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Dynamic movements of Ro52 cytoplasmic bodies along microtubules

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Abstract

The RING-finger protein Ro52/TRIM21 is known as an autoantigen and is recognized by anti-Ro/SSA antibodies, which are commonly found in patients with Sjögren's syndrome and systemic lupus erythematosus. Recently, Ro52 has been shown to localize to distinct structures called cytoplasmic bodies and function as an E3 ubiquitin ligase. However, the Ro52 cytoplasmic bodies have not been well characterized. In this study, we investigated the Ro52 cytoplasmic bodies using fluorescence microscopy. This analysis revealed that the Ro52 cytoplasmic bodies are diffusely located in the cytoplasm and exist independently of TRIM5 α cytoplasmic bodies. Our results further showed that the Ro52 cytoplasmic bodies are not stained with MitoTracker dye and are not colocalized with the proteasome subunit Rpt5, the caveolae component caveolin-1, the endosome markers (EEA1, Rab5, and Rab7), and the lysosome marker LAMP2. These results indicate that the Ro52 cytoplasmic bodies are not mitochondria, proteasome-enriched structures, caveolae, endosomes, or lysosomes. Importantly, the Ro52 cytoplasmic bodies are highly motile and are located along the microtubule network. These results suggest that the Ro52 cytoplasmic bodies are unidentified structures that are transported along the microtubule network.

Keywords

Ro52; TRIM21; Ubiquitin ligase; Cytoplasmic bodies; Microtubule

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Introduction

Ro52 (also known as TRIM21) is a RING-finger protein expressed in most tissues and cells (Sibilia 1998). Ro52 is well known as an autoantigen that is recognized by anti-Ro52 autoantibodies found in the sera of patients with several autoimmune diseases, especially Sjögren's syndrome and systemic lupus erythematosus (Sibilia 1998). Previously, we identified Ro52 as an E3 ubiquitin ligase (Wada and Kamitani 2006a). Ro52 has been shown to catalyze ubiquitination of several proteins, including Ro52 itself (Wada and Kamitani 2006a), Usp4 (also known as UnpEL or Unph) (Wada and Kamitani 2006b), IRF-8 (Kong et al. 2007), and TRIM5 α (Yamauchi et al. 2008). Recently, we found that Ro52 conjugates monoubiquitin to IKK β in cells expressing Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV-1) (Wada et al. 2009). Our observations suggest that Tax protein induces phosphorylation of IKK β , which leads to interaction of IKK β with Ro52 for monoubiquitination. Ro52 might play a role in the negative regulation of NF- κ B-dependent inflammatory signaling through ubiquitination of IKK β or other substrates. Indeed, using Ro52 knockout mice, Yoshimi et al. (2009) recently showed this possibility. Furthermore, Espinosa et al. (2009) also showed that Ro52-deficient mice develop uncontrolled inflammation and systemic autoimmunity as a consequence of minor tissue injury, suggesting that Ro52 plays a role in the negative regulation of inflammation and systemic auto-immunity.

Ro52 belongs to the *tripartite motif* (TRIM) protein family (also known as the RBCC family) because it possesses a RING-finger domain, a B-box domain, and two coiled-coil domains (Reymond et al. 2001; Wada and Kamitani 2006a). Individual TRIM family members were shown to localize to distinct, specific cell compartments (Reymond et al. 2001). For example, TRIM19 (also known as PML) localizes to small dots in the nucleus, called nuclear bodies (Ishov et al. 1999; Kamitani et al. 1998), whereas Ro52 and TRIM5 α localize to small dot- or rod-like structures in the cytoplasm, called cytoplasmic bodies (Campbell et al. 2007; Reymond et al. 2001; Rhodes et al. 2002; Wada et al. 2006b; Yamauchi et al. 2008). Although it is clear that Ro52 localizes to cytoplasmic bodies, they have not been well characterized. In this study, we performed immunofluorescence microscopy and live cell fluorescence microscopy to characterize the Ro52 cytoplasmic bodies in human cells.

Materials and methods

Cell lines and culture conditions

Human embryonic kidney (HEK) 293, human cervical carcinoma HeLa, and human lung fibrosarcoma HT1080 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies

Rabbit anti-caveolin-1 antibody (#sc-894), mouse monoclonal anti-Ro52 (D-12) antibody (#sc-25351), and rat monoclonal anti- α -tubulin (YL1/2) antibody (#sc-53029) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Rpt5 antibody was purchased from Affiniti Research Products Ltd. (Mamhead, Exeter, UK). Mouse anti-human EEA1 antibody (14/EEA1) was purchased from BD Transduction Laboratories (San Jose, CA). Mouse anti-human LAMP2 antibody (H4B4) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA).

Western blotting

Protein samples were treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β -mercaptoethanol. After SDS-polyacrylamide gel electrophoresis, Western

blotting was performed according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). As a secondary antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) was used.

