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*J Immunol* 2011; 186:539-548; Prepublished online 22 November 2010;
doi: 10.4049/jimmunol.1001650

http://www.jimmunol.org/content/186/1/539

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/11/22/jimmunol.1001650.DC1

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Negative Feedback Regulation of NF-κB Action by CITED2 in the Nucleus

Xiwen Lou, Shaogang Sun, Wei Chen, Yi Zhou, Yuefeng Huang, Xing Liu, Yufei Shan, and Chen Wang

NF-κB is a family of important transcription factors that modulate immunity, development, inflammation, and cancer. The biological activity of NF-κB is subjected to various spatial and temporal regulations. Bioinformatics analysis predicts that CITED2 is topologically close to NF-κB in the protein interaction networks. In this study, we show that ectopic expression or knockdown of CITED2 attenuates or potentiates, respectively, the expression of NF-κB–responsive genes. Mechanistically, CITED2 constitutively localizes inside the nucleus and interacts specifically with the coactivator p300. This prevents p65 from binding to p300, impairs p65 acetylation, and attenuates p65 binding to its cognate promoters. Furthermore, LPS induces CITED2 expression via NF-κB in macrophages. CITED2 sensitizes cells to TNF-α–induced apoptosis. Collectively, this study identifies CITED2 as a novel regulator of NF-κB in the nucleus, which reveals a negative feedback mechanism for NF-κB signaling. The Journal of Immunology, 2011, 186: 539–548.

Nuclear factor κB is an important family of transcription factors (1), including RelA (p65), RelB, C-Rel, p50 (p105), and p52 (p100) (2). The p65/p50 heterodimer is the predominant form of NF-κB in most cells, and it controls a wide array of genes critical in the immune response and inflammation (3). It might also play some potential roles in development (4, 5). The aberrant action of NF-κB is causally associated with cancer (6) and some autoimmune diseases (7).

The latent form of the NF-κB heterodimer is retained in the cytoplasm through binding to its inhibitor IκBα. IκBα is quickly phosphorylated, ubiquitinated, and degraded in response to a myriad of extracellular and intracellular stimuli, such as TNF-α, IL-1β, LPS, and so forth. This releases the NF-κB heterodimer, which then translocates from cytoplasm into nucleus to mediate the transcription of the corresponding target genes (2). The past two decades have witnessed the successful elucidation, at the molecular and cellular levels, of the major signaling pathways leading to IκBα degradation and NF-κB nuclear translocation.

As with any other pivotal molecules in various biological processes, the activity of NF-κB is controlled strictly in vivo. The modulation of NF-κB action inside the nucleus has recently attracted much attention, revealing unexpected multilayers of tight regulations (8, 9). For example, a dynamic equilibrium exists between promoter-bound and nucleoplasmic NF-κB, which enables the cells to retune quickly its transcriptional programs according to external stimuli (10). In addition, the modification status of histones on a given promoter could define the accessibility of the NF-κB dimers (11, 12). Furthermore, the modification status of NF-κB per se markedly influences its transcriptional activity (11, 13, 14). Nuclear p65 can be phosphorylated and acetylated on distinct sites by different kinases and acetyltransferases (15, 16). Promoter-bound NF-κB can also be ubiquitinated and degraded in certain conditions (17, 18). All of these provide an array of delicate mechanisms for the rapid and reversible integration of stress signals.

Recently, a growing list of novel molecules has been implicated in the modulation of nuclear NF-κB action via different mechanisms (19). For example, Akirin2 (20) and UXT (21) were recently demonstrated to function as essential coactivators in the NF-κB enhanceosome. Importantly, the transcriptional coactivator p300/CBP not only serves as a scaffold for the NF-κB enhanceosome but also directly acetylates both p65 and histones simultaneously (22). It is a great challenge to understand how these proteins cooperate both dynamically and temporally to remodel the NF-κB enhanceosome.

CITED2 (35 kDa), also known as MRG1 or p35srj, may function in several biological processes. Knockout analysis demonstrated that CITED2 plays crucial roles in mouse embryo development. The CITED2−/− embryos could not proceed beyond embryogenesis. Cardiac malformation, adrenal agenesis, neural crest defects, and exencephaly were observed in the embryos (23–25). Further studies revealed left–right patterning defects in these embryos, especially in co-isogenic C57BL/6J backgrounds (26). Moreover, CITED2 could selectively maintain adult hematopoietic stem cell functions, at least in part via Ink4a/Arf and Trp53 (27).

