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The Sjögren’s Syndrome-Associated Autoantigen Ro52 Is an E3 Ligase That Regulates Proliferation and Cell Death

Alexander Espinosa,* Wei Zhou,** Monica Ek,** Malin Hedlund,* Susanna Brauner,* Karin Popovic,† Linn Horvath,* Therese Wallerskog,* Mohamed Oukka,† Filippa Nyberg,† Vijay K. Kuchroo,‡ and Marie Wahren-Herlenius*

Patients affected by Sjögren’s syndrome and systemic lupus erythematosus (SLE) carry autoantibodies to an intracellular protein denoted Ro52. Although the serologic presence of Ro52 autoantibodies is used clinically for diagnostic purposes, the function of the protein or why it is targeted as an autoantigen in several rheumatic conditions has not been elucidated. In this study, we show that the expression of Ro52 is significantly increased in PBMC of patients with Sjögren’s syndrome and SLE, and demonstrate that Ro52 is a RING-dependent E3 ligase involved in ubiquitination. Overexpression of Ro52, but not of Ro52 lacking the RING domain, in a mouse B cell line lead to decreased growth in steady state and increased cell death after activation via the CD40 pathway. The role of Ro52 in activation-mediated cell death was further confirmed as a reduction in Ro52 expression restored cell viability. These findings suggest that the increased expression of the Ro52 autoantigen in patients may be directly involved in the reduced cellular proliferation and increased apoptotic cell death observed in Sjögren’s syndrome and SLE, and may thus contribute to the autoantigenic load and induction of autoimmune B and T cell responses observed in rheumatic patients. The Journal of Immunology, 2006, 176: 6277–6285.

Sjögren’s syndrome is a common systemic autoimmune disease, characterized by an autoimmune exocrinopathy involving in particular salivary and lacrimal glands. Extraglandular manifestations include severe fatigue, arthralgia, myalgia, and pulmonary and renal dysfunction. The syndrome may occur alone (primary Sjögren’s syndrome) or together with other rheumatic diseases such as systemic lupus erythematosus (SLE), and is then termed secondary Sjögren’s syndrome (1). In both Sjögren’s syndrome and SLE, the numbers of circulating apoptotic leukocytes are increased, and apoptosis of lymphocytes following in vitro activation is augmented (2–5). The increased apoptosis, together with a defective clearance of apoptotic material, has been suggested to contribute to the autoimmune pathogenesis by increasing the load of autoantigenic exposure (6).

B cell subpopulations are abnormal in Sjögren’s syndrome with a reduced number of memory B cells and altered B cell trafficking pattern (7, 8). There is also excess production of Igs with resulting hypergammaglobulinemia, which includes the disease-associated autoantibodies. The Ro52 protein is a main target for these autoantibodies in both Sjögren’s syndrome and SLE (9, 10), and the Abs may cause congenital heart block in the fetus of these mothers during pregnancy (11, 12). However, why autoantibodies target Ro52 in these rheumatic conditions, and whether there is a relationship between Ro52, increased apoptosis, and increased production of autoantibodies has not been studied. To understand this process and the potential link between Ro52 and the diseases in which Abs to Ro52 are induced, it is essential to understand the function of Ro52.

Although the Ro52 autoantibodies have been clinically used in the diagnosis of rheumatic disease for decades, the function of Ro52 is not known. Ro52 contains a RING-finger motif, a B-box, and a coiled-coil domain (13–15), placing it within the family of Ring-B-box-coiled-coil (RBCC) or tripartite motif (TRIM) proteins, wherefore Ro52 is also denoted TRIM 21 (13). Many RING-finger proteins have E3 ligase activity and are, as such, involved in the process of covalently modifying proteins with the 76-aa polypeptide ubiquitin. Ubiquitin is first bound and activated by an E1 enzyme and transferred to an ubiquitin-conjugating enzyme (E2 or Ubc). E3 ligases mediate the transfer of the activated ubiquitin from an E2/Ubc to a substrate protein, and, depending on whether the substrate is modified by poly- or monoubiquitination, it is degraded by the 26S proteasome, targeted for lysosomal degradation or functionally modified (16, 17).

In this study, we show that Ro52 is a RING-dependent E3 ligase and that its expression is increased in PBMC of patients with Sjögren’s syndrome and SLE. We also show that overexpression of Ro52 in a B cell line leads to reduced growth and increased apoptosis after activation. These data provide the first evidence that Ro52 is an E3 ligase and that it promotes cell death. Therefore, the increased expression of Ro52 in patients may be directly responsible for the increased apoptosis leading to autoantigenic exposure and promoting autoreactivity, including the generation of Ro52 autoantibodies.
Materials and Methods

Patients

The patients were included consecutively on the basis of testing positive for Ro/SSA Abs (positive serum sample 1996–2002 in Stockholm County), a diagnosis of Sjögren’s syndrome or SLE, and a willingness to participate in the study. Patients were clinically examined at the Department of Dermatology, Danderyd Hospital (Stockholm, Sweden), and blood was sampled for preparation of serum and cells. Samples were used fresh or stored in aliquots at −70°C until use. Patients with primary Sjögren’s syndrome (n = 20) met the revised European classification criteria (18), and patients with SLE (n = 18) met the American College of Rheumatology criteria (19). Eighteen age- and sex-matched healthy persons served as controls. The Ethics committee at the Karolinska Hospital approved the study, and written informed consent was obtained from all patients.

