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Downregulation of active IKK β by Ro52-mediated autophagy

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Abstract

Upon activation, NF- κ B translocates into the nucleus and initiates many biological events. This NF- κ B signaling is mainly induced by the protein kinase IKK β . Early in this signaling pathway, IKK β is phosphorylated for activation by several factors, such as pro-inflammatory cytokines and the Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV-1). In cells expressing Tax protein, IKK β is persistently phosphorylated, which chronically activates NF- κ B signaling. But the active IKK β is conjugated with a monoubiquitin by the E3 ubiquitin ligase Ro52, and the IKK β -induced NF- κ B signaling is downregulated. However, the mechanism of the downregulation has been unknown. Here, we show that Ro52-mediated monoubiquitination is involved in the subcellular translocation of active IKK β to autophagosomes. Furthermore, using reporter assays, we show that Ro52 suppresses IKK β -induced NF- κ B signaling and that this suppression is blocked by an autophagy inhibitor. These results suggest that Ro52-mediated monoubiquitination plays a critical role in the downregulation of active IKK β through autophagy.

Keywords

autophagy; trafficking; monoubiquitination; protein degradation; inflammation

1. Introduction

As a transcription factor, NF- κ B plays a role in many biological events. In resting cells, NF- κ B is sequestered in the cytoplasm by the inhibitory protein I κ B α , which binds to the nuclear localization sequence of NF- κ B (Hayden and Ghosh, 2004). In cells stimulated by various biological inducers through cell-surface receptors such as tumor necrosis factor (TNF) receptor, Toll-like receptor, lipopolysaccharide (LPS) receptor, and members of the immunoglobulin superfamilies, NF- κ B is transiently activated (Li and Verma, 2002). NF- κ B is also persistently activated in cells expressing the Tax protein of human T-cell leukemia virus type 1 (HTLV-1), which is an oncovirus (Giam and Jeang, 2007). Despite differences in the initiating step, inducers such as TNF- α , LPS, and Tax lead to activation of the kinase IKK β through its phosphorylation. In turn, the activated phospho-IKK β phosphorylates

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I κ B α , which is subsequently ubiquitinated and degraded by proteasomes. This degradation of I κ B α allows NF- κ B to translocate into the nucleus because its nuclear localization sequence becomes exposed. In the nucleus, NF- κ B promotes the transcription of many genes involved in inflammation, immunity, tumorigenesis, and apoptosis (Hayden and Ghosh, 2004). Thus, IKK β is a key positive-regulator in the NF- κ B signaling pathway.

The kinase activity of IKK β is stimulated by signals through cell-surface receptors that trigger its phosphorylation at Ser-177 and Ser-181 (Delhase et al., 1999). In contrast to its transient pattern of phosphorylation and activation in cells stimulated by these signals, IKK β is also chronically phosphorylated and activated in cells expressing Tax protein of HTLV-1, as described above (Carter et al., 2001). In human cells activated by Tax, a small amount of IKK β is conjugated with a single ubiquitin molecule (monoubiquitin). Importantly, this monoubiquitination is contingent upon Tax-induced phosphorylation of IKK β at Ser-177 and Ser-181 (Carter et al., 2003). Although this indicates an important mechanistic link between phosphorylation and monoubiquitination of IKK β , the biological relevance of monoubiquitination of IKK β remains unknown.

Ubiquitin, a 76-amino-acid polypeptide, is highly conserved in evolution, with a difference of only 3 amino acids between the human and yeast homologues (Yeh et al., 2000). In the presence of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase, ubiquitin becomes covalently attached to Lys residues of target proteins through the formation of isopeptide bonds (Yeh et al., 2000). In many cases, the internal Lys residue of ubiquitin further forms an isopeptide bond with the C-terminal Gly residue of another ubiquitin molecule to create a polyubiquitin chain. This chain serves as a proteasome-targeting signal (Yeh et al., 2000). In proteasomes, polyubiquitinated proteins are degraded in an ATP-dependent manner (Yeh et al., 2000). Thus, polyubiquitination of cellular proteins is well recognized as a targeting signal to proteasomes for degradation. However, ubiquitination has recently emerged as a regulatory signal for biological events other than proteasome-mediated degradation. Notably, monoubiquitination seems to play an intimate role in protein-protein interactions and trafficking of proteins between various cellular compartments (Mosesson and Yarden, 2006). Although IKK β is monoubiquitinated, whether this monoubiquitination is involved in these proteasome-unrelated biological events is still unclear.

Ro52 (also known as TRIM21) is a RING-finger protein expressed in most tissues and cells (Sibilia, 1998). Ro52 is well known as an autoantigen that is recognized by anti-Ro52 autoantibodies found in the sera of patients with several autoimmune diseases, especially Sjögren's syndrome and systemic lupus erythematosus (Sibilia, 1998). Recently, using Ro52 knockout mice, Yoshimi et al. showed the possibility that Ro52 plays a role in the downregulation of NF- κ B-dependent inflammatory signaling (Yoshimi et al., 2009). Furthermore, Espinosa et al. also showed that Ro52-deficient mice develop uncontrolled inflammation and systemic autoimmunity as a consequence of minor tissue injury (Espinosa et al., 2009), suggesting that Ro52 plays a role in the negative regulation of inflammation and systemic autoimmunity.

