Ubiquitin-specific Peptidase 21 Inhibits Tumor Necrosis Factor α-induced Nuclear Factor κB Activation via Binding to and Deubiquitinating Receptor-interacting Protein 1

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Ubiquitination and deubiquitination of receptor-interacting protein 1 (RIP1) play an important role in the positive and negative regulation of the tumor necrosis factor α (TNFα)-induced nuclear factor κB (NF-κB) activation. Using a combination of functional genomic and proteomic approaches, we have identified ubiquitin-specific peptidase 21 (USP21) as a deubiquitinase for RIP1. USP21 is constitutively associated with RIP1 and deubiquitinates RIP1 in vitro and in vivo. Notably, knockdown of USP21 in HeLa cells enhances TNFα-induced NF-κB ubiquitination, IkB kinase β (IKKβ), and NF-κB phosphorylation, inhibitor of NF-κB α (IκBα) phosphorylation and ubiquitination, as well as NF-κB-dependent gene expression. Therefore, our results demonstrate that USP21 plays an important role in the down-regulation of TNFα-induced NF-κB activation through deubiquitinating RIP1.

Transcription factor nuclear factor κB (NF-κB) plays an important role in controlling the expression of survival factors, cytokines, and proinflammatory molecules in a broad range of cellular responses (1–3). NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins called inhibitor of NF-κB (IκB) proteins in inactivated cells. Many intercellular stimuli are capable of triggering the activation of a signal transduction pathway that leads to the degradation of IκB proteins through the 26 S proteasome (4–6). Degradation of the IκB proteins allows NF-κB translocation from cytoplasm to the nucleus and activates the expression of the target genes (7).

Upon binding of tumor necrosis factor α (TNFα), TNF receptor 1 (TNFR1) recruits several adaptor proteins, including receptor-interacting protein 1 (RIP1/RIPK1) and TNF receptor-associated factor 2 (TRAF2), to form a complex (8, 9). This TNFR1-associated complex initiates the activation of IκB kinase (IKK), which phosphorylates IκB protein and activates NF-κB (10–17).

Protein ubiquitination is a crucial regulatory mechanism in various cellular processes, including cell cycle progression, the DNA damage response, and immune responses (18–20). In the TNFα-induced NF-κB signal transduction pathway, the Lys63-linked polyubiquitination of RIP1 protein mediated by TRAF2 E3 ligase is essential for TNFα-induced IKK/NF-κB activation, whereas phosphorylation of the IκB proteins by activated IKK leads to their Lys48-linked polyubiquitination, which labels it for its degradation by the 26 S proteasome (21).

Several deubiquitinating enzymes, including CYLD, A20, Cezanne, ubiquitin-specific peptidase 15 (USP15), and USP31, have been suggested to be involved in the down-regulation of TNFα-induced NF-κB activation (22–26). However, it remains unclear how deubiquitination plays a role in the down-regulation of TNFα-induced NF-κB activation.

The USPs belong to a subclass of the protein-deubiquitinat- ing enzyme (DUB) superfamily that are categorized into five subclasses based on their ubiquitin-protease domains in the human genome and have been shown to be involved in a broad range of biological activities (27). Even though the USP subclass of DUB represents the bulk of the deubiquitinating enzymes encoded in the human genome, the roles of USP proteins in the TNFα-induced NF-κB signal transduction pathway have not been studied in great detail.

In this study, we used a functional genomic approach to ident- ify the USPs that are involved in TNFα-induced NF-κB activation by screening a library of USPs whose overexpression inhibits TNFα-induced NF-κB activation. By combining with a proteomic approach, here we present evidence that USP21 functions as a RIP1 deubiquitinase that deubiquitinates RIP1 and down-regulates TNFα-induced NF-κB activation.

EXPERIMENTAL PROCEDURES

Identification of USP21-associated Proteins by Mass Spectrometry—HEK-293T cells were transfected with the empty vector control and FLAG-RIP1 vector and lysed. FLAG-
RIP1 was immunoprecipitated from cell lysates with anti-FLAG antibodies after preclearing with normal mouse IgG. The immunoprecipitates were separated on SDS-PAGE and stained with Coomassie Blue. Each lane was divided into 12 pieces, and proteins in which were identified with mass spectrometry as described (28).

**Cell Culture and Transfection**—HEK-293T and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (2 mM) supplemented with 10% fetal bovine serum and transfected with FuGENE 6 or FuGENE HD according to the manufacturer’s recommendation.