Plasmids

pFLAG-Ro52 (FLAG-Ro52) and pFLAG-Ro52RING (FLAG-Ro52RING) plasmids were made by amplifying base pairs 4-1412 of the mouse Ro52 coding cDNA sequence (GenBank no. NM_000277) with PCR using primers containing 5’-restriction sites: Ro52-F, 5’-CAAGAATTTTACCCACCTCACAACCTCAAATGG-3’; Ro52RING-F, 5’-CAAGAATTTCTGTTGAGAGGTAATATCCAGGTC-3’; and Ro52RING-R, 5’-CAGGTATGGCAACAATCTCACCTTTAGTGGCAAGACG-3’. The PCR products were cleaved with EcoRI and Sall and ligated into the pFLAG-CMV-6c vector (Sigma-Aldrich). The p6xHis-Ubiquitin plasmid (20) encodes a fusion of ubiquitin with a 6xHis-tag and enables purification of ubiquitin-modified proteins with Ni2+–NTA resin. The pEGFP-N1-Ro52 (Ro52-GFP) and pEGFP-N1-Ro52RING (Ro52RING-GFP) constructs were made by amplifying the corresponding coding sequence of mouse Ro52 by PCR using primers with 5’-restriction sites and ligating the PCR product, cleaved with EcoRI and AgeI, into the pEGFP-N1 vector (Clontech Laboratories); Ro52-GFP-F, 5’-CCGAATTCCATGATGTTCCACCTTCTTACA-3’; Ro52RING-GFP-F, 5’-CCGAATTCCATGATGTTCCACCTTCTTACA-3’; Ro52RING-GFP-R, 5’-CCAACCGTGAATCTTGTATGGCAAGC-3’. The pQF-09 Ro52 plasmid was a gift from T. Gordon (Flinders University, Adelaide, Australia) (21). All constructs were sequenced at the Kiseq core facility at Karolinska Institutet (www.cgb.ki.se/cgb/kiseq/).

Preparation of PBMC and subsets of lymphocytes

PBMC were separated by density gradient centrifugation using Ficoll-Paque plus (Amersham Biosciences) from heparin-treated blood and either stored at −70°C for later RNA extraction or used immediately for cell separation.

T cells, B cells, and monocytes were isolated by magnetic beads coated with anti-CD3, anti-CD19, and anti-CD14, respectively (Dynal Biotech) from PBMCs according to the manufacturer’s instruction. Briefly, PBMCs were resuspended to 10 × 106 cells/ml in cold PBS containing 2% FCS and divided to three tubes, and washed magnetic beads (25 μl/10 × 106 cells) were added to each tube and incubated at 4°C under rotation (anti-CD3 for 10 min, anti-CD19 for 20 min, and anti-CD14 for 50 min). Samples were washed three times in PBS/2% FCS using a Dynal MPC (Dynal Biotech) before extraction of RNA.

Subpopulations of B cells were isolated from PBMC samples. PBMCs were resuspended in PBS containing 5% human serum and FITC-anti-CD19, and PE-anti-CD27 and allophycocyanin-anti-CD138 (BD Biosciences) were added to the samples and incubated at 4°C in the dark for 10 min. After filtering through a 40-μm cell strainer (BD Biosciences), naive B cells (CD19+CD27−), memory B cells (CD19+CD27+), and plasma cells (CD138+) were isolated by sorting on a MoFlo cell sorter (DakoCytomation).

Relative quantification of Ro52 mRNA expression by real-time quantitative RT-PCR

Total RNA was extracted from PBMC or subsets of lymphocyte using an RNeasy mini kit (Qiagen) with on-column RNase-free DNase digestion (Qiagen). Reverse transcription was performed with 100 ng of random primer, 0.5 mM dNTP, 40 U of RnaseOUT, and 200 U of Superscript reverse transcriptase (Invitrogen Life Technologies).

