A Splice Variant of Stress Response Gene ATF3 Counteracts NF-κB-dependent Anti-apoptosis through Inhibiting Recruitment of CREB-binding Protein/p300 Coactivator*

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Activating transcription factor (ATF)3 plays a role in determining cell fate and generates a variety of alternatively spliced isoforms in stress response. We have reported previously that splice variant ATF3ΔZip2p, which lacks the leucine zipper region, is induced in response to various stress stimuli. However, its biological function has not been elucidated. By using cells treated with tumor necrosis factor-α and actinomycin D or cells overexpressing ATF3ΔZip2, we showed that ATF3ΔZip2 sensitizes cells to apoptotic cell death in response to tumor necrosis factor-α, at least in part through suppressing nuclear factor (NF)-κB-dependent transcription of anti-apoptotic genes such as cIAP2 and XIPAP. ATF3ΔZip2 interacts with a p65 (RelA)-cofactor complex containing CBP/p300 and HDAC1 at NF-κB sites of the proximal promoter region of the cIAP2 gene in vivo and down-regulates the recruitment of CBP/p300. Our study revealed that ATF3ΔZip2 counteracts anti-apoptotic activity of NF-κB, at least in part, by displacing positive cofactor CBP/p300 and provides insight into the mechanism by which ATF3 regulates cell fate through alternative splicing in stress response.

Alternative splicing of pre-mRNAs encoding transcription factors is one of the common mechanisms for generating the complexity and diversity of gene regulation (1). A variety of functionally distinct isoforms is generated from a single gene by use of different combinations of splice junctions, and may play role in coordinating gene regulation in response to cells to various environmental stimuli. However, the altered gene regulatory function and the biological implication of spliced variants still remain elusive.

Activating transcription factor (ATF)3 is a member of the ATF/CREB family of basic leucine zipper-type transcription factors. It is induced upon exposure of cells to a variety of physiological and pathological stimuli (2), and it is thought to have cell-detrimental effects, such as cell cycle arrest and apoptosis (2–5). Ectopic expression of ATF3 in heart, liver, and pancreatic β-cells causes cardiac enlargement, liver cell dysfunction, and diabetes, respectively (6–8), supporting cytopathic activity of ATF3. On the other hand, ATF3 is also rapidly induced in the regenerating liver (9) or in cells treated with serum or growth-stimulating factors (10). It can support cell survival of endothelial cells (11) and protect neuronal cells from c-Jun N-terminal kinase-induced cell death (12). Furthermore, we have shown recently that ATF3 is a target of the proto-oncogene c-myc in serum-induced cell proliferation (13). Thus, ATF3 may function differently depending on the cellular context.

ATF3 is composed of 181 amino acids, and the basic region and leucine zipper domain from 88 to 147 amino acids are required for dimer formation and specific DNA binding (14, 15). The homodimer of ATF3 represses transcription from various promoters with ATF sites (14–16), whereas heterodimers with c-Jun or JunB activate transcription (15). In addition to the heteromeric complexity, various spliced isoforms of ATF3 may further generate functional diversity in different cellular context. ATF3ΔZip has been isolated in serum-stimulated HeLa cells (14), whereas ATF3ΔZip2c and ΔZip3 were identified in amino acid-deprived cells (17). Another isoform, ATF3b, is implicated in mediating cAMP signaling of proglucagon transcription in pancreatic α-cells (18). We have isolated previously ATF3ΔZip2a and -b from cells treated with various stimuli such as A23187, TNF-α, endoplasmic reticulum stress, or oxidative stress (19). These two isoforms encode the C-terminally truncated protein of 135 amino acids, which shares the N-terminal 116 amino acids with the full-length ATF3 but contains novel 19 amino acids at the C terminus. ATF3ΔZip2 lacks the leucine zipper domain and thus is capable of DNA binding. It is localized in the nucleus and counteracts the transcriptional regulation by full-length ATF3 in a reporter assay (19). However, the functional role and biological significance of this spliced isoform in stress response are unknown.