**Expression Plasmids, USP cDNA Expression Library, Small Hairpin RNA Constructs, Small Interfering RNA**—Human or mouse USP cDNA clones were purchased from Open Biosystems Company (Huntsville, AL). A full-length cDNA sequence for each USP member containing an open reading frame was subcloned into pcDNA3.1 expression vector (Invitrogen). The NF-κB-dependent Firefly luciferase reporter plasmid and CMV promoter-dependent Renilla luciferase reporter were purchased from Clontech. Mammalian expression vectors for TRAF2, RIP1, CYLD, A20, and USP21 were constructed by subcloning cDNAs encoding the full-length wild type human proteins into the pcDNA3.1 vectors with an N-terminal Myc, FLAG, or HA tag. The USP21C221A mutant expression construct was generated using the QuikChange site-directed mutagenesis kit (Stratagene). Mammalian expression vectors for HA-IKKβ and FLAG-TRAF5 were obtained from Dr. Paul Chiao and Dr. Bryant Darnay, respectively (M. D. Anderson Cancer Center, Houston, TX). Lys48-only and Lys63-only ubiquitin with N-terminal HA tags were subcloned into pcDNA3.1 expression vector (Invitrogen). A pSUPER-retro vector was
used to generate shRNA plasmids for USP21. The following target sequences have been selected: 5'-H11032-AAGATGGCTCA-TCACACACTC-3' (shUSP21-1), 5'-H11032-AACTTAGCCCGTTC-CAAGTCT-3' (shUSP21-2). A scramble sequence 5'-H11032-AGC- H11032-GCGCTTTGTAGGATTCG-3' was used as a negative control, and the selected shRNA sequences against USP21 were submitted to a BLAST search against the human genome sequence to ensure specificity. The shA20 plasmid was obtained from Dr. Peter Storz (Mayo Clinic). Synthetic small interfering RNAs against human USP21 (target sequence, 5'-GCCGTTCAGTGTCGA-3') and A20 (target sequence, 5'-CAACGGCTACTGCAACGAA-3') were purchased from Ambion (Austin, TX).

Antibodies and Reagents—Anti-USP21 antibodies were purchased from Abcam and Santa Cruz Biotechnology. Monoclonal anti-A20 and RIP1 antibodies were purchased from BD Biosciences. Other antibodies used in this study include anti-FLAG M2 monoclonal and anti-actin antibodies (Sigma); anti-IkBα and secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology); anti-IKKγ (NEMO); anti-HA and anti-Myc epitope antibodies (Santa Cruz Biotechnology). Human recombinant Lys48-linked and Lys63-linked polyubiquitin wild type chains were purchased from Boston Biochem (Cambridge, MA). Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN).

Immunoprecipitation and Immunoblotting—Cells were washed with phosphate-buffered saline and lysed for 30 min at 4°C in lysis buffer containing 25 mM HEPES, pH 7.6, 135 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 g/ml aprotinin, 10 g/ml leupeptin, 1 mM benzamidine, 1 mM PMSF. Lysates were then cleared by centrifugation, and proteins were immunoprecipitated with affinity antibody and protein A-agarose beads at 4°C. Immunoprecipitates were washed four times with lysis buffer and boiled with sample buffer before being separated by SDS-PAGE and then transferred onto nitrocellulose membranes following standard procedures. After being probed with the appropriate antibodies, the appropriate IgG horseradish peroxidase-conjugated antibodies were used as the secondary antibodies, followed by detection with the ECL Plus Western blotting system (Amersham Biosciences) and visualized by autorography.

Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic extracts were prepared by adding buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1
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mm PMSF, 20 mm glycerophosphate, 0.1 mm Na3VO4, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) to cell pellets. The cells were then suspended and chilled on ice for 15 min followed by adding 25 µl of 10% Nonidet P-40 and vortexing vigorously for 10 s. Cytoplasmic extracts were collected after centrifugation at 12,000 \( \times \) g for 5 min. For the nuclear extracts, the nuclear pel-
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USP21 proteins were immunoprecipitated from the transfected HEK-293T cell lysates prepared with Nonidet P-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) Nonidet P-40, 1 mM PMSF, and 1 mM DTT). The immunoprecipitates were washed three times with washing buffer and then incubated with 1.25 μg of either Lys48-linked or Lys63-linked polyubiquitin substrates in 15 μl of 50 mM Tris-HCl buffer, pH 7.2, containing 1 mM DTT for 2 h or with 15 μl of cell lysates prepared from the FLAG-RIP1-transfected HEK-293T cells in Nonidet P-40 lysis buffer and incubated at 37 °C for 2 h. Reaction mixtures were spun down, and the supernatants were analyzed by silver staining and immunoblotting with anti-FLAG (M2) antibodies.