Primers for amplification of human GAPDH and Ro52 were as follows: GAPDH-R, 5’-AGGCTGCTTITTTAATCTGTTAAA-3’ and GAPDH-F, 5’-CATGATTACGGATGAACTACATCTGCTGTT-3’; Ro52-R, 5’-GAAATGCAGAGGTGGGTGATAA-3’ and Ro52-F, 5’-AGTTTCTGGAGAATATATCAAGGTC-3’. PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR Kit (Qiagen) and a three-step protocol (95°C 10 min, followed by 95°C 15 s, 60°C 30 s, 72°C 30 s for 40 cycles). The amplification of GAPDH and Ro52 was performed in a separate well on the same optical 96-well reaction plate (Applied Biosystems), and each reaction was run in duplicate. The relative amount of mRNA was calculated by the standard curve method. The standard curve was constructed using five 10-fold serial dilutions of pooled cDNA from all patients, and corresponding cycle of threshold value was calculated. GAPDH was used as a stable endogenous control. The expression level of Ro52 in each sample was calculated as the ratio between the relative amount of Ro52 mRNA and the relative amount of corresponding GAPDH mRNA.

Cell culture

Human embryonic kidney cells (HEK 293) were maintained in DMEM (Invitrogen Life Technologies) with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% heat-inactivated FCS. All cultures were grown at 37°C in a humidified incubator with a 5% CO2 atmosphere.

Immunofluorescence

HeLa cells were grown on coverslips and fixed in 4% aceton:methanol (1:3) for 10 min. After air drying and rinsing with PBS, a mouse mAb specific for human Ro52 (clone 7.8C7) was added and incubated for 40 min after rinsing PBS. After 10 min of incubation in PBS with 0.5% fat-free bovine serum albumin (FBS) and 2-ME, the coverslips were mounted in PBS-glycerol and analyzed in a fluorescence microscope (Leitz DM RBE; Leica). All steps were performed at room temperature (RT). The Ro52 monoclonal (Ab 7.8C7) was developed by standard protocols for fluor staining with goat antiserum from mice immunized with recombinant Ro52 (14) with SP 200 myeloma cells to generate a hybridoma producing mAbs. Resulting hybridomas were subcloned and characterized by Western blot and ELISA using Ro52 deletion constructs (14). The 7.8C7 monoclonal binds recombinant Ro52 and is specific for an epitope within aa 200–239 of the Ro52 protein. In Western blotting of HeLa cells, the 7.8C7 monoclonal binds a single band at 52 kDa (data not shown).

Expression and purification of Ro52

To produce functionally active Ro52, we expressed 6xHis-Ro52 in Escherichia coli and used nondenaturing buffers for purification. The E. coli strain M15 (pREP4) (Qiagen), transformed with pQE-9-Ro52, was grown in Luria Bertani medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Three hundred milliliters of culture was induced with 0.2 mM isopropyl β-thiogalactoside when an OD600 of ~0.6 was reached. The culture was pelleted after shaking for 16–20 h at 30°C, and 6xHis-Ro52 was purified with Ni2+–NTA under native conditions as follows: the pellet was dissolved in 50 mM NaPO4, pH 8.0, and dialyzed at 4°C in a dialysis bag containing an EDTA-free protein inhibitor mixture (Sigma-Aldrich), 50 mM NaH2PO4, 500 mM NaCl, 10 mM imidazole, 5% glycerol, and 20 mM 2-ME. After addition of 500 μl of lysosome (10 mg/ml), the lysate was kept on ice for 30 min. The lysate was sonicated at low intensity for 3 × 20 s and centrifuged at 15,000 × g for 15 min in 4°C. Ni2+–NTA resin (Qiagen) was added to the supernatant, and the mixture was rotated at 4°C for 1 h. The Ni2+–NTA resin was washed once in 10 ml of lysis buffer and twice in 10 ml of wash buffer (pH 8.0) containing 50 mM NaH2PO4, 500 mM NaCl, 25 mM imidazole, 3% glycerol, and 5 mM 2-ME. Protein elution was made with 10 × 500 μl of elution buffer (pH 8.0) with increasing imidazole concentration: 50 mM NaH2PO4, 300 mM NaCl, 50 mM imidazole, 1% glycerol, 5 mM 2-ME (fraction 1–3) and 50 mM NaH2PO4, 300 mM NaCl, 100 mM imidazole, 1% glycerol, 5 mM 2-ME (fraction 4–6) and 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, 1% glycerol, 5 mM 2-ME (fraction 7–10). All fractions were separated with SDS-PAGE, and proteins were visualized by Coomassie brilliant blue (R-250) staining.