Nuclear factor-κB (NF-κB) is a transcription factor that plays a critical role in the expression of genes involved in immune and inflammatory responses (20, 21). It is located in the cytoplasm of nonstimulated cells as a form bound to IκB, but it is released by degradation of IκBα and enters the nucleus in response to stimuli. There are five known members of the mammalian NF-κB/Rel family: p65 (RelA), c-Rel, RelB, p50, and p52. p65, RelB, and c-Rel are transcriptionally active, whereas p50 and p52 function as the DNA-binding subunits. Recent analysis of NF-κB-deficient mice and cells has further clarified its role in inhibiting apoptosis (22).

For instance, RelA-deficient mouse fibroblasts showed increased sensitivity to pro-apoptotic stimuli such as TNF-α, which by itself is a poor inducer of apoptosis unless accompanied by inhibitors of new RNA or protein synthesis (23, 24). NF-κB induces the expression of a number of genes including cIAPs, cFLIP, A1, TRAF1, and TRAF2, whose products can inhibit apoptosis (22, 25). Among them, cIAP2...
contains two functional κB sites in the promoter (26), and the cIAP2 protein directly binds and inhibits effector caspases, such as caspase 3 and 7, as well as prevents activation of pro-caspase 6 and 9 (27). Another cIAP, X chromosome-linked IAP (XIAP), also inhibits TNF-induced apoptosis of cells expressing the 1κBα super-repressor mutant (28). NF-κB has also been shown to inhibit apoptosis by DNA-damaging agents, including cancer therapeutics (29, 30).

It is well known that NF-κB-dependent transcription requires the recruitment of multiple coactivator proteins. CBP and its homologue cIAP2 is also required for the NF-κB-activated transcription (34), whereas steroid receptor coactivator-1 interacts with the p50 subunit of NF-κB to potentiate the transcription (35). Inversely, HDAC1/2 (36), SNIP1, and HDAC4 (37), and PIAS3 (38) interact with p65 to negatively regulate gene expression. More recently, the candidate tumor suppressor gene product Zip2 and a bacterial expression plasmid vector pMEFLAGATF3, which is generated after stress-activated alternative splicing, represses NF-κB activity and may play pro-apoptotic role in stress response.

In this report, we explored the functional role of ATF3ΔZip2 by using TNF-α-stimulated cells. It was demonstrated that ATF3ΔZip2, but not full-length ATF3, sensitizes cells to apoptotic cell death, partly by suppressing the expression of NF-κB-dependent anti-apoptotic genes cIAP2 and XIAP. ATF3ΔZip2 is further shown to bind directly to the p65 subunit of NF-κB and down-regulate the CBP/p300 recruitment. Our study provides evidence that ATF3ΔZip2, which is generated through stress-activated alternative splicing, represses NF-κB activity and may play pro-apoptotic role in stress response.

**EXPERIMENTAL PROCEDURES**

*Plasmids, Antibodies, and Reagents* — A mammalian expression plasmid pMEFLAGATF3ΔZip2 and a bacterial expression plasmid encoding the GST fusion of ATF3ΔZip2 were as described (19). Mammalian expression vectors for the p65 and p50 subunits of NF-κB were generous gifts from Dr. Fujita at the Tokyo Metropolitan Institute, Tokyo, Japan. Plasmid vector for expressing p65 in *Escherichia coli* was constructed by subcloning into pET21a (Novagen). Expression plasmids, pCI-cIAP2 and pCI-XIAP, were prepared by subcloning cDNAs encoding human cIAP2 (GenBank™ accession number NM182962) and XIAP (GenBank™ accession number NM001167) into the pCIneo vector (Clontech), respectively. A bacterial expression plasmid pGST-CBP2N encoding a GST fusion of the N-terminal 117–738 amino acids of CBP and a mammalian expression vector pCDNA3/mCBP-HA for mouse CBP (40, 41) were generous gifts from Dr. Nakajima, St. Marianna University School of Medicine, Tokyo, Japan. Rabbit anti-ATF3 (C-19), anti-ATF2 (N-96), anti-p65, anti-p50, anti-1κBα (FL), anti-cIAP2 (H-85), anti-cBP (C-20), anti-p300 (C-20), goat anti-HDAC1 (C-19), and monoclonal anti-GST (B-14) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-FLAG M2 and anti-β-tubulin antibodies were from Sigma. Anti-ATF3 antibody was also generated by immunizing rabbits with full-length ATF3 expressed in *E. coli*. Recombinant human NF-κB-α was purchased from Genzyme. Other chemicals were reagent grade.

