Mitochondrial ubiquitin ligase activator of NF-κB regulates NF-κB signaling in cells subjected to ER stress

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Abstract. The nuclear factor-κB (NF-κB) transcription factor family members control various biological processes, such as apoptosis and proliferation. The endoplasmic reticulum (ER) has emerged as a major site of cellular homeostasis regulation. The accumulation of misfolded protein in the ER causes stress and ER stress-induced NF-κB activation to protect cells from apoptosis. In this study, we found a putative ER stress-response element (ERSE) on the promoter of mitochondrial ubiquitin ligase activator of NF-κB (MULAN), and that MULAN expression was upregulated by ER stress. MULAN specifically activated NF-κB dependent gene expression in an E3 ligase activity-dependent manner. The ectopic expression of MULAN induced the nuclear translocation of endogenous p65 and the degradation of IκB. Binding assay revealed that MULAN was associated with transforming growth factor β-activated kinase (TAK1). The knockdown of MULAN using siRNA inhibited the activation of NF-κB in the cells subjected to ER stress. The findings of our study indicate that MULAN is an E3 ligase that regulates NF-κB activation to protect cells from ER stress-induced apoptosis.

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Abbreviations: TAK1, transforming growth factor β-activated kinase; MULAN, mitochondrial ubiquitin ligase activator of nuclear factor-κB; ERSE, endoplasmic reticulum stress-response element; TRAF, tumor necrosis factor receptor-associated factor; IκBα, inhibitor of κBα; IKK, IκB kinase

Key words: endoplasmic reticulum stress, nuclear factor-κB transcription, mitochondrial ubiquitin ligase activator of nuclear factor-κB, E3 ubiquitin ligase, transforming growth factor β-activated kinase

Introduction

Nuclear factor-κB (NF-κB) is a dimeric transcription factor that controls the expression of genes involved in multiple processes, including immunity, inflammation, apoptosis and cell cycle progression (1). Briefly, in resting cells, NF-κB is kept inactive in the cytoplasm by its association with the inhibitor of κBα (IκBα). Stimulating cells with an agonist, such as tumor necrosis factor α (TNFα) or interleukin (IL)-1β) activates the IκB kinase (IKK) complex, which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO. This signal-induced phosphorylation targets IκBα for Lys48-linked polyubiquitination and subsequent degradation by the ubiquitin-proteasome system (2,3). In this way, NF-κB is released from IκBα, and translocates into the nucleus to trigger the expression of its target genes. Recent studies have revealed several enzymes involved in the ubiquitination and deubiquitination of signaling proteins that mediate IKK activation through a degradation-independent mechanism (4). TNFR-associated factor (TRAF) proteins, ubiquitin E3 ligases (5) have a pivotal role in signaling pathways that are involved in the activation of NF-κB by many cell-surface receptors, including the TNFR superfamily, the IL-1 receptor (IL-1R) and Toll-like receptors (TLRs) (6). Following ligand binding to IL-1R or TLRs, TRAF6 is recruited to the receptor complexes and forms oligomers. TRAF6 oligomerization activates its ligase activity, leading to the Lys63-linked polyubiquitination of target proteins, including TRAF6 and NEMO. It is also known that receptor-interacting protein (RIP) is ubiquitinated by TRAF2 through the Lys63 linkage immediately following TNFα stimulation (7). Both the Lys63-linked polyubiquitylation of TRAF6 and RIP recruits transforming growth factor β (TGFβ)-activated kinase (TAK1) and two adaptor proteins, TAK1-binding protein (TAB)1 and TAB2. TAB2 contains a highly conserved novel zinc-finger domain that binds preferentially to polyubiquitin chains that are linked by Lys63 (8). TAB2-associated TAK1 phosphorylates IKKβ at two serine residues in the activation loop, thereby activating the IKK complex (9,10).

Ubiquitination has been shown to play important roles in the regulation of NF-κB signaling pathways, as well as in endoplasmic reticulum (ER) stress. There are two branches of ER quality control that address the situation. One is the unfolded protein response (UPR) pathway (11). The activation of UPR results in attenuation of protein synthesis, and upregulation of genes encoding chaperones that facilitate the protein folding process in the ER. Thus, UPR reduces the accumulation and aggregation of malfolded proteins, giving the cell the possibility of correcting the environment inside the ER (11,12). A second major response is known as the ER-associated degradation (ERAD) pathway to reduce the misfolded proteins in the ER, which involves retro-translocation, polyubiquitination and degradation in the cytosol through the 26S proteasome (13,14). Synoviolin, a representative ERAD-associated E3 ubiquitin ligase, is regulated by ER stress through the ER stress-response element (ERSE) which is a regulatory element located in many of these ER stress-response genes (15). Synoviolin, a mammalian homolog of Hrd1p/Der3p, was identified from the cDNA of rheumatoid synovial cells and is involved in the development of obesity, rheumatoid arthritis, fibrosis, limb girdle muscular dystrophy and liver cirrhosis (16-20).

