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Autoantigen Ro52 is an E3 ubiquitin ligase

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Abstract

Anti-Ro/SSA antibodies are classic autoantibodies commonly found in patients with Sjögren's syndrome, a chronic autoimmune disease characterized by dryness of the eyes and mouth. The autoantibodies recognize a RING-finger protein, Ro52, whose function is still unknown. Since many RING-finger proteins have been identified as E3 ubiquitin ligases, this study was designed to determine whether Ro52 functions as an E3 ubiquitin ligase. For this purpose, recombinant Ro52 was purified from bacterial lysate and used to investigate its activity of E3 ubiquitin ligase in vitro. Its enzymatic activity was also tested in HEK293T cells using wild-type Ro52 and its RING-finger mutant. Our results indicated that Ro52 ubiquitinates itself in cooperation with E2 ubiquitin-conjugating enzyme UbcH5B, thereby validating that Ro52 is a RING-finger-type E3 ubiquitin ligase. Importantly, this ubiquitin modification is predominantly monoubiquitination, which does not target Ro52 to the proteasome for degradation.

Keywords: Ro52; Sjögren's syndrome; Autoantigen; Autoantibody; Ubiquitin; TRIM; RING finger; RBCC; Ubiquitin ligase; E3

Anti-Ro/SS antibodies are antinuclear antibodies most commonly found in patients with Sjögren's syndrome. They play a pathogenic role in a variety of clinical manifestations, including skin lesions and congenital heart block. Autoantibodies to Ro recognize a ribonucleoprotein complex composed of small, single-stranded RNAs and of one or more peptides. The Ro autoantigen is found in most tissues and cells (e.g., erythrocytes, platelets), with differences in structure and quantity across tissues, species, and embryonic developmental stages [1]. Recent studies have shown that the Ro autoantigen is heterogeneous. There are two isoforms: the 60-kDa form (Ro60) and the 52-kDa form (Ro52) [2]. Although a few groups have investigated the function of the Ro autoantigen, its precise function remains unknown.

Ubiquitin, a 76-amino-acid polypeptide, is highly conserved in evolution, with only 3 amino acid differences between the human and yeast homologues [3]. The C-terminus of ubiquitin contains a conserved Gly residue, which is activated to form a thiol-ester linkage with the Cys residue of E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to E2 ubiquitin-conjugating enzyme to form another thiol-ester linkage. Subsequently, with the aid of E3 ubiquitin ligase, ubiquitin becomes covalently attached to Lys residues of target proteins through the formation of isopeptide bonds [3]. The internal Lys residue of ubiquitin can also form 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 [3]. In the proteasome, polyubiquitinated proteins are degraded in an ATP-dependent manner [3]. By targeting ubiquitinated proteins to the proteasome for degradation, ubiquitination plays a critical role in many biological events [3].

As described above, Ro52 is one of the Ro autoantigens. It is a RING-finger protein that belongs to a RBCC (RING-finger/B-box/coiled-coil) family (also known as TRIM (Tripartite motif) family) [4]. Recent results from several laboratories indicated that the RING-finger proteins recruit E2 ubiquitin-conjugating enzymes and act as E3 ubiquitin ligase [5,6]. It is possible, therefore, that

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Ro52 functions as an E3 ubiquitin ligase. Indeed, we previously showed that Ro52 is strongly monoubiquitinated when overexpressed in COS cells [7], suggesting that Ro52 ubiquitinates itself (self-ubiquitination). In this study, we determined whether Ro52 functions as an E3 ubiquitin ligase both in vitro and in vivo.

Materials and methods

Cell culture. HEK293T human embryonic kidney cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies. Mouse anti-HA antibody (16B12) was purchased from Covance (Richmond, CA). Mouse anti-RH antibody (specific for the amino acid sequences RGSHHHH and GGSHHHH) was purchased from Qiagen (Santa Clara, CA). Rabbit anti-MBP antibody was purchased from New England Biolabs (Beverly, MA).

Preparation of cDNAs. The cDNAs of ubiquitin, Ro52 [8], N-terminal fragment of HIF1α (HIF1α(ΔC)) [9], and E2 ubiquitin-conjugating enzymes, such as UbcH2, UbcH5B, UbcH7, UbcH10, and hCDC34, were amplified by polymerase chain reaction (PCR) using appropriate primers from a human testis or heart cDNA library (Life Technologies).

Site-directed mutagenesis. 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 [10]. The mutated cDNA was subcloned into pMAL-c2 or pcDNA3/RH-C (see below).