*Cell Culture and Transient Expression* — TGR1 or U2OS cells treated as indicated were harvested, washed in PBS, and resuspended in lysis buffer (50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 10 μg/ml each leupeptin and aprotinin, 200 μg sodium vanadate, 100 μM NaF, and 10% glycerol). After incubation on ice for 10 min, the cells were centrifuged at 10,000 rpm for 10 min, and the supernatants were taken as whole cell extract. The amounts of protein were measured by the Lowry method using bovine serum albumin as standard (44). Cell extracts (20 μg of protein) were separated on an SDS-PAGE, transferred onto a nitrocellulose membrane, and subjected to Western blot using the protocol of ECL kit (Amersham Biosciences).
Proapoptosis by Stress Response Gene ATF3 Splice Variant

Nuclear Translocation of NF-κB—For nuclear translocation of NF-κB, U2OS cells (1 × 10^5 cells) were infected with AdATF3ΔZip2 or AdLacZ at 25 m.o.i. for 24 h and then treated with 20 ng/ml TNF-α. At the time points indicated after TNF-α treatment, cells were harvested and suspended in buffer of 10 mM Hepes-KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 0.1% Nonidet P-40. Cytoplasmic fraction was obtained by centrifugation of cells at 10,000 rpm for 10 min. The resultant pellet containing nuclei was washed by the above buffer, dissolved in sample buffer for SDS-PAGE, and subjected to Western blot analysis.

Electrophoretic Mobility Shift Assay—U2OS cells (1.5 × 10^7 cells) were infected with 25 m.o.i. of AdATF3ΔZip2 or AdLacZ for 24 h and then treated with 20 ng/ml TNF-α for 3 h. Nuclear extracts were then prepared according to the method of Dignam et al. (45). Nuclear extracts (2 μg of protein) were incubated in 20 μl of binding buffer (10 mM Hepes-KOH, pH 7.9, 60 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 0.1 mM PMSF, 5 mM β-mercaptoethanol) containing 0.5 μg of poly(dI-dC) and 0.5 ng of radiolabeled DNA probe at room temperature for 30 min. For supershift assays, anti-p65 or anti-p50 antibody (0.1 μg each) was added and incubated for another 30 min. An oligonucleotide DNA probe for consensus NF-κB (5'-AGTTGAGGG-GACTTTCCCAGGC-3') was obtained from Santa Cruz Biotechnology and radiolabeled with 25 μCi of [γ-32P]ATP (6,000 Ci/mmol) and polynucleotide kinase. Mutant oligonucleotide (sc-2511) used for the competition experiment was also product of Santa Cruz Biotechnology.

Binding mixture was applied onto a 5% nondenatured polyacrylamide slab gel in Tris borate-EDTA buffer. After electrophoresis, the gel was stained with 0.1% Amido black and then destained. The DNA was transferred to a nylon membrane and autoradiographed. Nuclei were isolated by the method of Dignam et al. (45). Subsequently, nuclear extracts were incubated with recombinant NF-κB and GST-Zip2 fusion protein for 3 h. After further incubation with 30 μg/ml actinomycin D for 3 h, whole cell extracts were prepared and incubated with 0.5 μg each of anti-p65 or anti-FLAG antibody at 4 °C for 3 h, followed by incubation with 30 μl of protein G-Sepharose (Amersham Biosciences) for 2 h. The resulting immunocomplex was washed and subjected to Western blot analysis.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed according to the protocol supplied by Upstate (Charlottesville, VA). U2OS cells (4 × 10^6 cells) were infected with AdATF3ΔZip2 at 25 m.o.i. for 24 h and treated with 20 ng/ml TNF-α. At 8 h post-stimulation, cells were crosslinked with 1% formaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were collected and lysed with 2 ml of SDS lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) containing a protease inhibitor mixture (Roche Applied Science), and sonicated to DNA lengths of ~250 bp. After centrifugation at 13,000 rpm for 10 min, supernatants were diluted 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). Immunoprecipitations were then performed with the indicated antibodies. ChIP DNA was detected using standard PCR with the following primer pairs for the different regions of the cIAP2 gene promoter: the proximal promoter region at −260 to +55 5'-GATAAGTCGGCAGAACAGGCACAAG-3' and the distal promoter region at −538 to −260 5'-CTCCCTTACCTTCTG-3' and 5'-GGTGTCATATCCGCCGATTTAC-3'.