Mechanistically, CITED2 has been suggested as a multifunctional transcriptional cofactor. It can bind to the LIM domain of Lhx2 and stimulate the expression of the glycoprotein hormone α-subunit gene (28). It can also bind TFAP2 and potentiate its
transcription activity, which is a possible explanation for the embryo lethality of CITED2−/− mice (23). Notably, CITED2 can be a repressor of the transcription of HIF-dependent genes (29, 30).

To our knowledge, there is no investigation concerning the function of CITED2 in immunity and inflammation. Using bioinformatics analysis, we noticed that CITED2 is topologically close to NF-κB in the protein interaction networks. In this study, we demonstrate that CITED2 is inducible by LPS. It prevents p65 from binding to p300, impairs p65 acetylation, and weakens p65 binding to its cognate promoters, revealing that CITED2 is a novel repressor of nuclear NF-κB.

Materials and Methods

Cell culture and transfection

HEK293T, J774, Raw264.7, and MEF cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT). Jurkat cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. For the bone marrow-derived macrophages (BMDMs), femora were aseptically removed from 8-wk-old male C57BL/6 mice. The femora were then cut off on both ends, and bone marrow cells were flushed out with a 27-gauge needle attached to a syringe containing 3 ml medium (RPMI 1640 supplemented with 20% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 40 μg/ml macrolone colony stimulating factor). The medium was changed every 2 d and the cells differentiated to BMDMs after 1 wk. All cells were maintained in a humidified 5% CO2 incubator at 37˚C.

Transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The short hairpin RNA (shRNA) plasmids were introduced into J774 cells and BMDMs using recombinant adenovirus (Invitrogen). Adenovirus harboring the GFP gene was constructed via the AdEasy Adenoviral Vector System (Stratagene, Lexington, MA), and the titer of all the adenoviruses was determined by the multiplicity of infection (MOI) test. Plasmids and small interfering RNAs (siRNAs) were introduced into Jurkat cells using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA).

Plasmids and siRNAs

CITED2 was a gift from Dr. S. Bhattacharya (University of Oxford, Oxford, U.K.). CITED2-Δm (L243EP244E2/4L2/4E2/W2/4E2) was constructed by using the QuickChange XL Site-Directed Mutagenesis method (Stratagene). p300 was a gift from Dr. Warner C. Greene (University of California, San Francisco, CA). p65 tagged with or without FLAG, FLAGEtBSR, FLAGE-TRAF6, FLAGE-MyDb88, and the reporter gene 3×κB-luc have been described previously (21). The CITED2 (human) siRNA sequences were as follows: CITED2 (396), 5′-CCACCAUGACGCGCA-UUUG-3′; CITED2Δm (396m), 5′-CCACCAACAGCGCAAUUUG-3′. The non-specific siRNA sequence (NC) was 5′-UUUCGCGAUCUGCA-CGU-3′. The CITED2 siRNA-resistant form was generated by introducing two silent mutations (C396TC399T) into the siRNA CITED2 (396) target sequence. The shRNA sequence targeting CITED2 (mouse) was as follows: 5′-CACCGCTACACAAATCAGCGATT-TCGAAAATCCTGATTTGTCG-3′. The siRNA sequence targeting p300 was previously reported (31).

Reagents

CITED2 (JA22), p300 (N-15), p65 (C-20), p65 (F-6), IxBα, HA, eCIP1, and RNA polymerase II Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG and β-actin Abs were purchased from Sigma-Aldrich (St. Louis, MO). Acetyl–NF-κB p65 (Lys310) Ab was obtained from Cell Signaling (Danvers, MA). rhTNF-α was obtained from R&D Systems (Minneapolis, MN). PMA, ionomycin, LPS, PD98059, LY294002, and Bay11-7082 were purchased from Sigma-Aldrich. 

Reporter assays

Reporter assays were performed as described previously (21).

Real-time RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) were used for the reverse transcription of the purified RNA. The amount of transcript of the genes of interest was measured by real-time RT-PCR method using the StepOne real-time PCR system (Applied Biosystems, Carlsbad, CA). The primers used were as follows: A20: sense 5′-GGGTCGAGGCACACAGTTTC-3′; antisense 5′-GCAAAGCCCGGTTTTCAACAAA-3′; MCP-1: sense 5′-TGCCTCTAGCATGAGAG-TC-3′; antisense 5′-GGGATATGACAGCTGCG-3′; IL-8: sense 5′-GCAGCTCTGTCCTTTTGTC-3′; antisense 5′-GGTGGACCAAACTGCTCTAAGG-3′; IL-2: sense 5′-ACGGTACCTCCTGCGTGTA-3′; antisense 5′-CTGTTCTGCGTGTA-3′; MCP-1: sense 5′-TGGAGCTTCTGC-3′; antisense 5′-GGCACTTTGCTGCGGCT-3′.