Plasmid construction and transfection

The cDNAs of human Ro52 (Wada and Kamitani 2006a) and TRIM5 α (Yamauchi et al. 2008) were amplified using polymerase chain reaction (PCR) with appropriate primers from human cDNA libraries of testis and brain (Invitrogen, Carlsbad, CA), respectively. To express Ro52 and TRIM5 α fused with enhanced green fluorescent protein (EGFP) at the C-terminus in mammalian cells, we inserted the cDNAs of Ro52 and TRIM5 α into pEGFP-N1 (Clontech, Palo Alto, CA) and generated pRo52-EGFP and pTRIM5 α -EGFP, respectively. To express Ro52 fused with monomeric red fluorescent protein (mRFP) at the C-terminus in mammalian cells, we first inserted the mRFP cDNA (a gift from Dr. Roger Tsien, University of California San Diego, La Jolla, CA) into pcDNA3 and generated pcDNA3/mRFP-C. The Ro52 cDNA was then inserted into pcDNA3/mRFP-C to generate pRo52-mRFP for the expression of Ro52-mRFP. Mammalian expression plasmids containing cDNAs of DsRed-fused Rab5 and Rab7 were provided by Dr. David Marks (Mayo Clinic, Rochester, MN) through Addgene (Cambridge, MA). These plasmids were transfected into HEK293, HeLa, or HT1080 cells using FuGENE6 (Roche Applied Science, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Immunostaining of endogenous Ro52 in human cells

HEK293, HeLa, and HT1080 cells were cultured on a coverslip in a 3.5-cm dish. After 24 h, the cells were fixed in a 4% paraformaldehyde solution for 30 min, stained with 0.1 μ g/ml of 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche Diagnostics) for 10 min, and permeabilized with 0.1% TritonX-100 for 15 min at room temperature. The cells were first labeled with mouse monoclonal anti-human Ro52 (D-12) antibody at a dilution of 1:1,000 overnight at 10°C. After washing, the cells were labeled with Alexa Fluor 594-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) at a dilution of 1:2,000 for 1 h at room temperature. Finally, the cells were analyzed by an Axio Imager M1 fluorescence microscope (Zeiss). The localization of endogenous Ro52 was shown by the red fluorescence of Alexa Fluor 594.

XZ imaging of monolayer cells containing Ro52 cytoplasmic bodies

To obtain vertical images of monolayer cells containing Ro52 cytoplasmic bodies, we performed the XZ imaging method as described previously (Shelden et al. 2002). Briefly, HEK293 cells were cultured in a 6-cm dish and transfected with 5 μ g of pRo52-EGFP plasmid using FuGENE6. As much as 24 h after transfection, the cells were seeded onto a Millicell culture plate insert membrane (Millicell-CM; Millipore, Bedford, MA) in a 24-well culture plate. To label glycoproteins on the apical surface of cells, after 24 h culture on the Millicell membrane, the cells were stained with 0.25 μ g/ml of Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) (Molecular Probes, Eugene, OR) for 1 h at room temperature as described previously (Berkova et al. 2006). After three washes with PBS, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Membranes were excised into 2-mm pieces using a razor blade, transferred into a cryomold containing OCT compound (Tissue-Tek II), and snap-frozen in liquid nitrogen. Cryosections of WGA-stained monolayer cells on the Millicell membrane were then cut at 5 μ m and placed onto cleaned slides. Finally, after DAPI staining, the XZ images of monolayer cells were analyzed by fluorescence microscopy.

Staining of mitochondria with MitoTracker dye

HEK293 cells were cultured on a coverslip in a 3.5-cm dish and transfected with 2 μ g of pRo52-EGFP by FuGENE6. After 24 h, the cells were incubated for 35 min in the culture medium containing 100 nM of MitoTracker Red CMXRos (#M-7512; Molecular Probes). The cells were then washed with PBS twice and fixed in a 4% paraformaldehyde solution for 30 min. After washing with PBS, the cells were analyzed by fluorescence microscopy. The localization of Ro52-EGFP was shown by the green fluorescence of EGFP, and the location of mitochondria was shown by the red fluorescence of MitoTracker Red CMXRos.

Characterization of Ro52 cytoplasmic bodies using single immunostaining

To characterize Ro52 cytoplasmic bodies in cultured cells, we performed single immunostaining. Specifically, HEK293 cells were cultured on a coverslip in a 3.5-cm dish and transfected with 2 μ g of pRo52-EGFP by FuGENE6. After 24 h, the cells were fixed in a 4% paraformaldehyde solution for 30 min, stained with 0.1 μ g/ml of DAPI for 10 min, and permeabilized with 0.1% TritonX-100 for 15 min at room temperature. The cells were first labeled with one of the following primary antibodies overnight at 10°C: mouse anti-Rpt5 (1:20,000), rabbit anti-caveolin (1:500), mouse anti-EEA1 (1:1,000), or mouse anti-LAMP2 (1:1,000). After washing, the cells were labeled with Alexa Fluor 594-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes) at a dilution of 1:2,000 for 1 h at room temperature. The cells were then analyzed by fluorescence microscopy. The localization of Ro52-EGFP was shown by the green fluorescence of EGFP, and the localization of immunostained proteins was shown by the red fluorescence of Alexa Fluor 594. Their colocalization was examined by the merging of both fluorescent images.