Luciferase Reporter Assay—HEK-293T or HeLa cells were transiently co-transfected with an NF-κB-dependent Firefly luciferase reporter and effector plasmids along with the Renilla luciferase plasmid. The cells were harvested in passive lysis buffer after 24 h, and luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega). The relative luciferase activity was calculated through dividing the Firefly luciferase activity by the Renilla luciferase activity. Data represent three independent experiments performed in duplicate.

Establishment of the Stable USP21 Knockdown HeLa Cell Lines—The pSUPER USP21 shRNA retroviral vectors were transfected into the HEK-293T cells with retrovirus packaging vector Pgpam 3e and RFD vector using FuGENE 6. Viral supernatants were collected after 36 and 48 h. HeLa cells were then incubated with virus-containing medium in the presence of 4 μg/ml Polybrene. Stable cell lines were established after 7 days of puromycin (2 μg/ml) selection. Knockdown of the USP21 was confirmed by real time reverse transcription-PCR and immunoblotting analysis.

RESULTS

USP21 Inhibits TNFa-induced NF-κB Activation—Protein ubiquitination and deubiquitination have been suggested to play important roles in TNFa-induced NF-κB activation (20). To explore whether any member of deubiquitinases in the USP subclass is involved in regulation of TNFa-induced NF-κB activation, we first generated a library of mammalian expression vectors that encode 30 USP deubiquitinases. Then we used an NF-κB-dependent luciferase reporter assay to assess the effects of overexpression of each USP on TNFa-induced NF-κB luciferase reporter activity. To avoid the potential inhibitory effect of a tag sequence on deubiquitination enzymatic activity, we did not put any tag into the deubiquitinase protein coding sequence in this USP expression library. In this screen, as shown in Fig. 1A, USP21 significantly inhibited TNFa-induced NF-κB luciferase reporter activity whereas other USPs had no or less effect. To assess whether the inhibitory effect of overexpression of these USPs on the TNFa-induced NF-κB reporter activity is due to their deubiquitinase activity, we generated expression vectors encoding a USP21 deubiquitinase-deficient mutant by substitution of a cysteine residue in the USP active site with an alanine (C221A) and found that only USP21 wild type, but not deubiquitinase-deficient C221A mutant, abolished the TNFa-induced NF-κB activity in a reporter assay (Fig. 1B). This result suggests that USP21 deubiquitinase activity is required for its inhibitory role in the TNFa-induced NF-κB activation. To test further whether USP21 possesses the deubiquitinase activity and whether the cysteine to alanine mutation in the potential active site abrogates its deubiquitinase, we immunoprecipitated the Myc-tagged USP21 wild type and mutant proteins from the cell lysates of the transfected HEK-293T cells and performed an in vitro deubiquitination assay with both Lys48- and Lys63-linked ubiquitin substrates. In this assay, we found that USP21 wild type but not C221A mutant proteins catalyze the deubiquitination of both Lys48- and Lys63-linked ubiquitin (data not shown). Together, our data strongly indicate that USP21 is involved in the down-regulation of the TNFa-induced NF-κB activation through its deubiquitinase activity.

USP21 Is Associated with RIP1—To determine the molecular mechanism of USP21 function in TNFa-mediated NF-κB activation, we immunoprecipitated the FLAG-tagged USP21 from the lysate of the transfected HEK-293T cells with FLAG-USP21 expression vector and identified the co-immunoprecipitated proteins with FLAG-USP21 by mass spectrometry (Fig. 2A). We found that receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP1/RIPK1) was one of the major co-immunoprecipitated proteins with FLAG-USP21. The rest of the identified USP21-associated proteins, with the exception of ubiquitin, which are more abundant than RIP1, seem more likely to be nonspecific binding proteins (supplemental Table 1). This result suggests that
RIP1 may be a major USP21-binding protein. To validate the result from the above protein identification analysis by mass spectrometry, the FLAG-USP21 proteins from the HEK 293T cells transfected with FLAG-USP21 were immunoprecipitated from cell lysates with anti-FLAG antibody and immunoblotted with anti-RIP1 antibody. In this assay, we found that RIP1 was co-immunoprecipitated with FLAG-USP21 (Fig. 2B). The association between USP21 and RIP1 was also confirmed by co-immunoprecipitation of overexpressed HA-RIP1 and FLAG-USP21 in HEK 293T cells (Fig. 2C) and co-immunoprecipitation of endogenous RIP1 and USP21 in HeLa cells with and without TNFα stimulation (Fig. 2, D and E). Together, these results suggest that USP21 is a RIP1-associated protein.

