Overexpression of USP14 Protease Reduces I-κB Protein Levels and Increases Cytokine Release in Lung Epithelial Cells*

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The ubiquitin-proteasome system is the major pathway of non-lysosomal intracellular protein degradation, playing an important role in a variety of cellular responses including cell division, proliferation, and apoptosis. Ubiquitin-specific protease 14 (USP14) is a component of proteasome regulatory subunit 19 S that regulates ubiquitinated proteins entering inside the proteasome core 20 S. The role of USP14 in protein degradation is still controversial. Several studies suggest that USP14 plays an inhibitory role in protein degradation. Here, in contrast, overexpression of USP14 induced I-κB degradation, which increased cytokine release in lung epithelial cells. Overexpression of HA-tagged USP14 (HA-USP14) reduced I-κB protein levels by increasing the I-κB degradation rate in mouse lung epithelial cells (MLE12). I-κB polyubiquitination was reduced in HA-USP14-overexpressed MLE12 cells, suggesting that USP14 regulates I-κB degradation by removing its ubiquitin chain, thus promoting the deubiquitinated I-κB degradation within the proteasome. Interestingly, we found that USP14 was associated with RelA, a binding partner of I-κB, suggesting that RelA is the linker between USP14 and I-κB. Lipopolysaccharide (LPS) treatment induced serine phosphorylation of USP14 as well as further reducing I-κB levels in HA-USP14-overexpressed MLE12 cells as compared with empty vector transfected cells. Further, overexpression of HA-USP14 increased the LPS-, TNFα-, or Escherichia coli-induced IL-8 release in human lung epithelial cells. This study suggests that USP14 removes the ubiquitin chain of I-κB, therefore inducing I-κB degradation and increasing cytokine release in lung epithelial cells.

Intracellular protein degradation in the proteasome system involves covalent conjugation of ubiquitin chain(s) to the substrates that are recognized and degraded within the proteasome (1, 2). The proteasome consists of a barrel-shaped multimeric protein complex, the 20 S proteasome core particle (CP), the 19 S regulatory particle (RP), and numerous deubiquitinating enzymes (DUBs) (3–6). The 20 S CP contains three major proteolytic activities, including chymotrypsin-like, trypsin-like, and caspase-like activities, and functions as the catalytic center that cleaves peptides on the carboxyl side of hydrophobic, basic, and acidic amino acids (4–6). The 20 S CP is capped at each end by the 19 S RP, which controls channel opening and the remodeling of substrate proteins (4–6). DUBs disassemble ubiquitin chains to regulate protein stability. Three major DUBs, including RPN11, UCH37, and USP14, are associated with mammalian 19 S RP (4–6).

USP14, one of ubiquitin-specific peptidases, disassembles the ubiquitin chain from its substrate-distal tip (7, 8). The activity of USP14 is regulated by its interaction with 19 S RP because USP14 by itself remains relatively inactive; however, activity is greatly increased when co-incubated with 19 S RP (8). The USP14 mutant (USP14ΔUBL), which lacks the ability to bind to 19 S RP, lost its activity in the presence of the 19 S RP (8). Loss of a yeast USP14 ortholog Ubp6 rapidly reduced the free ubiquitin pool (9), suggesting a role of USP14 in regulation of ubiquitin recycling. In addition to its deubiquitination ability, USP14 regulates gate opening of 20 S CP (10). USP14 has been shown to function as an inhibitor of the proteasome because it was found to slow substrate degradation (11). The small molecule USP14 inhibitor IU1 enhanced proteasome activity and degradation of several substrates such as Tau and ataxin-3 (11).

Growing evidence suggests that USP14 regulates various biological responses including neural function (12, 13), chemokine signaling (14), and tumorigenesis (15, 16). The ataxia mutation (ax3) mice, a loss of Usp14 function strain, exhibited a reduced growth rate, ataxia, and hindlimb muscle wasting (12, 17). The neuron-specific overexpression of Usp14 restored viability and motor system function to the ax3 mice (13, 17). USP14 deubiquitinated chemokine receptor CXC4R, whereas both overexpression and RNAi-induced knockdown of USP14 blocked ligand-induced CXC4R degradation (14). Further, knockdown or inhibition of USP14 by siRNA or inhibitor (IU1) reduced murine norovirus-1 (MNV-1) nonstructural gene expression in RAW cells (18). These data indicate that USP14 is implicated in chemokine receptor signaling, virus infection, and immune responses. A recent study using an inhibitor of USP14, b-AP15, revealed that inhibition of USP14 resulted in accumulation of...
polyubiquitin, tumor cell apoptosis, a lower tumor progression rate, and organ infiltration in an acute myeloid leukemia model (16). These studies suggest that USP14 is a new drug target in anticancer and anti-infection.