In vitro ubiquitination assay. For the assay, we first expressed several recombinant proteins in bacteria, using eukaryotic expression vectors pMAL-c2 (New England BioLab) and pTrcHisB (Invitrogen, Carlsbad, CA). These proteins included MBP (maltose-binding protein)-fused Ro52 (MBP-Ro52), RH-tagged ubiquitin (RH-Ub), and poly-His-tagged E2 ubiquitin-conjugating enzymes. Next, bead-immobilized MBP-Ro52 was incubated with RH-Ub, E1 ubiquitin-activating enzyme (Boston Biochem, Cambridge, MA), and poly-His-tagged E2 ubiquitin-conjugating enzyme in reaction buffer [50 mM Tris–HCl (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, and 2 mM DTT] for 30 min at 37 °C. After reaction, the beads were washed by washing buffer [25 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.5% NP-40] and treated for 1 h at 50 °C in a sample treating solution containing 2% SDS and 5% β-mercaptoethanol. Finally, the solubilized MBP-Ro52 was analyzed by Western blotting using anti-RH antibody to detect ubiquitinated Ro52 and anti-MBP antibody to detect both unubiquitinated and ubiquitinated Ro52.

Plasmid construction and transfection. To express full-length Ro52 or N-terminal fragment (amino acids 1–330) of HIF1α [9] tagged with RH-epitope (RGSHHHHHH) at the C-terminus in HEK293T cells, the cDNA was ligated into pcDNA3/RH-C [11]. To express ubiquitin tagged with HA at the N-terminus, pcDNA3/HA-N was used [12]. The plasmids were transfected into HEK293T cells using FuGENE6 (Roche Applied Science, Indianapolis, IN). The transfected cells were harvested for TALON-bead precipitation 20 h after transfection.

Treatment with proteasome inhibitor. MG132 was purchased from Calbiochem (San Diego, CA) to treat cells as described previously [13]. In brief, 1×10^6 HEK293T cells were transfected by FuGENE 6. After overnight culture, the culture medium was replaced with fresh medium containing proteasome inhibitor MG132 (20 μM). The cells were further cultured at 37 °C with MG132 for 6 h. Then the cells were harvested, and the total cell lysates were prepared for TALON-bead precipitation.

TALON-bead precipitation. Because the sequence of the RH tag is RGSHHHHHH, the Ro52-RH or HIF1α(ΔC)-RH expressed in HEK293T cells was precipitated from the total cell lysate by using cobalt-ion-charged TALON beads (Clontech, Palo Alto, CA) under denaturing condition as described previously [14].

Western blotting. Protein samples were treated at 50 °C for 1 h in a sample treating solution containing 2% SDS and 5% β-mercaptoethanol. After SDS–PAGE, Western blotting was performed using the protocol provided with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). As a secondary antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used.

Results

E2 enzyme-dependent in vitro self-ubiquitination of Ro52

Ro52 possesses a RING-finger domain at its N-terminal region. Since many RING-finger proteins have been identified as E3 ubiquitin ligase [5,6], we hypothesized that Ro52 functions as an E3 enzyme. However, this hypothesis raised the question as to what the substrate of the Ro52-mediated ubiquitination is. Previously, we found that Ro52 is strongly ubiquitinated when overexpressed in COS cells [7]. Because several groups demonstrated that E3 enzymes also catalyze their own ubiquitination [15,16], our previous finding suggested that Ro52 acts as an E3 enzyme and ubiquitinates itself. To test this possibility, we performed an in vitro ubiquitination assay.

In the assay, MBP-Ro52 was expressed in bacteria and purified by using beads. MBP-Ro52 immobilized on the beads was then incubated with recombinant E1 enzyme and different recombinant E2 enzymes (UbcH2, UbcH5B, UbcH7, UbcH10, and hCDC34 produced in bacteria) in the presence of RH-tagged ubiquitin. In this in vitro system, MBP-Ro52 served as both a potential substrate and a potential E3 enzyme for its self-ubiquitination. After the incubation, MBP-Ro52 was solubilized and analyzed by Western blotting using anti-RH antibody and anti-

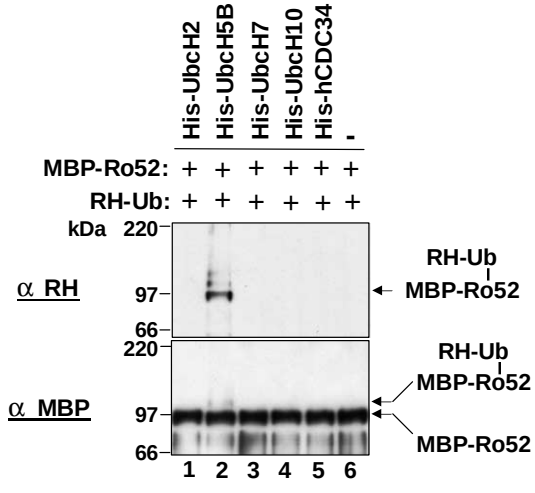

Fig. 1. UbcH5B-dependent in vitro self-ubiquitination of Ro52. MBP-fused Ro52 was purified by using beads and incubated with the reaction mixture containing RH-tagged ubiquitin, recombinant E1 enzyme, and poly-His-tagged various recombinant E2 enzymes (UbcH2, UbcH5B, UbcH7, UbcH10, and hCDC34). After reaction, MBP-Ro52 immobilized on beads was washed to remove the reaction mixture and solubilized in SDS solution. MBP-Ro52 was analyzed by Western blotting using anti-RH antibody to detect ubiquitinated MBP-Ro52 (upper panel) and anti-MBP antibody to detect both unubiquitinated and ubiquitinated MBP-Ro52 (lower panel). Molecular size markers are shown on the left in kilodaltons (kDa).