Previously, Ro52 was identified as an E3 ubiquitin ligase that catalyzes ubiquitination of several proteins, including Ro52 itself (Wada and Kamitani, 2006a), Usp4 (also known as UnpEL or Unph) (Wada and Kamitani, 2006b), IRF-8 (Kong et al., 2007), and TRIM5 α (Yamauchi et al., 2008). Most recently, we found that Ro52 conjugates a monoubiquitin to IKK β in cells expressing the Tax oncoprotein of HTLV-1 (Wada et al., 2009). Our observations suggested that Tax protein induces phosphorylation of IKK β , which leads to interaction of IKK β with Ro52 for monoubiquitination. Importantly, Ro52 seems to downregulate Tax-induced NF- κ B signaling by monoubiquitinating IKK β (Wada et al.,

2009). However, the reason why monoubiquitination of IKK β results in the suppression of NF- κ B signaling is unknown. To address these questions, cell biological assays and biochemical assays were performed. In this study, we show that autophagy is involved in the Ro52-mediated downregulation of NF- κ B signaling. We propose that Tax-activated IKK β is monoubiquitinated by Ro52, which triggers the translocation of the protein complex containing active IKK β and Ro52 to autophagosomes for sequestration and subsequent degradation.

2. Materials and Methods

2.1. Cell lines and culture conditions

Human embryonic kidney (HEK) 293 and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

2.2. Antibodies

Mouse anti-HA antibody (16B12) was purchased from Covance (Richmond, CA). Mouse anti-FLAG antibody (M2) was purchased from Sigma (St. Louis, MO). Rabbit anti-human LC3 antibody (#sc-28266) and mouse anti-human IKK β antibody (H-4; #sc-8014) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Rpt5 antibody was purchased from Affiniti Research Products Ltd. (Mamhead, Exeter, UK). Mouse anti-human LAMP2 antibody (H4B4) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Rabbit anti-phospho-IKK β (Ser177/181) antibody (#2694) and rabbit anti-IKK β (L570) antibody (#2678) were purchased from Cell Signaling Technology (Danvers, MA). Anti-Ro52 antibody is a gift from Dr. Eiji Sugiyama (University of Toyama, Toyama, Japan).

2.3. Preparation of cDNAs

The cDNAs of human ubiquitin (Kamitani et al., 1997a), Ro52 (Wada and Kamitani, 2006a), and IKK β (Wada et al., 2009) were amplified using polymerase chain reaction (PCR) with appropriate primers from a human testis, heart, or brain cDNA library (Invitrogen, Carlsbad, CA). The cDNAs of Tax (Tsukada et al., 1997) and mRFP were kindly provided by Dr. Junichi Tsukada (University of Occupational and Environmental Health, Kitakyushu, Japan) and Dr. Roger Tsien (University of California San Diego, La Jolla, CA), respectively. A mammalian expression plasmid inserted with cDNA of EGFP-fused LC3 was provided by Dr. William Jackson (Stanford University, Palo Alto, CA) through Addgene (Cambridge, MA).

2.4. Plasmid construction and transfection

To express proteins tagged with an epitope at the N-terminus in mammalian cells, the cDNAs were inserted into the plasmid vectors pcDNA3/HA-N (Kamitani et al., 1997c) or pcDNA3/FLAG-N (Wada et al., 2006) as described previously (Kamitani et al., 1997a). To express EGFP-fused Ro52 in HEK293 cells, we inserted the cDNA of Ro52 into pEGFP-N1 (Clontech, Palo Alto, CA) and generated pRo52-EGFP. To express proteins fused with mRFP at the C-terminus in HEK293 cells, we inserted the mRFP cDNA (a gift from Dr. Roger Tsien, University of California San Diego, La Jolla, CA) into pcDNA3 and generated pcDNA3/mRFP-C. The cDNA of IKK β .SE (see below) was then inserted into pcDNA3/mRFP-C to generate pIKK β .SE-mRFP for the expression of IKK β .SE-mRFP. The plasmids were transfected into HEK293 and HeLa cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.5. Site-directed mutagenesis

To abolish the ligase activity of Ro52, a Cys-to-Ala substitution was generated in Ro52 at Cys-16. The cDNA of wild-type Ro52 was mutated by PCR-based site-directed mutagenesis as described previously (Kamitani et al., 1997b). The mutated cDNA was subcloned into pcDNA3/RH-C (Kamitani et al., 1998b). To replace both Ser-177 and Ser-181 of IKK β with the phosphomimetic glutamic acid (IKK β .SE), the cDNA of wild-type IKK β was also mutated by PCR-based site-directed mutagenesis. The mutated cDNA was subcloned into pcDNA3/RH-N (Kamitani et al., 1998a), pcDNA3/FLAG-N (Wada et al., 2006), or pcDNA3/mRFP-C.