Immunoprecipitation assay and immunoblot analysis

For immunoprecipitation, whole-cell lysates were obtained by lysing cultured cells with lysis buffer containing 1% Triton X-100, 50 mM Tris·HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM NaVO3, and protease inhibitor mixture (Roche, Grenzacherstrasse, Basel); or nuclear extracts were obtained as previously described (32). The lysates were centrifuged, and the supernatant was collected and precleared with protein G-Sepharose (Santa Cruz Biotechnology) at 4˚C for 1 h. Then the precleared supernatant was incubated with the indicated Abs at 4˚C overnight. Protein A/G agarose beads were added after another 1 h, and the immunoprecipitate was washed extensively with wash buffer containing 0.5% Triton X-100. Finally, each sample was diluted with 1× SDS buffer, boiled for 5 min. For immunoblot analysis, all immunoprecipitates and whole-cell lysates were subjected to SDS-PAGE. The resolved proteins were then electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with skimmed milk and probed with indicated Abs. The protein bands were visualized by using a SuperSignal West Pico chemiluminescence ECL kit (Pierce, Rockford, IL).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Billerica, MA) was used according to the manufacturer’s instructions with some modifications. Formaldehyde was added directly into the culturing media to a final concentration of 1% at room temperature for cross-linking. Ten minutes later, the cells were harvested and washed and lysed with SDS lysis buffer. The lysates were sonicated until the chromatin was sheared to an average size between 200 and 1000 bp. The sonicated lysates were centrifuged under 12,000 × g for 10 min, and the supernatant was diluted 10-fold by ChIP dilution buffer and precleared by protein A/G agarose beads that were saturated with salmon sperm DNA and BSA for 1 h. Then, the precleared supernatant was incubated with the indicated Abs at 4˚C overnight. Protein A/G agarose beads were added after another 1 h, and the immunoprecipitates were washed sequentially and extensively with a series of wash buffer. Then, immunoprecipitates were extracted with elution buffer to release the immune complexes. The DNA and protein was de-cross-linked by heating at 65˚C under high salt conditions for more than 5 h. The RNA contaminants were removed by adding RNeasy A at 37˚C for 1 h, and the protein contaminants were removed by incubating with protease K at 45˚C for 2 h. Then, DNA was purified by phenol/chloroform extraction and precipitated with anhydrous ethanol and finally dissolved in sterile water. Then, PCR was performed to measure the amount of promoter DNA in each sample.

Immunofluorescence and microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, blocked using 1% BSA, and stained with the indicated primary Abs followed by incubation with a Cy5-conjugated anti-mouse IgG (Millipore, Billerica, MA). Nuclei were counterstained with DAPI (Sigma-Aldrich). Slides were mounted using Aqua-Poly/Mount (Polysciences, Warrington, PA). Images were captured at room temperature using a confocal microscope (TCS SP2 ACBS; Leica) with a ×63 (numerical aperture 1.4) oil objective. The acquiring software was TCS (Leica, Solms, Germany).

Apoptosis assay

The 293T cells were transfected with the indicated plasmids. Twenty-four hours later, cells were treated with 50 ng/ml TNF-α plus 5 μg/ml cytochrome C.
cloheximide (CHX) or left untreated for 24 h. Suspended and adherent cells were collected, trypsinized, and washed twice with PBS. Then, the cells were analyzed with a FACSCalibur (BD Biosciences) flow cytometer using annexin V–FITC (BD Biosciences) and propidium iodide (PI; Sigma-Aldrich) according to the manufacturer’s instructions.

Results
Identification of CITED2 as a potential regulator of NF-κB signaling

Some proteins have been shown to play dual roles in development and immunity (20, 33). As CITED2 is essential for development, we wondered if it could play a role in immunity. Notably, bioinformatics analysis with Cytoscape (http://www.cytoscape.org/) software predicts that CITED2 is topologically close to NF-κB, IFN regulatory factor 3 (IRF3), and AP-1 (c-Jun/ATF3) in the protein interaction networks (Fig. 1A).

To explore the potential relevance of this prediction, the corresponding specific luciferase reporters were used to test whether CITED2 had any impact on these transcription factors. We observed that ectopic expression of CITED2 apparently had little effect on either AP-1 or IRF3 reporter (Fig. 1B and Supplemental Fig. 1). In contrast, ectopic expression of CITED2 significantly attenuated the NF-κB–responsive luciferase reporter, which was stimulated by TNF-α (Fig. 1B) or Sendai virus (SeV; Supplemental Fig. 1). Consistently, this was also true for the IFN-β and E-selectin luciferase reporters, which are tightly regulated by NF-κB (Fig. 1C).