MBP antibody. As shown in Fig. 1, incubation of MBP-Ro52 in the reaction mixture containing UbcH2, UbcH7, UbcH10, or hCDC34 did not result in the ubiquitination of MBP-Ro52, whereas incubation of MBP-Ro52 in the reaction mixture containing UbcH5B yielded strong mono-ubiquitination and weak polyubiquitination of MBP-Ro52. These results indicated that Ro52 is ubiquitinated in vitro, and that this ubiquitination is catalyzed by UbcH5B but not by other E2 enzymes.

Role of Ro52 in its in vitro self-ubiquitination

In general, ubiquitin conjugates to the substrate in the presence of E1 enzyme, E2 enzyme, and E3 enzyme. These proteins are the minimum requirements for a ubiquitination reaction. To confirm whether these proteins are essential for the ubiquitination detected in Fig. 1, we performed another in vitro ubiquitination assay (Fig. 2). As a positive control, MBP-Ro52 was incubated in the complete reaction mixture containing RH-ubiquitin, recombinant E1 enzyme, and recombinant UbcH5B (E2 enzyme). In the other reactions, MBP-Ro52 was incubated in the incomplete reaction mixture lacking one of these components. After the incubation, MBP-Ro52 was solubilized and analyzed by Western blotting using anti-RH antibody and anti-MBP antibody. As shown in Fig. 2, incubation of MBP-Ro52 in the complete reaction mixture resulted in the ubiquitination of MBP-Ro52 (lane 4), whereas incubation of MBP-Ro52 in the incomplete reaction mixture lacking one component did not yield ubiquitination of MBP-Ro52 (lanes 1–3 and 5). These results indicated that ubiquitin, E1 enzyme, and UbcH5B (E2 enzyme) are minimum requirements for the in vitro ubiquitination of Ro52. Because reaction mixtures used in this assay did not contain any E3 enzymes other than Ro52, these results also indicated that Ro52 functions as an E3 enzyme and ubiquitinates itself.

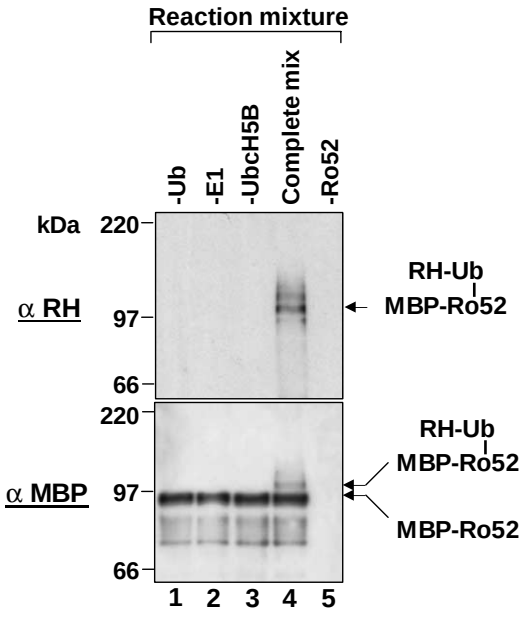

Fig. 2. Minimum requirements for the in vitro self-ubiquitination of Ro52. In the in vitro ubiquitination, the complete reaction mixture (lane 4) contained RH-ubiquitin, MBP-Ro52 both as a substrate and an E3 enzyme, recombinant E1 enzyme, and UbcH5B as an E2 enzyme. To confirm minimum requirements for the self-ubiquitination of Ro52, the incomplete reaction mixture lacking one of these components was prepared and used for the in vitro ubiquitination assay. After reaction, MBP-Ro52 immobilized on beads was solubilized and analyzed by Western blotting using anti-RH antibody to detect ubiquitinated MBP-Ro52 (upper panel) and anti-MBP antibody to detect both unubiquitinated and ubiquitinated MBP-Ro52 (lower panel). The incomplete reaction mixture shown in lanes 1–3 and 5 lacked RH-ubiquitin, recombinant E1, UbcH5B, and MBP-Ro52, respectively.

RING-motif-dependent in vitro self-ubiquitination of Ro52

Ro52 possesses a RING-consensus sequence (Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys-X_2-Cys-X_{4-48}-Cys-X_2-Cys) between amino acid residues 16 and 54 [17] (Fig. 3A). This sequence coordinates two Zn ions in a "cross-braced" fashion [17,18]. Recent results from several laboratories indicated that RING-finger proteins recruit E2 enzymes through their RING domain and act as E3 enzyme [5]. This E3 activity of RING-finger proteins has been shown to be abolished by a mutation of the conserved Cys or His residue described above [19,20]. To determine whether the E3 activity of Ro52 is dependent on its RING-finger domain, we substituted the conserved Cys-16 to Ala in the RING-finger domain to generate a Ro52 mutant (C16A) (Fig. 3A). Then, we tested whether this mutation abolishes the E3 activity of Ro52, using an in vitro ubiquitination assay.