In the present study, we searched for E3 ligases which are associated with ER stress and regulate NF-κB signaling. We identified an E3 ligase, mitochondrial ubiquitin ligase activator of NF-κB (MULAN). Previous studies have reported that MULAN is one of the NF-κB-activating genes by large scale screening (21), and regulates mitochondrial trafficking and morphology (22). In addition, mitochondrial hyperfusion promotes NF-κB activation in a TAK1- and IKK-dependent manner via MULAN (23). In this study, we found that the expression of MULAN is upregulated by ER stress. MULAN associates with TAK1 and activates NF-κB signaling in an E3 ligase activity-dependent manner. The knockdown of MULAN by siRNA resulted in the downregulation of NF-κB signaling in cells subjected to ER stress. Our results suggest that MULAN is an E3 ligase which regulates NF-κB signaling under conditions of ER stress.

Materials and methods

Plasmids. The coding sequences for full-length MULAN genes were PCR-amplified (primers, 5'-acagaattcATGGAGA GCGGAGGGC-3' and 5'-tgtgtcgacGCTGTTGTACAGGGGT ATCA-3') from reversed-transcribed HeLa cell RNA. PCR products were digested with *Eco*RI and *Sal*I, and ligated into pGEX-5X-1 (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA) for *in vitro* ubiquitin (Ub) assays. For expression in mammalian cells, the fragments of MULAN and mutants were inserted into pcDNA3 hemagglutinin antigen (HA) or pcDNA3 FLAG, which were constructed by inserting the HA sequence or FLAG sequence into pcDNA3 (Invitrogen, Carlsbad, CA, USA), respectively. Fragments of deletion mutants, termed MULAN-A, MULAN-B, MULAN-N and MULAN-C were obtained by PCR amplification. Fragments of additional deletion mutants, termed MULAN delta transmembrane (dTM) and MULAN 3C were generated by PCR-based methods. The sequences of all plasmids generated by PCR were confirmed by sequence analysis. The IKKβ, IKKβ K44A, TRAF6, TRAF6 dominant negative (DN; delta RING), TRAF2, TRAF2 DN (delta RING) and RIP1 plasmids used in this study were obtained by a PCR-based method. The ERSE-Luc reporter plasmid was also obtained by a PCR-based method and possesses 4 copies of synoviolin ERSE. The TAK1, TAK1 K63W and TAB1 plasmids were a kindly gif from Dr Kunihiro Matsumoto. The pcDNA3 HA-Ub expression plasmid, NF-κB-Luc reporter plasmids, Som-Luc and CMV-β-gal plasmid have been described in previous studies (16,24-26).

Cell culture and antibodies. HeLa cells and 293 cells [provided by RIKEN BRC through the National Bio-Resource Project of MEXT (Ibaraki, Japan)] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum plus penicillin and streptomycin at 37˚C in 5% CO_2. The following antibodies were used: anti-FLAG (M2; F3165) and anti-β-actin (A5441) from Sigma Chemical Co. (St. Louis, MO, USA), anti-HA [12CA5 (1583816) and 3F10 (1867423); Boehringer Mannheim GmbH, Mannheim, Germany], anti-IκB (sc-203) and anti-p65 (sc-372) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Induction of ER stress. To induce ER stress, the cells were stimulated with tunicamycin at concentrations of 2 μg/ml for 0, 3, 6 and 9 h and total RNA was collected following stimulation. ER stress was also induced by thapsigargin in luciferase assay (Fig. 5). The cells were stimulated with thapsigargin at concentrations of 2 μM for 4 h.

TNFα and IL-1β treatment. TNFα is known to activate NF-κB signaling by promoting the degradation of IκBα and the nuclear translocation of p65. We treated the cells with TNFα at 10 nM for 4 h as a positive control (Fig. 4) and an inducer of NF-κB signaling (Fig. 5). IL-1β was also used at 10 ng/ml for 4 h.

Transfection and immunofluorescence. For immunofluorescence experiments, trypsinized cells were seeded on a cover glass and incubated for 24 h prior to transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen) with 0.5 μg of MULAN expression plasmid, according to the manufacturer's instructions. The cells were incubated for a further 12 h after transfection, washed 3 times with phosphate-buffered saline (PBS), and fixed with 3.7% formaldehyde in PBS for 30 min, followed by permeabilization with 0.1% Triton X-100 for 30 min. The cells then were incubated with the primary antibody followed by staining with Alexa Fluor 488 anti-mouse second antibody or Alexa Fluor 594 anti-rabbit second antibody (Molecular Probes, Eugene, OR, USA). Samples were scanned with a Zeiss LSM 510 confocal laser scanning microscopy (Carl Zeiss Microscopy, Jena, Germany). Co-localization scatter diagrams were generated using LSM 510 software (27).