2.6. Plasmid construction for direct fusion of monoubiquitin to IKK β

For the monoubiquitin fusion, we used truncated ubiquitin (termed UbG), in which the last Gly residue at the 76th amino acid of wild-type ubiquitin was deleted. Because of this truncation, UbG-fused proteins are not hydrolyzed by ubiquitin C-terminal hydrolases. To amplify a cDNA encoding UbG, PCR was used. The cDNA of UbG was then inserted into pcDNA3/IKK β .SE-RH (Wada et al., 2009) and pcDNA3/IKK β .SE-mRFP to generate plasmids pUbG-IKK β .SE-RH and pUbG-IKK β .SE-mRFP for the expression of Ub-IKK β .SE-RH and Ub-IKK β .SE-mRFP, respectively.

2.7. Western blotting

Protein samples were treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β -mercaptoethanol. After SDS-polyacrylamide gel electrophoresis, Western blotting was performed according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). As a secondary antibody, horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was used.

2.8. Reporter assay

To investigate the involvement of autophagy in the Ro52-mediated downregulation of IKK β , we used 3-MA (Sigma), a specific inhibitor of autophagy (Herman-Antosiewicz et al., 2006) in reporter assays. In brief, as indicated in the figures, proteins were expressed in HEK293 cells transfected with pRL-TK plasmid (Promega, Madison, WI) and a 3 $\times$ NF- κ B luciferase reporter plasmid (NF- κ B-Luc), which was a gift from Dr. Jinke Cheng (The University of Texas M. D. Anderson Cancer Center). Four hours after transfection, the cells were treated with or without 10 mM 3-MA. Fifteen hours after transfection, the cell extract was prepared and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) (Tanaka et al., 2005) according to the manufacturer's instructions. The luciferase activity was measured using the Lumistar Galaxy automated microplate luminescence reader (BMG Labtechnologies, Durham, NC). The luciferase activity was determined and normalized according to the Renilla luciferase activity induced by pRL-TK transfection. The relative luciferase activity was determined using a representative transfection experiment performed in triplicate.

All of the values were presented as the mean $\pm$ standard error from at least three independent experiments. The statistical significance of the data was evaluated using the Student's *t*-test. Probability values below 0.05 ($P < 0.05$) were considered significant and probability values below 0.01 ($P < 0.01$) were considered highly significant.

2.9. Fluorescence microscopy

To investigate the subcellular location of Ro52 and IKK β in cultured cells, we performed fluorescence microscopy studies. HEK293 cells were cultured on a coverslip in a 3.5-cm dish and then transfected with pRo52-EGFP and/or pIKK β .SE-mRFP. After 20 h, the cells

were fixed with a 4% paraformaldehyde solution (pH 7.5) for 30 min at room temperature. The cells were then counterstained with 0.1 µg/ml of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche Diagnostics) for 10 min and analyzed under a BX60 fluorescence microscope (Olympus, Center Valley, PA) or an Axio Imager M1 fluorescence microscope (Carl Zeiss, Thornwood, NY).

To characterize the large cytoplasmic vesicles, in which monoubiquitin-fused IKKβ localized, we also performed fluorescence microscopy studies as described above. pUbG-IKKβ.SE-mRFP was transfected with a plasmid for the expression of EGFP-fused LC3 into HEK293 cells by FuGENE6. The localization of Ub-IKKβ.SE-mRFP and EGFP-LC3 were analyzed under a fluorescence microscope.

2.10. Live cell fluorescence microscopy

To observe live images of the large round-shaped structures that are generated by coexpression of Ro52 and IKKβ.SE, live cell fluorescence microscopy was performed as described previously (Campbell et al., 2007; Tanaka et al., 2010). HEK293 cells expressing Ro52-EGFP and IKKβ.SE were taken as images in a 46-frame sequence captured at 2-second intervals using live cell fluorescence microscopy with a 100× objective lens. These images were used to make movies (Supplemental Movie 1). Afterwards, the 11 frames were picked up to follow the movements of seven large round-shaped structures.

2.11. Immunostaining

To characterize the large cytoplasmic vesicles, in which Ro52 colocalized with IKKβ, we further performed single immunostaining. HEK293 cells were cotransfected with pRo52-EGFP and pIKKβ.SE-mRFP by FuGENE6. After 24 h, the cells were fixed and permeabilized as described above. The cells were first labeled with one of the following primary antibodies: mouse anti-Rpt5 (1:20,000), rabbit anti-LC3 (1:100), and mouse anti-LAMP2 (1:1,000). After washing, the cells were labeled with Alexa Fluor 750-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, OR) at a dilution of 1:1,000. The cells were then analyzed with a fluorescence microscope.

