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RESEARCH ARTICLE

RNF152, a novel lysosome localized E3 ligase with pro-apoptotic activities

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ABSTRACT

RING finger protein 152 (RNF152) is a novel RING finger protein and has not been well characterized. We report here that RNF152 is a canonical RING finger protein and has E3 ligase activity. It is polyubiqitinated partly through Lys-48-linked ubiquitin chains *in vivo* and this phenomenon is dependent on its RING finger domain and transmembrane domain. RNF152 is localized in lysosomes and co-localized with LAMP3, a lysosome marker. Moreover, over-expression of RNF152 in Hela cells induces apoptosis. These results suggest that RNF152 is a lysosome localized E3 ligase with pro-apoptotic activities. It is the first E3 ligase identified so far that is involved in lysosome-related apoptosis.

KEYWORDS RNF152, RING finger, ubiquitinate, apoptosis

INTRODUCTION

RING finger proteins (RNFs) are a family of proteins harboring the RING finger domain (Ikeda et al., 2005). The RING domain contains a conserved $Cys_3HisCys_4$ amino acid motif ($CX_2CX_{9-39}CX_{1-3}HX_{2-3}CX_2CX_{4-48}CX_2C$, where X can be any amino acid), which binds to two zinc cations (Barlow et al., 1994; Lorick et al., 1999). The cysteine and histidine residues of the RING finger domain yield a rigid, globular platform for protein-protein interaction as demonstrated by three-dimensional structures (Borden et al., 1995; Zheng et al., 2000).

The RING finger is generally located close to the amino or carboxyl terminus of the protein, and is mostly associated with other domains to define the function of the protein. For instance, the inhibitors of apoptosis gene family, IAPl, IAP2 and XIAP, have both a RING domain at their C-termini and BIR (baculovirus IAP repeat) domain at their N-termini (Takahashi et al., 1998; Liston et al., 2001). When the RING finger protein sequence motif was initially identified, the knowledge about its general function was very limited. Recently, accumulating evidence demonstrated that RING finger proteins could simultaneously bind to E2s and the substrates through their RING finger domains, and hence, act as E3 ligases in ubiqitination (Deshaies and Joazeiro, 2009). Ubiquitin (Ub) is a ubiquitously expressed 76-amino-acid protein that can be covalently attached to target proteins, leading to their ubiquitination (Hershko and Ciechanover, 1998). This process is catalyzed by a cascade of enzymatic reactions involving a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). Ubiquitinated proteins can change their functions and locations, or be degraded by a proteolytic complex (proteasome) (Weissman, 2001). Therefore, ubiquitination plays an important role in many cellular activities. Accordingly, the RING finger proteins having the activity of E3 ligase function in various biological processes, including the growth, differentiation, transcription, signal transduction, oncogenesis, apoptosis and so on. As for apoptosis, it has been reported that many proteins in cell apoptosis machinery undergo Ub modification (Yang and Yu, 2003). Some apoptosis regulatory proteins also contain RING finger domain and exhibit Ub ligase activity (Zhang et al., 2004). For example, the inhibitors of apoptosis proteins (IAPs) are responsible for self-degradation when an apoptotic signal is transduced, and their anti-apoptotic activity is lost when the RING domain is mutated (Yang et al., 2000).

RNF152 is a novel member of RING finger protein family. The full ORF of RNF152 has been sequenced and confirmed by the Mammalian Gene Collection program (Strausberg

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et al., 2002). Decreased expression of RNF152 was reported in some cancer cell lines (Yamamoto and Yamamoto, 2007). Our studies showed for the first time that RNF152 is an E3 ligase and is self-polyubiqutinated. It is localized in lysosomes and displays a pro-apoptosis activity when overexpressed.

