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Function and subcellular location of Ro52β

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Abstract

Autoantigen Ro52α was recently identified as an E3 ubiquitin ligase. Its splicing variant Ro52β, which lacks a leucine zipper, has not been characterized yet. We therefore characterized Ro52β in contrast to Ro52α. Our biochemical assays revealed that both Ro52α and Ro52β function as E3 ubiquitin ligases and self-ubiquitinate in cooperation with UbcH5B in vitro. In addition, both Ro52α and Ro52β are ubiquitinated when overexpressed with ubiquitin in HEK293T cells, suggesting that both function as E3 ligases and self-ubiquitinate in vivo. However, cytological studies revealed that Ro52α mainly localizes to the cytoplasmic rod-like structures, whereas Ro52β diffusely localizes to both the cytoplasm and the nucleus. Since the leucine zipper plays a role in the homodimerization and heterodimerization of Ro52α, the dimerization might be required for the localization of Ro52α to the rod-like structures. On the basis of these results, Ro52α and Ro52β appear to ubiquitinate their particular substrates at different locations.

Keywords: Ro52; Sjögren's syndrome; Ubiquitin; TRIM; RING finger; RBCC; Ubiquitin ligase; E3; UnpEL; Usp4

Anti-Ro/SSA antibodies are autoantibodies that are 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. Ro52 is one of the autoantigens recognized by the anti-Ro/SSA autoantibodies. So far, α and β isoforms of Ro52 have been reported. Ro52α, a comparatively large isoform (52 kDa), is a RING-finger protein that belongs to an RBCC (RING-finger/B-box/coiled-coil) family (also known as the TRIM family) [1]. Ro52β, a comparatively small isoform, is a product of mRNA derived from the alternative splicing of exon 3 to exon 5, skipping exon 4, which results in a smaller protein with a predicted molecular weight of 45 kDa [2]. Recently, we reported that Ro52α functions as an E3 ubiquitin ligase and ubiquitinates itself [3]. We also reported that Ro52α colocalizes with UnpEL to the cytoplasmic rod-like structures and is selectively deubiquitinated by UnpEL [4]. Thus, we have characterized the molecular function and subcellular location of Ro52α and identified its interacting protein UnpEL as a potential regulator. However, Ro52β has not yet been characterized to this extent.

Although Ro52β is a minor splicing variant in comparison with Ro52α [5], the expression of Ro52β seems to be clinically important. This was indicated by the finding that fetal congenital heart block caused by maternal anti-Ro/SSA autoantibodies might be triggered by the expression of Ro52β in the fetal heart. Since the expression of Ro52β is maximal at the time of cardiac ontogeny when maternal antibodies gain access to the fetal circulation, just prior to the clinical detection of brady-arrhythmia, Ro52β has been implicated in the development of congenital heart block [5]. It is therefore important to learn more about Ro52β in order to shed light on the pathogenesis of congenital heart block and perhaps other disorders in which anti-Ro/SSA antibodies play a pathogenic role. In the study described here, we investigated Ro52β using the same assay methods that we previously used to characterize Ro52α and compared Ro52β with Ro52α.

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Materials and methods

Cell culture. Human embryonic kidney HEK293 cells and HEK293T cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies. Mouse anti-FLAG antibody (M5) was purchased from Sigma Chemical Company (St. Louis, MO). Mouse anti-RH antibody (specific for the amino acid sequences RGSHHHH and GGSHHHH) was purchased from QIAGEN (Santa Clara, CA). Mouse anti-HA antibody (16B12) was purchased from Covance (Richmond, CA). Rabbit anti-MBP antibody was purchased from NewEngland Biolabs (Beverly, MA).

Preparation of Ro52β cDNA. The cDNA of Ro52β [2] was generated by a recombinant polymerase chain reaction [6] using the cDNA of Ro52α [7] as a template.

Plasmid construction and transfection. To express Ro52β tagged with RH-epitope (RGSHHHHHH) at the C-terminus in HEK293T cells, the cDNA of Ro52β was subcloned into pcDNA3/RH-C [8], as we previously did for the expression of Ro52α [7]. To express ubiquitin tagged with HA at the N-terminus, pcDNA3/HA-N was used [9]. The plasmids were transfected into HEK293T cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) and 20 h later, the transfected cells were harvested for TALON-bead precipitation.

In vitro ubiquitination assay. For the in vitro ubiquitination assay, we first expressed several recombinant proteins in bacteria using the eukaryotic expression vectors pMAL-c2 (New England BioLabs) and pTrcHisB (Invitrogen, Carlsbad, CA). These proteins include maltose-binding protein (MBP)-fused Ro52α (MBP-Ro52α) [3], MBP-Ro52β, and MBP-Ro52α (C16A) [3], RH-tagged ubiquitin (RH-Ub) [3], and poly-His-tagged E2 ubiquitin-conjugating enzyme UbcH5B [3]. Next, bead-immobilized MBP-Ro52 was incubated with RH-Ub, E1 ubiquitin-activating enzyme (Boston Biochem, Cambridge, MA), and poly-His-tagged UbcH5B 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 the reaction, the beads were washed with 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 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.