In the assay, MBP-fused wild-type Ro52 or its C16A mutant was expressed in bacteria and purified by using beads. MBP-Ro52 immobilized on the beads was then incubated with RH-ubiquitin, recombinant E1 enzyme, and recombinant E2 enzyme (UbcH5B). After the incubation, MBP-Ro52 was solubilized and analyzed by Western blotting using anti-RH antibody and anti-MBP antibody. As shown in Fig. 3B, the wild-type Ro52 ubiquitinated itself (lanes 1 and 3), whereas the C16A mutant did not ubiquitinate itself at all (lanes 2 and 4). These results indicated that the in vitro self-ubiquitination of Ro52 is dependent on its RING-finger domain. Thus, we confirmed that Ro52 is a RING-motif-dependent E3 enzyme.

In vivo self-ubiquitination of Ro52 in HEK293T cells

The E3 activity of Ro52 was determined by in vitro assays described in the preceding sections. Therefore, this raised the question whether Ro52 functions as an E3 enzyme in human cells. Although we previously detected the ubiquitination of Ro52 in COS cells [7], it might be catalyzed by the other E3 enzymes. To investigate this, we performed an in vivo ubiquitination assay, using the wild-type Ro52 and its RING-finger mutant (C16A). In brief, RH-tagged wild-type Ro52 or its C16A mutant was expressed with or without HA-tagged ubiquitin in HEK293T cells.

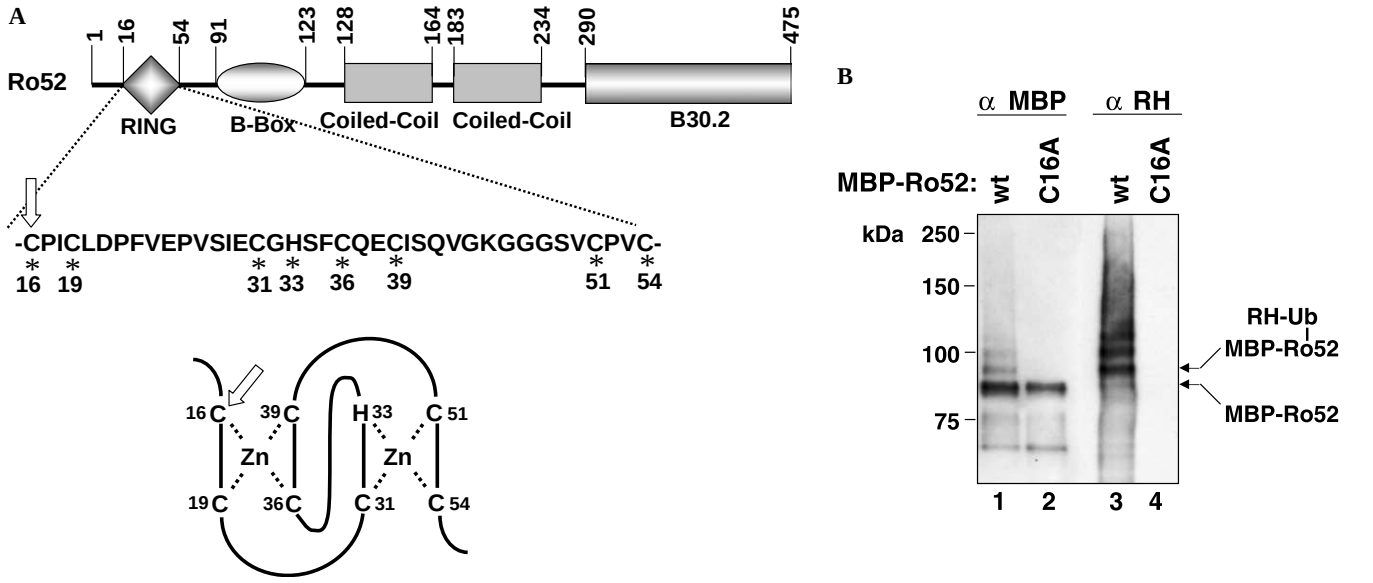

Fig. 3. E3 activity of wild-type Ro52 and its RING mutant in vitro. (A) Schematic presentation of Ro52 structure. The domain structure of the entire Ro52 molecule and the structure of its RING-finger domain are shown. Asterisks indicate conserved Cys and His residues in the RING-finger domain. Arrows indicate Cys-16, which was substituted to Ala to generate Ro52 (C16A). (B) In vitro ubiquitination assay using wild-type Ro52 and its RING mutant C16A. MBP-Ro52 (wild-type) or MBP-Ro52 (C16A) was purified by using beads and incubated with the reaction mixture containing RH-ubiquitin, recombinant E1 enzyme, and poly-His-tagged UbcH5B. After reaction, MBP-Ro52 immobilized on beads was solubilized and analyzed by Western blotting using anti-MBP antibody to detect both unubiquitinated and ubiquitinated MBP-Ro52 (lanes 1 and 2) and anti-RH antibody to detect ubiquitinated MBP-Ro52 (lanes 3 and 4).