Transient transfection assay. Transient transfection assays were performed using the 293 cells, as previously described (24). The cells were lysed with cell lysis buffer (Toyo Ink, Tokyo, Japan) 24 h following transfection and luciferase activities were measured. The recorded activity was normalized to the β-gal activity from CMV-β-gal. All experiments were performed in triplicate. The 293 cells were transfected with 100 ng of NF-κB-Luc, 50 ng of CMV-β-gal control plasmid, and 0, 50 and 100 ng of MULAN or mutants, and/or DN-mutants plasmid, or truncated forms of MULAN expression vector. To

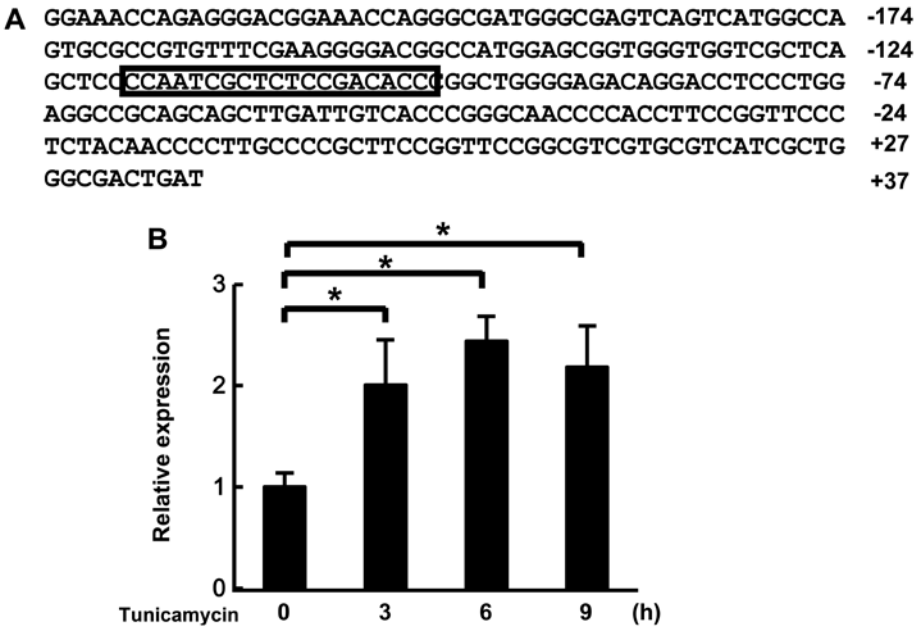

Figure 1. Induction of mitochondrial ubiquitin ligase activator of nuclear factor-κB (MULAN) expression by endoplasmic reticulum (ER) stress. (A) Nucleotide sequence of the 5'-region of the *MULAN* gene. The numbers on the right indicate the nucleotide position with respect to the transcription start site (+1). Putative ER stress-response elements (ERSEs) are boxed. (B) HeLa cells were treated with 2 μg/ml tunicamycin for the indicated periods of time. Following stimulation, total RNA was collected, and RT-PCR and real-time PCR were performed. The signals were calculated by normalization to human GAPDH. Data were analyzed using the Student's t-test and represent the means ± SD (n=3 experiments). *P<0.05.

ensure an equal amount of DNA, empty plasmids were added in each transfection.

Immunoprecipitation assay. The HeLa cells were transfected with HA TAK1, HA TAB1, HA RIP1, HA IKKβ, and/or MULAN/FLAG expression vector. After 12 h of transfection, the cells were lysed in 1 ml of lysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol and protease inhibitors). The lysates were mixed with 1 μg of anti-FLAG antibody (M2) conjugated to protein G-sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden). Following 4 h of incubation at 4˚C, the beads were washed 3 times with lysis buffer. Bound proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and examined by western blot analysis.

Western blot analysis. The protein extracts were resolved by 10% SDS-PAGE, transferred onto a PVDF membrane, and incubated with primary antibodies [anti-FLAG M2 (F3165), anti-β-actin (A5441), anti-HA 3F10 (1867423), anti-IκB (sc-203) and anti-p65 (sc-372)] followed by horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were visualized using an ECL detection system (Promega, Madison, WI, USA).

RNA interference assay, RT-PCR and real-time PCR. siRNAs (Stealth RNAi) for MULAN and GFP were purchased from Invitrogen. The sequence of the MULAN siRNA-1 was 5'-UUUCCACAAACUGGCUGUUAAGCGU-3', and that of control siRNAwas 5'-AAGAAGUCGUGCUGCUUCAUGUGGU-3'. Transfection with siRNAs was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Isogen was used for total RNA isolation and RT-PCR was performed with ReverTra Ace (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA preparation. Real-time PCR was carried out using the Universal ProbeLibrary system (Roche Diagnostic, Mannheim, Germany). Signals from each sample were normalized to values obtained for the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, which was assayed simultaneously with the experimental samples. Analyses were performed with a sequence detector (model 7500; Applied Biosystems, Foster City, CA, USA).

Statistical analysis. The non-paired Student's t-test was used to analyze mean differences. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

MULAN is regulated by ER stress. Recent studies have revealed that ER stress induces the activation of NF-κB (36,37). It is well known that ubiquitination is critical in both the response to ER stress and NF-κB activation. Therefore, we wished to determine whether E3 ligases, which are related to ER stress, regulate NF-κB signaling. We found the putative ERSE located at positions -118 to -100 on the promoter region of MULAN, which is one of the NF-κB-activating genes (Fig. 1A). First, we analyzed the mRNA expression of MULAN in cells subjected to ER stress by real-time PCR. MULAN mRNA expression was increased by the induction of ER stress by stimulation with tunicamycin (Fig. 1B). These results suggest that the expression of MULAN is regulated by ER stress.