USP21 Acts as a RIP1 Deubiquitinase—The Lys63-linked ubiquitination of RIP1 plays an essential role in TNFα-induced NF-κB activation (21). Our results suggest that the inhibitory effect of USP21 on TNFα-induced NF-κB activation could be through its association with RIP1 and regulation of RIP1 ubiquitination. To test this hypothesis, FLAG-RIP1 and HA-ubiquitin expression vectors were co-transfected with vector control or expression vectors encoding Myc-tagged USP21 wild type or deubiquitinase-deficient C221A mutant as well as CYLD into the HEK 293T cells. FLAG-RIP1 proteins were immunoprecipitated with the antibody for FLAG epitope and immunoblotted with anti-HA antibody to detect the presence of ubiquitinated RIP1. As shown in Fig. 3A, USP21 wild type abrogated the RIP1 overexpression-induced ubiquitination of RIP1, whereas the USP21 deubiquitinase-deficient mutant and CYLD failed to do so. Furthermore, overexpression of Myc-USP21 wild type had no inhibitory effect on FLAG-TRAF2 and FLAG-TRAF5 polyubiquitination (supplemental Fig. S2). To confirm the
Knockdown of USP21 expression enhances TNF-α-induced IL-6 activation. 

**A.** IL-6 expression in USP21 stable knockdown HeLa cell lines

![Graph showing IL-6 expression over time](image)

**B.** IL-6 production in USP21 stable knockdown HeLa cell lines

![Bar graph showing IL-6 production](image)

**Figure 4.** Knockdown of USP21 expression enhances TNF-α-induced RIP1 polyubiquitination and IKK/β/NF-κB activation. A. Knockdown of USP21 expression enhances TNF-α-induced RIP1 polyubiquitination and IkB-α/NF-κB activation. The sh-control and sh-USP21 HeLa cell lines were either untreated or treated with TNF-α (10 ng/ml) for the time points indicated. The knockdown effect of USP21 expression was examined by both quantitative reverse transcription-PCR and immunoblotting with anti-USP21 antibodies. The data are presented as the average of three independent experiments ± S.D. (error bars). B. Knockdown of USP21 expression enhances TNF-α-induced IL-6 production. The sh-control and sh-USP21 HeLa cell lines were either untreated or treated with TNF-α (2 ng/ml) for 24 h. The supernatants from these cell cultures were collected and subjected to the human IL-6 enzyme-linked immunosorbent assay analysis according to the manufacturer’s instructions.

**Figure 5.** USP21 negatively regulates TNF-α-mediated IL-6 gene expression. A. Knockdown of USP21 expression enhances the TNF-α-induced NF-κB-dependent IL-6 gene expression. The sh-control and sh-USP21 HeLa cell lines were either untreated or treated with TNF-α (10 ng/ml) for the time points indicated. Total RNAs from these cells were extracted. IL-6 transcript levels in the sh-control and sh-USP21 cell lines were measured using quantitative reverse transcription-PCR normalized to glyceraldehyde-3-phosphate dehydrogenase. The data are presented as the average of three independent experiments ± S.D. (error bars). B. Knockdown of USP21 expression enhances TNF-α-induced IL-6 production. The sh-control and sh-USP21 HeLa cell lines were either untreated or treated with TNF-α (2 ng/ml) for 24 h. The supernatants from these cell cultures were collected and subjected to the human IL-6 enzyme-linked immunosorbent assay analysis according to the manufacturer’s instructions.

Above result further, the immunoprecipitated Myc-USP21 wild type and deubiquitinase-deficient mutant proteins from the transfected HEK 293T cell lysates were co-incubated with FLAG-RIP1-transfected HEK 293T cell lysates in vitro. In this assay, USP21 wild type but not deubiquitinase-deficient C221A mutant abrogated the ubiquitination of RIP1 (Fig. 3B).