To determine the localization of ubiquitin in the large cytoplasmic vesicles, in which Ro52 colocalizes with IKKβ, we immunostained the HA epitope in cells expressing HA-ubiquitin. Briefly, HEK293 cells were cotransfected with pRo52-EGFP, pIKKβ.SE-mRFP, and pcDNA3/HA-ubiquitin (Kamitani et al., 1997a) by FuGENE6. After 24 h, the cells were fixed and permeabilized as described above. The cells were then labeled with mouse anti-HA antibody (1:2,000). After washing, the cells were labeled with Alexa Fluor 750-conjugated goat anti-mouse IgG at a dilution of 1:1,000. The cells were then analyzed under a fluorescence microscope.

To characterize the large cytoplasmic vesicles, in which monoubiquitin-fused IKKβ localizes, we immunostained endogenous LAMP2 in the transfected cells. HEK293 cells were cotransfected with pUbG-IKKβ.SE-mRFP and pEGFP-LC3 by FuGENE6. After 24 h, the cells were fixed and permeabilized as described above. The cells were then labeled with mouse anti-LAMP2 antibody (1:1,000). After washing, the cells were labeled with Alexa Fluor 750-conjugated goat anti-mouse IgG at a dilution of 1:1,000. Next, the cells were analyzed with a fluorescence microscope.

3. Results

3.1. Subcellular translocation of active IKK β .SE when coexpressed with Ro52

We recently demonstrated that Ro52 strongly interacts with IKK β .SE (Wada et al., 2009), which is a constitutively active form of IKK β due to substitutions with the phosphomimetic glutamic acid at Ser-177 and Ser-181 (Carter et al., 2001; Carter et al., 2003; Mercurio et al., 1997). On the basis of this observation, we wondered whether Ro52 colocalizes with IKK β .SE in cells. To address this question, we performed fluorescence microscopy studies (Fig. 1). We first investigated the subcellular location of Ro52 by expressing EGFP-fused Ro52 alone in HEK293 cells. As shown in panels A1 and A2, Ro52-EGFP was mainly localized to rod-like structures called cytoplasmic bodies, which we and other groups reported previously (Reymond et al., 2001; Rhodes et al., 2002; Tanaka et al., 2010; Wada et al., 2006). Next, we expressed monomeric red fluorescent protein (mRFP)-fused IKK β .SE alone in HEK293 cells to determine the subcellular location of IKK β .SE. As shown in panels B1 and B2, we found that IKK β .SE is diffusely located in the cytoplasm. Finally, we coexpressed Ro52-EGFP with IKK β .SE-mRFP in HEK293 cells to examine their colocalization (panels C1–4). As shown in panel C1, Ro52 was relocated to the cytoplasm diffusely and to large round-shaped structures in the cytoplasm. IKK β .SE was detected in the cytoplasm diffusely and in large round-shaped structures in the cytoplasm (panel C2). Importantly, IKK β .SE was clearly colocalized with Ro52 in the large round-shaped structures (panels C3 and C4). These results suggest that the coexpression of Ro52 and IKK β .SE leads to the subcellular translocation of both proteins.

3.2. Live images of large round-shaped structures

To investigate the large round-shaped structures generated by coexpression of Ro52 and IKK β .SE, we performed live cell fluorescence microscopy. In brief, Ro52-EGFP and IKK β .SE were coexpressed in HEK293 cells. Then, images of Ro52-positive structures were rapidly captured in sequence at the shortest possible time interval compatible with obtaining a sufficiently long exposure to record the movements of large round-shaped structures. Supplemental Movie 1 shows such a 46-frame sequence captured at 2.0-second intervals. There are 7 large round-shaped structures in one cell (Fig. 2; *a–g*). Importantly, structures *f* and *g* show a ring form. In structures *a*, *b*, *c*, *d*, and *e*, a shiny region exists at the periphery and looks membranous, suggesting that the large round-shaped structures are vesicles and that Ro52 predominantly localizes to their membrane. Another important thing is that all the round-shaped structures are motile. Two types of movements can be observed; namely, fusion and fission.

As shown in Fig. 2 and Supplemental Movie 1, structure *a* moves to structure *b* (time 00:26–00:32). At time 00:34, structure *a* looks to be fused with structure *b*. Interestingly, the fused structure throws out structure *a'* at time 00:36 (fission). Afterwards, structure *a'* travels to structure *c* (time 00:38–01:14). At time 01:16, they fuse together. In Fig. 2 and Supplemental Movie 1, another fusion can be observed. Specifically, large round-shaped structures *d* and *e* are separated (time 00:00–00:38). However, they fuse together (time 00:48–01:14). Thus, large round-shaped structures are motile vesicles and show fusion and fission in cells, suggesting that these structures are late endosomes, autophagosomes, or lysosomes, but not protein aggregates.