MULAN activates NF-κB gene expression in an E3 ligase activity-dependent manner. To determine whether MULAN

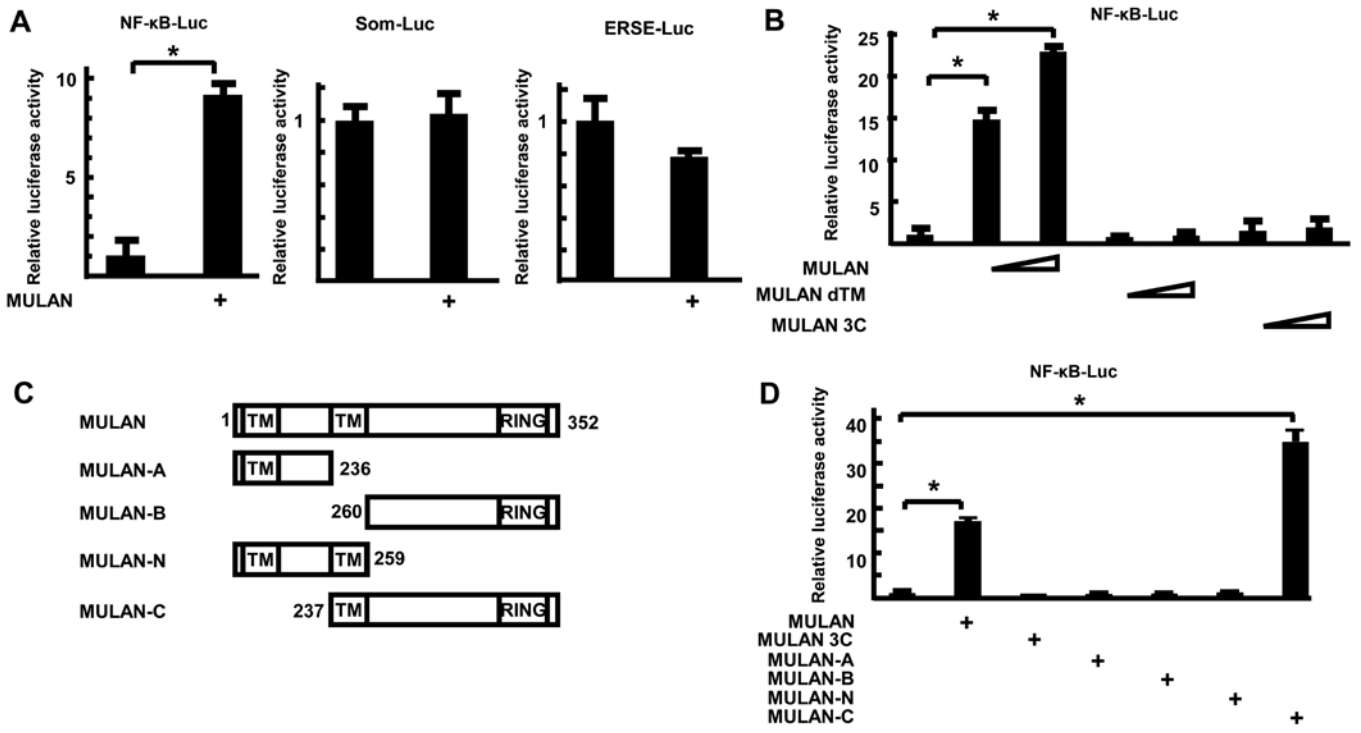

Figure 2. Mitochondrial ubiquitin ligase activator of nuclear factor-κB (MULAN) activates nuclear factor-κB (NF-κB)-dependent transcription via E3 ligase activity and the transmembrane domain (TM). (A) Reporter assays of 293 cells using NF-κB-Luc (left panel), Som-Luc (middle panel), ER stress-response element (ERSE)-Luc (right panel). The luciferase activity of cells transfected with pcDNA3 HA vector alone was designated 1. Data were analyzed with the Student's t-test and represent the mean ± SD (n=3). *P<0.05. (B and D) HEK-293 cells were transfected with NF-κB-Luc reporter, and expression vectors of wild-type MULAN or MULAN mutants. The luciferase activity of cells transfected with pcDNA3 HA vector alone was designated as 1. Data were analyzed using the Student's t-test and represent the means ± SD (n=3 experiments). *P<0.05. (C) Schematic representation of deletion mutants of MULAN. RING, RING finger domain.