To make it more physiologically relevant, we explored, via real-time RT-PCR, whether CITED2 influenced the expression of endogenous NF-κB target genes induced by TNF-α. Notably, ectopic expression of CITED2 markedly impaired the expression of these genes (A20, IL-8, MCP-1, and GRO-1) in 293T cells (Fig. 1D). We also constructed CITED2(4m) by introducing four amino acid changes (L243E/P244E/L246E/W247E) into the CITED2 sequence. In contrast, expression of CITED2(4m) could barely influence the induction of these genes. Ectopic expression of CITED2 also decreased the induction of NF-κB–regulated genes (IL-2 and IL-8) in Jurkat T cells (Fig. 1E), suggesting that CITED2 could be a negative regulator of NF-κB signaling.

Knockdown of CITED2 potentiates NF-κB signaling

Alternatively, an RNA interference knockdown approach was used to investigate whether the endogenous CITED2 regulated NF-κB

FIGURE 1. Identification of CITED2 as a novel negative regulator of NF-κB activation. A, CITED2-associated proteins are determined from the known protein–protein interaction networks using Cytoscape. B, 293T cells were co-transfected with either empty vector or HA–CITED2 with AP-1, IRF3, and NF-κB reporter genes as indicated. Twenty-four hours after transfection, cells were treated with 10 ng/ml PMA or SeV (MOI, 0.5) or 10 ng/ml TNF-α for 10 h before the luciferase assays were performed. C, Empty vector or HA–CITED2 and the indicated reporter genes were transfected into 293T cells. After 24 h, the cells were treated with SeV (MOI, 0.5) or 10 ng/ml TNF-α for 10 h. The cells were assayed as described in B, D, 293T cells were transfected with CITED2 or its loss-of-function mutant CITED2(4m). Twenty-four hours later, the cells were treated with 10 ng/ml PMA plus 1.5 μM ionomycin for 3 h. Twenty-four hours later, the cells were treated with 50 ng/ml PMA plus 1.5 μM ionomycin for 3 h. The endogenous mRNA expression levels of IL-2 and IL-8 were measured by real-time RT-PCR. E, Jurkat cells were transfected with CITED2 or its loss-of-function mutant CITED2(4m). Twenty-four hours later, the cells were treated with 50 ng/ml PMA plus 1.5 μM ionomycin for 3 h. The endogenous mRNA expression levels of IL-2 and IL-8 were measured by real-time RT-PCR. Data in B–E are represented as means ± SD (error bars) of at least three independent experiments. *p < 0.05; **p < 0.01.
signaling. The siRNA oligonucleotide was screened out and determined to be efficient in reducing both the exogenous and endogenous CITED2 protein levels. At the same time, nonspecific (NC) and mutant (mCITED2) siRNAs were used as negative controls (Fig. 2A). Knockdown of endogenous CITED2 modestly potentiated NF-κB target genes in HEK293T cells upon TNF-α treatment (Fig. 2B) and in Jurkat T cells after PMA/ionomycin stimulation (Fig. 2C).

To rule out the potential off-target effect of the CITED2 siRNA, an RNA interference-resistant construct of CITED2, CITED2(FLr), was generated, which contained silent mutations in the corresponding siRNA target region of CITED2 without changing the protein sequence. HEK293T cells were first transfected with the control or CITED2 siRNA followed by transfection of the control or CITED2(FLr) plasmids as indicated. Then, the induction of NF-κB target genes was measured by real-time RT-PCR after TNF-α treatment. As shown in Fig. 2D, knockdown of endogenous CITED2 potentiated the expression of NF-κB target genes, which was reversed by the ectopic expression of CITED2(FLr).

We then went on to test the function of CITED2 in macrophages (J774 and BMDM). The CITED2 siRNA oligonucleotide used above did not knock down well in murine macrophages, so we screened out another shRNA oligonucleotide and introduced it into macrophages via a recombinant adenovirus. An shRNA targeting the lacZ gene was used as negative control (Fig. 2E). Adenovirus-GFP was used to confirm the transduction efficiency, which was ~50–60% (Supplemental Fig. 2). Consistently, a decrease in endogenous CITED2 expression significantly boosted the induction of NF-κB target genes stimulated by LPS (Fig. 2F, G). Taken together, these data indicated that CITED2 is a novel repressor of NF-κB signaling.