Fluorescence microscopy for the detection of both DsRed-fused Rab5 and Rab7

To examine the colocalization of Ro52 with Rab5 or Rab7 in cultured cells, we performed fluorescence microscopy studies. In these studies, pRo52-EGFP was transfected with a plasmid for the expression of DsRed-fused Rab5 or Rab7 into HEK293 cells. The localization of Ro52-EGFP was shown by the green fluorescence of EGFP, and the localization of DsRed-Rab5 and DsRed-Rab7 was shown by the red fluorescence of DsRed.

Double immunostaining for α -tubulin and Ro52

HT1080 cells were cultured on a coverslip in a 3.5-cm dish. After 24 h, the cells were rinsed once in PBS, fixed 5 min in methanol at -20°C , and permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature. The cells were then washed 3 times in PBS and blocked for 30 min at room temperature in PBS containing 5% horse serum. The cells were first labeled with rat monoclonal anti- α -tubulin (YL1/2) antibody at a dilution of 1:1,000 and mouse monoclonal anti-Ro52 (D-12) antibody at a dilution of 1:1,000 overnight at 10°C. After washing, the cells were labeled with Alexa Fluor 488-conjugated goat anti-rat IgG for α -tubulin and Alexa Fluor 555-conjugated goat anti-mouse IgG for Ro52 at a dilution of 1:1,000 for 1 h at room temperature. These secondary antibodies were highly cross-absorbed before use (Molecular Probes). Finally, after DAPI staining, the cells were analyzed by fluorescence microscopy. The localization of endogenous α -tubulin was shown by the green fluorescence of Alexa Fluor 488. The localization of endogenous Ro52 was shown by the red fluorescence of Alexa Fluor 555. Their colocalization was examined by the merging of both fluorescent images.

Live cell fluorescence microscopy

To observe live images of Ro52 cytoplasmic bodies, live cell fluorescence microscopy was performed as described previously (Campbell et al. 2007). HT1080 cells expressing Ro52-EGFP were taken as images in a 60-frame sequence captured at 2-s intervals using live cell

fluorescence microscopy with a 100× objective lens. These images were used to make movies (Supplemental Movies 1 and 2). To follow the tracks of movements of Ro52 cytoplasmic bodies manually, the 12 frames (10-s intervals) were combined into one image using an image addition function of Adobe Photoshop CS3. Each track was manually verified as authentic by comparison with the original movies before further analysis.

Fluorescence microscopy to detect cytoplasmic bodies of Ro52 and TRIM5α

To examine cytoplasmic bodies of Ro52 and TRIM5α in cultured cells, we performed fluorescence microscopy studies. pRo52-mRFP was cotransfected with pTRIM5α-EGFP for the coexpression of mRFP-fused Ro52 and EGFP-fused TRIM5α in HT1080 cells. The localization of Ro52-mRFP was shown by the red fluorescence of mRFP, and the localization of TRIM5α-EGFP was shown by the green fluorescence of EGFP.

Results

Subcellular localization of endogenous Ro52 and exogenously expressed Ro52-EGFP

Ro52 has been shown to localize to cytoplasmic bodies (Reymond et al. 2001; Rhodes et al. 2002; Wada et al. 2006a, b; Yamauchi et al. 2008). However, these Ro52 cytoplasmic bodies have not been well characterized. In this study, we investigated the cytoplasmic bodies using fluorescence microscopy. First, we detected endogenous Ro52 using mouse monoclonal anti-human Ro52 antibody (clone D-12) that recently became commercially available. To determine whether this antibody is specific to Ro52, we performed Western blotting using total cell lysates of three human cell lines (HeLa, HT1080, and HEK293). As shown in the upper panel of Fig. 1a, this antibody strongly detected a single band just below a 55-kDa marker without any other bands in all three lysates, indicating that the antibody reacts with Ro52 only in the total cell lysates.

Using this antibody, we next immunostained endogenous Ro52 expressed in HeLa, HT1080, and HEK293 cells. As shown in Fig. 1b, endogenous Ro52 localized to cytoplasmic bodies in all three cell lines, which has previously been reported by several groups as described above. Thus, we confirmed that this antibody (D-12) can be used for immunostaining of endogenous Ro52.