Consistent with this result, co-transfection of FLAG-TRAF2 or FLAG-RIP1 with Myc-USP21 wild type but not the deubiquitinase-deficient mutant in HEK-293T cells resulted in a decreased TRAF2 and RIP1-induced NF-κB activities in an NF-κB-dependent luciferase reporter assay, whereas co-overexpression of USP21 failed to inhibit TRAF6-induced NF-κB activation (Fig. 3C). Furthermore, overexpression of USP21 had no inhibitory effect on interleukin 1 (IL-1)-induced NF-κB activation (supplemental Fig. S3). These results strongly suggest that USP21 is a bona fide RIP1 deubiquitinase and inhibits TNF-α but not IL-1-induced NF-κB activation.

**USP21 Down-regulates TNF-α-Induced RIP1 Ubiquitination and IKK/β/NF-κB Activation—** TNF-α induces a rapid RIP1 ubiquitination, which is required for TNF-α-mediated NF-κB activation (21). To determine whether USP21 is involved in down-regulating TNF-α-induced RIP1 ubiquitination, we generated USP21 stable knockdown HeLa cell lines using a retroviral transduction system (Fig. 4A). We then analyzed the effect of USP21 knockdown on TNF-α-induced RIP1 ubiquitination. In this assay, we found that TNF-α induced a higher level of the RIP1 ubiquitination at the early time points and that this higher level of RIP1 ubiquitination was sustained at the later time points of the TNF-α stimulation in the USP21 knockdown cells compared with the control cells (Fig. 4A). This result demonstrates that USP21 is involved in terminating TNF-α-induced RIP1 ubiquitination in the cells.
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TNFα induces a strong NF-κB activation through the phosphorylation and activation of IKK (29). To evaluate the role of USP21 in TNFα-induced IKK/NF-κB activation, we analyzed the effect of USP21 knockdown on TNFα-induced IKK phosphorylation, IκBα phosphorylation, ubiquitination, and degradation, as well as NF-κB RelA subunit phosphorylation at Ser536. As shown in Fig. 4B, TNFα induced an increased level of IKK phosphorylation at the early time points of stimulation, an increased level of IκBα phosphorylation and ubiquitination, as well as an increased level of RelA phosphorylation in the USP21 knockdown cells compared with the control cells. We also found that TNFα induced a higher level of c-Jun N-terminal kinase phosphorylation at the earlier time points of stimulation in the USP21 knockdown cells compared with the control cells (Fig. 4B).

Consistent with the above results, TNFα also induced a higher level of the NF-κB-dependent luciferase reporter activity in the USP21 knockdown cells compared with the control cells (Fig. 4C). Taken together, these results suggest that USP21 inhibits TNFα-induced IKK/NF-κB activation by suppressing RIP1 ubiquitination.

**USP21 Inhibits TNFα-Induced NF-κB-Dependent IL-6 Gene Expression**—NF-κB activation is necessary for TNFα-induced IL-6 expression (30–32). To determine the role of USP21 in the regulation of TNFα-induced IL-6 gene expression, we extracted total RNA from the control and USP21 knockdown HeLa cell lines treated with TNFα for the time points indicated and performed quantitative reverse transcription-PCR to examine the TNFα-induced IL-6 expression levels in the cells. As shown in Fig. 5A, TNFα induced a higher level of IL-6 expression in USP21 knockdown cells compared with the control cells. Consistently, TNFα also induced a higher level of IL-6 protein in the cell medium from USP21 knockdown cells compared with the control cells in ELISA (Fig. 5B). These results suggest that USP21 down-regulates TNFα-induced gene expression by inhibiting TNFα-induced RIP1 ubiquitination.

**DISCUSSION**

Lys63-linked RIP1 ubiquitination is an essential step in TNFα-induced IKK/NF-κB activation (20, 33). Following TNFα stimulation, RIP1 is promptly ubiquitinated at Lys6377 by TRAF2 E3 ligase within 5 min. RIP1 will be quickly deubiquitinated at the later time points of stimulation, suggesting that tight control of RIP1 ubiquitination is critical for normal TNFα-induced cellular responses. However, the mechanism of RIP1 deubiquitination following TNFα stimulation to attenuate TNFα-induced RIP1 ubiquitination and downstream IKK-NF-κB activation remains to be clearly defined. In this study, we identify USP21 as a major deubiquitinase of RIP1 in the TNFα-induced NF-κB activation. By using a combination of functional genomic screening and proteomic approaches, we demonstrate that USP21 is critical to modulate TNFα-induced RIP1 ubiquitination as well as downstream IKK/NF-κB activation through constitutive association with RIP1 and suppression of Lys63-linked RIP1 ubiquitination. Our studies suggest that USP21 serves as another level of critical yin-yang regulatory control to maintain a delicate balance in TNFα-induced inflammatory responses by targeting RIP1.