CITED2 modulates NF-κB signaling inside the nucleus

A key regulatory node in NF-κB signaling is the phosphorylation and ubiquitin-proteasome-mediated degradation of its inhibitor,
We observed that neither ectopic expression nor knockdown of CITED2 could affect the degradation of IκBα (Fig. 3A, 3B). In addition, ectopic expression of MyD88, TRAF6, IKKβ, and p65 respectively could activate NF-κB signaling, and all of which were attenuated by the coexpression of CITED2 (Fig. 3C). Given the hierarchical relations of these molecules in NF-κB pathway, we reasoned that CITED2 modulates NF-κB inside the nucleus. This was further substantiated by the observation that CITED2 is constitutively expressed inside the nucleus (Fig. 4A).

**CITED2 prevents p65 from binding to its cognate promoters**

It was apparent that CITED2 did not interact directly with either NF-κB p65 or p50, although IκBα co-immunoprecipitated strongly with them (Fig. 4B). Besides, UXT was recently uncovered as an integral component of NF-κB enhanceosome (21). Neither could CITED2 promote the export of NF-κB from the nucleus to the cytoplasm or actively retain NF-κB in the nucleus, whereas IκBα (SR) (Fig. 4C) or UXT (Fig. 4D) could do so, respectively.

Consequently, ChIP assay was used to investigate the status of NF-κB on its cognate promoters. Notably, ectopic expression of CITED2 could apparently decrease the amount of p65 on the promoters of A20 and IL-8. This was also true for RNA polymerase II on these promoters (Fig. 4E, 4G). In contrast, an increase of CITED2 expression had no obvious effect on the GAPDH transcription complex, which is not an NF-κB target gene (Fig. 4E, 4G). Furthermore, ectopic expression of CITED2 displayed no observable effect on IRF3 in terms of its corresponding promoters (X. Lou and C. Wang, unpublished data). Consistently, knockdown of the endogenous CITED2 protein level led to a moderate increase in the amount of p65 and RNA polymerase II on the promoters of A20 or IL-8 (Fig. 4F, 4H). Knockdown of CITED2 had no effect on the GAPDH promoter. Taken together, these data indicate that CITED2 regulates the affinity of NF-κB toward its cognate promoters but does not directly interact with NF-κB.

**CITED2 attenuates p65 acetylation by preventing its access to coactivator p300**

There exists research suggesting that CITED2 interacted with the transcriptional coactivator p300/CBP (29). Also, previous studies indicated that p65 bound p300 specifically upon stimulation, and this interaction is essential for NF-κB enhanceosome assembly (34). We confirmed that CITED2 could interact with p300 directly and tightly, via immunoprecipitation and GST pull-down (Fig. 5A). Notably, the CH1 domain of p300 has been implicated, via a yeast two-hybrid approach, to bind to CITED2, p65, Hif-1α, and so forth (35). So we speculated whether CITED2 could influence the specific interaction between p65 and p300.

To test this possibility, we mapped the C terminus of CITED2 (aa 221–270) to explore the binding site of p300 (Fig. 5B). A series of mutagenesis analyses uncovered four amino acid residues of CITED2 to be critical for this interaction. Therefore, CITED2 (L243E/P244E/L246E/W247E), named as CITED2(4m), could barely interact with p300 (Fig. 5B), whereas CITED2(L243E/L246E) could partially do so (X. Lou and C. Wang, unpublished data). Notably, ectopic expression of CITED2(4m) was unable to attenuate the activation of the NF-κB reporter gene (Fig. 3C) or the induction of NF-κB responsive genes (Fig. 1D, 1E). Neither could CITED2(4m) expression impair p65 to bind its cognate promoters (Fig. 4E, 4G). We noticed that expression of CITED2 (4m) could, to a lesser extent, influence the binding of RNA polymerase II to its cognate promoters (Fig. 4E). These data indicated that the CITED2 and p300 interaction is functionally important for modulating NF-κB activity inside the nucleus.

Furthermore, we carried out a mammalian two-hybrid assay to explore whether CITED2 competed against p65 for binding to p300. The N terminus (aa 1–800) of p300 was fused with the yeast GAL4 binding domain (BD). As expected, ectopic expression of this fusion protein and p65 induced the expression of a luciferase reporter harboring five tandem repeats of the GAL4 binding site. Notably, introduction of CITED2 into this system significantly reduced the induction of the GAL4 reporter. In contrast, ectopic expression of CITED2(4m) slightly potentiated the induction of the GAL4-reporter by p300 N800-BD and p65, possibly due to the dominant negative effect of CITED2(4m) (Fig. 5C). These data indicate that CITED2 disrupted the interaction between p300 and p65.