Binding Assay of Zip2 Complex—For in vivo binding, U2OS cells (1 × 10^7 cells) were infected with AdATF3ΔZip2 at 25 m.o.i. for 24 h and then treated with 20 ng/ml TNF-α for 3 h. Alternatively, U2OS cells stably expressing ATF3ΔZip2 siRNA or control GFP siRNA were treated with 10 ng/ml TNF-α and 0.05 μg/ml actinomycin D for 3 h. Whole cell extracts were prepared and incubated with 0.5 μg each of anti-p65 or anti-FLAG antibody at 4 °C for 3 h, followed by incubation with 30 μl of protein G-Sepharose (Amersham Biosciences) for 2 h. The resulting immunocomplex was washed and subjected to Western blot analysis.

RESULTS

Ectopic Expression of ATF3ΔZip2-sensitized Cells to TNF-α-induced Cell Death—Full-length ATF3 and its splice variant ATF3ΔZip2 are induced in response to various stimuli (19). To investigate the role of
ATF3ΔZip2 in determining cell fate, we first overexpressed it by adenovirus-mediated gene transfer, and we examined cell viability after treatment with TNF-α. As shown in Fig. 1A, expression of full-length ATF3 or control LacZ had no effect on the viability of TGR1 cells upon TNF-α stimulation, consistent with previous observations that TNF-α does not induce cell death if not provided with RNA or protein synthesis inhibitors. By contrast, viability of TNF-α-treated TGR1 cells was significantly decreased when ATF3ΔZip2 was expressed. However, no change in cell viability was observed when ATF3ΔZip2 was expressed without TNF-α treatment, suggesting that ATF3ΔZip2 alone is not sufficient to trigger cell death. A microscopic study of the cells expressing ATF3ΔZip2 revealed an appearance of cells with fragmented nuclei (Fig. 1B, left panel), and the analysis of genomic DNA showed the formation of nucleosomal DNA ladder (Fig. 1B, right panel), providing evidence for apoptotic cell death. Specific cleavage of poly(ADP-ribose) polymerase, which is known to occur in apoptotic cells, was also observed in these cells (data not shown). Next, we further expressed ATF3ΔZip2 by using plasmid expression vector to minimize nonspecific effects of adenoviral gene transfer. As shown in Fig. 1C, the ATF3ΔZip2 expression caused the appearance of cells with condensed or fragmented nuclei in transfected GFP-positive cells. In control cells transfected with empty vector, only a basal level of apoptotic cells was observed compared with empty vector, *, p < 0.05. In the right panel, GFP fluorescence, DAPI staining, and their merge were shown for cells transfected with empty (upper panel) or pMEFLAGATF3ΔZip2 vector (lower panel), respectively.

NF-κB Was Normally Activated in ATF3ΔZip2 Cells after TNF-α Stimulation—NF-κB is one of the major anti-apoptotic transcription factors that inhibit TNF-α-induced apoptosis (22–24). Thus, we next examined the activation of NF-κB in ATF3ΔZip2 cells after TNF-α stimulation. Fig. 2A, upper panel, showed that IκBα was rapidly degraded in ΔZip2 cells with kinetics similar to control LacZ cells.
Nuclear import of the p65 subunit of NF-κB was then examined. As illustrated in Fig. 2A, lower panel, p65 was efficiently translocated from cytoplasmic to nuclear fraction in ΔZip2 cells, and its kinetics showed no difference from control LacZ cells. These data suggested that NF-κB is normally activated and translocated into nuclei in cells expressing ATF3ΔZip2 after TNF-α stimulation. In Fig. 2B, DNA binding activity of NF-κB was examined. In both control and ΔZip2 cells, the binding activity of NF-κB significantly increased in response to TNF-α, and its extent of activation was apparently similar in both cells. Anti-p65 antibody caused a supershift of the NF-κB complex, whereas anti-p50 antibody induced a slight supershift. These data indicate that NF-κB activation, including IkBα degradation, nuclear translocation, and DNA binding of NF-κB, is not affected by ATF3ΔZip2 in TNF-α-stimulated cells.