In vivo ubiquitination assays

HEK293 cells were cultured in 9-cm petri dishes (Nunc). Twenty-four hours before transfection, 1 × 105 cells were seeded per petri dish. Cells were transfected with 30 μg of p6xHis-Ubiquitin and 50 μg of pFLAG-Ro52 or 50 μg of pFLAG-Ro52RING, using the calcium phosphate precipitation method as described in Ref. 23. The proteasome inhibitor MG132 (Sigma-Aldrich) was added to the cultures (10 μM) 24 h after transfection.
transfection. Six hours later, the cells were washed in PBS and harvested into Eppendorf tubes. The cells were pelleted and lysed in 1 ml of a de
naturating lysis buffer (pH 8.0) containing 6 M guanidinium hydrochloride, 0.1 M NaH2PO4, and 10 mM imidazole. After sonication and centrifugation, 15,000 × g for 10 min, the lysates were incubated with 75 µl of Ni2+ -NTA (Qiagen) with rotation for 4 h at 4°C. The resins were washed twice with 1 ml of lysis buffer followed by washing twice with 1 ml of an 1:3 mix of lysis buffer with a buffer (pH 6.8) containing 25 mM Tris-Cl and 20 mM imidazole. To remove guanidine-HCl, the resins were washed twice with 1 ml of wash buffer (pH 6.8) containing 25 mM Tris-Cl and 20 mM imidazole. 6×His-ubiquitin-modified proteins were eluted by boiling 3 min with 1 ml of wash buffer containing 250 mM Tris-Cl and 200 mM NaCl. The eluted proteins were separated with SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting against FLAG or 6×His.

**In vitro ubiquitination assays**

In the in vitro ubiquitination assays, 1 µg of 6×His-Ro52, expressed in E. coli and purified as described above, was mixed with 100 ng of E1, 500 ng of Ubc, and 2.5 µg of ubiquitin (Boston Biosciences) in a buffer containing 50 mM Tris-Cl, 2.5 mM MgCl2, 0.5 mM DTT, and 2 mM ATP. The total volume of the reactions was 20 µl, and they were incubated for 2 h at 30°C. Reactions were terminated by addition of 5 µl of SDS-PAGE sample buffer containing 100 mM Tris-Cl, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, and 500 mM DTT. Proteins were separated with SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting against ubiquitin.

**Stable transfection of A20 cells**

A total of 10 × 10⁶ A20 cells was transfected with 10 µg of pEGFP-N1, pEGFP-N1-Ro52, or pEGFP-N1-Ro52ARING-GFP; the plasmids were linearized with DraIII before transfection to increase the frequency of stable transfected cells. Transfection was made by electroporation (Gene Pulser; Bio-Rad) using the settings 960 µF, 390 V, and 400 Ω. After 48 h, G418 (Invitrogen Life Technologies) was added to the cultures to a concentration of 1 mg/ml. After 3–4 wk of selection, single GFP-positive cells were sorted on a MoFlo cell sorter (DakoCytomation). Anti-FLAG M2 (Sigma-Aldrich) diluted 1/1000 in PBS with 0.05% Tween 20 was added to the cultures to increase the frequency of stable transfected cells. Transfection was made by electroporation (Gene Pulser; Bio-Rad) using the settings 960 µF, 390 V, and 400 Ω. After 48 h, G418 (Invitrogen Life Technologies) was added to the cultures to a concentration of 1 mg/ml. After 3–4 wk of selection, single GFP-positive cells were sorted on a MoFlo cell sorter (DakoCytomation). The clones were collected using the ECL system (Amersham Biosciences). To verify protein expression, cell extracts were made, and proteins were separated with SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting against ubiquitin.

**Immunoblotting**

In the in vivo ubiquitination assay, nitrocellulose membranes were incubated with anti-FLAG M2 (Sigma-Aldrich) diluted 1/1000 in PBS with 0.05% Tween 20 in PBS (TPBS) at 4°C overnight, followed by washing in TPBS and anti-mouse-Ig-HRP (DakoCytomation) (1/1000) at RT for 1 h. In the in vitro ubiquitination assay, nitrocellulose membranes were incubated with anti-ubiquitin 6C1 (Sigma-Aldrich) diluted in TPBS (1/1500), followed by washing and incubation with anti-mouse-Ig-HRP (1/1,000) (DakoCytomation) at RT for 1 h. For verification of the Ro52-GFP and GFP expression in stable transfected A20 cells, nitrocellulose membranes were incubated with anti-GFP (1/1,500) (Clontech Laboratories) diluted 1/1000 (Sigma-Aldrich) was used to control input amount. Patient cell extracts were prepared by pooling PBMCs from four patients and separating CD3⁺, CD19⁺, and CD14⁺ cells as described above. All nitrocellulose membranes were blocked with 5% (w/v) fat-free milk and 1% (w/v) BSA in TPBS before incubation with Abs, and all blots were developed with the ECL system (Amersham Biosciences).

**Proliferation assay**

A [3H]Thymidine incorporation assay was used to measure the proliferation of stable transfected A20 cells and the parental cell line. A total of 0.2 × 10⁸ cells of five independent clones each of A20.GFP, A20.Ro52ARING-GFP, and A20.Ro52ARING-GFP were seeded in triplicates on 96-well plates. The parental A20 cell line was used as a non-GFP-expressing control. A total of 10 µg/ml anti-CD40 Abs (1C10) (24) was added in the proliferation assays with anti-CD40 treatment. Twenty hours before counting of incorporated radioactivity, 0.5 µCi of [3H]Thymidine was added to each well. Cells were harvested with a microplate harvester (Torrance), and radioactivity was measured with a microplate liquid scintillation counter (Wallace Micro BioSolutions; PerkinElmer Life Sciences). The Mann-Whitney U test was used for statistical analysis of differences in radioactivity incorporation between A20.GFP, A20.Ro52ARING-GFP, A20.Ro52-GFP, and the parental A20 cell line.