Finally, we detected cytoplasmic bodies that were generated by exogenously expressed Ro52-EGFP. In brief, Ro52-EGFP was expressed in HeLa, HT1080, and HEK293 cells by transfection. Using a fluorescence microscope, we then observed the green fluorescence of Ro52-EGFP to determine its subcellular location. As shown in Fig. 1c, Ro52-EGFP localized to cytoplasmic bodies. However, the cytoplasmic bodies of Ro52-EGFP were larger than those of endogenous Ro52 (see Fig. 1b). The number of cytoplasmic bodies of Ro52-EGFP was less than that of endogenous Ro52 (Fig. 1c vs. b). Interestingly, the cytoplasmic bodies of Ro52-EGFP showed a rod-like shape as described previously (Rhodes et al. 2002; Wada et al. 2006a,b), while the shape of the cytoplasmic bodies of endogenous Ro52 was round or irregular.

XZ images of monolayer cells expressing Ro52-EGFP

Ro52 localized to cytoplasmic bodies. Previously, Rhodes et al. (2002) transiently expressed epitope-tagged Ro52 in HeLa cells to determine the intracellular distribution of Ro52 by immunofluorescence microscopy. They reported that the cytoplasmic bodies are located directly underneath the plasma membrane. To confirm this, we performed fluorescence microscopy using a XZ imaging method (Shelden et al. 2002). Briefly, HEK293 cells expressing Ro52-EGFP were cultured onto a membrane. The apical surface of cells was then labeled with red fluorescent dye-conjugated WGA. Then, the cells were fixed. Cell-attached

membranes were then transferred into a cryomold containing OCT compound and were snap-frozen in liquid nitrogen. Next, cryosections of WGA-stained monolayer cells on the membrane were cut and placed on slides. Finally, the XZ images of monolayer cells were analyzed by fluorescence microscopy. Figure 2 shows XZ images of 4 individual cells. It is clear that Ro52 cytoplasmic bodies are located not only underneath the plasma membrane but also diffusely in the cytoplasm.

Relation of Ro52 cytoplasmic bodies to mitochondria, proteasomes, caveolae, early endosomes, and lysosomes

What exactly are Ro52 cytoplasmic bodies? Because of their shape, we initially hypothesized that they were mitochondria, proteasome-enriched structures (Johnston et al. 1998; Kopito 2000; Tanji et al. 2006), caveolae (Parton and Simons 2007), early endosomes, or lysosomes. To test this hypothesis, we first stained mitochondria with MitoTracker dye. As shown in the bottom panel of Fig. 3, Ro52 cytoplasmic bodies did not merge with stained mitochondria. Next, we performed immunofluorescence microscopy using antibodies against Rpt5, caveolin-1, EEA1, and LAMP2. Rpt5 is a subunit of PA700 (also called 19S proteasome) and localizes to proteasome-enriched structures (Tanji et al. 2006). Caveolin-1 is a principal component of caveolae (Parton and Simons 2007). EEA1 is a marker protein for early endosomes because it colocalizes to the early endosomes with transferrin receptor and Rab5 but not with the late endosome-localizing Rab7. LAMP2 is a marker protein for lysosomes as is LAMP1. By immunostaining these marker proteins, we determined the location of proteasome-enriched structures, caveolae, early endosomes, and lysosomes. As shown in Fig. 4, the shapes of the immunostained structures were very similar to those of the Ro52 cytoplasmic bodies (panels b, e, h, and k). However, Ro52 cytoplasmic bodies did not merge with immunostained structures (panels c, f, i, and l). These staining results indicate that the cytoplasmic bodies are not mitochondria, proteasome-enriched structures, caveolae, early endosomes, or lysosomes.

Relation of Ro52 cytoplasmic bodies to Rab5- or Rab7-positive endosomes

Although the Ro52 cytoplasmic bodies were EEA1-negative, we thought that these structures might be endosomes of the other populations. To examine the broad populations of endosomes, we examined endosomes containing the small GTPases Rab5 and Rab7, which are key determinants of the early and late endosomes, respectively. Basically, the early endosomes containing Rab5 fluctuate dynamically by fusion and fission events. They progressively become fewer and larger endosomes that migrate from the cell periphery to the center, where Rab5 is rapidly replaced with Rab7 (Rink et al. 2005). To determine whether Ro52 cytoplasmic bodies are Rab5- or Rab7-positive, we coexpressed Ro52-EGFP with DsRed-fused Rab5 or Rab7 in HEK293 cells. As shown in Fig. 5, Ro52 cytoplasmic bodies did not merge with Rab5- or Rab7-positive endosomes.