specifically activates NF-κB-Luc, the luciferase reporter plasmids were co-transfected with MULAN/HA-expressing plasmid into the 293 cells. Twenty-four hours after transfection, the luciferase activities were measured. MULAN enhanced the reporter activity of NF-κB-Luc, but not that of Som-Luc and ERSE-Luc (Fig. 2A). To further examine the mechanism of induction of NF-κB-dependent gene expression by MULAN, we utilized several mutants of MULAN and performed reporter assays. MULAN increased NF-κB-dependent gene expression by 21.9-fold in a dose-dependent manner (Fig. 2B). By contrast, dTM, which lacks the second transmembrane domain, and MULAN 3C, which includes a cysteine to serine substitution in the RING finger domain and lacks E3 ubiquitin ligase activity, did not stimulate the reporter activity. To determine which domains contributes to the activation of NF-κB-Luc, several truncations of MULAN were expressed as HA fusion proteins (Fig. 2C) and examined by reporter assay. MULAN induced the reporter activity 16.4-fold. Among these 4 mutants, only MULAN-C markedly activated transcription 33.4-fold, whereas MULAN-A, MULAN-B and MULAN-N did not (Fig. 2D). These results suggest that both the transmembrane domain (TM) and RING finger domain are important for the MULAN-dependent activation of NF-κB.

The mechanism of MULAN-dependent NF-κB activation. To further investigate the target of MULAN, we utilized dominant negative (DN) mutants of major factors in NF-κB signals, such as IKKβ, TAK1, TRAF6 and TRAF2. First, we examined the inhibitory effects of these DN mutants in TNFα-treated cells, and we found that they suppressed the reporter activity of NF-κB-Luc in a dose-dependent manner. As shown in Fig. 3A, the introduction of DN-IKKβ plasmids markedly inhibited the MULAN-induced activation of NF-κB in a dose-dependent manner. In addition, transfection with DN-TAK1 also yielded the same result (Fig. 3B). Conversely, DN-TRAF2 and DN-TRAF6 failed to suppress the MULAN-dependent activation of NF-κB (Fig. 3C and D). These results suggest that MULAN plays a role in NF-κB activation upstream of TAK1 and downstream of TRAF2 and TRAF6. These results prompted us to search the interactants of MULAN. To determine whether MULAN interacts with TAK1 TAB1, RIP1 and IKKβ, we transiently transfected HeLa cells with HA TAK1, HA TAB1, HA RIP1, HA IKKβ expression plasmids and/or MULAN/FLAG expression plasmids. We performed an immunoprecipitation assay following western blot analysis. As shown in Fig. 3E (upper panel), HA TAK1 was detected in the immunoprecipitate with anti-FLAG antibody, but HA TAB1, HA RIP1 and HA IKKβ were not. These results suggest that TAK1 may be one of the targets of MULAN through which it induces the activation of NF-κB.

MULAN activates endogenous NF-κB signals. It is well known that the NF-κB signaling pathway can be activated through various processes; however, the release of cytoplasmic NF-κB proteins from IκB proteins and nuclear translocation are pivotal for signal transduction (28). To determine whether MULAN induces endogenous NF-κB activation, we observed the translocation of endogenous p65 into the nucleus. MULAN/FLAG or MULAN 3C/FLAG were transiently transfected into the HeLa

