknowledgment—We thank Dr. Esta Sterneck for critical reading of the manuscript.

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