Inhibition of NF-κB Acetylation and its Transcriptional Activity by Daxx

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We propose a biochemical mechanism by which Daxx modulates NF-κB transcriptional activity. Both chromatin immunoprecipitation (ChIP) assay and electrophoretic mobility shift assay (EMSA) have confirmed Daxx-mediated repression of transcriptional competence of NF-κB in HeLa cells. Overexpression of Daxx repressed the expression of NF-κB-regulated genes such as IkBα and IL8. Co-immunoprecipitation assay revealed the existence of intermolecular association between endogenous Daxx and p65 subunit of NF-κB stimulated by TNFα. Here, we suggest that Daxx-mediated repression of NF-κB transactivation correlates with the inhibition of p65 acetylation by Daxx. Based on the finding that the Daxx binding N-terminal side of p65 includes the major sites of acetylation mediated by p300/CBP, we further propose that the physical interaction between Daxx and p65 provides a functional framework for the inhibition of p65 acetylation by p300/CBP and subsequent repression of NF-κB transcriptional activity.

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Introduction

Daxx was initially identified as a protein associated with the death domain of Fas receptor and thought to enhance Fas-mediated apoptosis.¹ While the function of Daxx in apoptosis as a pro-apoptotic or anti-apoptotic molecule remains a matter of dispute, the ability of Daxx to function as a transcriptional repressor has been presented. Hollenbach et al.² have first shown that Daxx binds to transcriptional factor Pax3 and represses its transcriptional activity. The depletion of Daxx by means of RNA interference has also suggested the ability of Daxx to repress the transcriptional activities of NF-κB and E2F1 transcription factors.³

Abbreviations used: ChIP, chromatin immunoprecipitation; ESMA, electrophoretic mobility shift assay; HDAC3, histone deacetylase 3; GST, glutathione S transferase; CBP, CREB binding protein; FITC, fluorescence isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

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The NF-κB transcription factor is involved in the expression of numerous genes participating in cell growth, development, apoptosis, carcinogenesis, inflammation and immune response.⁴ The long list of NF-κB-regulated gene products includes cytokines, chemokines, inducible enzymes, adhesion molecules, anti-microbial peptides and anti-apoptotic proteins.⁵ NF-κB proteins comprise a group of structurally related and evolutionally conserved proteins. The five known mammalian NF-κB genes encode p65 (also known as RelA), p105/50, p100/52, RelB and c-Rel proteins. These members of NF-κB family proteins exist in homo- or heterodimers to form a transcriptionally competent or repressive complex and are generally referred to as NF-κB.⁶ NF-κB activation is regulated by a family of NF-κB inhibitors, such as IκBα, IκBβ, IκBε, IκBγ and Bcl-3.⁴ In an inactive state, IκB is present in the cytoplasm as a heterodimer consisting of p65, p105/p50 and IκB subunits. In response to an activation signal, for example, TNFα signal, IκBα is phosphorylated by IκB kinase (IKK), ubiquitinated and degraded through the proteasomal pathway. Degradation of IκBα liberates the NF-κB heterodimer and unmasks the nuclear localization signal present on the p65 subunit of NF-κB leading to the rapid translocation of the active NF-κB heterodimer to the nucleus.
of NF-κB complex into the nucleus. Following the nuclear import of NF-κB, NF-κB is subjected to bind to a specific sequence in DNA in order to initiate transcription of NF-κB-regulated genes.4,6

Although the degradation of IκBα and nuclear import of NF-κB are important for the regulation of NF-κB activation, the capacity of nuclear NF-κB to drive transcription is also regulated by post-translational modifications of NF-κB, particularly p65. Following the degradation of IκBα, p65 is phosphorylated either in the cytoplasm or in the nucleus by different kinases including PKA7, CKII,8,9 PCK,10 MSK111 and IKKs.12,13 Suggested roles of p65 phosphorylation in transcriptional activation of NF-κB target gene expression involve the recruitments of p300/CREB binding protein (CBP) and PCAF (p300/CBP-associated factor) to nuclear NF-κB. Multiple studies have demonstrated that both p300/CBP and PCAF are transcriptional co-activators that enhance NF-κB transcriptional activity by