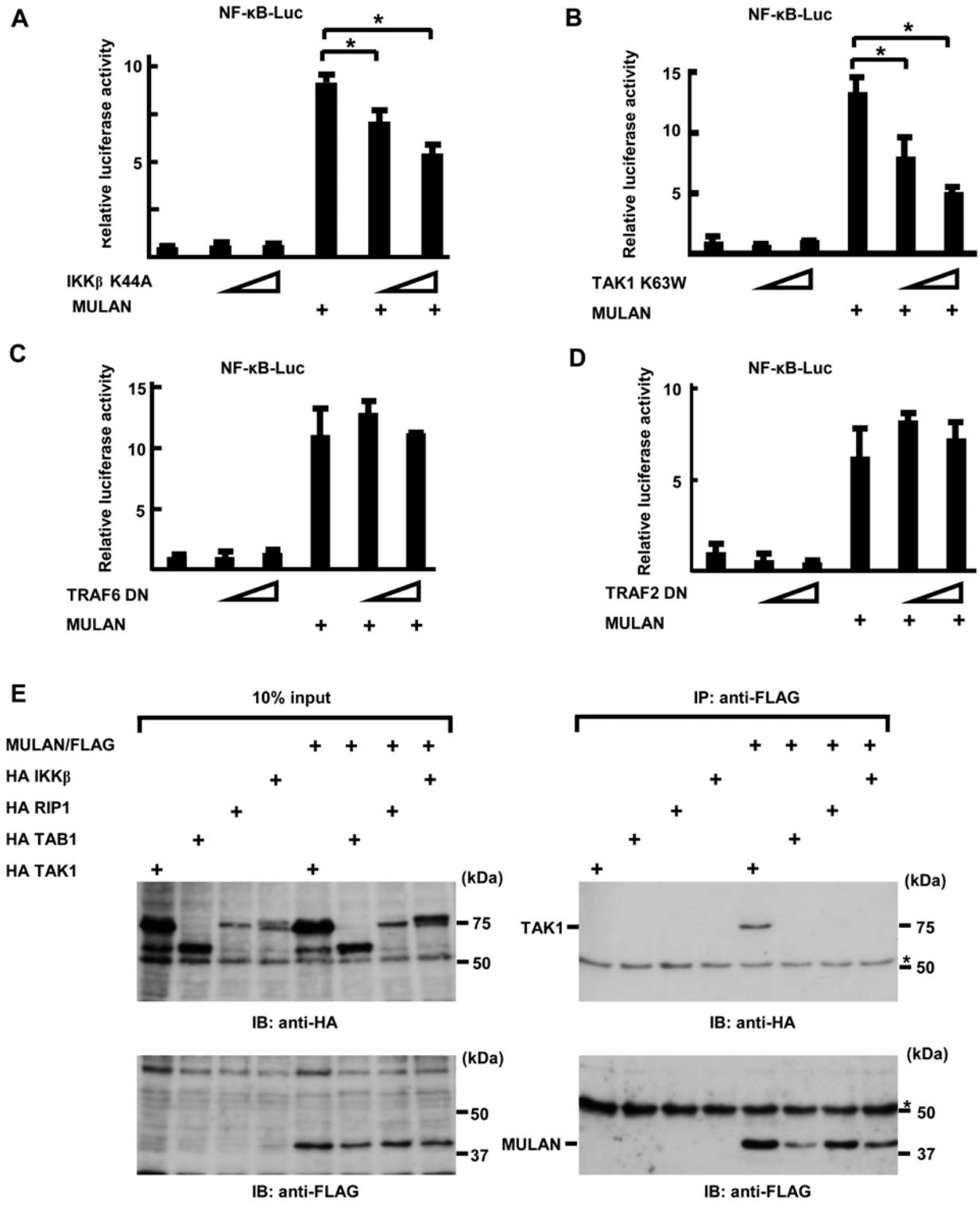

Figure 3. Mitochondrial ubiquitin ligase activator of nuclear factor-κB (MULAN) associates with TGFβ-activated kinase (TAK1). (A-D) 293 cells were transfected with (A) NF-κB-Luc reporter, MULAN expression plasmids and/or IκB kinase (IKK) K44A expression plasmids, (B) TAK1 K63W expression plasmids, (C) TNFR-associated factor 6 (TRAF6) DN expression plasmids, or (D) TRAF2 DN expression plasmids. The luciferase activity of cells transfected with empty vector alone was designated as 1. Data were analyzed using the Student's t-test and represent the means ± SD (n=3 experiments). *P<0.05. (E) Interaction of MULAN with TAK1 *in vivo*. HeLa cells were transfected with HA TAK1, HA TAK1-binding proteins 1 (TAB1), HA RIP1, HA IKKβ, and/or MULAN/FLAG expression plasmids. After 12 h, the cells were harvested and lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-HA antibody. The asterisks indicates the immunoprecipitated IgG heavy chain.

cells. The nuclear localization of endogenous p65 was only observed in the cells that expressed MULAN/FLAG (Fig. 4A). To further confirm the nuclear localization of p65, we used nuclear extraction and western blot analysis with anti-p65 specific antibody. In the cells transfected with the pcDNA3 HA vector or MULAN 3C/HA expression vector, a small amount of p65 was found in the nuclear extracts. On the other hand, stimulation with TNFα and the expression of MULAN led