Dynamic movements of Ro52 cytoplasmic bodies

Ro52 is an E3 ubiquitin ligase that belongs to the TRIM family (Torok and Etkin 2001; Wada and Kamitani 2006a). Previously, we showed that a TRIM family member, TRIM5 α , is structurally similar to Ro52 and functions as an E3 ubiquitin ligase (Yamauchi et al. 2008). Importantly, although their shape is different, TRIM5 α also localizes to cytoplasmic bodies (Yamauchi et al. 2008). Recently, using live cell microscopy, Campbell et al. (2007) showed that TRIM5 α cytoplasmic bodies are highly motile in human cells. On the basis of the similarities between Ro52 and TRIM5 α , we hypothesized that Ro52 cytoplasmic bodies are also highly dynamic structures in cells. To test this, we performed live cell fluorescence microscopy (Campbell et al. 2007). In brief, images were rapidly captured in sequence at the shortest possible time interval compatible with obtaining a sufficiently long exposure to record

the movement of Ro52 cytoplasmic bodies. Supplemental Movie 1 shows such a 60-frame sequence captured at 2.0-s intervals. Almost all cytoplasmic bodies are motile. Two types of movements can be observed; namely, multidirectional short-distance movements and rapid long-distance movements. These movements are very similar to those of TRIM5 α that have been reported previously (Campbell et al. 2007).

Using an image addition process, the 12 frames (10-s intervals) from Supplemental Movie 1 were combined into one image (Fig. 6a). This process clearly shows a number of tracks, which can be easily verified by Supplemental Movie 1. Several tracks with long distance are identified in Fig. 6a and Supplemental Movie 1. To clearly visualize the tracks, we identified the cytoplasmic bodies showing long tracks in the original 60 frames (2-s intervals). The individual cytoplasmic bodies were then labeled with 5 different colors and another movie was made (Supplemental Movie 2). Then, using an image addition process, the 12 frames (10-s intervals) from Supplemental Movie 2 were combined into one image (Fig. 6b). The 12 frames used for Fig. 6b are shown in Fig. 6c.

As shown in Fig. 6b, c, and Supplemental Movie 2, the Ro52 cytoplasmic bodies are shaped like rods, peanuts, or dumbbells, and move in the cytoplasm like earthworms or inchworms. A cytoplasmic body colored with purple locates at the perinuclear region at the beginning (time 00:00–00:10). Although it moves to the direction of the nuclear membrane (time 00:20), the direction is quickly changed to the opposite to move to the peripheral region (time 00:20–01:30). The cytoplasmic body then changes the direction again to move to the center (time 01:40). However, it rapidly changes the direction to the peripheral region (time 01:40–02:00). Thus, this cytoplasmic body moves to and fro in the cytoplasm. A cytoplasmic body colored with blue locates at the peripheral region and shows multidirectional short-distance movements from the beginning (time 00:00–01:40). However, it rapidly changes the direction to the nucleus and moves at high speed toward the end of the movie (time 01:40–02:00). A cytoplasmic body colored with red locates at the peripheral region at the beginning and shows a “three-point turn” (time 00:00–00:30). Then, it changes the direction to the nucleus (time 00:30–01:00) followed by multidirectional short-distance movements (time 01:00–02:00). A cytoplasmic body colored with green shows an unusual movement. It bends the peanut-shaped body, and the center of the body looks to be pulled by something (time 00:50–01:00) followed by multidirectional short-distance movements (time 01:00–02:00). A cytoplasmic body colored with yellow shows multidirectional, short-distance movements (time 00:00–01:20). Then, it moves a long distance to the peripheral region (time 01:20–01:40) and makes a 90° turn (time 01:40–02:00).

In summary, Ro52 cytoplasmic bodies are highly motile in cells. They show rapid long-distance movements and/or multidirectional short-distance movements in the cytoplasm.

Ro52 cytoplasmic bodies located along microtubules

As described above, we found that several Ro52 cytoplasmic bodies rapidly moved long distances, suggesting that an active mechanism, such as a transport system along microtubules, is involved in their movements. This observation prompted us to compare the distributions of Ro52 cytoplasmic bodies and the microtubule network. For this purpose, we double-immunostained endogenous Ro52 for cytoplasmic bodies and endogenous α -tubulin for the microtubule network in flat extended HT1080 cells and determined their distributions by fluorescence microscopy. As shown in Fig. 7, α -tubulin formed a fine network structure of microtubules (Fig. 7a), while Ro52 was observed as cytoplasmic bodies (Fig. 7b). When the images were merged, the microtubule network was abundantly decorated with Ro52 cytoplasmic bodies (Fig. 7c). Importantly, magnified images clearly showed that Ro52 cytoplasmic bodies are located along the microtubules (Fig. 7d, e).