Our study here suggests that functional genomic screening strategy is a powerful tool to identify the new genes involved in the regulation of TNFα-induced NF-κB activation. However, in this type of screening, we cannot guarantee that USPs are overexpressed at the same level. Therefore, this screening serves only as an initial step to identify the genes whose overexpression significantly inhibits TNFα-induced NF-κB activation.

Interestingly, we found that both A20 and USP21 efficiently inhibited RIP1 overexpression-induced ubiquitination, and both FLAG-tagged USP21 and A20 co-immunoprecipitated...
with endogenous RIP1, whereas USP21 pulled down more endogenous RIP1 and was a more efficient RIP1 deubiquitinase in our in vitro deubiquitination assay conditions compared with A20 (supplemental Fig. S1, A, B, and C). Furthermore, A20 expression and binding to RIP1 can only be detected after 30 min of TNFα stimulation in HeLa cells, whereas USP21 is constitutively expressed and bound to RIP1 (supplemental Fig. S1D). Transient knockdown of USP21 but not A20 expression enhanced TNFα-induced RIP1 polyubiquitination in HeLa cells within 15 min of stimulation (supplemental Fig. S1E). Notably, TNFα-induced RIP1 polyubiquitination peaks at 5 min and rapidly goes down to the basal level within 30 min (Fig. 2E). However, A20 cannot be detected in HeLa cells within 30 min of TNFα stimulation (supplemental Fig. S1D), suggesting that the polyubiquitinated RIP1 induced by TNFα is deubiquitinated by a DUB in the absence of A20 in the cells. Interestingly, knockdown of both USP21 and A20 further enhanced TNFα-induced NF-κB activation with single knockdown of USP21 or A20 (supplemental Fig. S1F). Together, these results strongly suggest that USP21 is mainly responsible for RIP1 deubiquitination at the early time points of TNFα stimulation, whereas A20 is induced at later time points of stimulation and plays an important “gatekeeper” role in the termination of persistent NF-κB activation in HeLa cells.

The ubiquitin-editing enzyme A20 (also called TNFAIP3) has previously been shown to be a potent inhibitor of NF-κB signaling that consists of an N-terminal deubiquitinating domain of the OTU family and a C-terminal ubiquitin ligase domain and targets RIP1 by cleaving Lys63-linked polyubiquitin chains and promoting Lys48-linked polyubiquitination that triggers proteasome-mediated RIP1 degradation (22, 34). Furthermore, A20 has been suggested to be a central gatekeeper in inflammation and immunity (35, 36). In contrast, as a member of the USP subclass, USP21 only contains a DUB domain (27). Recently, A20 has been shown to form a ubiquitin-editing complex with TAXBP1/Itch/RNF11 proteins to modulate the TNFα-induced RIP1 ubiquitination (37–39). Future studies are needed to determine whether USP21 needs other scaffolding molecules to mediate its function to target RIP1 in TNFα-mediated NF-κB activation. In addition, Cezanne has been reported to be involved in the modulation of RIP1 ubiquitination process through binding to RIP1 in a TNFα-inducible manner (23). Future studies should be conducted to determine how USP21 cooperates with A20 and Cezanne to modulate the TNFα-induced RIP1 ubiquitination process.

Previous reports demonstrate that USP21 can be a NEDD8 protease (40) and a functional deubiquitinase of histone H2A in the regulation of transcriptional initiation (41). These results suggest that USP21 may target proteins other than RIP1. In conclusion, our results provide direct evidence that USP21 interacts with RIP1 physically and acts as a RIP1 deubiquitinase in the TNFα-mediated NF-κB activation. In view of the data presented here and in previous reports, we propose a working model (Fig. 6) in which TNFα induces RIP1 ubiquitination and NF-κB activation as well as A20 expression. Lys63-linked RIP1 would be rapidly deubiquitinated by USP21 at the early time points of stimulation, followed lately by cooperative action of the A20 ubiquitin-editing complex, including TAXBP1/Itch/RNF11, to deubiquitinate Lys63-linked RIP1 completely and ubiquitinate RIP1 in the form of Lys48-linked ubiquitin to promote RIP1 degradation and terminate the persistent TNFα-mediated IKK/NF-κB activation.

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