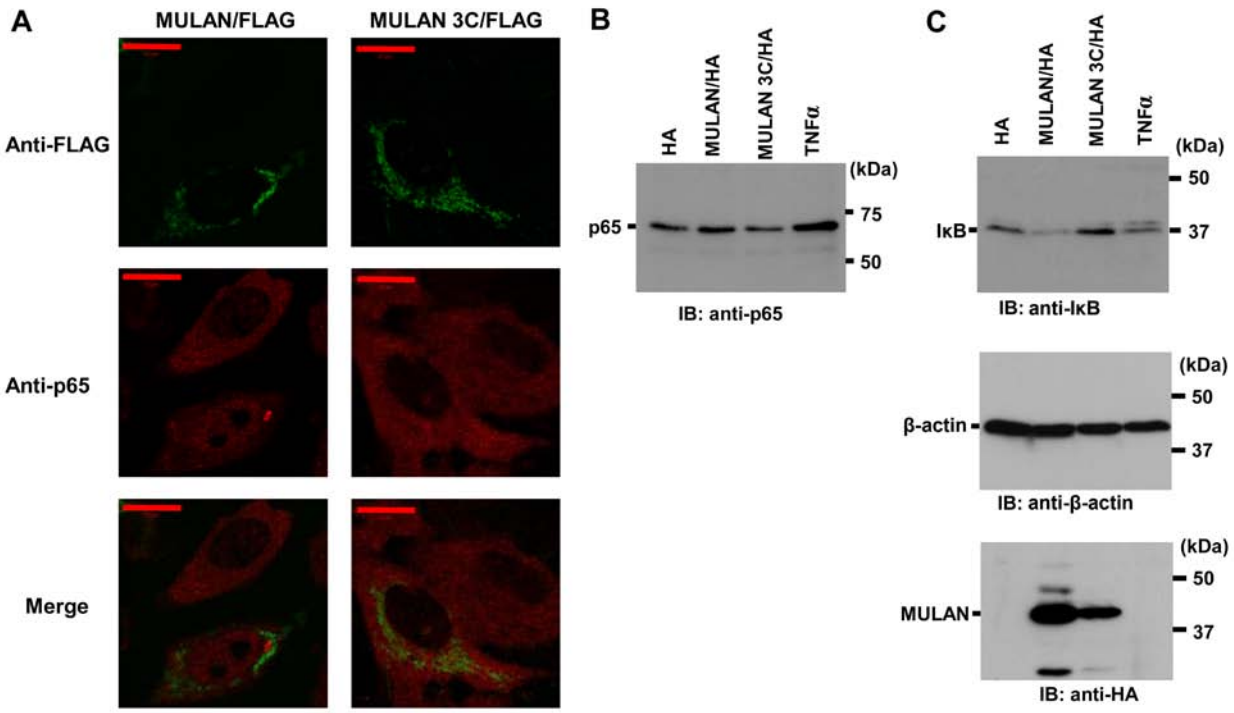

Figure 4. Mitochondrial ubiquitin ligase activator of nuclear factor-κB (MULAN) induces endogenous nuclear factor-κB (NF-κB) activation. (A) Intracellular localization of MULAN and endogenous p65. HeLa cells transfected with the plasmids of MULAN/FLAG or MULAN 3C/FLAG, which includes a cysteine to serine substitution in the RING finger domain, were fixed at 12 h post-transfection and stained with antibody specific to p65. Bar, 20 μm. (B and C) HeLa cells were transfected with MULAN/HA or MULAN 3C/HA. After 12 h of transfection, the cells were harvested. (B) Nuclear extracts were immunoblotted with anti-p65 antibody. (C) Whole cell lysates were immunoblotted with anti-IκB, anti-β-actin and anti-HA antibody.

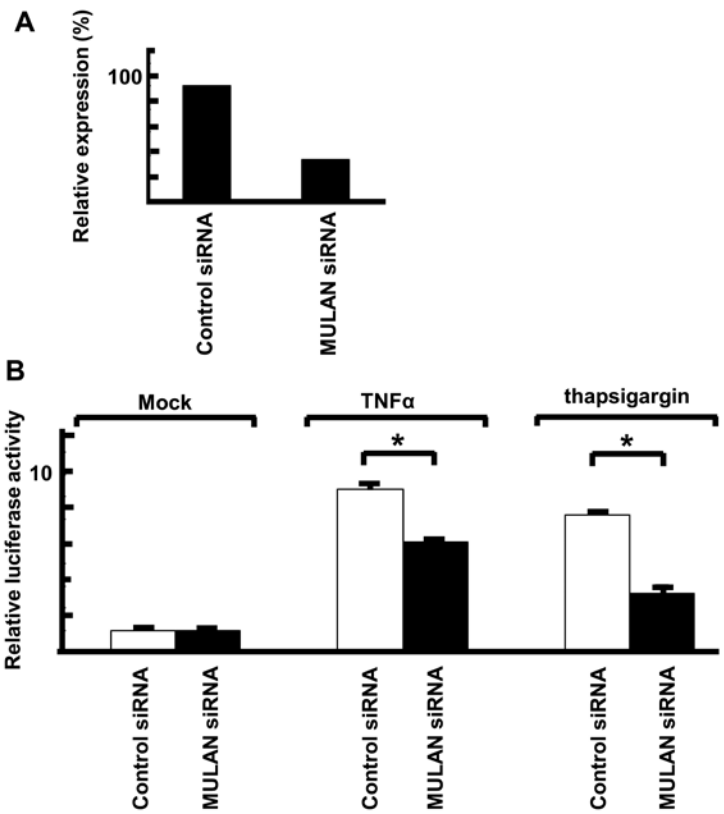

Figure 5. Downregulation of mitochondrial ubiquitin ligase activator of nuclear factor-κB (MULAN) leads to the reduced induction of NF-κB activation in endoplasmic reticulum (ER) stress-stimulated cells. (A) HeLa cells were transiently transfected with MULAN siRNA or control siRNA. Real-time PCR assays were performed. The expression of GAPDH was used as an internal control. (B) HeLa cells were transiently transfected with MULAN siRNA or control siRNA. After 72 h, NF-κB-Luc reporter plasmids were transfected, and cells were further incubated 24 h. The cells were then stimulated with tumor necrosis factor α (TNFα) (10 nM), or thapsigargin (2 μM) for 4 h. The luciferase activity of the cells transfected with empty vector alone was designated as 1. Data were analyzed using the Student's t-test and represent the means ± SD (n=3 experiments). *P<0.05.

to an increase in p65 levels in the nucleus (Fig. 4B). We then examined the degradation of endogenous IκBα. We transfected MULAN/HA or MULAN 3C/HA into the HeLa cells, and cell lysates were prepared 12 h following transfection. In the cells transfected with pcDNA3 HA vector or MULAN 3C/HA, hardly any IκBα degradation was observed, whereas noticeable degradation was detected in the cells expressing wild-type MULAN (Fig. 4C). These results indicate that MULAN activates NF-κB-dependent gene expression in an enzymatic activity-dependent manner.