Distinction between Ro52 and TRIM5 α cytoplasmic bodies

Because cytoplasmic bodies of Ro52 are very similar to those of TRIM5 α with regard to the shape and movement (Campbell et al. 2007; Yamauchi et al. 2008), we hypothesized that Ro52 shares the same cytoplasmic bodies with TRIM5 α . To test this, fluorescence microscopy was performed. Briefly, Ro52-mRFP was coexpressed with TRIM5 α -EGFP in HT1080 cells. Cells were then fixed and analyzed by fluorescence microscopy. As shown in Fig. 8, TRIM5 α (panel a) and Ro52 (panel b) localized to cytoplasmic bodies. In the merged image (panel c), Ro52 did not exist at the TRIM5 α cytoplasmic bodies, and vice versa. Although there were several yellow spots in panel c, these appeared to be generated by overlapping of both cytoplasmic bodies. To clarify this point, we chose three flat, extended areas of the cell and magnified the images. As shown in panels d–f, it was easy to distinguish between cytoplasmic bodies of Ro52 and those of TRIM5 α . In other words, it was clear that Ro52 does not share the same cytoplasmic bodies with TRIM5 α .

Discussion

Ro52 cytoplasmic bodies were previously investigated by several groups including us. Reymond et al. (2001) reported that the cytoplasmic bodies are not immunostained with antibodies to transferrin (a marker of recycling endosomes), cytochrome *c* (a marker of mitochondria), and mtHSP70 (a marker of mitochondria). Rhodes et al. (2002) reported that the cytoplasmic bodies are not immunostained with antibodies to AP2 (a marker of clathrin-coated endocytic vesicles), EEA1 (a marker of early endosomes), and LAMP1 (a marker of lysosomes). We also reported that ubiquitin is not immunostained at the Ro52 cytoplasmic bodies (Wada et al. 2006b). In this study, we showed that the Ro52 cytoplasmic bodies are not stained with Mito-Tracker dye. Furthermore, we examined colocalization of Ro52 with caveolin-1 (a marker of caveolae), EEA1, Rab5 (a marker of endosomes), Rab7 (a marker of endosomes), LAMP2 (a marker of lysosomes), and Rpt5 (a marker of proteasomes). Unexpectedly, Ro52 did not colocalize with these structure-specific markers at the cytoplasmic bodies. These results suggest that Ro52 cytoplasmic bodies are not mitochondria, caveolae, clathrin-coated endocytic vesicles, recycling endosomes, early endosomes, Rab5-positive endosomes, Rab7-positive endosomes, lysosomes, ubiquitin-enriched structures, or proteasome-enriched structures. However, we found that Ro52 cytoplasmic bodies are located along microtubules. This and previous studies are summarized in Table 1. Based on the results shown in Table 1, the Ro52 cytoplasmic bodies seem to be unidentified structures along the microtubule network.

The *ro52* gene is located on human chromosome 11p15 with a cluster of other *trim* genes including the *trim5* gene (Yamauchi et al. 2008), suggesting that the *ro52* and *trim5* genes were generated by amplification from a single gene on chromosome 11p15. In addition, Ro52 is structurally similar to TRIM5 α . Both proteins possess RING-finger and B-box domains in the N-terminal region. In the central region, Ro52 contains two separated coiled-coil domains, while TRIM5 α contains a fused coiled-coil domain. In the C-terminal region, both proteins contain a B30.2 domain. Thus, the domain structure of Ro52 is almost identical to that of TRIM5 α (Yamauchi et al. 2008). Moreover, both Ro52 and TRIM5 α function as E3 ubiquitin ligases and localize to cytoplasmic bodies along the microtubule network (Campbell et al. 2007; Yamauchi et al. 2008). These similarities between Ro52 and TRIM5 α raised the question as to whether Ro52 shares the same cytoplasmic bodies with TRIM5 α . In this study, we clearly showed that Ro52 cytoplasmic bodies do not contain TRIM5 α , and vice versa. Previously, Reymond et al. (2001) showed that Ro52 interacts with Ro52 (self-association) but not with other TRIM family members, including TRIM1, 3, 5, 6, 8, 9, 10, 11, 23, 24, 25, 26, 29, 31, and 32. Recently, Ro52 was shown to oligomerize into a trimer due to self-association (Rhodes and Trowsdale 2007). Similarly, TRIM5 α forms a trimer due to self-association (Mische et al.

2005). Because Ro52 does not form a heteromer with TRIM5 α , Ro52 may not be able to share cytoplasmic bodies with TRIM5 α .

