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Anti-Ro52 Autoantibodies from Patients with Sjögren’s Syndrome Inhibit the Ro52 E3 Ligase Activity by Blocking the E3/E2 Interface*

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Ro52 (TRIM21) is an E3 ligase of the tripartite motif family that negatively regulates proinflammatory cytokine production by ubiquitinating transcription factors of the interferon regulatory factor family. Autoantibodies to Ro52 are present in patients with lupus and Sjögren’s syndrome, but it is not known if these autoantibodies affect the function of Ro52. To address this question, the requirements for Ro52 E3 ligase activity were first analyzed in detail. Scanning a panel of E2 ubiquitin-conjugating enzymes, we found that UBE2D1–4 and UBE2E1–2 supported the E3 ligase activity of Ro52 and that the E3 ligase activity of Ro52 was dependent on its RING domain. We also found that the N-terminal extensions in the class III E2 enzymes affected their interaction with Ro52. Although the N-terminal extension in UBE2E3 made this E2 enzyme unable to function together with Ro52, the N-terminal extensions in UBE2E1 and UBE2E2 allowed for a functional interaction with Ro52. Anti-Ro52-positive patient sera and affinity-purified anti-RING domain autoantibodies inhibited the E3 activity of Ro52 in ubiquitination assays. Using NMR, limited proteolysis, ELISA, and Ro52 mutants, we mapped the interactions between Ro52, UBE2E1, and anti-Ro52 autoantibodies. We found that anti-Ro52 autoantibodies inhibited the E3 ligase activity of Ro52 by sterically blocking the E2/E3 interaction between Ro52 and UBE2E1. Our data suggest that anti-Ro52 autoantibodies binding the RING domain of Ro52 may be actively involved in the pathogenesis of rheumatic autoimmune disease by inhibiting Ro52-mediated ubiquitination.

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4 The abbreviations used are: SS, Sjögren’s syndrome; SSA, SS type A; IRF, interferon regulatory factor; SLE, systemic lupus erythematosus; TRIM, tripartite motif; MaBP, maltose-binding protein; Ni-NTA, nickel-nitritriacetic acid; CSP, chemical shift perturbation; RBL, RING-B-box linker.
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These results might have important functional and clinical implications indicating a role for RING-specific anti-Ro52 autoantibodies in autoimmune exocrinopathy by blocking the E3 activity of Ro52.

EXPERIMENTAL PROCEDURES

**Patient Sera and Monoclonal Ro52 Antibodies**—Sera from 75 Ro/SSA-positive patients were included. Forty two were diagnosed with Sjögren’s syndrome and 33 with SLE. Blood was drawn at outpatient visits, and serum was stored in aliquots at −70 °C. The diagnosis of SS was based on the criteria proposed by the American-European consensus group (19), and the diagnosis of SLE by the American College of Rheumatology criteria (20, 21). Sera from 12 patients with sicca symptoms but no inflammatory activity or autoantibodies were used as control sera. The study was approved by the Regional Human Ethics Committee at the Karolinska University Hospital, and informed consent was obtained from all subjects. Monoclonal antibodies against human Ro52 were generated by standard hybridoma protocols and characterized as described previously (1, 22).

**Plasmids**—Plasmids encoding human Ro52 and Ro52 mutants, fused to maltose-binding protein (MaBP), were generated as described previously (23). pMAL-cRI (New England Biolabs) plasmids encoding mouse Ro52 and mutants were generated by subcloning Ro52-coding DNA from pcMV-FLAG6c-mRo52, pCMV-FLAG6c-mRo52ΔRING, pCMV-FLAG6c-Ro52mut1, and pCMV-FLAG6c-Ro52mut2 into pMAL-cRI using compatible EcoRI and Sall sites. pMAL-Ro52b and pMAL-Ro52ΔRING were made by subcloning the Ro52-coding fragment from pGBK7-Ro52β and pGBK7-Ro52ΔRING into pMAL-c-RI using compatible EcoRI and Xhol/Sall sites. Constructs encoding the RING-RBL (residues 1–91 in the human Ro52 amino acid sequence) and RBL-B-box (residues 55–128 in the human Ro52 amino acid sequence) proteins were generated by PCR amplification of Ro52 using primers with engineered restriction enzyme restriction sites (22). The PCR fragments were digested by Ndel and EcoRI and inserted into the pETMCIII-vector containing an N-terminal His6 tag. For structural studies, the RING-RBL was inserted into the pET28b-vector containing a thrombin cleavage site subsequently to the His6 tag. The E2 constructs were made by inserting E2 coding cDNA into the PET15 or PET28 vectors. To produce MaBP-tagged RING-RBL, we generated pMAL-cRI-RING-RBL and mutants using PCR cloning and overlap extension PCR-mediated mutagenesis. Plasmid and primer sequences are available on request.