**Induced Expression of cIAP2 and XIAP Was Down-regulated in ATF3ΔZip2 Cells after TNF-α Treatment**—We next performed a microarray analysis of cDNAs to search for gene(s) responsible for increased sensitivity to TNF-α-induced apoptosis of ATF3ΔZip2-expressing cells. Among the 21,576 genes examined, 1133 genes (5.2%) and 398 genes (1.8%) were down- and up-regulated in ΔZip2 cells with more than a 2-fold difference from control LacZ cells after TNF-α treatment. The analysis revealed that two anti-apoptotic genes, cIAP2 and XIAP, were significantly down-regulated in ΔZip2 cells. As illustrated in Fig. 3A, RT-PCR analysis confirmed that expression of these two genes was remarkably inhibited in ΔZip2 cells. On the other hand, the expression of other anti-apoptotic factors, TRAF2 and FLIP, was less affected.

**Suppression by ATF3ΔZip2 of cIAP2 gene promoter-dependent and NF-κB-dependent reporter activity.** U2OS cells (5 × 10⁶ cells) were transfected with 2 μg of pMEFLAGATF3ΔZip2 and 0.3 μg of pCMV-GFP with or without indicated the amounts of pMEFLAGATF3ΔZip2. In the left panel, cells were stimulated by TNF-α and assayed for luciferase activity as under “Experimental Procedures.” Cells were also cotransfected with 0.25 μg each of p65 and p50 expression vectors, and the luciferase activity was assayed (right panel). Data were means ± S.D. of three independent experiments. In all assays, significant inhibition of reporter activity was observed by ATF3ΔZip2; *p < 0.05.

**Recruitment of ATF3ΔZip2 to the proximal promoter region of cIAP2 gene in TNF-α-stimulated cells.** U2OS cells (4 × 10⁷ cells) were infected with AdLacZ or AdATF3ΔZip2 at 25 m.o.i. for 24 h and treated with TNF-α. Cells were harvested before (left lane) and after TNF-α treatment for 8 h (right lanes) and cross-linked with formaldehyde, and ChIP assay was performed using anti-FLAG, anti-p65 antibodies, or control IgG as under “Experimental Procedures.” Immunoprecipitated DNA was amplified by PCR for the proximal (upper panels) or distal (lower panel) promoter regions of the human cIAP2 gene.
ATF3\textsuperscript{Zip2} Suppresses cIAP2 Gene and NF-\kappaB-dependent Reporter Activity—Human cIAP2 gene contains at least three NF-\kappaB-binding motifs in the proximal promoter region, and two of them are responsible for NF-\kappaB-dependent transcription in response to TNF-\alpha (26). Therefore, we examined if the cIAP2 induction by TNF-\alpha was affected by ATF3\textsuperscript{Zip2} by using reporter assay. Fig. 4A, upper panel, illustrates the cIAP2 gene promoter from -500 to +30 containing three NF-\kappaB sites and a TATA sequence. As shown in Fig. 4A, lower left panel, ATF3\textsuperscript{Zip2} had no effect on the basal activity of the cIAP2 gene promoter in the absence of TNF-\alpha. In the presence of TNF-\alpha, the activity was markedly increased, but this activity was significantly inhibited by coexpression of ATF3\textsuperscript{Zip2} as well as various C-terminal deletions of ATF3 as GST fusions, and their activity of binding to p65 was assayed by GST pulldown as in B, left panel. Input was 10% of total.

Assays using the NF-\kappaB-dependent reporter gene in which four tandem repeats of \kappaB sites were located upstream of the thymidine kinase gene promoter. As in Fig. 4B, the reporter activity was significantly suppressed by ATF3\textsuperscript{Zip2} in both TNF-\alpha-stimulated and p65/p50-cotransfected cells. From these data, it is indicated that ATF3\textsuperscript{Zip2} downregulates the TNF-\alpha-induced cIAP2 gene activation, most likely through inhibiting NF-\kappaB transactivation.