3.3. Characterization of large round-shaped structures containing IKK β .SE and Ro52

Next, we characterized large round-shaped structures containing IKK β .SE and Ro52 by using immunofluorescence microscopy. To examine whether these structures are proteasome-enriched structures, late endosomes, lysosomes, or autophagosomes, we coexpressed Ro52-EGFP with IKK β .SE-mRFP in HEK293 cells to generate large round-

shaped structures containing Ro52 and IKK β .SE. We then immunostained endogenous Rpt5 for proteasome-enriched structures, LC3 for autophagosomes, and LAMP2 for lysosomes in the cells. As shown in Fig. 3A, the large round-shaped structures did not contain the proteasome subunit Rpt5, indicating that the structures are not proteasome-enriched structures. As shown in Fig. 3B, however, all of these structures were positive for LC3, suggesting that they are autophagosomes. In the immunostaining for LAMP2, we found that about 90% of the large round-shaped structures were negative for LAMP2 and that about 10% were positive. In Fig. 3C, we showed two large round-shaped structures in a cell. The left structure was LAMP2-positive. Interestingly, the immunostaining of LAMP2 showed a ring shape, suggesting that the structure was surrounded by a LAMP2-positive membrane. In contrast, the right structure was LAMP2-negative. These results indicate that the LAMP2-negative structures (such as the right structure in Fig. 3C) are autophagosomes and that the LAMP2-positive structures (such as the left structure in Fig. 3C) are autophagolysosomes, which are autophagosomes fused with lysosomes.

Because Ro52 monoubiquitinates active IKK β , such as IKK β .SE and Tax-induced phospho-IKK β , in HEK293T cells (Carter et al., 2003; Wada et al., 2009), we hypothesized that the monoubiquitinated form of active IKK β translocates to autophagosomes. To test this, we further examined whether ubiquitin localizes to the IKK β .SE-positive autophagosomes. We expressed Ro52-EGFP, IKK β .SE-mRFP, and HA-tagged ubiquitin in HEK293 cells and immunostained the cells with anti-HA antibody to determine the location of HA-ubiquitin. As shown in Fig. 3D, HA-ubiquitin was stained as ring forms at the IKK β .SE-positive autophagosomes, suggesting that ubiquitin or ubiquitinated proteins localize to the membranes of autophagosomes.

3.4. Subcellular location of monoubiquitin-fused IKK β .SE in HEK293 cells

Since ubiquitin is strongly detected at the membranes of the IKK β .SE-positive autophagosomes, the monoubiquitination of IKK β .SE might be a targeting signal to autophagosomes. To test this possibility, we examined whether the direct fusion of a monoubiquitin to IKK β .SE causes its translocation. In the molecule of monoubiquitin-fused protein, however, the monoubiquitin links to the N-terminal Met residue of the protein via an α -peptide bond. This linkage is artificial, not natural, in cells. In the naturally monoubiquitinated protein in cells, the monoubiquitin links to the target Lys residue of the protein via an isopeptide bond. Thus, the monoubiquitin fusion is different from the natural monoubiquitination, exactly, but they are structurally similar. Therefore, using the direct fusion of monoubiquitin to target proteins, we are able to investigate the biological relevance of their monoubiquitination (Wu et al., 2006).

To determine whether monoubiquitin-fused IKK β .SE (Ub-IKK β .SE) localizes to autophagosomes or autophagolysosomes, we expressed mRFP-fused Ub-IKK β alone in HEK293 cells and analyzed by fluorescence microscopy. As expected, we found that Ub-IKK β .SE locates to large round-shaped structures without coexpression of Ro52 (data not shown). Next, we coexpressed mRFP-fused Ub-IKK β .SE with EGFP-fused LC3 in HEK293 cells to characterize the structures. As shown in Fig. 4A (middle panel), we observed the generation of large Ub-IKK β .SE-positive structures in these cells. Importantly, LC3 colocalized to all of these structures (left and right panels), suggesting that the large Ub-IKK β .SE-positive structures are autophagosomes. To further characterize these structures, we immunostained them with an antibody to the lysosome marker LAMP2. Briefly, we coexpressed mRFP-fused Ub-IKK β .SE with EGFP-fused LC3 in HEK293 cells and then immunostained the cells with anti-LAMP2 antibody. About 5% of the structures were LAMP2-positive, whereas about 95% were LAMP2-negative. In Fig. 4B, a LAMP2-positive structure, which contains both LC3 and Ub-IKK β .SE, is shown as an example. These results suggest that most large Ub-IKK β -positive structures are autophagosomes but

about 5% are autophagolysosomes. In addition these results support the hypothesis that the monoubiquitination of IKK β .SE is a targeting signal to autophagosomes.