Alternatively, p65 was co-transfected respectively with CITED2, CITED2(4m), or empty vector. Then, the cell extracts were
FIGURE 4. CITED2 reduces the amount of p65 on its cognate promoters. A, Subcellular localization of endogenous and exogenous CITED2 in 293T cells. 293T cells were transfected with (bottom) or without (top) HA–CITED2. Immunofluorescence was performed with the indicated Abs. Scale bar, 20 μm. Original magnification ×63. B, 293T cells were transfected with FLAG–p65 (left) or FLAG–p50 (right) together with empty vector, HA–CITED2, or HA–IκBα. Twenty-four hours after transfection, the whole-cell lysate was prepared, immunoprecipitated, and immunoblotted as indicated. C, 293T cells grown on coverslips were transfected with plasmids as mentioned. Twenty-four hours later, the cells were treated with 10 ng/ml TNF-α for 30 min or left untreated. Then, the cells were stained with an anti-p65 primary Ab and a Cy5-conjugated secondary Ab. The nucleus was counterstained with DAPI. Scale bar, 20 μm. Original magnification ×63. Stains in A, C, and D are Cy5 (red) and DAPI (blue). E and F, 293T cells were transfected with plasmids or siRNAs as indicated. Twenty-four hours or 48 h later, the cells were treated with 10 ng/ml TNF-α for 30 min or left untreated. Then, the cells were immediately cross-linked with formaldehyde and applied to ChIP assay in terms of A20, IL-8, and GAPDH promoters using the corresponding Abs and primers. G and H, Cells were applied to ChIP assay as in E and F and analyzed using real-time RT-PCR.
subjected to a GST pull-down assay with recombinant GST-p300 CH1. Again, in the absence of CITED2, p65 was pulled down by GST-p300 CH1. Notably, in the presence of CITED2, much less amount of p65 was pulled down, whereas CITED2(4m) did not influence p65 pull-down (Fig. 5D). Importantly, co-immunoprecipitation assays indicated that overexpression of CITED2 could impair the endogenous interaction between p300 and p65, whereas knockdown of CITED2 could potentiate the interaction between p300 and p65 upon TNF-α stimulation (Fig. 5E, 5F).

p65 is reported to be acetylated by p300 on the enhanceosome. In particular, acetylation of lysine 310 is critical for NF-κB transcriptional activity (16). Therefore, p65 and p300 were co-transfected into 293T cells along with CITED2 or its mutants as indicated. Expectedly, p300 could catalyze the acetylation of p65. This modification was sharply reduced in the presence of CITED2, whereas CITED2 mutants failed to do so (Fig. 5G). This further substantiated the finding that CITED2 prevents p65 from binding to p300. CITED2 is a coactivator for p65, and we hypothesized that it may promote p65 binding to its cognate promoters. We confirmed this by transfecting cells with siRNA targeting p300, followed by ChIP experiments (Supplemental Fig. 3). We observed that knockdown of p300 markedly decreased the amount of p65 and RNA polymerase II on the A20 and IL-8 promoters upon TNF-α stimulation. This finding is consistent with the above results (Fig. 4E–H) that CITED2 could block the binding of p65 to its cognate promoters. CITED2 sensitizes cells to TNF-α-induced apoptosis

TNF-α with a low concentration of CHX (5 μg/ml) is often used to induce cell apoptosis (36, 37). NF-κB signaling antagonizes this apoptosis by inducing the transcription of antiapoptotic genes, such as c-FLIP, cIAP-1, and cIAP-2 (38, 39). Blockade of NF-κB activation can amplify the indicated apoptosis. Consequently, ectopic expression of CITED2 apparently sensitized cells to apoptosis induced by TNF-α plus CHX, as evidenced by a differential interference contrast microscope (Fig. 6A) and flow cytometry (Fig. 6B, 6C).

At the concentration of ≤10 μg/ml, CHX only reduces but does not block de novo protein synthesis (37). We verified this result by showing the expression of CITED2 and its mutant in the presence of a low dose of CHX (5 μg/ml) (Fig. 6E). We also demonstrated