** Colony-forming assay**

A total of 5 × 10⁵ A20.GFP, A20.Ro52-GFP, A20.Ro52ARING-GFP, A20.Ro52-GFP, and parental A20 cells was seeded in triplicates on six-well plates. On days 5 and 7, the cells were washed carefully in PBS and fixed for 10 min in ice-cold methanol. The colonies were stained with 1% Giemsa in PBS for 10 min, rinsed in water, and dried.

**Propidium iodide (PI) exclusion assay**

A total of 1 × 10⁶ A20.Ro52-GFP, A20.Ro52ARING-GFP, A20.Ro52-GFP, and parental cells were seeded in 25-cm² culture flasks and were cultured for 5 days with or without 10 µg/ml anti-CD40. Floating and adherent cells were harvested and pooled in ice-cold PBS. A total of 5 µg/ml PI (Sigma-Aldrich) was added, and the cells were analyzed for PI fluorescence with flow cytometry (FACS Calibur; BD Biosciences).

The level of Ro52 in A20.Ro52-GFP cells was decreased by seeding them in 12-well plates (1 × 10⁵ cells/well) and culturing them for 8 days with different concentrations of G418 (1.0, 0.5, and 0 mg/ml). As controls, A20.GFP and parental cells were subjected to the same procedure. During the last 4 days of culturing, the cells were grown in the presence of anti-CD40 (10 µg/ml). The cells were then collected and analyzed for PI fluorescence as described above.

**Results**

Expression of the Ro52 autoantigen is increased in patients with primary Sjögren’s syndrome and SLE

Autoantibodies to the Ro52 protein are included in the criteria for classification of Sjögren’s syndrome (18), and detected in 30–40% of patients with SLE (19). However, neither the function of the Ro52 protein nor expression pattern of this autoantigen in patients with Sjögren’s syndrome or SLE is known. To investigate the expression of Ro52 in patients with Ro52 autoantibodies, we collected PBMC from Ro/SSA-positive patients with primary Sjögren’s syndrome and SLE (Table I). The expression of Ro52 was significantly increased both in patients with Sjögren’s syndrome (p < 0.0001) and SLE (p < 0.0001) compared with healthy controls as determined by quantitative PCR (Fig. 1A). To identify which cell type of the PBMCs accounted for the increased expression, Ro52 levels were determined in cells separated by the cell surface expression of CD19 (B cells), CD3 (T cells), and CD14 (monocytes). B cells expressing CD19 showed the highest Ro52 mRNA expression (Fig. 1B), whereas monocytes expressed lower levels of Ro52, followed by T cells. To determine the B cell subset that expressed most Ro52, we further sorted B cell subsets from the PBMCs into naive, memory, and plasma cells using CD19, CD27, and CD138 as markers; the most pronounced Ro52 mRNA expression was observed in naive CD19⁻CD27⁻B cells (Fig. 1C). Analysis of Ro52 expression at the protein level in Western blot, however, did not reveal increased detectable Ro52 protein in CD19⁺ cells; rather, the levels were lower than in CD3⁺ and CD14⁺ cells (Fig. 1D).

**Table I. Clinical characteristics of patients included in the study**

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DLE, Discoid lupus erythematosus; RA, rheumatoid arthritis; ANA, antinuclear Abs.
To understand the function of Ro52, we first investigated its intracellular localization. Conflicting data have been published in the literature as to which subcellular compartment Ro52 localizes (25–27), and to address this issue we made constructs encoding full-length Ro52 (Ro52-GFP), or a deletion mutant lacking the RING-finger (Ro52 encoding full-length Ro52 (Ro52-GFP), or a deletion mutant of GFP (Fig. 1A). HeLa cells transfected with Ro52-GFP showed a predominantly cytoplasmic localization, whereas the mutant lacking the RING domain was not (Fig. 2A), suggesting that Ro52 may indeed be an E3 ligase.

A characteristic feature of many E3 ligases is their potential to modify themselves with ubiquitin via autoubiquitination. To determine whether Ro52 has E3 ligase activity, autoubiquitination of Ro52 and c-Cbl, BRCA1, and RNF4, indicating that Ro52 may be an E3 ligase.