3.5. Role of autophagy in Ro52-mediated downregulation of NF- κ B signaling

Using reporter assays and biochemical assays, we previously demonstrated that Ro52 downregulates Tax-induced NF- κ B signaling by monoubiquitinating IKK β (Wada et al., 2009). However, we could not explain the reason why monoubiquitination of IKK β resulted in the suppression of NF- κ B signaling. In the prior sections, we showed that IKK β .SE translocates to autophagosomes in the presence of overexpressed Ro52. In addition, we showed that monoubiquitin-fused IKK β .SE translocates to autophagosomes in the absence of overexpressed Ro52 (see above). On the basis of these results, we hypothesized that active IKK β , such as IKK β .SE and Tax-activated phospho-IKK β , is monoubiquitinated by Ro52, which triggers the translocation of the protein complex containing both Ro52 and active IKK β to autophagosomes for its sequestration and subsequent degradation, resulting in the suppression of NF- κ B signaling.

To prove this hypothesis, we used 3-methyladenine (3-MA) (Herman-Antosiewicz et al., 2006), a specific inhibitor of autophagy, in reporter assays (Fig. 5A). Specifically, empty vector, wild-type IKK β , or IKK β .SE was coexpressed with empty vector or Ro52 (wild-type) in HEK293 cells transfected with the NF- κ B reporter plasmid. The cells were then treated with or without 3-MA. Afterwards, the cell extract was assayed for luciferase activity. As shown in Fig. 5A (left panel), IKK β (wild-type) weakly promoted the NF- κ B reporter activity. In contrast, IKK β .SE strongly promoted it. However, as we showed previously (Wada et al., 2009), the promoted activity was reduced to ~30% when co-expressed with Ro52. Most importantly, this Ro52-mediated reduction was blocked by 3-MA (right panel), suggesting that autophagy is involved in the reduction in reporter activity.

Previously, we demonstrated that the monoubiquitin fusion of IKK β .SE leads to reduction in its NF- κ B reporter activity (Wada et al., 2009). In the prior section, we showed that monoubiquitin-fused IKK β .SE localizes to autophagosomes or autophagolysosomes. These observations suggest that the monoubiquitin-fused IKK β .SE is downregulated by autophagy, resulting in a reduction in NF- κ B signaling. To prove this, we examined whether the autophagy inhibitor 3-MA blocks the reduction in NF- κ B reporter activity of monoubiquitin-fused IKK β .SE (Fig. 5B). In brief, wild-type IKK β (control), IKK β .SE, or monoubiquitin-fused IKK β .SE was expressed in HEK293 cells transfected with the NF- κ B reporter plasmid. The cells were then treated with or without 3-MA. Afterwards, the cell extract was assayed for luciferase activity. As shown in Fig. 5B, monoubiquitin fusion of IKK β .SE led to a reduction in reporter activity (left panel). However, this reduction was blocked by 3-MA (right panel). Thus, autophagy is involved in the activity reduction caused by the monoubiquitin fusion.

3.6. Ro52-induced downregulation of endogenous phospho-IKK β in HeLa cells expressing Tax

Ro52-mediated monoubiquitination seems to play a role in the translocation of active IKK β (such as IKK β .SE and Tax-induced phospho-IKK β) to autophagosomes. If this is correct, overexpression of Ro52 should lead to reduction in the expression of active IKK β through autophagy. To examine this, we overexpressed FLAG-tagged Ro52 (wild-type) or its mutant C16A (as a control) in HeLa cells expressing Tax. We then prepared cell lysates, followed by Western blot analysis to detect expression levels of endogenous total IKK β and phospho-IKK β , which is phosphorylated at Ser-177 and Ser-181. As shown in Fig. 6, the expression level of total IKK β was not changed by overexpression of wild-type Ro52 or C16A (lanes 2–4, top panel). In contrast, as shown in the second panel, phospho-IKK β induced by Tax

was downregulated by overexpression of wild-type Ro52 (lanes 2 vs. 3), but not by C16A (lanes 2 vs. 4). These results suggest that the ubiquitin-ligase activity of Ro52 is involved in the reduction in endogenous phospho-IKK β induced by Tax. In other words, Ro52-mediated monoubiquitination might decrease the amount of the Tax-induced active IKK β .

4. Discussion

The E3 ubiquitin ligase Ro52 conjugates a monoubiquitin to active IKK β , such as IKK β .SE and Tax-induced phospho-IKK β in cells (Wada et al., 2009). Because monoubiquitination plays roles in proteasome-unrelated biological events, including protein trafficking (Di Fiore et al., 2003; Haglund et al., 2003; Miyase et al., 2005; Polo et al., 2003), we hypothesized that monoubiquitination is involved in the trafficking of active IKK β . To test this hypothesis, we first determined the subcellular location of IKK β .SE after Ro52 was overexpressed in cells. The results showed that IKK β .SE relocates to autophagosomes along with Ro52. These results suggest that Ro52-mediated monoubiquitination of active IKK β is a targeting signal for the protein complex containing active IKK β and Ro52 to translocate to autophagosomes.