Our live cell microscopy clearly showed that Ro52 cytoplasmic bodies are highly motile in HT1080 cells. Interestingly, some cytoplasmic bodies rapidly moved long distances, suggesting the involvement of a microtubule-mediated transportation system in the movements. If this is the case, what is the biological relevance of the transportation of Ro52 cytoplasmic bodies? What is the role of Ro52 in transportation of the cytoplasmic bodies? Ro52 was previously shown to interact with Usp4, which is a deubiquitinating enzyme (Di Donato et al. 2001). Recently, we demonstrated that Usp4 colocalizes with Ro52 at the cytoplasmic bodies (Wada et al. 2006b). These observations suggest that, although Ro52 (an E3 ubiquitin ligase) and Usp4 (a deubiquitinating enzyme) have opposing activities, they form a heteromeric protein complex at cytoplasmic bodies to regulate both the ubiquitination and deubiquitination of their substrates (Wada et al. 2006b). Most likely, the microtubule network allows the Ro52–Usp4 heteromeric complex to be efficiently transported and to show dual activities in the cytoplasm. Another possibility is that the Ro52–Usp4 complex regulates the movements of the cytoplasmic bodies by ubiquitination and deubiquitination. However, common substrates of Ro52 and Usp4 have not been identified. Further studies are needed to clarify the biological relevance of the transportation of Ro52 cytoplasmic bodies and to define the role of Ro52 and Usp4 in the movements of the cytoplasmic bodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HTLV-1	Human T-cell leukemia virus type 1
TRIM	Tripartite motif
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
EGFP	Enhanced green fluorescent protein
PCR	Polymerase chain reaction
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
WGA	Wheat germ agglutinin

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Fig. 1.

Subcellular localization of Ro52 in HeLa, HT1080, and HEK293 cells. **a** Expression levels of endogenous Ro52 in human cells. Total cell lysates were prepared from indicated cell lines. Endogenous Ro52 in the lysates was then detected by Western blotting using mouse monoclonal anti-Ro52 antibody D-12 (*upper panel*). To demonstrate equal loading amount of total cell lysates, Western blotting using anti-actin antibody was also performed (*lower panel*). Endogenous Ro52 and actin are indicated by *open arrowheads*. Molecular size markers are shown in kilodaltons. **b** Subcellular localization of endogenous Ro52 in human cells. Cells of the indicated cell lines were fixed in a 4% paraformaldehyde solution, stained with DAPI, permeabilized with 0.1% TritonX-100, and immunostained with mouse monoclonal anti-Ro52 antibody D-12. After washing, cells were labeled with Alexa Fluor 594-conjugated goat anti-mouse antibody. The cells were then analyzed by fluorescence microscopy. The localization of endogenous Ro52 is shown by the *red* fluorescence of Alexa Fluor 594. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. A *scale bar* indicates 10 μm. **c** Subcellular localization of Ro52-EGFP expressed by transfection. Cells of the indicated cell lines were fixed in a 4% paraformaldehyde solution. After washing, the cells were stained with DAPI. The cells were then analyzed by fluorescence microscopy. The localization of Ro52-EGFP is shown by the *green* fluorescence of EGFP. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. A *scale bar* indicates 10 μm.

XZ imaging

Fig. 2.

XZ images of monolayer cells expressing Ro52-EGFP. HEK293 cells were transfected for the expression of Ro52-EGFP and seeded onto a membrane. The apical surfaces of the cells were then stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA). After fixation with paraformaldehyde, the membrane was transferred into a cryomold containing OCT compound and snap-frozen in liquid nitrogen. Next, cryosections of WGA-stained monolayer cells on the membrane were cut as shown in the schema. After DAPI staining, the XZ images of the monolayer cells were analyzed by fluorescence microscopy. Ro52 cytoplasmic bodies are shown by the *green* fluorescence of EGFP, and the apical surfaces stained with WGA are shown by the *red* fluorescence of Alexa Fluor 594. Nuclear counter-staining is shown by the *blue* fluorescence of DAPI. A *scale bar* indicates 10 μ m

Fig. 3. Staining of mitochondria with MitoTracker dye in cells expressing Ro52-EGFP. HEK293 cells were transfected to express Ro52-EGFP. The cells were then stained with MitoTracker dye. After DAPI staining, cells were analyzed by fluorescent microscopy. Ro52 cytoplasmic bodies are shown by the *green* fluorescence of EGFP (*panel a*). The localization of mitochondria is shown by the *red* fluorescence of MitoTracker dye (*panel b*). The merged image of both fluorescences is shown in *panel c*. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. A *scale bar* indicates 10 μm

Fig. 4.

Immunostaining of Ro52-EGFP expressing cells. HEK293 cells were transfected to express Ro52-EGFP. The cells were then fixed with para-formaldehyde, permeabilized, and immunostained with primary antibodies against various proteins such as the proteasome subunit Rpt5 (*panels a–c*), the caveolae component caveolin-1 (*panels d–f*), the early endosome marker EEA1 (*panels g–i*), and the lysosome marker LAMP2 (*panels j–l*). Afterwards, cells were labeled with Alexa Fluor 594-conjugated secondary antibody. After DAPI staining, cells were analyzed by fluorescence microscopy. Ro52 cytoplasmic bodies are shown by the green fluorescence of EGFP (*panels a, d, g, and j*). The localization of immunostained proteins is shown by the red fluorescence of Alexa Fluor 594 (*panels b, e, h, and k*). The merged images of both fluorescences are shown in *panels c, f, i, and l*. Nuclear counterstaining is shown by the blue fluorescence of DAPI. A scale bar indicates 10 μ m

Fig. 5.