ATF3\textsuperscript{Zip2} Binds to cIAP Gene Promoter in TNF-\alpha-stimulated Cells—Data above suggested that ATF3\textsuperscript{Zip2} may inhibit cIAP2 gene expression by interacting with the gene promoter region. Thus, we examined whether ATF3\textsuperscript{Zip2} is recruited to the cIAP2 gene promoter in TNF-\alpha-stimulated cells by ChIP assay. In Fig. 5, the proximal promoter region containing NF-\kappaB sites, but not the distal promoter region, was efficiently immunoprecipitated by anti-p65 antibody in TNF-\alpha-stimulated cells, consistent with the report that the NF-\kappaB element(s) are involved in TNF-\alpha-mediated induction of cIAP2 (26). When anti-FLAG antibody was used to immunoprecipitate FLAG-tagged ATF3\textsuperscript{Zip2}, the
proximal region, but not the distal region, was also efficiently immunoprecipitated. In the absence of TNF-α treatment, however, neither anti-p65 nor anti-FLAG antibody did not immunoprecipitate the promoter, suggesting that the recruitment of ATF3ΔZip2 as well as p65 is TNF-α-dependent. Taken together, it is indicated that ATF3ΔZip2 is recruited to the proximal region of the clAP2 gene in response to TNF-α treatment.

ATF3ΔZip2 Is Recruited but CBP/p300 Is Displaced from the p65-Cofactor Complex in TNF-α-stimulated Cells—NF-κB forms the cofactor complex consisting of positive and negative regulators, including CBP/p300 and HDAC, thereby regulating NF-κB-dependent transcription. Thus, we next examined whether ATF3ΔZip2 interacts with this complex. For this purpose, U2OS cells expressing ATF3ΔZip2 were stimulated with TNF-α, and their cell extracts were subjected to immunoprecipitation assay. As shown in Fig. 6A, left panel, FLAG-tagged ATF3ΔZip2 as well as CBP/p300 and HDAC1 were immunoprecipitated specifically with anti-p65 antibody but not with control IgG, indicating that ATF3ΔZip2 interacts with the p65-cofactor complex. More intriguingly, the amount of CBP/p300 in the complex, but not of HDAC1, was significantly lower in ΔZip2 cells than in control LacZ cells, despite the fact that there was no difference in the total amount of CBP/p300 in the cell extracts. When ATF3ΔZip2 was inversely immunoprecipitated by using anti-FLAG antibody, p65 was specifically immunoprecipitated (Fig. 6A, right panel). These data strongly support that ATF3ΔZip2 is a component of the p65-cofactor complex, and CBP/p300 is displaced from this complex in TNF-α-stimulated cells.

ATF3ΔZip2 Directly Interacts with p65 in Vitro—We next sought to identify the component(s) of the NF-κB-cofactor complex with which ATF3ΔZip2 directly interacts in TNF-α-stimulated cells. To this end, an in vitro binding study was performed using recombinant ATF3ΔZip2 and p65 proteins. As shown in Fig. 6B, GST-ATF3ΔZip2 and His-tagged p65 directly interacted each other, as detected by GST-pulldown (left panel) or nickel-agarose beads (right panel) assay. We further performed a deletion study of ATF3 to delineate the region responsible for p65 binding. As illustrated in Fig. 6C, ΔZip2 bound p65 inversely as compared with control ATF3 Zip2, whereas dC34 mutant that deletes the C-terminal 34 amino acids but shares the basic region with ATF3ΔZip2 bound p65 only weakly. By contrast, the full-length ATF3 and the C-terminal deletions dC96 and dC141 showed no significant p65 binding. The data combined strongly indicate that ATF3ΔZip2 directly binds p65, and the unique C-terminal 19-amino acid sequence of ATF3ΔZip2 plays a role in the interaction.
ATF3Zip2 Counteracts CBP in p65 Binding, Reporter Assay, and Cell Death—It has been shown that the p65 subunit of NF-κB directly interacts with CBP or p300 (31, 32), and the data above showed that ATF3Zip2 also binds to p65. By contrast, no significant binding of ATF3Zip2 with CBP was observed in our assay (data not shown). Therefore, we examined whether ATF3Zip2 affects the association of p65 with CBP in vitro. For this purpose, p65 was incubated with GST-CBP2N, a GST fusion with the N-terminal portion of CBP from 117 to 738 amino acids, in the absence or presence of ATF3Zip2. As shown in Fig. 7A, left panel, CBP2N specifically interacted with p65 in the absence of ATF3Zip2 as reported (31). In the presence of ATF3Zip2, however, the binding of p65 to CBP was suppressed in a dose-dependent manner, as detected by GST-pulldown (Fig. 7A, upper right panel) or nickel-agarose beads (lower right panel) assay. Furthermore, under this assay condition, ATF3ΔZip2 was recruited into the p65 complex. By contrast, the control LacZ protein had no effect. These results indicated that ATF3ΔZip2 interferes with the association of p65 and CBP, possibly through its direct binding to p65. We next determined whether CBP functionally antagonizes ATF3ΔZip2 activity in both reporter and cell death assay. Fig. 7B showed that coexpression of CBP alleviated the repression of reporter activity by ATF3ΔZip2 and restored the p65-dependent reporter activity. In the cell death assay, CBP relieved the cell death induced by ATF3ΔZip2 in TNF-α-stimulated cells (Fig. 7C). These results, taken together, strongly indicate that the transcriptional repression by ATF3ΔZip2 depends on its ability to compete with CBP for p65 binding, and this suppression is responsible for the pro-apoptotic activity of ATF3ΔZip2.