The Ro52 autoantigen is a RING-dependent E3 ligase

Ro52 belongs to the TRIM family of proteins (13) of which several members have recently been identified as E3 ligases, acting as intracellular regulators of processes such as apoptosis, proliferation, and signal transduction (28, 29). The TRIM proteins belong to the RING-dependent class of E3 ligases, which includes Casitas B-lineage lymphoma c (c-Cbl), and RING-finger protein 4 (RNF4). The Ro52 RING-domain sequence was aligned with RING-domain sequences of known E3 ligases (Fig. 1G). Critical cysteine and histidine residues are conserved between Ro52 and BRCA1, Casitas B-lineage lymphoma c (c-Cbl), and RING-finger protein 4 (RNF4).

FIGURE 1. Ro52 is a TRIM protein that is overexpressed in patients with Sjögren’s syndrome and SLE. A, Quantitative real-time SYBR RT-PCR was used to determine Ro52 mRNA levels in PBMC from patients and controls. Ro52 mRNA levels are shown relative to GAPDH mRNA, and each point represents one individual. Primary Sjögren’s syndrome (pSS; n = 20), SLE (n = 18), and healthy controls (n = 18). B, Comparison of the Ro52 expression as detected by quantitative SYBR RT-PCR between B cells (CD19+), T cells (CD3+), and monocytes (CD14+) in 12 patients (pSS; n = 4) (SLE; n = 8). C, In B cell subsets, Ro52 expression was slightly higher in naive B cells (CD19+CD27−) compared with memory (CD19+CD27+) and plasma cells (CD138+). Samples from nine patients (pSS; n = 3) (SLE; n = 6) were analyzed. D, Western blot of CD3+ and CD19+ and CD14+ cells. Ro52 was detected by a Ro52 monoclonal (7.8C7). Anti-β actin was used to verify equal loading of extracts. E, TRIM/RBCC-proteins are characterized by a RING-finger, one or two B-boxes, and a coiled-coil domain containing a leucine zipper motif. The schematic representation of Ro52 also shows the B30.2 domain. Full-length and mutant Ro52 clones were generated for localization and ubiquitination experiments. F, Ro52-GFP and Ro52ΔRING-GFP were expressed in HeLa cells. Endogenous Ro52 was visualized by immunofluorescence in untransfected HeLa cells using a monoclonal anti-Ro52 Ab. G, The Ro52 RING-domain sequence was aligned with RING-domain sequences of known E3 ligases. Critical cysteine and histidine residues are marked in bold and are conserved between Ro52 and BRCA1, Casitas B-lineage lymphoma c (c-Cbl), and RING-finger protein 4 (RNF4).
some experiments (Fig. 2C), possibly due to a remaining weak interaction with an E2/Ubc.

To further verify the E3 ligase activity of Ro52, we established an in vitro ubiquitination assay. Ubiquitination relies on the presence of E1, E2/Ubc, and E3 ligases, ATP and ubiquitin. Purified E1, E2/Ubc, and Ro52 were added to ubiquitin and ATP to exclude the possibility that Ro52 polyubiquitination was dependent on additional cellular proteins. We tested the purified Ro52 with several different E2/Ubc:s to investigate which E2/Ubc supported Ro52-mediated ubiquitination. Polyubiquitination was detected with UbcH2, UbcH5a-c, UbcH6, and UbcH7 (Fig. 2D) but was most pronounced with UbcH6. Because Ubc:s are occasionally polyubiquitinated in an E3 ligase-independent manner (36), the in vitro assay was performed with and without Ro52 (Fig. 2E). Some E3 ligase-independent ubiquitination of UbcH6 was detected; however, in the presence of Ro52, polyubiquitination was dramatically increased. As expected, the in vitro polyubiquitination was also dependent on E1, E2/Ubc, and ATP (Fig. 2F), because absence of either of these reagents resulted in loss of polyubiquitination. Together, these data clearly demonstrate that the Ro52 autoantigen is a RING-dependent E3 ligase.

A20 B cells stably transfected with Ro52 have reduced colony-forming properties

In our study, patients with primary Sjögren’s syndrome and SLE showed increased mRNA but not protein expression of Ro52 in B cells. Therefore, to investigate functional consequences of increased cellular expression of Ro52 in B cells, mouse Ro52-GFP, Ro52ΔRING-GFP, or GFP was stably expressed in the mouse B cell lymphoma line A20 (Fig. 3A). Five independent clones of A20.Ro52-GFP, A20.Ro52ΔRING-GFP, and A20.GFP, respectively, were expanded for functional studies. GFP was distributed evenly all through the cell, whereas Ro52-GFP was detected mainly in the cytoplasm, confirming the data obtained with the transiently transfected HeLa cells (Figs. 1F and 3A). To confirm the protein expression, lysates from A20 and the GFP, Ro52-GFP-, and A20.Ro52ΔRING-GFP-transfected cells were subjected to immunoblotting with anti-GFP (Fig. 3B). Expression of Ro52 was similar between the five generated Ro52-GFP clones, and quantitative SYBR-PCR showed an ~7-fold increase in expression of Ro52 in the stably transfected clones compared with the parental A20 cell line (data not shown).
To begin analyzing the function of Ro52, we first studied growth and expansion of the A20 B cell tumor line expressing Ro52 in comparison to the wild-type A20, Ro52\(^{-}/H9004\) RING-GFP, and GFP-expressing A20 cells in a colony-forming assay. The plating efficiency and colony size was significantly decreased in Ro52-GFP-expressing cells compared with the Ro52\(^{-}/H9004\) RING-GFP- and GFP-expressing as well as parental A20 cells (Fig. 3, C and D), indicating that expression of Ro52 may inhibit expansion and growth of a tumor cell line at the steady-state level.