The cells were then harvested and lysed under denaturing condition. Afterwards, Ro52-RH (wild-type or C16A) in the lysate was precipitated by TALON beads, solubilized, and then analyzed by Western blotting using anti-HA antibody to detect ubiquitinated Ro52-RH and anti-RH antibody to detect both unubiquitinated and ubiquitinated Ro52-RH. As shown in Fig. 4, the wild-type Ro52 was strongly monoubiquitinated and weakly polyubiquitinated when overexpressed with HA-ubiquitin in HEK293T cells (lane 3). In contrast, the C16A mutant was faintly ubiquitinated when overexpressed with HA-ubiquitin in HEK293T cells (lane 4). This faint ubiquitination of Ro52 (C16A) might be catalyzed by wild-type Ro52, which was endogenously expressed in HEK293T cells. These results indicated that Ro52 ubiquitinates itself through the function of its RING-finger domain in HEK293T cells.

In vivo self-ubiquitination of Ro52 unrelated to proteasomal degradation

In the previous sections, we demonstrated that Ro52 ubiquitinated itself mainly with a single ubiquitin in vitro and in vivo. Although Ro52 also ubiquitinated itself with a polyubiquitin chain *in vitro* and in vivo, the polyubiquitination was faint. Generally, a polyubiquitin chain is assembled through the formation of isopeptide bonds involving specific Lys residues in ubiquitin. Substrates with a polyubiquitin chain, linked via Lys-48 or Lys-29, are targeted for degradation by the proteasome [21]. In contrast, the conjugation of a single ubiquitin, or the ligation of a

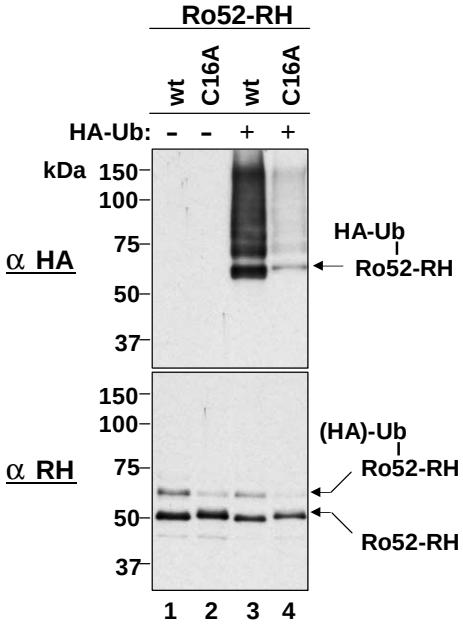

Fig. 4. E3 activity of wild-type Ro52 and its RING mutant in HEK293T cells. RH-tagged wild-type Ro52 or its RING mutant C16A was expressed with or without HA-tagged ubiquitin in HEK293T cells by plasmid transfection. Twenty hours after transfection, the cells were harvested and lysed under denaturing condition. Ro52-RH (wild-type or C16A) in the lysate was precipitated by cobalt-coated TALON beads and solubilized in 2% SDS solution. The solubilized Ro52-RH was then analyzed by Western blotting using anti-HA antibody to detect ubiquitinated Ro52-RH (upper panel) and anti-RH antibody to detect both unubiquitinated and ubiquitinated Ro52-RH (lower panel).

K63-linked polyubiquitin chain, does not target the substrates for proteasomal degradation [22,23]. To determine whether the self-ubiquitination of Ro52 leads to its degradation by the proteasome, we performed an in vivo ubiquitination assay. Specifically, using the proteasome inhibitor MG132, we inhibited the proteasomal degradation in HEK293T cells to test whether the ubiquitinated Ro52 was accumulated. Briefly, Ro52-RH was coexpressed with HA-ubiquitin in HEK293T cells in the presence or absence of proteasome inhibitor MG132. The cells were then harvested and lysed under denaturing condition. Afterwards, Ro52-RH in the lysate was precipitated by TALON beads, solubilized, and then analyzed by Western blotting using anti-HA antibody to detect ubiquitinated Ro52-RH and anti-RH antibody to detect both unubiquitinated and ubiquitinated Ro52-RH. As positive control for the effect of MG132 on proteasomal degradation of ubiquitinated proteins, we used HIF1α(ΔC), which is an N-terminal fragment (amino acids 1–330) of HIF1α. This is because we had previously detected the clear effect of MG132 on the proteasomal degradation of HIF1α(ΔC) [9]. As shown in Fig. 5A, the treatment with MG132 did not increase the expression of either ubiquitinated Ro52-RH or unubiquitinated Ro52-RH (lane 3 vs. lane 6). These results suggested that the self-ubiquitination of Ro52 does not lead to its proteasomal degradation. In contrast, a positive control HIF1α(ΔC) was stabilized by the treatment with MG132. As shown in Fig. 5B, the treatment with MG132 increased the expression of HIF1α(ΔC)-RH approximately threefold (lane 3 vs. lane 6, lower panel). Furthermore, the treatment increased the polyubiquitinated HIF1α(ΔC)-RH (lane 3 vs. lane 6, upper panel), because MG132 inhibited the proteasomal degradation of the polyubiquitinated HIF1α(ΔC)-RH, resulting in its accumulation. These results suggested that the polyubiquitination of HIF1α(ΔC) targets it to proteasomal degradation.