The role of USP14 in the regulation of lung inflammation has not yet been studied. Here, we show that USP14 regulates I-κB stability and cytokine release in lung epithelial cells. This is the first study demonstrating that overexpression of USP14 reduces I-κB ubiquitination as well as its protein levels, thus increasing interleukin-8 (IL-8) release. Further, we demonstrate that USP14 interacts with phosphorylated ReLA. This study will provide a basis for developing a new therapeutic strategy to treat lung inflammatory diseases.

MATERIALS AND METHODS

Cell Culture and Reagents—Murine lung epithelial (MLE12) cells (ATCC, Manassas, VA) were cultured with medium supplemented with hydrocortisone, insulin, transferrin, estrogen, selenium (HITES), 10% fetal bovine serum (FBS), and antibiotics at 37 °C in 5% CO2 incubator. Human lung epithelial (Beas2B) cells (ATCC, Manassas, MA) were cultured in BEGM medium with 10% FBS and antibiotics. HA tag, FLAG tag, I-κB, RelA, phospho-JNK, and ubiquitin antibodies were obtained from Cell Signaling (Danvers, MA). Cycloheximide, USP14 shRNA, and β-actin antibody were obtained from Sigma. USP14 antibody, immobilized protein A/G beads, and control IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). HA-USP14 plasmid was kindly provided by Wade Harper (Harvard Medical School). FLAG-RelA was kindly provided by Thomas Gilman (Boston University). Escherichia coli isolated from chronic obstructive pulmonary disease patients were kindly provided by Rama Mallampalli (University of Pittsburgh). All materials used in the experiments are in the highest grades and are commercially available.

Plasmid Transfection—MLE12 cells were nucleofected with plasmids. 1 × 10⁶ MLE cells were suspended in 120 μl of nucleofection buffer and well mixed with 3 μg of plasmid DNA in an electroporation cuvette. Electroporation was performed in the Nucleofector™ II system (Lonza, Gaithersburg, MD), and the cells were cultured in 2 ml of complete HITES (hydrocortisone, insulin, transferrin, estrogen, selenium) medium for 48 h. Beas2B cells were transfected with FuGENE HD transfection reagent (Roche Applied Science). Briefly, 2 μg of plasmid were mixed with 3 μl of FuGENE HD reagent in BEBM blank medium for 10 min. The mixture was added into Beas2B cell culture medium to grow for a further 2 days.

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—Cell lysates in 120 μl of lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) were sonicated on ice for 12 s and centrifuged at 500 × g for 5 min at 4 °C in a microcentrifuge. For immunoprecipitation, equal amounts of cell lysates (1 mg) were incubated with 2 μg/ml specific primary antibodies overnight at 4 °C followed by the addition of 40 μl of protein A/G-agarose for 2 h at 4 °C. For Western blotting, equal amounts of supernatant (20 μg) were subjected to 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, blocked with 5% (w/v) nonfat milk in TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20) for 1 h, and incubated with primary antibodies in 5% (w/v) BSA in TBST for 1–2 h. The membranes were washed at least three times with TBST at 10-min intervals followed by a 1-h incubation with mouse, rabbit, or goat horseradish peroxidase-conjugated secondary antibody (1: 2,000). The membranes were developed with an enhanced chemiluminescence detection system according to manufacturer’s instructions.

Immunofluorescence Staining—MLE12 cells grown on glass bottom dishes were fixed with 3.7% formaldehyde for 20 min, immunostained with USP14, HA tag, or RelA antibody, washed three times, and incubated with fluorescent conjugated secondary antibodies. Images were captured by Nikon ECLIPSE TE 300 inverted microscope.

Cytokine ELISA Measurement—Cell culture media were replaced with blank media before LPS treatment. At the end of the experiment, cell supernatants were collected, centrifuged at 1,000 × g for 5 min at 4 °C, and frozen at −80 °C for later analysis of IL-8 by ELISA kit according to the manufacturer’s instructions.

Statistical Analyses—All results were subjected to statistical analysis using one-way analysis of variance and, wherever appropriate, the data were also analyzed by Student-Newman-Keuls test and expressed as mean ± S.D. Data were collected from at least three independent experiments, and p < 0.05 was considered significant.