Reduced colony-forming ability may relate to several different properties. Already at the stage of expanding the single-cell clones, a difference in proliferation rate was detected, in that the Ro52-GFP-expressing clones proliferated at a lower rate than the GFP-expressing clones. To verify this observation, \(^{3}\text{H}\) thymidine incorporation was investigated with the five independent Ro52-GFP-, Ro52\(^{-}/H9004\) RING-GFP-, and GFP-expressing clones (Fig. 4 A).

The proliferation of A20.Ro52-GFP cells was markedly decreased \((p < 0.01)\) compared with Ro52\(^{-}/H9004\) RING-GFP, A20.GFP, and A20 parental cells (Fig. 4A). Thymidine incorporation at various time points verified the observation of decreased proliferation in Ro52-overexpressing cells (Fig. 4B).

**Overexpression of Ro52 mediates activation-induced cell death in a dose-dependent manner**

To examine the effect of Ro52 overexpression on cell growth and proliferation following activation, we used an agonistic anti-CD40 Ab for stimulation of the five Ro52-GFP, Ro52\(^{-}/\text{RING}-\text{GFP}\), and five GFP-expressing clones. Compared with steady-state propagated Ro52-expressing clones, the addition of anti-CD40 Ab to the cells expressing Ro52 led to further inhibition of proliferation (Fig. 4, D and E). Anti-CD40 has been described to increase cell death in a number of transformed cell lines (37), and we therefore analyzed whether the decreased proliferation in the stimulated cultures related to the extent of cell death. As expected, the cell death in all the A20-based stable clones (Ro52-GFP, Ro52\(^{-}/\text{RING}-\text{GFP}\), and GFP expressing) increased after CD40-mediated activation, but was significantly more increased in Ro52-GFP-expressing clones (Fig. 4, C and F). Proliferation of the five clones stably transfected with Ro52\(^{-}/\text{RING}-\text{GFP}\) was not significantly different from A20.GFP transfected clones either in steady state or after anti-CD40-mediated activation (Fig. 4, A–D), demonstrating the importance of the RING domain of Ro52 also in mediating CD40-induced cell death.

To further demonstrate that the degree of cell death was related to the level of Ro52 expression in the transfectants, we tapered the selecting drug G418, which resulted in a gradual loss of expression with commensurate reduction in the expression of GFP (Fig. 5). With reduced Ro52-GFP expression, the amount of cell death as detected by PI-positive staining was also reduced. The reduction of cell death was dose dependent in that the highest Ro52-expressing cells also showed the highest (67%) cell death, whereas the lowest Ro52-expressing cells showed lower (24%) cell death. A20.GFP cells, in contrast, did not
show any change in the level of cell death with the reduction of the amount of G418 (16, 15, and 17%, respectively). These data show that the CD40-mediated cell death is directly related to Ro52 expression and that even in the same clone, reduction of Ro52 increases cell viability. Together, our data show that Ro52 suppresses cellular proliferation and that Ro52 increases cell death after anti-CD40-mediated activation, indicating that the decreased proliferation and increased apoptosis of PBMCs in SLE and Sjögren’s syndrome may relate to their high expression of Ro52.

Discussion

The Ro52 protein is frequently targeted by Abs in patients with Sjögren’s syndrome and SLE. Although these autoantibodies are clinically used for diagnostic purposes, the function of Ro52 and why it becomes a target autoantigen in rheumatic diseases is not well understood. In this study, we show that 1) patients with Sjögren’s syndrome and SLE have an increased expression of Ro52 in PBMCs, 2) Ro52 is an E3 ligase dependent on its RING domain for activity, and 3) increased experimental expression of Ro52 decreases growth and induces cell death.

Ro52 has been implicated as a substrate for ubiquitination but was not previously identified as an E3 ligase. It was reported that Ro52 is modified by ubiquitin after ectopic expression of Ro52 and ubiquitin (38), and it has been demonstrated that Ro52 interacts with the deubiquitinating enzyme UnpEL in a coiled-coil-dependent manner (39). Both of these pieces of data indirectly support our finding that Ro52 is an E3 ligase. Our results further demonstrate an important role of the RING domain in the Ro52-mediated ubiquitination; full-length Ro52 was modified with polyubiquitin, whereas the Ro52-mutant lacking the RING domain (Ro52ΔRING) was modified with monoubiquitin only. These data are consistent with other E3 ligases of the RING family, where this domain has been shown to mediate the interaction with E2/Ubc.