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

TALON-bead precipitation of RH-tagged Ro52. To investigate the in vivo ubiquitination of Ro52α and Ro52β, HA-tagged ubiquitin was coexpressed with Ro52α-RH or Ro52β-RH in HEK293T cells by the cotransfection method. Since the sequence of the RH tag is RGSHHHHHH, RH-tagged proteins can be purified by cobalt-immobilized resin beads (TALON beads; Clontech, Palo Alto, CA) [3,4]. The total cell lysate of the transfectants expressing RH-tagged Ro52 (α or β isoform) and HA-ubiquitin was prepared in lysis buffer (20 mM Tris–HCl [pH 8.0], 6 M guanidine–HCl, and 100 mM NaCl). DNA in the sample was sheared with a 22-gauge needle, and then the lysate was centrifuged at 100,000*g* for 30 min at 15 °C. The supernatant was incubated with TALON beads for 1 h at room temperature. The beads were washed once with lysis buffer and then washed twice with washing buffer (20 mM Tris–HCl [pH 7.0], 15 mM imidazole, 8 M urea, and 100 mM NaCl). Finally, the beads were washed twice with phosphate-buffered saline (PBS) and treated for 1 h at 50 °C in sample treating solution containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol, followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

In vivo deubiquitination assay. To investigate the isopeptidase activity of UnpEL against ubiquitinated Ro52α and Ro52β, we performed an in vivo deubiquitination assay, as described previously [4]. Briefly, FLAG-tagged UnpEL was expressed in HEK293T cells with HA-ubiquitin and an RH-tagged substrate (Ro52α-RH or Ro52β-RH) using plasmid cotransfection method. Twenty hours after transfection, cells were lysed in the lysis buffer containing 6 M guanidine–HCl (see above description). The RH-tagged substrate in the lysate was then precipitated with cobalt-coated TALON beads, washed, and solubilized in sample treating solution containing 2% SDS and 5% β-mercaptoethanol (see above). Finally, the RH-tagged substrate was analyzed by Western blotting using anti-RH antibody to detect all derivatives of the substrate and anti-HA antibody to detect the substrate conjugated with HA-ubiquitin.

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

Immunocytochemistry. To investigate the subcellular location of Ro52α, Ro52β, and UnpEL in cultured cells, we performed immunocytochemical studies, as described previously [4]. Briefly, HEK293 cells were cultured on a coverslip in a 3.5-cm dish, and 1 μg of pEGFP-N1 [4], pEGFP-N1/Ro52α [4], or pEGFP-N1/Ro52β (pEGFP-N1 inserted with the Ro52β cDNA) was transfected with or without 1 μg pcDNA3/FLAG-UnpEL [4]. After 24 h, the cells were fixed with a 4% paraformaldehyde solution [pH 7.5] for 30 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. The cells cotransfected with pcDNA3/FLAG-UnpEL were first labeled with mouse anti-FLAG antibody. After washing, the cells were labeled with Alexa Fluor 594-conjugated anti-mouse IgG antibody (Molecular Probes, Eugene, OR). Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/ml PBS) for 5 min and analyzed under a fluorescence microscope. The localization of enhanced green fluorescent protein (EGFP) alone, Ro52α-EGFP, and Ro52β-EGFP was shown by the green fluorescence of EGFP, and the localization of FLAG-UnpEL was shown by the red fluorescence of Alexa Fluor 594. Their colocalization was shown by the merging of both fluorescences.

Results and discussion

Ro52β structurally lacks the leucine zipper

To compare human Ro52α and Ro52β, we first summarized their domain structure, which was previously reported [2]. As shown in Fig. 1, Ro52α possesses a RING finger

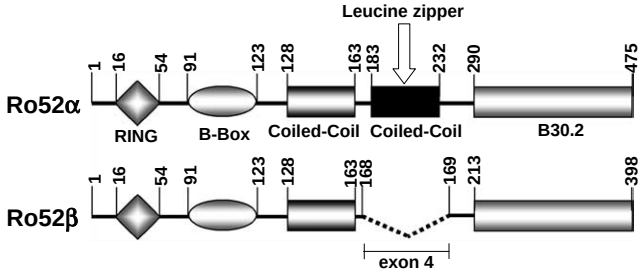

Fig. 1. The protein domains of human Ro52α and Ro52β. The two isoforms are products of alternative splicing of a single gene. Ro52α (52 kDa) possesses a RING finger and a B box at the N-terminal region, two coiled coils in the central domain with the second as a leucine zipper encoded in exon 4, and a B30.2 domain at the C-terminus. Ro52β (45 kDa) is identical to Ro52α but lacks 77 amino acids (amino acid residues 169–245) inclusive of the second coiled-coil domain with the leucine zipper.