Coexpression of Ro52-EGFP with DsRed-fused endosome markers. HEK293 cells were cotransfected to express Ro52-EGFP with DsRed-Rab5 (*panels a–c*) or DsRed-Rab7 (*panels d–f*). Cells were then fixed in a 4% paraformaldehyde solution and stained with DAPI. Afterwards, the cells were analyzed by fluorescence microscopy. The Ro52 cytoplasmic bodies are shown by the *green* fluorescence of EGFP (*panels a* and *d*). The localization of Rab5 and Rab7 is shown by the *red* fluorescence of DsRed (*panels b* and *e*). The merged images of both fluorescences are shown in *panels c* and *f*. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. A *scale bar* indicates 10 μ m

Fig. 6.

Dynamic movements of Ro52 cytoplasmic bodies. **a** Original tracking image of Ro52 cytoplasmic bodies. A live HT1080 cell expressing Ro52-EGFP was imaged over 60 frames at 2.0-s intervals (2-min total time) to generate a movie (Supplemental Movie 1). Afterwards, the 12 individual frames (10-s intervals, 2-min total time) were picked up from 60 frames and summed using Adobe Photoshop CS3. The tracks of a number of cytoplasmic bodies are shown. Five tracks showing long distances are indicated by *lines*. A *scale bar* indicates 5 μm . **b** Tracking image of Ro52 cytoplasmic bodies showing prominent movement. Five prominent tracks were identified for further analysis. These tracks were labeled with five different colors (*purple, yellow, red, green, and blue*). The directionality and authenticity of these tracks were confirmed by comparison with Supplemental Movies 1 and 2. A *scale bar* indicates 5 μm . *Nuc* nucleus, *Cyt* cytoplasm. **c** Location of Ro52 cytoplasmic bodies at each time point (12 frames at 10-s intervals; from time point 10 s to 2 min). A *scale bar* indicates 10 μm

Fig. 7.

Double immunostaining of microtubules and Ro52 in HT1080 cells. Cells were fixed in cold methanol, permeabilized with 0.1% Tri-tonX-100, and immunostained with both rat monoclonal anti- α -tubulin antibody and mouse monoclonal anti-human Ro52 antibody. After washing, the cells were labeled with both Alexa Fluor 488-conjugated goat anti-rat antibody and Alexa Fluor 555-conjugated goat anti-mouse antibody. The cells were then stained with DAPI and analyzed by fluorescence microscopy. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. The localization of microtubules is shown by the *green* fluorescence of Alexa Fluor 488 (*panel a*), and the localization of endogenous Ro52 is shown by the *red* fluorescence of Alexa Fluor 555 (*panel b*). The merged image is shown in *panel c*. Two areas in *panel c* are magnified (*panels d* and *e*). Scale bars indicate 10 μ m in *panel a* and 3 μ m in *panel e*

Fig. 8.

Distinction between Ro52 and TRIM5 α cytoplasmic bodies in HT1080 cells. Cells were cotransfected to express both TRIM5 α -EGFP and Ro52-mRFP. The cells were then fixed with paraformaldehyde and stained with DAPI. Afterwards, cells were analyzed by fluorescent microscopy. TRIM5 α cytoplasmic bodies are shown by the *green* fluorescence of EGFP (*panel a*). Ro52 cytoplasmic bodies are shown by the *red* fluorescence of mRFP (*panel b*). The merged image of both fluorescences is shown in *panel c*. Nuclear counterstaining is shown by the *blue* fluorescence of DAPI. A scale bar in *panel c* indicates 10 μ m. Three flat, extended areas in *panel c* were magnified (*panels d, e, and f*). Scale bars in these panes indicate 2 μ m

Table 1

Colocalization of Ro52 cytoplasmic bodies with structure-specific markers

Structure	Marker	References		
		Ref. 1	Ref. 2	Ref. 3 This study
Caveolae	Caveolin-1			No
Endocytic vesicles	AP2		No	
Recycling endosomes	Transferrin	No		
Early endosomes	EEA1		No	No
Rab5 (+)-endosomes	Rab5			No
Rab7 (+)-endosomes	Rab7			No
Lysosomes	LAMP1		No	
	LAMP2			No
Mitochondria	MitoTracker			No
	Cytochrome c	No		
	mtHSP70	No		
Ubiquitinated structures	Ubiquitin			No
Proteasomes	Rpt5			No
Microtubules	α -tubulin			Yes

Results of previous studies (References 1–3) and this study are summarized

Ref. 1: Reymond et al. (2001) EMBO J 20:2140–2151

Ref. 2: Rhodes et al. (2002) Immunology 106:246–256

Ref. 3: Wada et al. (2006b) Biochem Biophys Res Commun 339:731–736