RESULTS

RNF152 is a highly conserved RING finger protein

Human RNF152 gene is located on 18q21.33 chromosomal region (Yamamoto and Yamamoto, 2007) and encodes a 203-amino-acid protein with predicted molecular weight of 22 kDa. RNF152 protein contains two predicted functional domains: a RING finger motif at N-terminus, with the sequence resembling a canonical RING motif ($CX_2CX_{14}CX_1HX_2CX_2CX_{12}CX_2C$), and a transmembrane motif at the C-terminus (Fig. 1A). Homology analysis comparing the orthologs from chimpanzee, mouse, rat, dog, chicken and frog revealed that the RNF152 protein is highly conserved among these species (Fig. 1A), suggesting that RNF152 plays an essential role in certain biological activities of vertebrates. Phylogenetic analysis using NTI software (Fig. 1B) showed that RNF152 does not share close similarities to other members of RING finger protein family.

To determine the expression pattern of RNF152, we analyzed the mRNA levels of RNF152 in various cell lines using RT-PCR (Fig. 1C). RNF152 mRNA expression was detectable in all the tested cell lines, and the monocyte cell line THP-1 showed highest level of RNF152 mRNA.

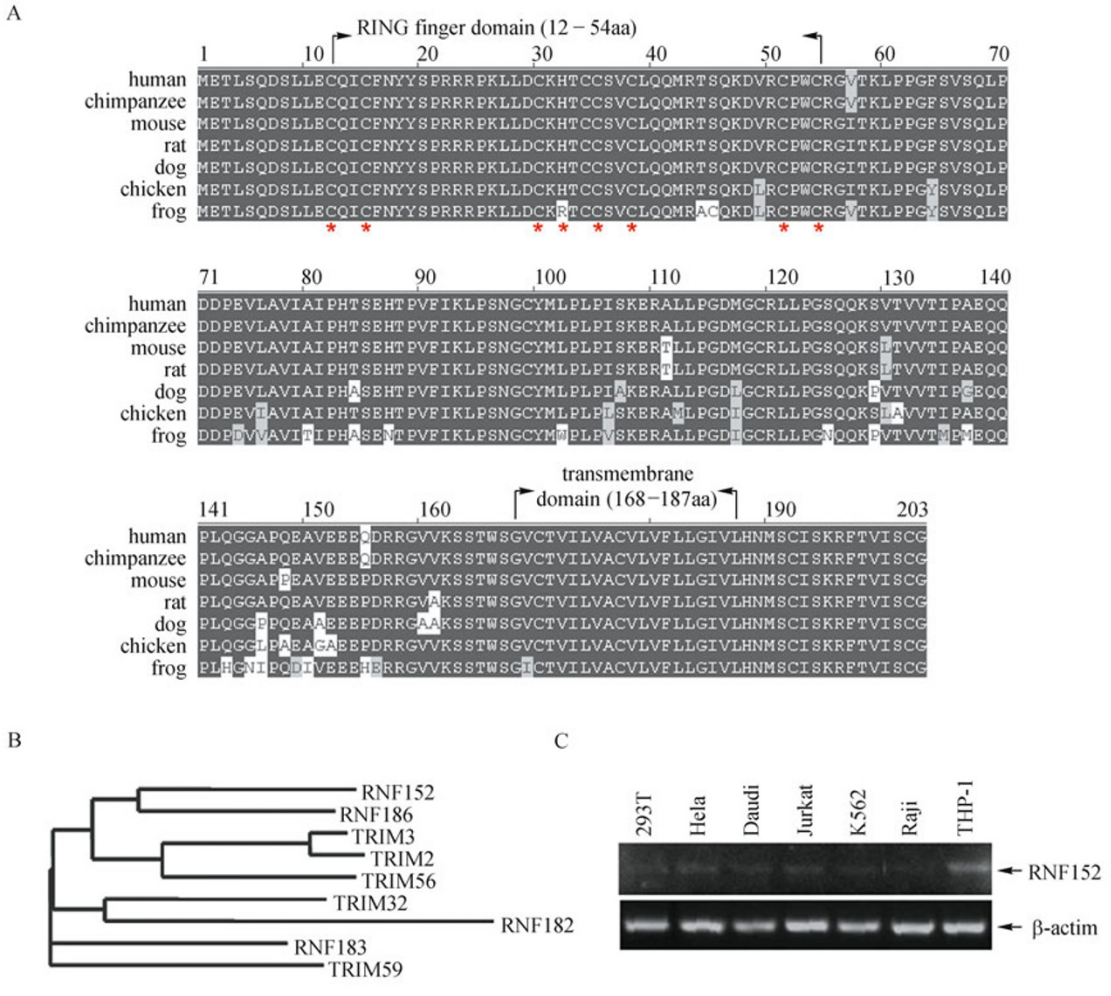

Figure 1. RNF152 protein sequence analysis, phylogenetic analysis and gene expression levels in cell lines. (A) RNF152 proteins are evolutionally conserved among vertebrates. The orthologs from chimpanzee, mouse, rat, dog, chicken and frog were compared with human RNF152. Red asterisks indicate the conserved residues in a typical RING domain. (B) Phylogenetic analysis of RNF152 and other members of RING finger protein family. (C) Relative expression levels of the genes for RNF152 in indicated cell lines were determined by RT-PCR. β-actin mRNA expression was analyzed as an internal control. The template cDNA of β-actin was 10-fold diluted, and the cycle number was 24 for β-actin and 30 for RNF152.

RNF152 is self-polyubiquitinated and degraded in proteasome