RESULTS AND DISCUSSION

Overexpression of USP14 Reduces I-κB Expression—The nuclear factor NF-κB pathway has long been considered a pro-inflammatory signaling pathway that regulates the expression of proinflammatory genes including cytokines and chemokines (19). To investigate the role of USP14 in inflammatory lung diseases, we first tested whether USP14 regulates I-κB stability, which has been implicated in NF-κB activity (19). MLE12 cells were overexpressed with an HA-tagged human USP14 (HA-USP14) plasmid for 48 h. Cell lysates were analyzed for I-κB and Iκκα expression. As shown in Fig. 1A, overexpression of HA-USP14 reduced I-κB level without altering IKκα and β-actin expression. Consistently, overexpression of HA-USP14 decreased levels of I-κB in a dose-dependent manner (Fig. 1B). The overexpressed HA-USP14 was confirmed with antibodies to USP14 and HA tag (Fig. 1, A and B). To further determine the role of USP14 in I-κB stability, I-κB levels were examined in USP14-overexpressed and USP14 knockdown MLE cells in the presence of cycloheximide (20 μg/ml), a protein synthesis inhibitor. Cycloheximide-mediated I-κB degradation was attenuated by USP14 shRNA-mediated knockdown of USP14, whereas overexpression of HA-USP14 promotes the I-κB degradation (Fig. 1C). Transfection of USP14 shRNA significantly reduced USP14 level (Fig. 1C). USP14 has been considered as an inhibitor of the proteasome because USP14 stabilized ubiquitinated cyclin B protein in vitro (11) and Tau and TDP-43 proteins in Usp14−/− murine embryonic fibroblasts (11). Nagai et al. (20) have shown that overexpression of USP14 inhibited the endoplasmic reticulum-associated degradation pathway, and knockdown of USP14 reversed the effect. In contrast to these findings,
Peth et al. (10) found that USP14 controls 20 S gate opening, thus activating the proteasome, in a deubiquitination enzyme activity-independent manner. In addition, loss of USP14 function increased GABA<sub>A</sub> receptor levels in the Purkinje cells from the ax<sup>−</sup> mice (17). The present study provides new evidence that USP14 promotes certain protein degradation, such as that of I<sup>B</sup>. Given that USP14 is the deubiquitination enzyme, we hypothesized that USP14 is responsible for the removal of the ubiquitin chain bound to I<sup>B</sup>. To further investigate whether USP14 regulates deubiquitination of I<sup>B</sup>, ubiquitinated proteins were immunoprecipitated from proteasome inhibitor (MG-132)-treated vector alone or HA-USP14-overexpressed MLE12 cell lysates followed by I<sup>B</sup>-150K immunoblotting. In addition, I<sup>B</sup> was immunoprecipitated followed by immunoblotting analysis with a ubiquitin antibody. Fig. 1<sup>D</sup> shows that MG-132-induced accumulation of polyubiquitinated I<sup>B</sup> was reduced by overexpression of HA-USP14. The data reveal that USP14 reduces I<sup>B</sup>-150K polyubiquitination. Sun et al. (21) had reported that USP11 targets I<sup>B</sup> for its deubiquitination. Here, we report that USP14 mediates I<sup>B</sup>-deubiquitination and stability. USP14 is a component of proteasome 19 S RP and controls ubiquitin recycling and proteasome gate opening (8–10). Deubiquitination of I<sup>B</sup> by USP11 increases I<sup>B</sup>-150K stability (21), whereas ubiquitin removal by USP14 promotes I<sup>B</sup> entrance into the 20 S CP for degradation. USP14 has been known to regulate gate opening of the 20 S CP (10). A future study will investigate whether the gate opening function of USP14 contributes to the regulation of I<sup>B</sup>-150K stability.