The monoubiquitination of Ro52ΔRING is possibly due to a remaining weak interaction with an E2/Ubc, enabling a decreased level of autoubiquitination despite the absence of a RING-domain. Such weak interaction with an E2/Ubc could potentially be mediated via the B-box and coiled-coil domain. An alternative explanation is that Ro52 is a substrate for another E3 ligase. Interestingly, overexpression of the E3 ligase Siah-2 leads to decreased levels of Ro52 and other TRIM proteins (40), raising the possibility that the observed monoubiquitination of Ro52ΔRING is mediated by Siah-2, although Ro52 may also be the substrate for other E3 ligases.
Ro52-driven polyubiquitination was mediated together with several E2/Ubc:s in vitro, and as observed for other E3 ligases, Ro52 probably has several Ubc partners. In our experiments, the strongest signal was observed with UbcH6, whereas UbcH3 and UbcH10 did not support any polyubiquitination in vitro. It is interesting to note that, in addition to the Ubc core domain, UbcH6 has an extra N-terminal domain, and modification of UbcH6 with ubiquitin triggers nuclear import via importin 11 (41). In our localization studies, we detected Ro52 both in the cytoplasm and the nucleus. Because Ro52 does not contain any of the identified nuclear localization signal sequences that can account for its nuclear presence, this suggests a possibility of Ro52 being imported into the nucleus via interaction with UbcH6.

Ectopic expression of Ro52, but not Ro52ΔRING, in the B cell line A20 resulted in slowing of growth in this tumor cell line, suggesting that Ro52 may function in inhibiting cell growth at steady-state proliferation and that it may act as a tumor suppressor gene. Notably, the Ro52 gene is located on the short arm of chromosome 11 in the human genome (11p15.5), in a region proposed to contain several tumor suppressor genes. Allelic loss in this genomic region in humans has been associated with a number of adult cancers (42, 43). These observations, together with the inhibition of tumor growth we observed in the Ro52-overexpressing tumor B cell line in our experiments, indeed suggest that Ro52 may have tumor suppressor activity. Furthermore, many TRIM proteins are involved in regulating cell death and proliferation (28, 29), strengthening the idea that Ro52 is a protein with an important role in these processes.

In addition to slowing the growth of A20 cells, ectopic expression of Ro52 enhanced activation-induced cell death when the Ro52-expressing cell line was activated with an agonistic anti-CD40 Ab. CD40-mediated cell death, and inhibition of proliferation, has been observed in several transformed cell lines (37, 44) and is probably the effect of a phenomenon similar to activation-induced cell death. Although CD40 is a member of the TNFR family, it has no death domain and is therefore in our experiments not likely directly involved in inducing apoptosis after engagement. Our data support that the anti-CD40-mediated cell death is due to a direct consequence of Ro52 expression, because when the expression of Ro52 was reduced the anti-CD40-induced cell death also decreased proportionally. Ro52 as an E3 ligase might target survival genes induced during CD40-mediated activation or alternatively may enhance functions of genes mediating apoptosis and cell death by relieving them from endogenous repression. The potential mechanism by which Ro52 promotes activation-induced cell death can only be clarified once the target protein/proteins of Ro52-driven ubiquitination is identified.

In the investigated anti-Ro52-positive patients with Sjögren’s syndrome and SLE, we found an increased expression of Ro52 in PBMCs. Whereas Ro52 expression at the mRNA level was highest in the CD19+ B cells, Western blotting showed that protein expression was higher in the CD3+ and CD14+ cells. This suggests that there is greater production of mRNA for Ro52 in B cells, but that there is relatively less mature protein accumulation in this cell type. Because Ro52 is an E3-ligase and itself is polyubiquitinated, this might indicate a rapid degradation of the Ro52 protein in B cells such that their protein expression is not proportionate to the relatively higher mRNA expression. Both decreased proliferation of lymphocytes and increased apoptotic activity has been shown in patients with SLE and Sjögren’s syndrome. In addition, resident cells in the affected organs of the patients, e.g., keratinocytes and fibroblasts in lupus, show both decreased proliferation and increased apoptosis (45, 46). In combination with a defect clearance of apoptotic material (6), this has been suggested to produce an increased load of autoantigens available to the immune system, and trigger or restimulate autoantigenic responses to multiple Ags found in the apoptotic bodies, including the Ro52 Ag. Our finding that Ro52 has increased expression in both patients with Sjögren’s
syndrome and SLE, and that overexpression of this E3 ligase may lead to apoptosis after activation, thus identifies a potential molecular basis for the decreased cellular proliferation and increased apoptosis in these patients.

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Disclosures

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References