Discussion

Anti-Ro52 autoantibody has been clinically well investigated. Although the autoantigen Ro52 is classic, its function has been reported by only a few groups. Sibilia [1] proposed that the function of the Ro autoantigen is associated with RNA transcription processes, because the Ro autoantigen shares homologies with gene-regulation proteins and binds to nucleic acids. Ishii et al. [24] reported that Ro52 promotes CD28-mediated IL-2 production in Jurkat T cells. Despite these reports, the precise molecular function of Ro52 is still unclear.

Ro52 is a RING-finger protein that belongs to the RBCC/TRIM family [4]. Many RING-finger proteins have been reported to recruit E2 ubiquitin-conjugating enzymes and act as E3 ubiquitin ligase [5,6]. Furthermore, some groups recently reported that RBCC/TRIM family mem-

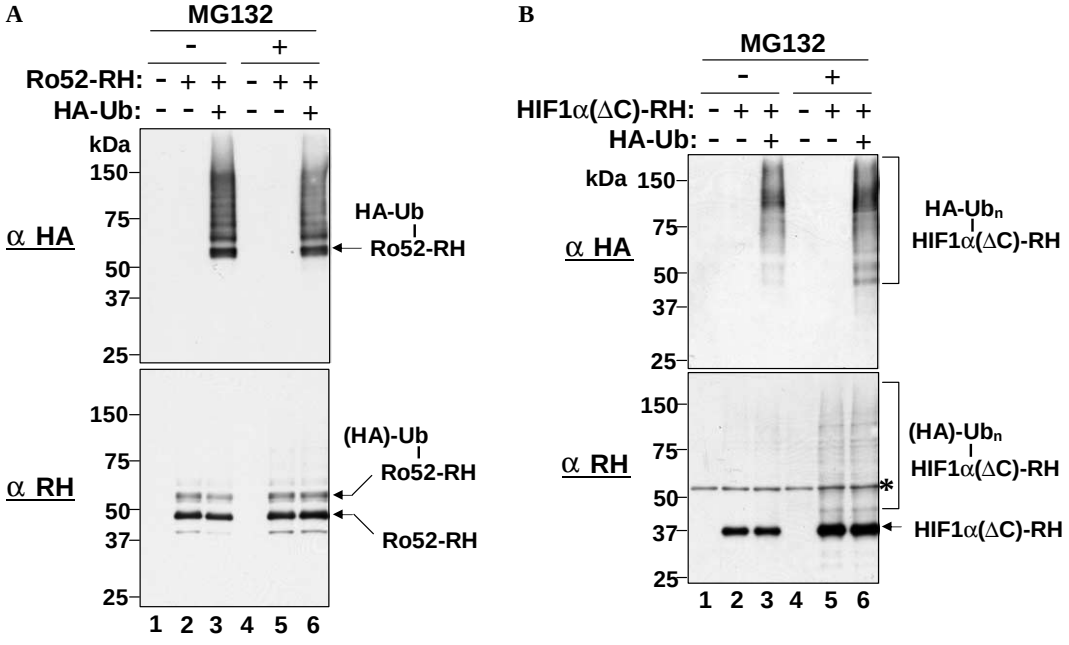

Fig. 5. Effect of proteasome inhibitor on the expression of Ro52 and HIF1α in HEK293T cells. (A) The expression of RH-tagged Ro52 in MG132-treated cells. (B) The expression of RH-tagged HIF1α N-terminal fragment, HIF1α(ΔC), in MG132-treated cells. Ro52-RH or HIF1α(ΔC)-RH was coexpressed in HEK293T cells with empty vector (lanes 2 and 5) or HA-tagged ubiquitin (lanes 3 and 6). The HEK293T cells were cultured for 6 h in the absence (lanes 1–3) or presence (lanes 4–6) of proteasome inhibitor MG132. After incubation, the cells were harvested and lysed under denaturing condition. Ro52-RH or HIF1α(ΔC)-RH in the lysate was precipitated by cobalt-coated TALON beads and solubilized in 2% SDS solution. The solubilized Ro52-RH or HIF1α(ΔC)-RH was then analyzed by Western blotting in which the ubiquitinated form of Ro52-RH or HIF1α(ΔC)-RH was detected by anti-HA antibody (upper panel), while both unubiquitinated and ubiquitinated forms were detected by anti-RH antibody (lower panel). A non-specific band is indicated by an asterisk.

bers, such as TRIM5δ [25], ARD1 [26], and TRIM37 [27], function as E3 ubiquitin ligase. Based on these reports, we hypothesized that Ro52 is also an E3 ubiquitin ligase. In the study presented here, we tested this hypothesis using both in vitro and in vivo ubiquitination assays. As expected, Ro52 ubiquitinates itself in vitro and in vivo in the presence of E1 enzyme and E2 enzyme UbcH5B, indicating that Ro52 functions as E3 enzyme for its self-ubiquitination. These results, however, raised two questions: what role does the self-ubiquitination play in the function of Ro52? And what are the substrates, other than Ro52 itself, in the Ro52-mediated ubiquitination?