The existence of RING finger domain at N-terminus suggests that RNF152 may function as an E3 ligase. To test this hypothesis, *in vivo* ubiquitination assays were performed to examine the E3 ligase activity of RNF152. Flag-tagged wild-type RNF152 was co-transfected with or without HA-tagged Ub into Hela cells, and RNF152 in cell lysates was precipitated by anti-Flag antibody and analyzed by Western blotting. Anti-HA antibody was used to detect ubiquitinated RNF152. As shown in Fig. 2A, RNF152 was intensively polyubiquitinated when it was co-expressed with Ub in Hela cells. Once polyubiquitinated, proteins are generally taken to the proteasome for degradation. When the proteasome inhibitor MG132 (Lee and Goldberg, 1998) was used, more ubiquitinated RNF152 was accumulated in cell lysates, suggesting that the ubiquitinated RNF152 was degraded in proteasome (Fig. 2B).

RNF152 is polyubiquitinated through Lys-48 linkage

A polyUb chain is formed by conjugating Ub to the substrate protein through different linkages. To assess which type of Ub linkage exists in RNF152, different Myc-tagged Ubs (wild-type, UbK29R, UbK48R or UbK63R) were individually co-transfected with Flag-tagged RNF152 into Hela cells. The immunoprecipitation results in Fig. 2C showed that RNF152 could be modified by the mutants UbK29R, UbK63R as well as by wild-type Ub; however, UbK48R mutation down-regulated polyubiquitinated RNF152, implying that K48 is important for the polyubiquitination of RNF152.

The RING finger domain and transmembrane domain of RNF152 are both responsible for its E3 ligase activity

To assess the roles of RING domain and transmembrane domain on the E3 ligase activity of RNF152, two Flag-tagged mutants, RNF152C-S (carrying two Cys to Ser mutations at positions 12, 15) and RNF152ΔTm (with deletion of the C-terminal transmembrane domain) were also constructed. As shown in Fig. 2D, ubiquitination of RNF152C-S was significantly decreased compared to wild-type RNF152, suggesting that *in vivo* self-ubiquitination of RNF152 was dependent on its RING domain. Interestingly, the RNF152 mutant lacking the transmembrane domain almost completely lost self-ubiquitination, suggesting that RNF152 membrane localization is also important for its E3 ligase activity.