**Figure 1.** Daxx-mediated repression of NF-κB transcriptional activity. (a) HeLa cells (5×10⁵) were co-transfected with expression vector for p65 (0.1 μg) and increasing amounts of expression vector for Flag-Daxx (0.1, 0.2 and 0.4 μg) together with luciferase reporter plasmid (0.1 μg). Total amounts of transfected DNA were kept constant by adding carrier vector. After incubation for 24 h, the activities of luciferase reporter driven by the NF-κB responsive element were determined. All the values were normalized for transfection efficiencies based on β-galactosidase activities and were presented as the percent of maximal activity. The transcriptional activity of transiently expressed p65 in the absence of Daxx was set to 100%. Error bars represent the standard deviation from at least three independent experiments. (b) HeLa cells (5×10⁵) were co-transfected with expression vector for Flag-Daxx (0.1, 0.2 and 0.4 μg) and luciferase reporter plasmid (0.1 μg). At 18 h after co-transfection, HeLa cells were treated with 20 ng/ml of TNFα for 6 h. The transcriptional activity of endogenous NF-κB induced by TNFα in the absence of Daxx was set to 100%. (c) HeLa cells (2×10⁶) were transfected with varying amounts of expression vector for Flag-Daxx (2, 4 and 8 μg). At 24 h after transfection, cells were treated with 20 ng/ml of TNFα for 1 h. The binding of 32P-labeled oligonucleotide probe to NF-κB was examined by EMSA. (d) NF-κB binding to endogenous promoters of IκBα and IL8 was assessed by ChIP assay. HeLa cells (2×10⁶) were transfected with the Flag-Daxx expression vector (2 μg). After incubation for 24 h, cells were treated with 20 ng/ml of TNFα for 30 min and processed for ChIP assay using p65 antibody. Immunoprecipitated chromatin was analyzed by PCR using the specific primer for IκBα or IL8. The image of PCR products was presented in reversed black and white in which the DNA band is in black.
acetylated p65 then subsequently associates with distinct histone deacetylase 3 (HDAC3). Deacetylation of p65 by HDAC3 allows its binding to the newly synthesized IκBα induced by NF-κB. IκBα binding to deacetylated NF-κB leads to a rapid nuclear export of NF-κB complex, thereby terminating NF-κB transactivation.14

Here, we propose a biochemical pathway in which Daxx interacts with NF-κB, inhibits NF-κB acetylation and represses NF-κB transcriptional activity.

Results

Repression of NF-κB transcriptional activity by Daxx

The effects of Daxx expression on NF-κB transcriptional activity were examined by measuring luciferase activity driven by the synthetic NF-κB response element (3×κB). Overexpression of Daxx repressed transcriptional activity of the p65 sub-unit of NF-κB in co-transfected HeLa cells (Figure 1(a)). Transcriptional activity of endogenous NF-κB was also repressed by ectopically expressed Daxx in TNFα-treated cells (Figure 1(b)). The effects of Daxx expression on DNA binding ability of NF-κB were examined by electrophoretic mobility shift assay (EMSA). Daxx inhibits NF-κB binding to its cognate promoter DNA sequence (Figure 1(c)). To further examine whether Daxx has the ability to repress NF-κB DNA binding in cells, we performed chromatin immunoprecipitation (ChIP) assays to examine p65 binding to cellular promoters of NF-κB-regulated genes. Daxx expression repressed the binding of p65 to the promoters of IκBα and IL8 in TNFα-treated cells (Figure 1(d)). Daxx-mediated transcriptional down-regulation of NF-κB-regulated genes IκBα and IL8 was ascertained by Northern blot analysis (Figure 2).

Effects of Daxx on cellular levels of NF-κB

In an attempt to determine whether or not Daxx expression controls the expression of the NF-κB gene or the stability of NF-κB gene products, the effects of Daxx expression on the cellular levels of NF-κB were examined (Figure 3). Western blot analysis reveals that Daxx interferes with the levels of ectopically expressed p65 nor the levels of endogenous p65 induced by TNFα.

Effects of Daxx on NF-κB nuclear transport

The early phase of NF-κB activation involves cytoplasmic events that activate IKKs and promote the degradation of IκBα. These events lead to the translocation of the liberated NF-κB complex into the nucleus. Therefore, it was necessary to determine whether Daxx-mediated repression of NF-κB transcriptional activity resulted from the perturbed translocation of NF-κB into the nucleus by Daxx. Data in Figure 4 show that Daxx expression is irrelevant to the translocation of NF-κB in TNFα-treated cells. Sub-cellular distribution of p65 in transfected cells overexpressing Daxx was virtually the same as that of non-transfected cells expressing only endogenous Daxx.