Knockdown of Endogenous ATF3ΔZip2 Restored CBP/p300 Binding and Attenuated Cell Death in TNF-α/Actinomycin D-Treated Cells—To test whether the endogenous level of ATF3ΔZip2 plays a role in vivo, U2OS cells were treated with TNF-α and actinomycin D. As shown in Fig. 8A, the ATF3Zip2 expression was remarkably increased, and cell death was also concomitantly induced in these cells. Next, an RNA interference approach was employed to determine the effects of depletion of endogenous ATF3ΔZip2. U2OS cells stably expressing ATF3ΔZip2 siRNA showed a significant decrease of adenovirus-driven expression of ATF3ΔZip2 protein but not full-length ATF3. Control cells for empty vector or GFP siRNA had no effect (Fig. 8B), demonstrating specific knockdown of ATF3ΔZip2 by RNA interference. In Fig. 8C, cells were treated with TNF-α and actinomycin D. ATF3ΔZip2 expression was specifically reduced in cells harboring ATF3ΔZip2 siRNA, whereas control empty or GFP siRNA cells induced ATF3ΔZip2 protein. Under this condition, cell death was partly but significantly suppressed in ATF3ΔZip2 siRNA cells, compared with control empty or GFP siRNA cells. We further examined the effect of ATF3ΔZip2 knockdown on the p65-cofactor complex. As shown in Fig. 8D, the addition of actinomycin D reduced the recruitment of CBP/p300 compared with TNF-α-treated cells, but it had no effect on the recruited amount of HDAC1. In contrast, the amount of CBP/p300 was significantly restored in cells with ATF3ΔZip2 siRNA, whereas control empty or GFP siRNA cells showed no such effect. The data combined clearly indicated that the endogenous level of ATF3ΔZip2 protein plays a role in regulating p65 binding to CBP/p300 and cell death in response to TNF-α and actinomycin D.