RNF152 induces apoptosis of Hela cells

To investigate the biological functions of RNF152, we investigated its activity using a variety of cell-based assays. We found that Hela cells transiently transfected with RNF152 underwent apoptotic cell death. In FACS analysis, plasmids encoding green fluorescence protein (GFP)-fused RNF152: RNF152WT, RNF152C-S and RNF152ΔTm were generated and transfected into Hela cells. Approximately 54% of Hela cells overexpressing GFP-RNF152WT displayed annexin V-positive staining in the absence of any apoptotic stimuli, whereas only 9% of control vector expressed cells were annexin V-positive (Fig. 3A and 3B). However, compared to wild-type RNF152, the percentage of apoptotic cells was lower when the two RNF152 mutants were overexpressed in Hela cells: RNF152C-S was about 33%, RNF152ΔTm was about 20% (Fig. 3A and 3B). These results suggested that both domains of RNF152 are required for its pro-apoptotic activity while the transmembrane domain is more important. In parallel, poly (ADP-ribose) polymerase (PARP) degradation was detected by Western blotting to confirm the pro-apoptotic activity of RNF152. PARP is one of the cleavage targets of caspase-3, which is the main executor of apoptosis *in vivo*. When the apoptotic signal pathway is activated, PARP is cleaved to an 89-kDa fragment from the 116-kDa full-length form. In accordance with the FACS data, wild type RNF152 induced clear degradation of PARP, and the C-S mutant and ΔTm mutant degraded PARP to a reduced extent (Fig. 3C).

RNF152 is localized in lysosomes

To determine the subcellular localization of RNF152, GFP-fused RNF152 was transiently transfected into Hela cells, which were stained with organelle-specific fluorescent red dyes for mitochondria, lysosome, golgi or endoplasmic reticulum. The results showed that RNF152 was localized in lysosomes (Fig. 4, upper panel). C-S mutation had no effect on the lysosomal localization of RNF152, but RNF152ΔTm was diffused in cytoplasma (data not shown). To further confirm the results, RNF152 was co-transfected with RFP-fused LAMP3 (lysosome-associated membrane protein 3), a lysosomal membrane protein, and these two proteins colocalized well in Hela cells (Fig. 4, lower panel).

DISCUSSION

The RING finger proteins belong to a large protein family. Bioinformatic analyses identified more than 300 human genes encoding RING domain proteins (Li et al., 2008). The E3 ligase activity of the RING domain confers to the RING finger proteins important roles in the degradation and translocation of their substrate proteins (Petroski and Deshaies, 2005). RNF152 is a RING finger protein with relatively simple structure, containing a hydrophobic transmembrane domain at the C-terminal and a RING finger domain at the N-terminal. In the present study, we found that RNF152 has E3 ligase activities and undergoes self-polyubiquitinated *in vivo* in a RING finger domain-dependent manner. Moreover, the transmembrane domain of RNF152 is