Inhibition of NF-κB acetylation by Daxx

We tested a hypothesis that Daxx represses NF-κB transcriptional activity by inhibiting its acetylation (Figure 5). HeLa cells were co-transfected with expression vectors for Flag-Daxx and HA-CBP possessing acetyltransferase activity. The levels of p65 acetylation in co-transfected cells were assessed by immunoprecipitation using anti-p65 antibody followed by Western blot analysis using a specific antibody that recognizes acetylated-Lys residues. Overexpression of CBP effectively induced p65 acetylation. Moreover, p65 acetylation was inhibited by ectopically expressed Daxx in co-transfected cells. Data also show that Daxx-mediated inhibition of p65 acetylation is not affected by treatment of the
HDAC inhibitor TSA, suggesting that the reduced p65 acetylation by Daxx is not the result of enhanced deacetylation of NF-κB by HDAC3.

Effects of Daxx on p65 phosphorylation

We tested the possibility that Daxx interferes with p65 phosphorylation and suppresses the DNA-binding property of NF-κB with the concomitant repression of NF-κB transactivation.15 (Figure 6). Our data show that the phosphorylation of endogenous p65 at Ser536 and 276 was not affected by Daxx expression, implicating a dispensable role of Daxx on p65 phosphorylation.

Effects of Daxx on CBP recruitment to p65

Since p300/CBP, a main effector of p65 acetylation, has been known to be recruited to p65 for its acetylation,16 it was necessary to determine whether Daxx expression interferes with the recruitment of CBP to p65 in co-transfected cells in which p65 was induced by TNFα treatment. Data in Figure 7 demonstrate that Daxx expression does not interfere with p65 interaction with CBP.

Figure 3. Daxx expression does not change cellular levels of p65. (a) HeLa cells (5×10⁵) were co-transfected with expression vectors for Flag-Daxx (0.1, 0.2 and 0.4 μg) and p65 (0.1 μg). After incubation for 24 h, cell lysate proteins were examined for the expression of p65, Daxx or actin by Western blot analysis. (b) HeLa cells (5×10⁵) were transfected with expression vectors for Flag-Daxx (0.1, 0.2 and 0.4 μg). After incubation for 18 h, transfected cells were treated with 20 ng/ml of TNFα for 6 h. Cell lysate proteins were examined for the expression of p65 by Western blot analysis as for (a).

Figure 4. Daxx plays a dispensable role on sub-cellular distribution of p65. HeLa cells (5×10⁵) were transfected with an expression vector for Flag-Daxx (1 μg). After incubation for 24 h, cells were treated with 20 ng/ml of TNFα for 30 min. Ectopically expressed Daxx was detected by immunostaining with anti-Daxx primary antibody and visualized using FITC conjugated secondary antibody (left). TNFα-induced p65 was immunostained with anti-p65 primary antibody and visualized using TRITC conjugated secondary antibody (middle). Cells were imaged by confocal microscopy. An overlay of the two channels is shown (right). Large arrows indicate transfected cells overexpressing Flag-Daxx. Small arrows indicate non-transfected cells.

Figure 5. Inhibition of p65 acetylation by Daxx. HeLa cells (10⁶) were co-transfected with expression vectors for Flag-Daxx (1 and 2 μg) and HA-CBP (1 μg). At 8 h after transfection, cells were treated with 400 nM TSA for 16 h. Intracellular p65 prepared from co-transfected cells was immunoprecipitated using anti-p65 antibody. The immune complex was analyzed by SDS-10% PAGE and p65 acetylation mediated by CBP was examined by Western blot analysis using anti-acetylated-Lys antibody (top). The expressions of HA-CBP (bottom) and Flag-Daxx (third panel from the top) were shown. The amounts of immunoprecipitated p65 were also monitored by Western blot analysis using anti-p65 antibody (second panel from the top).
Intracellular association between p65 and Daxx