**FIGURE 5.** CITED2 blocks the interaction of p65 and p300, attenuating p65 acetylation. **A**, The nuclear extracts of 293T cells were applied to immunoprecipitation with either an anti-CITED2 or anti-p300 Ab (top). 293T cells were transfected with the indicated plasmids, and then the cells were harvested and applied to immunoprecipitation with an anti-FLAG Ab (middle) or GST pull-down with GST or the GST–CH1 protein (right). Finally, they were analyzed by immunoblot analysis. PD, pull-down. **B**, Schematic of CITED2 and its mutants (top). CITED2 or its mutants as mentioned earlier were overexpressed in 293T cells. Then, cell lysate was prepared and used for GST pull-down assay with the GST–CH1 protein (bottom). (CITED2 and CITED2(1–221) were tagged with HA, and CITED2(4m) was tagged with FLAG.) **C**, The mammalian two-hybrid reporter gene pFR–luc was transfected into 293T cells with the plasmids as indicated. Twenty-four hours after transfection, the cells were collected and analyzed by a luciferase assay. Data represent means ± SD (error bars) of at least three independent experiments. **D**, The plasmids as indicated were transfected into 293T cells. The cells were harvested and used for GST pull-down assay with the GST–CH1 protein and then analyzed by immunoblotting. **E** and **F**, 293T cells were transfected with either plasmids as indicated in **E** or siRNAs as indicated in **F**. Twenty-four hours or 48 h later, the cells were treated with TNF-α for 1 h, and the nuclear extracts were obtained for immunoprecipitation with an anti-p65 Ab and then analyzed by immunoblotting. **G**, 293T cells were transfected with plasmids as indicated. After 24 h, the whole-cell lysates were prepared and immunoblotted with the indicated Abs, including an Ab that could detect acetyl lysine 310 of p65.
that the protein level of cIAP-1 was lower when CITED2 was overexpressed, compared with CITED2(4m) or vector, under TNF-α/CHX treatment (Fig. 6D).

LPS robustly induces CITED2 expression via NF-κB

LPS, also known as endotoxin, is a potent inducer of NF-κB activation. The NF-κB activation is quickly downregulated to avoid the detrimental side effects toward the host such as chronic inflammation. To address the potential role of CITED2 in this negative regulation, we challenged the murine macrophage cell lines J774, Raw 264.7, and BMDM with LPS. Notably, the CITED2 mRNA was quickly induced by LPS in these cells (Fig. 7A). The protein level of CITED2 was also elevated (Fig. 7B).

Given that LPS activates several signaling pathways, we used specific inhibitors to probe how CITED2 was induced in the murine macrophage. We observed that a MAPK kinase inhibitor (PD98059) and a PI3K inhibitor (LY294002) could not impair the induction of CITED2 upon LPS treatment. In contrast, an NF-κB signaling inhibitor (Bay11-7082) significantly attenuated the induction of CITED2 by LPS (Fig. 7C). Furthermore, wild-type MEF and p65−/− MEF cells were respectively challenged with LPS. Induction of CITED2 was scarcely observed in p65−/− MEF cells compared with that in the wild-type MEF cells (Fig. 7D).

Consistently, bioinformatics analysis identified an authentic κB site on the promoter of CITED2. ChIP assay revealed that p65 bound to this particular site after LPS stimulation, indicating that NF-κB regulates the expression of CITED2 per se (Fig. 7E).

Taken together, these data uncovered a novel negative feedback mechanism for regulating NF-κB inside the nucleus.

Discussion

Given the physiological and pathological roles of NF-κB in immunity, cancer, and autoimmune diseases, it is necessary for NF-κB to be subjected to meticulous checks and balances. Recently, much attention has been focused on the regulation of NF-κB inside the nucleus. Several nuclear coactivators have been demonstrated as critical in the regulation of NF-κB activity and the corresponding enhanceosome, via specific mechanisms. For example, p300/CBP is an essential coactivator for NF-κB, which simultaneously serves as a scaffold protein for the NF-κB enhanceosome and catalyzes the acetylation of p65 (22). In addition, the nuclear proteins UXT (21), CARM1 (40), protein kinase C βII (41), Akirin2 (20), and so on are essential for NF-κB to induce a subset of target genes.

Notably, our understanding of the mechanisms that decrease nuclear NF-κB activity has been more rudimentary than that of those that lead to its activation. A few corepressors have been suggested to impair NF-κB activity in the nucleus. For example, PIAS1 can interact with p65 and block its interaction with the promoters of NF-κB–responsive genes induced by TNF-κB and LPS (42). PDLIM2 interacts directly with p65 in certain conditions, which promotes the relocation of p65 to insoluble nuclear bodies and its subsequent ubiquitin-mediated degradation (43). These observations nicely indicate that the strength and duration of NF-κB response inside the nucleus is modulated through more complicated mechanisms than previously expected.