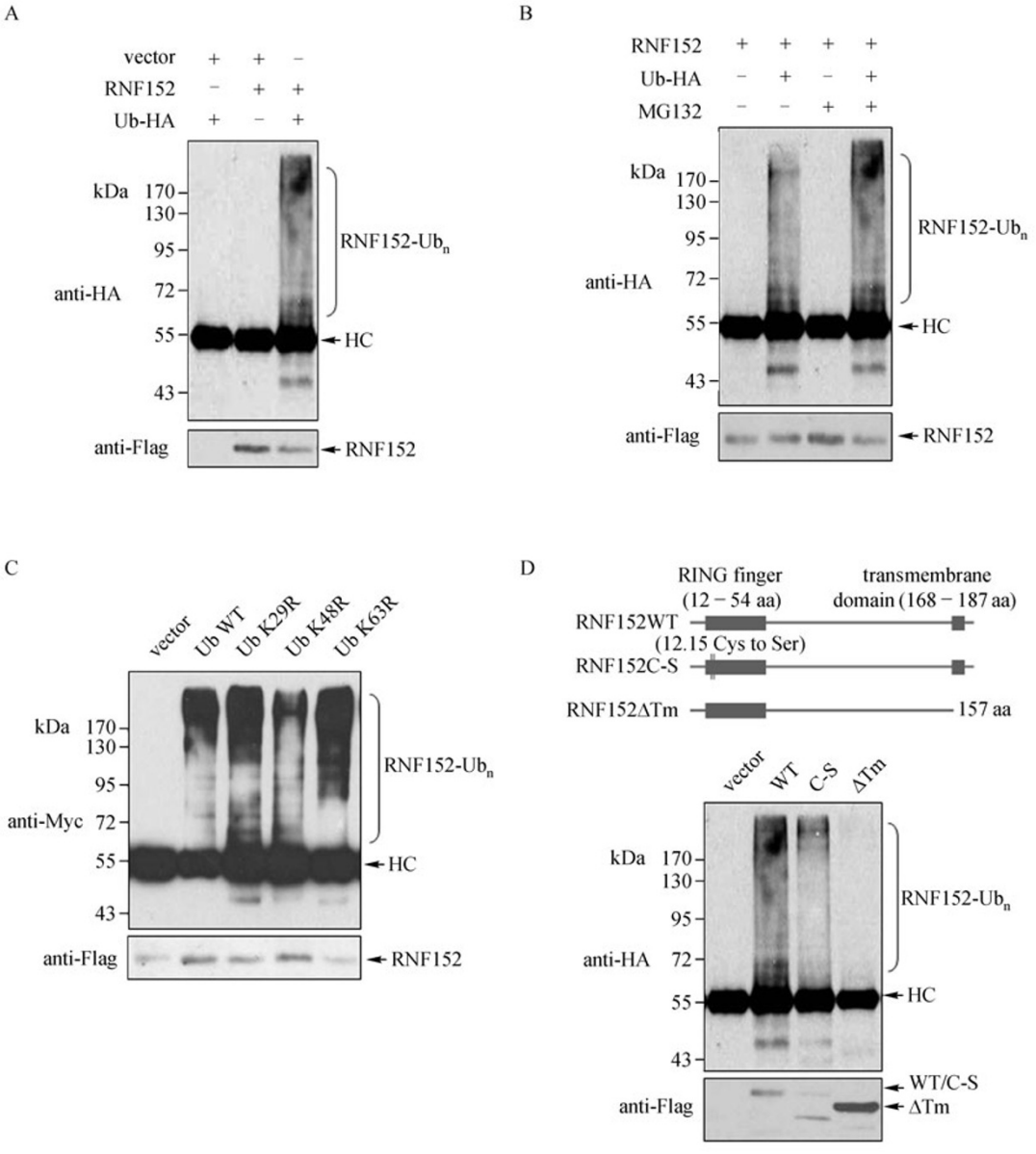

Figure 2. Self-ubiquitination of RNF152 *in vivo*. (A) RNF152 was polyubiquitinated. Flag-tagged wild-type RNF152 was co-transfected with or without HA-tagged Ub into Hela cells. Twenty-four hours later, MG132 was supplemented into cell cultures, and the cells were further cultured for 4 h and harvested for immunoprecipitation. Ubiquitinated RNF152 was analyzed by Western blotting with the anti-HA antibody (upper panel). The expression of RNF152 in the precipitates was detected by anti-Flag antibody (lower panel). HC represents heavy chain of anti-Flag antibody (55 kDa). (B) MG132 (10 μM) inhibited the degradation of polyubiquitinated RNF152. Twenty-four hours after transfection, cells were treated with proteasome inhibitor MG132 or untreated. (C) K48R mutation affected RNF152 polyubiquitination. Wild-type RNF152 was transfected into Hela cells with the Myc-tagged Ub or its variants K29R, K48R, K63R. The polyubiquitination of RNF152 was detected by Western blotting with anti-Myc antibody. (D) RING finger domain and transmembrane domain were both responsible for RNF152 self-ubiquitination. Wild-type, C-S mutant, ΔTm mutant of RNF152 or empty vector were co-transfected with Ub into Hela cells. Immunoprecipitation was performed as described above.

also responsible for its self-ubiquitination, implying that the membrane localization was necessary for the function of this protein.