Knowing that the inhibition of p65 acetylation by Daxx is irrelevant to both p65 phosphorylation and the recruitment of CBP to p65, we explored the possibility that the interaction between Daxx and p65 might provide steric hindrance to CBP-mediated acetylation of p65 at Lys residues. To investigate whether Daxx is capable of interacting with p65 in cells, co-immunoprecipitation experiments were carried out using total cell extract isolated from HeLa cells in which endogenous p65 was activated by the treatment of 20 ng/ml TNFα for 30 min. We confirmed the intracellular association between Daxx and p65 in TNFα-treated HeLa cells. Results also reveal that endogenous levels of Daxx and p65 in cell lysates are not affected by TNFα treatment (Figure 8(a) and (b)). The data showing the co-localization of p65 and Daxx to the nucleus of TNFα-treated cells implicate that Daxx associates with activated NF-κB in the nucleus (Figure 8(c)). A glutathione S transferase (GST) pull-down assay shows that the Daxx binding region of p65 contains multiple acetylation sites (Figure 8(d)).

Discussion

Here, we report physical and functional links between Daxx and NF-κB. Our results imply that overexpression of Daxx inhibits DNA binding ability of NF-κB to the promoters of NF-κB-regulated genes (Figure 1(c) and (d)) and represses NF-κB transcriptional activity (Figure 1(a) and (b)). The inhibition of NF-κB DNA binding activity by Daxx was confirmed both by EMSA (Figure 1(c)) and ChIP assay (Figure 1(d)). The repression of NF-κB transcriptional activity by Daxx resulted in the reduced mRNA expression of NF-κB-regulated genes such as IκBα (Figure 2(a)) and IL8 (Figure 2(b)).

The exact mechanism accounting for the regulation of transcriptional activities of various transcription factors by Daxx is largely unknown. In the following are plausible scenarios for the repression of NF-κB transcriptional activity by Daxx. First, Daxx may regulate NF-κB transcriptional activity by controlling the expression of NF-κB transcription factor or its stability. However, our data demonstrate that the levels of both ectopically expressed (Figure 3(a)) and TNFα-induced p65 (Figure 3(b)) were not affected by the expression of Daxx. Second, Daxx may control nuclear transport of NF-κB and regulate NF-κB transcriptional activity. Following the degradation of IκBα bound to NF-κB, NF-κB is rapidly transported into the nucleus in order to activate NF-κB-related gene expression.17 It is unlikely that Daxx represses NF-κB transcriptional activity by inhibiting the nuclear transport of NF-κB, since our immunofluorescence analysis reveals that Daxx does not interfere with the sub-cellular distri-
bution of p65 in TNFα-treated HeLa cells (Figure 4). Third, Daxx may influence NF-κB post-translational modifications and regulate the nuclear function of NF-κB. Our data in Figure 5 illustrate that Daxx overexpression inhibits p65 acetylation. Acetylation of p65 has been known to be required for the full transactivation function of NF-κB. At least five acetylation sites have been identified within p65: Lys122, 123, 218, 221 and 310. Studies have shown that the acetylation of p65 at Lys221 and 310 is important in the regulation of transcriptional activity of NF-κB. Acetylation at Lys221 enhances NF-κB DNA binding and impairs its assembly with IκBα, resulting in the augmentation of transcriptional competence of NF-κB. Conversely, acetylation of p65 at Lys310 does not affect NF-κB DNA binding or its assembly with IκBα, but is required for the overall transcription activity of NF-κB, possibly by recruiting yet unidentified transcription co-activators. The p300/CBP represents acetyltransferase that has been implicated as the major player of p65 acetylation both in vitro and in vivo. Acetylated p65 is subjected to deacetylation by HDAC3. The reversible acetylation of p65 by HDAC3 has been