USP14 Interacts with RelA—DUBs remove the ubiquitin chain via direct interaction with its ubiquitinated substrates or the ubiquitin chain (22). For instance, USP22 directly interacts with and mediates deubiquitination of Sirt1 (23). To investigate the mechanism by which USP14 regulates I<sup>B</sup> stability, we examined whether USP14 directly interacts with I<sup>B</sup> or its binding partner RelA. Cell lysates from MLE12 cells transfected with an empty vector alone or HA-USP14 plasmid were subjected to immunoprecipitation with an HA tag antibody followed by immunoblotting with antibodies to RelA and HA tag. Input lysates were subjected to immunoprecipitation with an HA tag antibody followed by immunoblottings with antibodies to RelA and HA tag. Cell lysates were subjected to immunoprecipitation with antibodies to USP14, FLAG tag, and β-actin. As shown in Fig. 1<sup>E</sup>, HA-USP14 was associated with RelA. Further, cells were transfected with the FLAG-RelA plasmid. The overexpressed FLAG-RelA was immunoprecipitated followed by USP14 immunoblotting analysis. Consistent with the data from Fig. 1<sup>E</sup>, USP14 was associated with overexpressed FLAG-RelA. Co-immunoprecipitation failed to detect the association between USP14 and I<sup>B</sup> (data not shown). The association between endogenous USP14 and RelA was confirmed by co-immunofluorescence staining with antibodies to USP14 and RelA. As shown in Fig. 1<sup>G</sup>, both USP14 and RelA were localized in the cytoplasm. A portion of the co-localized USP14 and RelA was detected. The ubiquitinated I<sup>B</sup>-150K degradation in the proteasome has been well studied, whereas the detailed understanding of precisely how the NF-κB-I<sup>B</sup>-150K complex interacts with the proteasome remains unclear. The present study indicates that RelA might play a novel role in recruiting the RelA-I<sup>B</sup>-150K complex to USP14 on the proteasome 19 S RP, therefore regulating I<sup>B</sup>-150K remodeling and promoting its entrance through...
the 20 S gate for degradation. The RelA binding domain within USP14 will need to be identified in future studies.

**USP14 Promotes Cytokine Release**—The role of USP14 in cytokine release has not yet been studied. First, we examined whether LPS treatment induced post-translational modification, such as phosphorylation, of USP14. MLE12 cells were transfected with an empty vector alone or the HA-USP14 plasmid for 48 h prior to LPS treatment followed by immunoprecipitation with an antibody to phospho-serine and immunoblotting with an HA tag antibody. Interestingly, the LPS treatment increased serine phosphorylation of USP14 at 15 min (Fig. 2A). The post-translational modification of DUBs has not yet been well demonstrated. A recent study from Zhang et al. (24) reports that USP4 is phosphorylated by AKT, which mediates its stability. Growing evidence suggests that phosphorylation of the components of the 19 S RP regulates proteasome activity. Subunits of proteasomes including α subunits C8 (α7), C9 (α3), and ATPase subunit S4 (rpt2) have been shown to be modified by phosphorylation (25). Djakovic et al. (26) reported that phosphorylation of the 19 S ATPase subunit, Rpt6, by the calcium/calmodulin-dependent protein kinase II α (CaMKIIα) may promote the tethering of proteasomes to scaffolds and cytoskeletal components. This study is the first to demonstrate a post-translational modification of USP14. The phosphorylation site and role of the serine phosphorylation in the regulation of USP14 activity will need to be further investigated. Further, we found that overexpression of USP14 promoted LPS-induced degradation of I-κB without altering phosphorylation of JNK1/2 (Fig. 2B), suggesting that USP14 is involved in LPS-induced NF-κB pathway, but not JNK signaling. To investigate the involvement of USP14 in LPS-induced cytokine release, human lung epithelial cells (Beas2B) were transfected with HA-USP14 plasmid prior to LPS treatment. The IL-8 levels in cell culture media were measured by a human IL-8 ELISA kit. Consistently, LPS treatment significantly increased IL-8 production. Interestingly, overexpression of HA-USP14 promoted LPS-induced IL-8 release (Fig. 2C). Further, overexpression of HA-USP14 increased TNFα or E. coli-induced IL-8 secretion (Fig. 2D). The data demonstrate that USP14 promotes antigen-induced I-κB degradation as well as cytokine release in lung epithelial cells.

This study is the first demonstrating that USP14 regulates cytokine release via modulating I-κB stability. This reveals a new mechanism by which the NF-κB pathway is regulated by USP14 in lung epithelial cells and suggests that the association between USP14 and RelA is the link between the NF-κB complex and the proteasome. The present study will provide a new therapeutic target to diminish lung inflammation. Several inhibitors have been developed to inhibit USP14 activity (11, 16, 18); however, their specificity remains unclear. The implications of these inhibitors in lung inflammation will likely be examined in future studies. In addition, an isoform of USP14, the short form (USP14SF), has been reported (11). Its expression in the lung cells and its involvement in the NF-κB pathway and inflammatory responses will be investigated.

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