It is well-characterized that the polyubiquitination of a protein often serves as a signal for its degradation in proteasome. To investigate the outcome of ubiquitinated

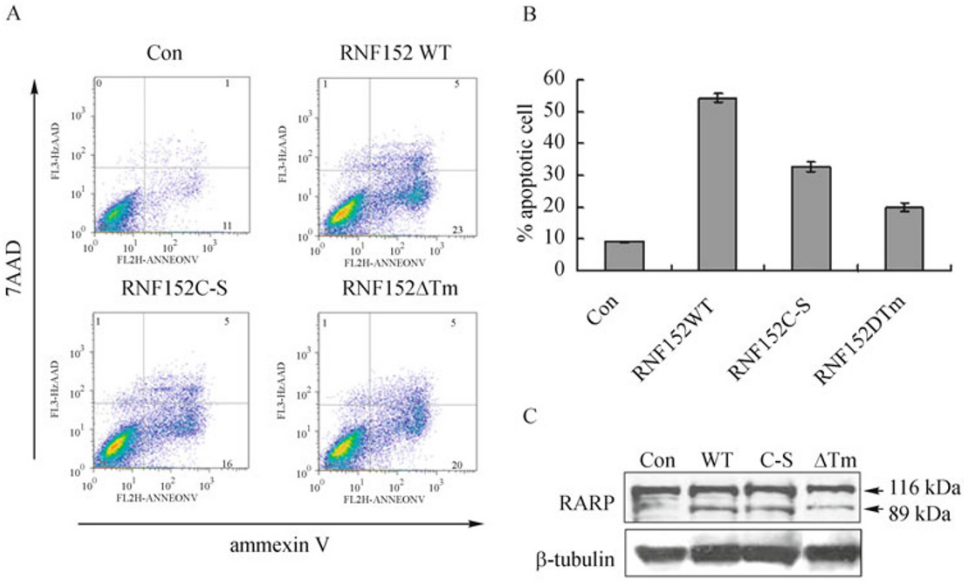

Figure 3. RNF152 induced apoptosis in Hela cells. (A and B) Hela cells were transfected with GFP-RNF152 (wild-type, C-S mutant, ΔTm mutant) or GFP as the negative control. Twenty-four hours after transfection, the cells were collected and stained with PE annexin V and 7-AAD for flow cytometry analysis. (C) PARP degradation was detected by Western blotting.

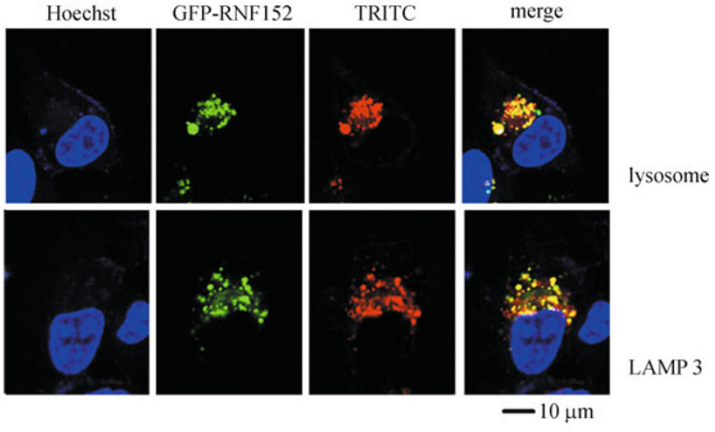

Figure 4. Lysosomal localization of RNF152. GFP-RNF152 was transfected into Hela cells, or GFP-RNF152 and RFP-LAMP3 were co-transfected into Hela cells (lower panel). Twenty-four hours post-transfection, cells were stained with lysoTracker Red at 37°C for 30 min (upper panel). Intracellular localization of RNF152 was examined by two-color confocal microscopy analysis under a confocal laser-scanning microscope. The nuclei were counterstained with Hoechst 33258.