Figure 8. Daxx interaction with p65 in the region of its acetylation. (a) HeLa cells (2 × 10⁶) were treated with 20 ng/ml of TNFα for 30 min. Daxx bound to p65 was immunoprecipitated using anti-p65 antibody. Immunoprecipitated proteins were separated by SDS–10% PAGE and analyzed by Western blotting using anti-Daxx antibody. Cell lysate proteins were examined for the co-existence of endogenous Daxx and NF-κB by Western blot analysis using antibodies against Daxx and p65. (b) Immunoprecipitation was carried out using anti-Daxx antibody. p65 bound to Daxx was examined by Western blot analysis using anti-p65 antibody. (c) Untreated HeLa cells (upper) or cells treated with 20 ng/ml of TNFα for 30 min (lower) were examined for the sub-cellular distribution of Daxx and NF-κB. Daxx was detected by immunostaining with anti-Daxx antibody and visualized using FITC secondary antibody (left). NF-κB was immunostained with anti-p65 antibody and visualized using TRITC conjugated secondary antibody (middle). Cells were imaged by confocal microscopy. An overlay of the two channels is shown (right). (d) HeLa cells (10⁶) were co-transfected with expression vectors for GST-fused p65 deletion derivatives (1 μg) and Flag-tagged full-length Daxx (1 μg). At 24 h after transfection, cells were treated with 20 ng/ml of TNFα for 30 min and lysed. Cell lysates were purified by GST pull-down followed by SDS–8% PAGE and Western blotting using anti-Flag antibody (top). Cell lysate proteins were examined for the expressions of Flag-Daxx (middle) and GST-fused p65 deletion derivatives (bottom).
reported to be important for the regulation of NF-κB transcriptional responses. However, our experimental procedures rule out the possibility that Daxx controls HDACs activity and regulates NF-κB deacetylation, since the treatment of TSA, HDACs inhibitor, failed to restore p65 acetylation in HeLa cells co-expressing Flag-Daxx and HA-CBP (Figure 5).

This study proposes a biochemical link between repression of NF-κB transcriptional activity and inhibition of p65 acetylation by Daxx (Figure 5). Although the acetylation of p65 is an important biochemical event that participates in the regulation of NF-κB transcriptional activity, the underlying mechanism for the control of p65 acetylation has not been elucidated. Following the degradation of IκBα and subsequent translocation into the nucleus, post-translational modifications activate the NF-κB transcription factor complex or relevant histones surrounding NF-κB target genes. Phosphorylations of p65 at Ser276, 311, 529 and 536 by multiple kinases have been identified. A mechanical link between phosphorylation and acetylation has been known to exist for NF-κB function. Studies have shown that phosphorylation of p65 at Ser276 or 536 enhances the recruitment of p300/CBP to NF-κB and p300/CBP acetylates both p65 and the histone tails surrounding NF-κB-regulated cellular genes. However, our data implicate that p65 phosphorylation and subsequent recruitment of CBP to NF-κB are irrelevant to the inhibition of p65 acetylation by Daxx. Our results show that Daxx influences neither NF-κB phosphorylation nor the recruitment of CBP to NF-κB (Figures 6 and 7). It is also unlikely that Daxx modulates NF-κB transcriptional activity by interfering with other post-translational modifications of NF-κB. Daxx contains a SUMO-interacting motif (SIM) and is known to participate in the transcriptional control of SUMO-modified transcription factors such as AR and Smad4. Transcriptional activity of NF-κB is also known to be regulated by Pin1-mediated prolyl isomerization of the p65 subunit. In an independent experiment, we performed Western blotting to determine whether p65 is modified by sumoylation and found that overexpressed p65 is not sumoylated in transfected HeLa cells expressing Flag-tagged SUMO (data not shown). A study by Ryo et al. has shown that Pin1-dependent modification of p65 correlates with translocation of NF-κB into the nucleus and maintenance of its stability. Whether or not Pin1-dependent modification of p65 is affected by Daxx demands additional studies. However, it seems unlikely that Daxx participates in the modulation of NF-κB transcriptional activity by inhibiting Pin1-dependent modification of p65, because Daxx did not influence the sub-cellular distribution of p65 (Figure 4) and its stability (Figure 3).