To answer the first question, we determined whether the ubiquitination of Ro52 targets it to the proteasome for degradation. This is because the proteasomal degradation is one of the well-known roles of ubiquitination. Using an in vivo ubiquitination assay, we found that the ubiquitination of Ro52 does not lead to the proteasomal degradation, probably because of the type of ubiquitination. In the ubiquitination of Ro52, the predominant type was monoubiquitination, not polyubiquitination. Because monoubiquitination does not target the substrates for proteasomal degradation and is thought to be involved in non-proteasomal events, including endocytosis, trafficking, or lysosomal degradation [23], the monoubiquitination of Ro52 might play a role in these non-proteasomal events.

As described above, the polyubiquitination of Ro52 was also detected in this study by using anti-HA antibody and anti-RH antibody. The polyubiquitination was clearly detected by anti-HA antibody (see upper panels in Figs. 4 and 5), whereas it was almost undetectable by anti-RH antibody (lower panels in Figs. 4 and 5). Why did the use of these two antibodies produce such discrepant results in the detection of polyubiquitination? A polyubiquitin chain on Ro52-RH consists of multiple molecules of HA-ubiquitin. Because each of these molecules reacts with an anti-HA antibody, the polyubiquitin chain is labeled with multiple molecules of anti-HA antibody. This is the reason why anti-HA antibody detects the polyubiquitin chain much more strongly than actual. In contrast, detection of the polyubiquitinated Ro52-RH by anti-RH antibody reflects the actual level of expression because the anti-RH antibody reacts only with a single RH-epitope of the polyubiquitinated Ro52-RH. Based on this, the actual population of polyubiquitinated Ro52 is very small in comparison with that of monoubiquitinated Ro52. Despite the minor population, the polyubiquitination, as well as the monoubiquitination, might play a role in the function of Ro52. Interestingly, the polyubiquitinated Ro52 was not accumulated in HEK293T cells treated with the proteasome inhibitor, suggesting that these polyubiquitin chains do not target Ro52 for proteasomal degradation. This is probably because the polyubiquitin chains are assembled with linkage through Lys residues other than Lys-48 and are independent from proteasomal degradation.

Although we identified Ro52 as an E3 ubiquitin ligase, we could not determine its substrates other than itself. So far, several proteins have been reported to interact with Ro52. These include a multifunctional calcium-binding protein Calreticulin [28], an oncogenic protein UnpEL (also known as Usp4) [29,30], and a molecular chaperone Grp78 (also known as BiP) [31]. These Ro52-interacting proteins are thought to be candidates for the substrates ubiquitinated by Ro52. To elucidate Ro52-mediated biological events, such as RNA transcription processes [1] and IL-2 production in Jurkat T cells [24], the identification of substrates in the Ro52-mediated ubiquitination is important. This is because Ro52 seems to modify the function or stability of its substrates through ubiquitination, and this modification might result in the Ro52-mediated biological events.

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References

[1] J. Sibilia, Ro(SS-A) and anti-Ro(SS-A): an update, Rev. Rhum. [Engl. Ed.] 65 (1998) 45–57.
[2] Y. Itoh, M. Reichlin, Autoantibodies to the Ro/SSA antigen are conformation dependent. I: anti-60 kD antibodies are mainly directed to the native protein; anti-52 kD antibodies are mainly directed to the denatured protein, Autoimmunity 14 (1992) 57–65.
[3] E.T.H. Yeh, L. Gong, T. Kamitani, Ubiquitin-like proteins: new wines in new bottles, Gene 248 (2000) 1–14.
[4] M. Torok, L.D. Etkin, Two B or not two B? Overview of the rapidly expanding B-box family of proteins, Differentiation 67 (2001) 63–71.
[5] P.K. Jackson, A.G. Eldridge, E. Freed, L. Furstenthal, J.Y. Hsu, B.K. Kaiser, J.D. Reimann, The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases, Trends Cell Biol. 10 (2000) 429–439.
[6] Y.C. Liu, Ubiquitin ligases and the immune response, Annu. Rev. Immunol. 22 (2004) 81–127.
[7] T. Fukuda-Kamitani, T. Kamitani, Ubiquitination of Ro52 autoantigen, Biochem. Biophys. Res. Commun. 295 (2002) 774–778.
[8] K. Itoh, Y. Itoh, M.B. Frank, Protein heterogeneity in the human Ro/SSA ribonucleoproteins. The 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes, J. Clin. Invest. 87 (1991) 177–186.
[9] K. Kanaya, T. Kamitani, pVHL-independent ubiquitination of HIF1alpha and its stabilization by cobalt ion, Biochem. Biophys. Res. Commun. 306 (2003) 750–755.
[10] T. Kamitani, H.P. Nguyen, E.T.H. Yeh, Activation-induced aggregation and processing of the human Fas antigen-detection with cytoplasmic domain specific antibodies, J. Biol. Chem. 272 (1997) 22307–22314.
[11] T. Kamitani, H.P. Nguyen, K. Kito, T. Fukuda-Kamitani, E.T.H. Yeh, Covalent modification of PML by the sentrin family of ubiquitin-like proteins, J. Biol. Chem. 273 (1998) 3117–3120.
[12] T. Kamitani, K. Kito, H.P. Nguyen, E.T.H. Yeh, Characterization of NEDD8, a developmentally down-regulated ubiquitin-like molecule, J. Biol. Chem. 272 (1997) 28557–28562.
[13] T. Kamitani, K. Kito, T. Fukuda-Kamitani, E.T.H. Yeh, Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1, J. Biol. Chem. 276 (2001) 46655–46660.
[14] H. Wada, E.T.H. Yeh, T. Kamitani, A dominant negative Ubc12 mutant sequesters NEDD8 and inhibits NEDD8-conjugation in vivo, J. Biol. Chem. 275 (2000) 17008–17015.