RNF152, we used MG132, an inhibitor of the proteasome (Lee and Goldberg, 1998), to treat cells, and found that there was increased accumulation of RNF152. This data suggested the proteolysis of self-ubiquitinated RNF152 through ubiquitin-proteasome pathway in physiologic conditions.

As for polyubiquination, there are at least eight different polyUb linkages. It seems that different linkages have distinct biological functions. The manner by which ubiquitin is linked to proteins generally determines the fate of the ubiquitinated targets. PolyUbs linked through Lys-48 are the primary targeting signals for proteasomal degradation, whereas polyUbs linked through Lys-63 recruit other binding partners and exhibit many functions such as kinase activation, protein synthesis and DNA repair (Weissman, 2001). To explore which kind of the polyUb linkage is used by RNF152, we determined the effect of the Lys-29, Lys-48 and Lys-63

mutations of Ub on polyubiquitination of RNF152. Our results showed that RNF152 was polyubiquitinaed to a less extend when it was co-transfected with Lys-48 mutant of Ub comparing with the wild-type Ub, which indicated that RNF152 was modified at least by Lys-48-based polyubiquitination, implying that RNF152 may function in ubiquitin-proteasome pathway.

Further investigation showed that RNF152 could promote apoptosis when it was overexpressed in Hela cells. The data generated by FACS analysis and the degradation of PARP detected by Western blotting confirmed that RNF152 had a pro-apoptotic activity in a RING finger domain-dependent and transmembrane domain-dependent manner. Since the transmembrane domain was necessary for both E3 ligase and pro-apoptotic activity of RNF152, we then examined the subcellular localization of RNF152. Overexpressed RNF152 in Hela cells was evidently localized in lysosomes instead of in mitochondria—the key apoptotic organelle (data not shown). Recently, accumulating evidences suggest the existence of a lysosomal pathway for apoptosis (Guicciardi et al., 2004). The lysosomal membrane permeabilization (LMP) that leads to the release of lysosomal proteases into the cytosol may initiate the apoptotic program in several models of apoptosis (Chwieralski et al., 2006; Stoka et al., 2007; Boya and Kroemer, 2008), such as TNFα-induced apoptosis (Autefage et al., 2009). Our studies revealed the first E3 ligase that localizes to lysosmes and exhibits pro-apoptotic activities at the same time. These results underline the uniqueness and novelty of RNF152.

In conclusion, our studies showed that RNF152 is a novel E3 ligase and is involved in apoptosis. Its RING finger domain and the transmembrane domain contribute to the activities of RNF152. Further studies are underway to identify the substrate of RNF152 in biological conditions in order to shed light on the precise mechanism of RNF152-induced apoptosis.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

Full length cDNA of RNF152 was amplified by PCR from Hela cell cDNA that was reverse-transcribed from total RNA. The primers were derived from NCBI Reference Sequence: NM_173557.1; the upper primer was 5′-GCACGAATTCATGGAGACGCTGTCCCAGGA-3′ and the lower primer was 5′-CTATGTCGACGCCACAGGATATCA-CAGTG-3′. The generated amplicons were subsequently cloned into pCI-neo vectors (Promega) that were constructed with the tags of Flag, HA, Myc, GFP, RFP to generate mammalian expression plasmids. The C-S, ΔTm mutants of RNF152 were obtained based on the wild-type plasmid; the internal primers for C-S mutant were 5′-CACCTGCTGTTCAGTGTGCCTGCAG-3′ and 5′-TCTGCTGCAGG-CACACTGAACAGCA-3′, the lower primer for ΔTm mutant was 5′-CTATGTCGACCGACCAGGTGGAGCTTTTC-3′. LAMP3, Ub and its K to R mutants were all constructed with pCI-neo vectors harboring the corresponding tags.