Autophagosomes are cytoplasmic vesicles, in which autophagy takes place. In general, the autophagy-lysosome pathway degrades organelles and cytoplasmic macromolecules at their final stage (Klionsky and Emr, 2000). Although researchers have long thought that autophagy is involved in bulk, non-selective protein degradation mechanism, but recently, there has been much evidence that protein degradation selectively occurs in autophagy (Yu et al., 2008; Yu et al., 2006). For instance, α -synuclein, which is a major component of Lewy bodies found in patients with Parkinson's disease, was detected inside the vesicles with autophagic morphology (Webb et al., 2003). Furthermore, treatment with an autophagy inhibitor promoted the accumulation of monoubiquitinated forms of α -synuclein (Rott et al., 2008). These observations suggest that monoubiquitination targets α -synuclein to autophagosomes, similar to the monoubiquitination of active IKK β . Autophagy might selectively regulate monoubiquitinated proteins other than α -synuclein and IKK β .

In this study and our previous studies, we mainly found four things regarding monoubiquitination of IKK β . First, Ro52 interacts with active IKK β and monoubiquitinates it (Wada et al., 2009). Second, the monoubiquitination targets active IKK β to autophagosomes (see Fig. 3). Third, active IKK β is decreased in cells overexpressing Ro52 (see Fig. 6). Fourth, NF- κ B signaling is induced by active IKK β but downregulated by Ro52 through autophagy (see Fig. 5). To explain these findings by one mechanism, we propose the model shown in Fig. 7: Ro52-mediated monoubiquitination triggers the translocation of active IKK β to autophagosomes for autophagic events. In other words, active IKK β , which is monoubiquitinated by Ro52, is sequestered in autophagosomes or further degraded in autophagolysosomes, resulting in the downregulation of NF- κ B signaling (Fig. 7).

NF- κ B is activated by various inducers through phosphorylation of IKK β . In mammalian cells, cell-surface receptors, such as TNF receptor, transiently transduce NF- κ B signaling (Li and Verma, 2002). Specifically, the cytokine TNF leads to rapid phosphorylation and activation of IKK β , followed by dephosphorylation and inactivation within 30–60 min (Delhase et al., 1999). Previously, we and other researchers reported that the TNF-induced transient response does not enable the stable formation and/or detection of monoubiquitination of IKK β , even in cells overexpressing Ro52 (Carter et al., 2005; Wada et al., 2009). This is probably because TNF-induced phospho-IKK β is rapidly dephosphorylated and is not able to interact with Ro52.

In contrast to the transient phosphorylation of IKK β , the oncoprotein Tax derived from HTLV-1 induces persistent phosphorylation and activation of IKK β (Carter et al., 2003), which allows Ro52 to stably interact with IKK β and efficiently monoubiquitinate it (Wada et al., 2009) for subsequent translocation and autophagic events (see Fig. 7). Taken together, Ro52-mediated monoubiquitination might play a crucial role only in elimination of persistently activated IKK β , but not transiently activated IKK β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TNF	tumor necrosis factor
HTLV-1	human T-cell leukemia virus type 1
HEK	human embryonic kidney
HA	hemagglutinin
RH	RGS-poly His
PCR	polymerase chain reaction
mRFP	monomeric red fluorescent protein
EGFP	enhanced green fluorescent protein
PBS	phosphate-buffered saline
3-MA	3-methyladenine

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Fig. 1. Subcellular localization of IKK β and Ro52 in HEK293 cells

(A) Localization of EGFP-fused Ro52. (B) Localization of mRFP-fused IKK β .SE. (C) Colocalization of Ro52-EGFP and IKK β .SE-mRFP. Ro52-EGFP alone (panels A1 and A2), IKK β .SE-mRFP alone (panels B1 and B2), or both Ro52-EGFP and IKK β .SE-mRFP (panels C1-C4) were expressed in HEK293 cells by plasmid transfection. After 24 h, the cells were fixed and then stained with DAPI. Afterwards, the cells were analyzed using a fluorescence microscope. The localization of Ro52-EGFP was shown by the green fluorescence of EGFP, and the localization of IKK β .SE-mRFP was shown by the red fluorescence of mRFP. Their colocalization was shown by the merging of both fluorescences (panels C3 and C4). The nuclear counterstaining was shown by the blue fluorescence of DAPI (panels A2, B2, and C4). Scale bars indicate 10 μ m.

Fig. 2. Dynamic movements of the large round-shaped structures

To generate the large round-shaped structures, Ro52-EGFP and IKK β .SE were coexpressed in HEK293 cells. The structures were then observed by live cell fluorescence microscopy. Specifically, a live HEK293 cell expressing Ro52-EGFP and IKK β .SE was imaged over 46 frames at 2.0-second intervals (92-sec total time) to generate a movie (Supplemental Movie 1). Afterwards, the 11 individual frames (time points 00:00, 00:08, 00:26, 00:32, 00:34, 00:36, 00:38, 00:48, 01:14, 01:16, and 01:18) were picked up from 46 frames. Seven large round-shaped structures are indicated by letters (a–g). Scale bars indicate 10 μ m.