**DISCUSSION**

The stress response gene *ATF3* is induced by various stress stimuli, and its activation is associated with cell death (2–5), survival (11, 12), and cell proliferation (9, 10, 13). Thus, ATF3 can function differently depending on cellular context. In this study, we reported that ATF3ΔZip2, a splice variant, gains a novel activity of suppressing NF-κB-dependent transcription, thereby sensitizing cells to apoptosis by TNF-α and actinomycin D. ATF3ΔZip2 inhibited the p65 binding to coactivator CBP in both overexpressed or endogenous levels, thus attenuating κB-dependent transcription. Because ATF3ΔZip2 is not capable of binding to DNA (19), this inhibitory activity of ATF3ΔZip2 is considered to be different from the repressive effect of the full-length ATF3 (14–16). Homo- or heterodimers of full-length ATF3 bind to the ATF/CRE motif of gene promoters and stabilize the repressive cofactors as proposed in the inhibitory cofactor model (14). By contrast, ATF3ΔZip2 but not full-length ATF3 directly interacted with the p65 subunit of NF-κB, and this
interaction suppressed the p65 binding with coactivator CBP (Figs. 6 and 7). ATF3ΔZip2 lacks the C-terminal 65-amino acid region of the full-length ATF3 but instead has novel sequence of 19 amino acids at the C terminus as shown in Fig. 6C (19). Our deletion study delineated the p65 binding activity of ATF3ΔZip2 to the C-terminal region, suggesting a role of the C-terminal unique sequence, whereas ΔC34 mutant bound p65 very weakly (Fig. 6C). It is interesting to note that ATF3ΔZip2, at the C-terminal junction, creates the LQKLp sequence that resembles the coregulator signature motif LXXLL that is present at the N terminus of PIAS3, another p65-binding repressor (38). It may be possible that ATF3ΔZip2 interacts with p65 through this novel sequence. Alternatively, ATF3ΔZip2 lacks the ability of dimer formation because of its loss of the leucine zipper region. This conformational change of ATF3ΔZip2 may expose a novel structural surface for p65 binding, which is otherwise masked in the dimer structure of the full-length ATF3. Indeed, we found no significant interaction between the full-length ATF3 and p65 in vitro (Fig. 6C), consistent with a previous report (46). Thus, it is highly likely that ATF3ΔZip2 gains novel structure for p65 binding through alternative splicing.

The coactivators CBP/p300 interact with a variety of transcriptional activators and repressors to provide a physical link between these factors and the basal transcription machinery. CBP/p300 also recruits other histone acetyltransferases such as p/CAF, whose activity is required for NF-κB-dependent transcription (34), and coordinate transcriptional pathways with chromatin remodeling via their intrinsic histone acetyltransferase activity. In this study, we showed that ATF3ΔZip2 inhibited the association of CBP with p65 both in vivo and in vitro. On the other hand, there was no difference in HDAC1 binding to the p65 complex in ΔZip2 cells, compared with control (Fig. 6A). This may represent one of mechanisms by which ATF3ΔZip2 causes repression of NF-κB-dependent transcription, because the balance between CBP/p300 and HDAC1/2 is critical for transcriptional outcome. We speculate that recruitment of ATF3ΔZip2 into the p65 complex inhibits the association of CBP but not of HDAC1. This imbalance of CBP against HDAC1 may result in dominant histone deacetylase activity and thereby lead to repression of gene transcription. However, at this moment, it is not clear how the binding of ATF3ΔZip2 to p65 causes impaired interaction of CBP/p300. CBP/p300 interacts with p65 at their N- and C-terminal sequences, and the binding at the N terminus is observed at a higher salt concentration than that at the C terminus (31, 32). As in Fig. 7A, ATF3ΔZip2 efficiently displaced the binding of CBP at the N terminus, suggesting that ATF3ΔZip2 may competitively bind to the same binding site of p65 as CBP/p300. Other possibility is that ATF3ΔZip2 binding causes conformational change of p65 and inhibits its interaction with CBP. Detailed molecular mechanism for CBP displacement by ATF3ΔZip2, however, must await further investigation.

The regulation of NF-κB-dependent gene transcription through p65 binding is also reported for several factors. For instance, SNIP1 (37) and PIAS3 (38) bind to p65 and down-regulate NF-κB-dependent gene transcription. SNIP1, an inhibitor of the transforming growth factor-β signaling pathway that depends on CBP/p300, also binds to the C/H domain of CBP/p300 and thereby competes with each other for binding. In contrast, we could not find significant binding of ATF3ΔZip2 to CBP (data not shown). Therefore, ATF3ΔZip2 and SNIP1 may recognize p65 in a different mechanism. More recently, candidate tumor suppressor gene protein ING4 (39) and arginine methyltransferase CARM1 (47) are also reported to bind to p65 and repress and activate the κB-dependent gene expression, respectively. Our study further supports the crucial role of p65 in regulating the association of multiple cofactors mediating between the κB site(s) of gene promoter and transcription machinery.
Proapoptosis by Stress Response Gene ATF3 Splice Variant