Knockdown of MULAN induces a decrease in ER stress-dependent NF-κB activity. To examine the upper signal of MULAN-dependent NF-κB activation, we performed reporter assay with siRNA against MULAN. To confirm the effects of siRNAs, MULAN siRNA and control siRNA were transiently transfected into the HeLa cells. After 48 h, total RNA was isolated and the mRNA levels were measured by real-time PCR. MULAN siRNA induced the downregulation of MULAN (Fig. 5A). We then performed a reporter assay with NF-κB-Luc. The cells stimulated with TNFα and transfected with control siRNA exhibited an enhanced reporter activity by 7.3-fold and the MULAN siRNA-transfected cells exhibited a low reporter activity (Fig. 5B). We also observed no difference in reporter activity when the cells were stimulated with IL-1β (data not shown). When the cells were treated with thapsigargin, a decrease in reporter activity of approximately 50% was observed in the MULAN siRNA-transfected cells compared with the cells transfected with control siRNA. These results indicate that the activation of NF-κB by MULAN plays an important in cells subjected to ER stress.

Discussion

MULAN is regulated by ER stress. We found putative ERSE on the promoter region of MULAN gene (Fig. 1). ERSE is found in many of these ER stress-response genes and is regulated by the ER responsive transcriptional factors, activating transcription factor 6 (ATF6) and X-box binding protein 1 (XBP1). ERSE, with a consensus sequence of CCAAT-N9-CCACG, is a *cis*-acting element that is necessary and sufficient for transcriptional induction of ER chaperone genes (29-31). The putative ERSE of MULAN, CCAAT-N9-ACACC, differs slighlty from the consensus sequence of ERSE. Many ER stress-responsive genes also have a different ERSE from a consensus sequence; for example, Bip/Grp78, CCAAT-N9-CCAAC and PDI, CCAGT-N9-ACAGC. In addition, the expression of MULAN was induced by ER stress (Fig. 1). However, we cannot not rule out possibility of the contribution of other elements on the MULAN promoter. These results suggest that the putative ERSE of MULAN may function as an ERSE and it is certain that MULAN plays a role in the response to ER stress.

The mechanism of MULAN-induced NF-κB activation. Ubiquitination is important in NF-κB activation. It has been demonstrated that TRAF2, which is recruited to the TNFR complex, ubiquitinates RIP, and polyubiquitinated RIP associates with TNFR as well as with TAB2 (8,32,33). These studies suggest that the polyubiquitination of RIP recruits the TAK1-TAB2 complex, which subsequently activates the IKK complex. It has been shown that TRAF6 recruited to the receptor complexes polyubiquitinates these complexes, and polyubiquitinated TRAF6 associates with the TAB2/TAB1/TAK1 complex (8). In this study, we revealed that MULAN activated NF-κB dependent transcription via E3 ubiquitin ligase activity. The activation of NF-κB via MULAN was inhibited by IKK DN and TAK1 DN, but not by TRAF2 and TRAF6. In addition, our binding assay revealed that MULAN associated with TAK1. TAK1 has been reported to be induced by polyubiquitination. TAK1 has Lys63-linked ubiquitination sites and the polyubiquitination of TAK1 induces IK-dependent NF-κB activation (34,35). Therefore, it is possible that MULAN may induce the polyubiqutination of TAK1 to activate NF-κB signaling.

MULAN-induced NF-κB activation in cells subjected to ER stress. The ER stress-induced activation of NF-κB has been demonstrated to be involved in both the protection of cells from ER stress-induced apoptosis (36) and in the induction of apoptosis (37). TRAF2 mediates the activation of both the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and the NF-κB pathways following ER stress (38,39). Therefore, TRAF2 simultaneously mediates the activation of the NF-κB survival pathway and the pro-apoptotic JNK pathway, and the fate of the cell would be determined by the interplay between these opposing signals. TAK1 also activates both the NF-κB survival pathway and the pro-apoptotic JNK pathway. The activation of TAK1 is induced by K63-linked polyubiquitination reactions by TRAF2 or TRAF6 at the K158, K34 and K209 residues (35). Recently, Lys562 of TAK1 was identified as a novel Lys63-linked ubiquitination site. The accumulation of polyubiquitination at Lys562 induces IKK-dependent NF-κB activation, but not JNK/p38 pathway activation (34). In this study, MULAN activated NF-κB dependent gene expression, and the expression of MULAN was regulated by ER stress. In addition, the MULAN-dependent NF-κB activation was TAK1-dependent and MULAN interacted with TAK1. We thus hypothesized that MULAN may induce NF-κB activation via TAK1 for cell survival following ER stress; MULAN may activate NF-κB-dependent gene expression by the induction of *de novo* MULAN by ER stress.

In conclusion, in the present study we provide evidence that MULAN is an E3 ligase that mediates NF-κB-dependent gene expression in cells subjected to ER stress. Further analysis of MULAN will be helpful in order to broaden our understanding of the physiological significance of MULAN and of the mechanisms of NF-κB activation by MULAN.

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