Fig. 3. Characterization of the large round-shaped structures containing Ro52 and IKK β

(A) Localization of the proteasome subunit Rpt5. (B) Localization of the autophagosome marker LC3. (C) Localization of the lysosome marker LAMP2. (D) Localization of ubiquitin. In A, B, and C, HEK293 cells were transfected to express both Ro52-EGFP and IKK β .SE-mRFP. In D, HEK293 cells were transfected to express Ro52-EGFP, IKK β .SE-mRFP, and HA-ubiquitin. The cells were then fixed, permeabilized, and immunostained with antibodies against Rpt5 (A), LC3 (B), LAMP2 (C), and the HA-epitope for HA-ubiquitin (D). Afterwards, cells were labeled with Alexa Fluor 750-conjugated secondary antibody. After DAPI staining, cells were analyzed using a fluorescence microscope. The localization of Ro52-EGFP was shown by the green fluorescence of EGFP. The localization

of IKK β .SE-mRFP was shown by the red fluorescence of mRFP. The localization of Rpt5 (A), LC3 (B), LAMP2 (C), and HA-ubiquitin (D) was determined by the fluorescence of Alexa Fluor 750 and shown by the purple color. The nuclear counterstaining was shown by the blue fluorescence of DAPI. Their colocalization was shown by the merging of fluorescences as indicated. Scale bars indicate 10 μ m.

Fig. 4. Characterization of the large round-shaped structures containing monoubiquitin (Ub)-fused IKKβ

(A) Localization of LC3 to the large round-shaped structures containing Ub-fused IKKβ. HEK293 cells were transfected to express Ub-fused IKKβ.SE-mRFP and EGFP-LC3. The cells were then fixed, stained with DAPI, and analyzed using a fluorescence microscope. (B) Localization of LAMP2 to the large round-shaped structures containing Ub-IKKβ and LC3. HEK293 cells were transfected to express Ub-IKKβ.SE-mRFP and EGFP-LC3. The cells were then fixed, permeabilized, and immunostained with anti-LAMP2 antibody. Afterwards, cells were labeled with Alexa Fluor 750-conjugated secondary antibody. After DAPI staining, cells were analyzed using a fluorescence microscope. The localization of EGFP-

LC3 was shown by the green fluorescence of EGFP. The localization of Ub-IKK β .SE-mRFP was shown by the red fluorescence of mRFP. The localization of LAMP2 was determined by the fluorescence of Alexa Fluor 750 and shown by the purple color. The nuclear counterstaining was shown by the blue fluorescence of DAPI. Their colocalization was shown by the merging of fluorescences as indicated. Scale bars indicate 20 μ m in A and 10 μ m in B.

Figure 5A **Figure 5B** **Fig. 5. Role of autophagy in Ro52-mediated downregulation of NF- κ B signaling**

(A) Involvement of autophagy in Ro52-mediated downregulation of IKK β activity. To examine whether Ro52 downregulates the IKK β activity through autophagy, IKK β (wild-type or SE mutant) was coexpressed with Ro52 in HEK293 cells transfected with the NF- κ B reporter plasmid. Four hours after transfection, the cells were treated with or without the autophagy inhibitor 3-MA. Fifteen hours after transfection, the cell extract was assayed for luciferase activity. (B) Involvement of autophagy in decrease of the activity of monoubiquitin-fused IKK β . To determine whether monoubiquitin fusion leads to a decrease in the activity of IKK β through autophagy, IKK β (wild-type), IKK β .SE, or Ub-fused IKK β .SE was expressed in HEK293 cells transfected with the NF- κ B reporter plasmid. Four

hours after transfection, the cells were treated with or without the autophagy inhibitor 3-MA. Fifteen hours after transfection, the cell extract was assayed for luciferase activity. Each bar represents the mean $\pm$ standard error (* $P < 0.01$, ** $P > 0.05$).

Fig. 6. Effect of Ro52 overexpression on levels of phospho-IKKβ in HeLa cells expressing Tax
 HeLa cells were transfected with or without expression vectors for FLAG-tagged Ro52 (wild-type or C16A) and Tax as indicated. Total cell lysates were then prepared and analyzed by Western blotting to detect total IKKβ, phospho-IKKβ (p-IKKβ), and Ro52. To demonstrate an equal loading amount of total cell lysates, Western blotting using anti-actin antibody was also performed (bottom panel). FLAG-Ro52 is indicated by an *open arrowhead*, and Ro52 without epitope tag is indicated by a *closed arrowhead* on the right.

Fig. 7. Model for downregulation of active IKK β by Ro52-mediated autophagy

In cells infected with HTLV-1, Tax is expressed and persistently phosphorylates IKK β . However, the active IKK β is downregulated by the E3 ubiquitin ligase Ro52 in our model. Briefly, Ro52 interacts with the phospho-IKK β to conjugate a monoubiquitin. The Ro52-mediated monoubiquitination targets the phospho-IKK β to autophagosomes for sequestration and subsequent lysosomal degradation (autophagy), resulting in the downregulation of NF- κ B signaling.