Daxx is a large nuclear protein that is present in most cell types. Daxx interacts with various nuclear proteins of which the typical example is promyelocytic leukemia protein (PML) in PML oncogenic domains (PODs) within the nucleus. The ability of Daxx to interact with nuclear transcription factors seems to provide a functional framework for the regulation of the activities of transcription factors by Daxx. Thus far, Daxx is known to interact with various transcription factors including ETS1, Pax3, Pax5, p73, androgen receptor (AR), heat shock factor 1 (HSF1), mineralocorticoid receptor (MR) and glucocorticoid receptor and regulate their transcriptional activities. A recent study by Croxton et al. has emphasized the important role of Daxx binding to the RelB subunit of NF-κB on the repression of NF-κB-mediated transactivation of apoptosis regulatory genes. In Figure 8, we determined whether Daxx interacts with p65 of which the acetylation was shown to be inhibited by Daxx. Co-immunoprecipitation and immunofluorescence analysis revealed the intra-nuclear interaction between Daxx and p65 in HeLa cells (Figure 8(a) and (b)). While Daxx predominantly exists in the nucleus, p65 resides both in the cytoplasm and nucleus. Upon stimulation by TNFα, p65 and Daxx became co-localized in the nucleus (Figure 8(c)). We also found that the portion of p65 that interacts with Daxx contains multiple acetylation sites (Figure 8(d)). Accordingly, Daxx interaction with p65 is thought to interfere with p65 acetylation and impair NF-κB transcriptional activity. It seems unlikely that the intermolecular association between Daxx and p65 interferes with CBP binding to p65, since CBP has been known to majorly interact with C-terminal side of p65, whereas Daxx interacts with the N-terminal side of p65 (Figure 8(d)).

**Materials and Methods**

**Cell culture and transfection**

Human HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum (Gibco) and maintained at 37 °C in 5% (v/v) CO2 under a humidified atmosphere. Cells were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer’s procedures.

**Plasmids and antibodies**

cDNA encoding Daxx was cloned into EcoRI and BamHI sites of pFlag-CMV2 (Sigma) to produce pFlag-Daxx vector. p65 was cloned into EcoRV and XbaI sites of pcDNA3 (Invitrogen) to produce the pcDNA3-p65 vector. Expression vectors for GST-fused p65 deletion mutants were generated by sub-cloning the respective genes into BamHI and Clal sites of pEBG. HA-CBP expression vector (pCMV-HA-CBP) was provided by Dr Choe J. Antibodies against Daxx, HA, and actin were purchased from Santa Cruz. Anti-p65 monoclonal antibody was also from Santa Cruz. Polyclonal antibodies against p65 and acetylated-Lys residues were from Upstate. Anti-phosphospecific p65 antibodies, which recognize phosphory-
lated p65 at Ser536 or Ser276 were from Cell Signaling. Anti-GST and anti-Flag antibodies were from Sigma.

**Luciferase assay**

HeLa cells were transfected with expression vectors for p65 and Flag-Daxx together with the synthetic NF-κB response element (3×κB) luciferase reporter plasmid. After 24 h, cells were harvested for luciferase reporter assay. To measure transcriptional activity of endogenous NF-κB, cells co-transfected with the Flag-Daxx expression vector and luciferase reporter plasmid were treated with 20 ng/ml of TNFα (Sigma) for 6 h and harvested for luciferase reporter assay. Cells were washed with phosphate-buffered saline (PBS) and lysed in Passive Lysis Buffer (Promega). Luciferase activities were measured using Luciferase Assay System (Promega) and the values were normalized relative to β-galactosidase activity using the Luminescent β-galactosidase detection kit II (Clontech).

**Electrophoretic mobility shift assay (EMSA)**

DNA binding ability of NF-κB to its recognition site was examined by EMSA. The reaction mixture (20 μl) containing 30 mM Hepes (pH 7.9), 0.3 mM EDTA, 100 mM NaCl, 10% (v/v) glycerol, 1.5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSE, 1 μg/ml of leupeptin, nuclear extract (10 μg) prepared from TNFα-stimulated HeLa cells overexpressing Daxx and 32P–end-labeled oligonucleotide probe (–105 c.p.m.) was incubated at 4 °C for 15 min. 32P–end-labeled double-stranded NF-κB oligonucleotide had the following sequence: 5′-GATTCAGAGGGACATTCCGAGAGA-3′ and its complementary strand. The binding complex was analyzed by eletrophoresis on 5% (w/v) native polyacrylamide gels in 0.5×Tris-borate electrophoresis buffer. The gels were fixed in 10% (v/v) acetic acid, dried and visualized by autoradiography.