Cell culture and transfection

Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (*v/v*) heat-inactivated fetal bovine serum, 2 mM glutamine in a humidified 5% CO_2 containing atmosphere at 37°C. Cells were kept in logarithmic growth phase and were plated 24 h prior to transfection. For transient assays, Hela cells were transfected at 70%–90% confluence with the indicated plasmids using the Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) according to the protocol. Two micrograms of the total RNA was reverse-transcribed to cDNA by M-MLV (Promega) using Oligo-dT as primers. Then cDNA was amplified by PCR and analyzed in agarose gels. The cDNA as the template of β-actin was 10-fold diluted. The annealing temperatures and the cycle numbers were: β-actin, 60°C, 24 cycles; RNF152, 56°C, 30 cycles. Primers were: β-actin, the upper primer, 5′-CCAACCGCGAGAAGATGA-3′, the lower primer 5′-GGAAG-GAAGGCTGGAAGAGT-3′; RNF152, the same as the primers for cloning.

Immunoprecipitation

Hela cells were lysed on ice in IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 10% glycerol, 1× protease inhibitor cocktail added freshly) after transfection. Total preclearing protein lysate gotten from a 60 mm-dish was incubated with 1.5 μg anti-Flag antibody at 4°C for 4 h, then 15 μL protein A/G agarose beads (Pierce) were supplemented. Following the incubation with protein lysate for 2 h at 4°C, the beads were collected and washed five times with lysis buffer. Immunoprecipitated proteins were subjected to SDS-PAGE and analyzed by Western blotting.

In vivo ubiquitination assays

Various RNF152 recombinant plasmids (wild-type, C-S mutant, ΔTm mutant) were transfected into Hela cells with or without Ub-HA/Myc expression plasmid. Twenty-four hours post-transfection, Hela cells were treated with proteasome inhibitor MG132 (10 μM) for 4 h and then cells were harvested and immunoprecipitated. The immunoprecipitates were washed and resolved on SDS-PAGE, followed by immunoblotting with anti-HA/Myc antibody to detect ubiquitinated proteins.

Western blotting

Cell lysates for immunoprecipitation was prepared as previously described. To detect PARP degradation, Hela cells were transfected with the plasmid of GFP-tagged RNF152 (wild-type, C-S mutant, ΔTm mutant). Twenty-four hours after transfection, cells were lysed on ice in RIPA buffer supplemented with 1 × protease inhibitors cocktail. Cell lysates were resolved by SDS-PAGE (10%–12%). The primary antibodies used as following: anti-Flag (Sigma), anti-HA (Santa Cruz), anti-Myc (Santa Cruz), anti-PARP (Cell Signaling), anti-β-tubulin (Sigma). The anti-PARP antibodies detect both full-length (116 kDa) and the cleaved form (89 kDa).

Flow cytometry

After indicated treatments, Hela cells were digested by typsin and collected, then washed with precooled 1 × PBS for 3 times. Cells were resuspended in prediluted binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.0) and stained with PE annexin V, 7-amino-actinomycin (7-AAD) for 15 min at room temperature, protected from light. The cell suspension was immediately analyzed by flow cytometry. The percentage of viable and dead cells were determined from 10,000 cells per sample that were gated for GFP positive.

Immunofluorescence microscopy

Hela cells were cultured on overlips of the confocal dishes, transfected with GFP-tagged RNF152 or co-transfected with GFP-tagged RNF152 and RFP-tagged LAMP3. Twenty-four hours later, cells transfected with RNF152 alone were stained with trackers of various subcellular organelles (LysoTracker Red DND99, L7528 Invitrogen; MitoTracker Red FM, M22415 Invitrogen; ER-Tracker Red Invitrogen, C1041 Beyotime) according to the protocols. Cells were washed with 1 × PBS for 5 times to clear the dyes. After staining with Hoechst 33258 (Santa Cruz) at 37°C for 10 min, cells were observed by fluorescence confocal microscopy (LSM Confocal Microscope, Carl Zeiss).

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ABBREVIATIONS

7-AAD; 7-amino-actinomycin BIR, baculovirus IAP repeat; IAP, inhibitors of apoptosis proteins; LAMP3, lysosome-associated membrane protein 3; PARP, poly (ADP-ribose) polymerase; RNF, RING finger proteins; Ub, ubiquitin

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