[15] U. Nuber, S.E. Schwarz, M. Scheffner, The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate, Eur. J. Biochem. 254 (1998) 643–649.
[16] Y. Yang, S. Fang, J.P. Jensen, A.M. Weissman, J.D. Ashwell, Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli, Science 288 (2000) 874–877.
[17] A.J. Saurin, K.L. Borden, M.N. Boddy, P.S. Freemont, Does this have a familiar RING? Trends Biochem. Sci. 21 (1996) 208–214.
[18] S. Fang, K.L. Lorick, J.P. Jensen, A.M. Weissman, RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer, Semin. Cancer Biol. 13 (2003) 5–14.
[19] L. Deng, C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, Z.J. Chen, Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, Cell 103 (2000) 351–361.
[20] C. Uchida, S. Miwa, K. Kitagawa, T. Hattori, T. Isobe, S. Otani, T. Oda, H. Sugimura, T. Kamijo, K. Ookawa, H. Yasuda, M. Kitagawa, Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation, EMBO J. 24 (2005) 160–169.
[21] A. Hershko, A. Ciechanover, The ubiquitin system for protein degradation, Annu. Rev. Biochem. 61 (1992) 761–807.
[22] E.W. Doss-Pepe, L. Chen, K. Madura, Alpha-synuclein and parkin contribute to the assembly of ubiquitin lysine63-linked multiubiquitin chains, J. Biol. Chem. (2005).
[23] L. Sun, Z.J. Chen, The novel functions of ubiquitination in signaling, Curr. Opin. Cell Biol. 16 (2004) 119–126.
[24] T. Ishii, K. Ohnuma, A. Murakami, N. Takasawa, T. Yamochi, S. Iwata, M. Uchiyama, N.H. Dang, H. Tanaka, C. Morimoto, SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production, J. Immunol. 170 (2003) 3653–3661.
[25] L. Xu, L. Yang, P.K. Moitra, K. Hashimoto, P. Rallabhandi, S. Kaul, G. Meroni, J.P. Jensen, A.M. Weissman, P. D'Arpa, BTBD1 and BTBD2 colocalize to cytoplasmic bodies with the RBCC/tripartite motif protein, TRIM5delta, Exp. Cell Res. 288 (2003) 84–93.
[26] A. Vichi, D.M. Payne, G. Pacheco-Rodriguez, J. Moss, M. Vaughan, E3 ubiquitin ligase activity of the trifunctional ARD1 (ADP-ribosylation factor domain protein 1), Proc. Natl. Acad. Sci. USA 102 (2005) 1945–1950.
[27] J. Kallijarvi, U. Lahtinen, R. Hamalainen, M. Lipsanen-Nyman, J.J. Palvimo, A.E. Lehesjoki, TRIM37 defective in mulibrey nanism is a novel RING finger ubiquitin E3 ligase, Exp. Cell Res. 308 (2005) 146–155.
[28] S.T. Cheng, T.Q. Nguyen, Y.S. Yang, J.D. Capra, R.D. Sontheimer, Calreticulin binds hYRNA and the 52-kDa polypeptide component of the Ro/SS-A ribonucleoprotein autoantigen, J. Immunol. 156 (1996) 4484–4491.
[29] F. Di Donato, E.K. Chan, A.D. Askanase, M. Miranda-Carus, J.P. Buyon, Interaction between 52 kDa SSA/Ro and deubiquitinating enzyme UnpEL: a clue to function, Int. J. Biochem. Cell Biol. 33 (2001) 924–934.
[30] T.A. Soboleva, D.A. Jans, M. Johnson-Saliba, R.T. Baker, Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme, J. Biol. Chem. 280 (2005) 745–752.
[31] A.W. Purcell, A. Todd, G. Kinoshita, T.A. Lynch, C.L. Keech, M.J. Gething, T.P. Gordon, Association of stress proteins with autoantigens: a possible mechanism for triggering autoimmunity? Clin. Exp. Immunol. 132 (2003) 193–200.