**Chromatin immunoprecipitation (ChIP assay)**

HeLa cells were transfected with expression vector for Daxx and incubated for 24 h. Cells were treated with 20 ng/ml of TNFα for 30 min. Cells were then fixed with 4% (v/v) formaldehyde, permeabilized with PBS containing 0.5% (v/v) Triton X-100 at 4 °C and blocked with 2% (w/v) bovine serum albumin (BSA) and 0.24% (v/v) goat serum albumin (Sigma) for 60 min. Cells were incubated with antibodies against Daxx and p65 in PBST containing 2% BSA for 10 h at 4 °C. The antigen–primary antibody complex was detected using a DIG-labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch) and tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-mouse secondary antibody (Jackson ImmunoResearch). Microscopic observation was performed under a confocal laser scanning microscope.

**Post-translational modification of NF-κB p65**

To determine the effects of Daxx expression on p65 acetylation, HeLa cells were co-transfected with expression vectors for Daxx and HA-CBP. At 8 h after transfection, cells were treated with 400 nM TSA (Sigma) for 16 h and washed with ice-cold PBS and lysed in lysis buffer A (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride). The whole cell extracts were incubated with anti-p65 antibody for 2 h and precipitated using protein G Sepharose beads (Amersham Pharmacia Biosciences). After incubation for 8 h, immune complexes were washed with lysis buffer A, boiled with SDS sample buffer (Tris–HCl, glycerol, SDS, 2-mercaptoethanol and bromphenol blue) for 5 min, resolved on SDS–10% (w/v) PAGE and analyzed by Western blotting using an antibody that recognizes acetylated-Lys residues. To measure the effects of Daxx expression on p65 phosphorylation, HeLa cells were transfected with an expression vector for Flag-Daxx. After incubation for 24 h, transfected cells were treated with 20 ng/ml of TNFα for the specified times and lysed. Phosphorylation of p65 was examined by Western blot analysis using phospho-specific antibody that recognizes phosphorylated p65 at Ser276 or Ser336.

**Northern blot analysis**

HeLa cells were transfected with expression vector for Flag-Daxx. After incubation for 24 h, cells were treated with 20 ng/ml of TNFα for 1 h and harvested. Total RNAs were extracted using Trizol Reagent (Molecular Research Center, Inc) according to the manufacturer’s instruction. RNA samples (3 μg each) were fractioned on 1.2% agarose–formaldehyde gels and transferred to a positively charged nylon membrane. The specific primers for the lκBα gene (forward primer: 5′-CGGCCGCTGACTCAGAA-3′; reverse primer: 5′-TGCCCCAGTAGCCATGGGA-3′) and IL8 gene (forward primer: 5′-ATGACTTCAGC- TGGCCGTCG-3′; reverse primer: 5′-TTATGATTTCCGACCTTCTC-3′) were used for PCR to produce DIG-labeled probes. The membrane was hybridized with a DIG-labeled probe according to the protocols specified by the manufacturer (Roche Molecular Biochemicals). The levels of mRNAs were detected by hybridization with DIG-labeled PCR probe at 50 °C for 8 h.

**Co-immunoprecipitation**

HeLa cells were treated with 20 ng/ml of TNFα for 30 min and lysed in lysis buffer A. To examine the interaction between Daxx and p65, anti-Daxx antibody or anti-p65 antibody (1 μg each) was added to the cell lysates followed by agitation for 2 h at 4 °C. To the cell preparations was added 20 μl of 50% protein A Sepharose 6B resin (Amersham Biosciences). The mixture was incubated for
an additional 8 h at 4 °C. Resin was washed four times with lysis buffer A. The immune complexes were released from the resin by boiling in SDS sample buffer for 5 min. Immunoprecipitated proteins were separated by SDS–10% PAGE followed by Western blot analysis using antibodies against Daxx and p65. To examine the CBP interaction with p65, cell lysates from co-transfected HeLa cells were immunoprecipitated using anti-p65 antibody. CBP bound to p65 was analyzed by Western blotting using an antibody that recognizes HA-tagged CBP.

**GST pull-down assay**

HeLa cells were transiently co-transfected with expression vectors for GST-fused p65 derivatives and Flag-directed against each respective epitope. Following incubation for 24 h, cell lysates were incubated with glutathione-Agarose (Sigma) at 4 °C for 30 min and lysed in lysis buffer A. Pellets were resuspended in SDS sample buffer. Bound proteins were washed four times with lysis buffer A. Cells were immunoprecipitated using anti-p65 antibody. Bound proteins were analyzed by SDS–8% PAGE followed by Western blot analysis using antibodies directed against each respective epitope.

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