Using bioinformatics analysis, we noticed that CITED2 is topologically close to NF-κB in the protein interaction networks. In this study, we characterized CITED2 as a novel corepressor for the NF-κB enhanceosome. Several lines of observation support this argument (1). Ectopic expression of CITED2 attenuated NF-κB reporter genes in response to multiple stimuli. Epistatic analysis supported that CITED2 functioned in the NF-κB enhanceosome, which was consistent with its nuclear localization. Furthermore, ectopic expression of CITED2 reduced the transcription of NF-κB target genes (2). Conversely, knockdown of endogenous CITED2 resulted in an apparent increase of the expression of NF-κB–responsive genes (2). Conversely, knockdown of endogenous CITED2 resulted in an apparent increase of the expression of NF-κB–responsive genes (2).
genes, stimulated by TNF-α or PMA/ionomycin or LPS. Notably, this increase could be dampened by an siRNA-resistant exogenous CITED2 (3). CITED2 did not influence the molecular events leading to NF-κB translocation into the nucleus or the amount of nuclear NF-κB. However, CITED2 markedly impaired the ability of NF-κB to bind its cognate promoters (4). CITED2 is unable to interact directly with NF-κB. Notably, CITED2 could tightly bind to the p300 CH1 domain, which was also recognized by p65. The antagonism between CITED2 and p65 was substantiated by mammalian two-hybrid, GST pull-down, and co-immunoprecipitation assays (5). Disruption of the p65 and p300 interaction led to a reduction in p65 acetylation catalyzed by p300, a strong correlation with the decreased presence of NF-κB on its cognate promoters (6).

The expression of CITED2 sensitized cells to TNF-α–induced apoptosis. This result is consistent with the antiapoptotic role of NF-κB. Taken together, a tentative model is proposed in which CITED2 seems to compete against NF-κB for binding to p300, thus attenuating p65 acetylation and its binding to the cognate promoters. CITED2 is a member of the CREB/p300-interacting transactivator with Glu/Asp-rich C-terminal domain (CITED) protein family, which has four members (CITED1–4). CITED1, CITED2, and CITED4 have been found in human and mouse. CITED2 localizes exclusively in the nucleus and functions as a transcription coactivator or corepressor depending on the context. For example, CITED2 is a coactivator for HNF4α in the development of the liver (44) and for TFAP2A in the neural tube formation and cardiac development (23). In contrast, CITED2 is an inducible and negative regulator of HIF-1α under hypoxia conditions. In this study, we demonstrated that CITED2 is a negative regulator of NF-κB. Besides, we did not observe any regulatory effects of CITED2 on other signaling pathways important in innate immunity such as IRF3 or AP-1.

The apparent multifunctionality of CITED2 can be understood by the observation that CITED2 interacts strongly with p300/CBP. A large number of transcription factors bind to p300/CBP on their different regions; therefore, depending on the topological orientations of p300/CBP, CITED2, and the corresponding transcription factors, CITED2 and the relevant transcription factor could antagonize or cooperate in terms of binding to p300/CBP, thus attenuating or promoting the transcriptional activity, respectively. For instance, Bhattacharya et al. (29) proposed a model in which CITED2 competes against HIF-1α for the CH1 domain of p300, thus CITED2 serves as a negative regulator for HIF-1α. Also, Bhattacharya et al. reported that the C terminus of CITED2 (aa 224–255) is sufficient for binding of CITED2 with p300 CH1. In this study, we refined the model and showed that the amino acids L243, P244, L246, and W247 are all important residues for CITED2 to bind to p300 CH1. This mechanism also can occur between other transcription factors. For example, STAT2 and NF-κB cross-inhibit each other by competing for p300/CBP at the CH1 binding site (45), indicating that the amount of nuclear p300/CBP is very limited. This finding is consistent with our observation that ectopic expression of p300/CBP was sufficient to reverse the inhibitory effects of CITED2 on NF-κB (data not shown). It is possible that CITED2 serves as a versatile valve to control dynamically the access of important transcription factors to p300/CBP, given that CITED2 is inducible under stress conditions. This hypothesis is substantiated by the observation that CITED2 per se was significantly induced by LPS, and the induction was NF-κB dependent.
In summary, the current study characterizes CITED2 as a negative regulator of NF-kB in the nucleus. This represents a novel feedback mechanism for NF-kB signaling. In future studies, it will be intriguing to generate conditional knockout mice and explore the in vivo functions of CITED2 in immunity and inflammation.

Acknowledgments

We thank Dr. S. Bhattacharya (University of Oxford, Oxford, U.K.) and Dr. Warner C. Greene (University of California, San Francisco, CA) for providing plasmids.

Disclosures

The authors have no financial conflicts of interest.

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