**Human E2 Panel**—The following were used: UBE2s* (NP_055316); UBE2d2 (NP_86282); UBE2d3 (NP_871619); UBE2d1 (NP_003329); UBE2d4 (NP_057067); UBE2e2 (NP_689866); UBE2e3 (NP_872619); UBE2t* (NP_054895); UBE2n (NP_003339); HIP2 (NP_005330); UBE2l6 (NP_004214); UBE2g1 (NM_003342); CDC34 (NM_004359); UBE2r2 (NM_017811); UBE2e1 (P51965) UBE2l3 (NM_003338); UBE2m (NM_003969); UBE2f (NM_080678); UBE2h (NM_003344); UBE2c (NM_007019); UBE2u* (NM_152489); FTS* (NP_022476); UBE2w* (NP_018299); UBE2v2 (NM_003350); UBE2v1 (NM_001032288); FLJ25076* (XM_05969); UBE2q2* (NM_173469); UBE2Q1* (NM_017582); UBE2a (NM_003336); UBE2j2* (NM_058167); UBE2j1* (NM_016021); UBE2z2 (NM_023079); UBE2b (NM_003337); UBE2i (NM_003345); and UBE2g2 (NM_003343). The E2 proteins highlighted with an asterisk were expressed as the ubiquitin-conjugating core domain only. The resulting His6 fusion proteins were expressed in the Escherichia coli strain BL21 (Codon plus™, Stratagene) and induced with 1 mm isopropyl 1-thio-β-D-galactopyranoside. Bacterial pellets were resuspended in cold buffer E (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 2 mM imidazole) and lysed with sonication. The lysates were clarified by centrifugation at 15,000 rpm at 4 °C for 1 h. After extensive washing (20-bed volume buffer) with cold buffer E, the proteins were eluted from the resin with buffer F (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 500 mM imidazole) and dialyzed against buffer G (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM PMSF, 1 mM DTT) overnight at 4 °C. The E2 proteins were frozen in buffer G with 10% glycerol at −80 °C until use.

**Protein Expression and Purification**—Expression and purification of MaBP fusion proteins were performed as described previously (23). For His-tagged constructs, pET28b-RING-RBL, pET28b-RING-B-box (22), and pET28b-UBE2E1 were transformed into E. coli strain BL21 (Codon plus™, Stratagene). pET28b-RBL-B-box was expressed and purified as described previously (22). Expression was induced with 0.8 mm isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C and overnight at 22 °C. RING-RBL was induced at an absorbance of 0.7, whereas UBE2E1 was induced at an absorbance of 1.0. To ensure stable RING-RBL, 20 μM ZnCl2 was added after induction. After expression, cells were spun down at 3000 rpm at 4 °C for 30 min. Pellets were resuspended in lysis buffer (20 mM Tris, 150 mM KCl, 5 mM DTT, 10 mM ZnCl2, pH 8), and the lysate was incubated on ice for 30 min prior to sonication (six times with 10-s bursts with 10-s breaks). After centrifugation at 10,000 rpm at 4 °C for 30 min, the supernatants were purified under native conditions using Ni-NTA resin before eluting antibodies with 250 μM imidazole. The resulting His6 fusion proteins were expressed in the Escherichia coli strain BL21 (Codon plus™, Stratagene) and induced with 1 mm isopropyl 1-thio-β-D-galactopyranoside. Bacterial pellets were resuspended in cold buffer E (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 2 mM imidazole) and lysed with sonication. The lysates were clarified by centrifugation at 15,000 rpm at 4 °C. After extensive washing (20-bed volume buffer) with cold buffer E, the proteins were eluted from the resin with buffer F (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 500 mM imidazole) and dialyzed against buffer G (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM PMSF, 1 mM DTT) overnight at 4 °C. The E2 proteins were frozen in buffer G with 10% glycerol at −80 °C until use.

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**Immunoglobulin Fraction Preparation and Affinity Purification of Antibodies**—Two hundred μl of patient serum was incubated with 100 μl of 50% protein-A-Sepharose (GE Healthcare) slurry with binding buffer (50 mM Tris, pH 8) for 1 h at room temperature. The beads were washed six times with 1 ml of binding buffer before eluting antibodies with 2× 50 μl of 0.1 M glycine, pH 2.8. The eluate was immediately neutralized by adding 2.5 μl of 1 N Tris, pH 9. Protein concentration was measured by the Bradford assay. For affinity purification of anti-RING-RBL antibodies, purified RING-RBL protein was separated by
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15% SDS-polyacrylamide gels. The protein was transferred to nitrocellulose filters, and the RING-RBL protein was refolded on the membrane in refolding buffer (100 mM Tris, 50 mM KCl, 10 mM DTT, pH 6.8) for 60 min before blocking with 5% (w/v) fat-free milk in PBS, 0.05% Tween (TPBS) for 30 min. The membrane areas with bound RING-RBL, identified by immunoblotting of outer strips cut from the membrane, were excised and incubated for 2 h with patient serum diluted 1:250 in TPBS. After washing the membrane with TPBS, the antibodies were eluted with 0.1 M glycine, pH 2.8, and transferred into 1 M Tris, pH 9, in a 1:1 ratio.

ELISA—ELISA was performed as described previously (24). Briefly, high binding 96-well plates (Nunc) were coated with 1 μg of protein diluted in carbonate buffer, pH 9.6, per well. Plates were blocked with 5% milk powder before addition of sera or monoclonal antibodies, diluted 1:50 and 1:500, respectively, in TPBS 1% milk powder and incubated overnight. Bound antibodies were detected by alkaline phosphatase-conjugated rabbit anti-mouse IgG or rabbit anti-human IgG antibody (Dakopatts). Phosphatase substrate tablets (Sigma) in diethanolamine buffer, pH 9.6, were used as substrate, and absorbance was measured at 405 nm.

Ubiquitination Assays—The ubiquitination assays were performed as described previously (7). In short, 0.5 μM MaBPRo52 or MaBPRo52 mutant proteins were used in each reaction. The reaction further contained 100 ng of E1, 500 ng of E2, and 2.5 μg of ubiquitin (Boston Biochem) in ubiquitination assay buffer (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 reactions were incubated for 1 h at room temperature. Reactions were terminated by boiling after addition of 20 μl of 2× SDS-PAGE sample buffer containing 10 mM Tris-Cl, 10% (w/v) SDS, 0.5% (w/v) bromphenol blue, and 500 mM DTT. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. The inhibitory effect of monoclonal antibodies on the ubiquitination process was investigated using a 4:1 molar ratio (antibody/Ro52). For testing the inhibitory effect of serum, Ig fractions and affinity-purified patient antibodies, the volume of water in the ubiquitination reactions, 12 ml, was replaced with 12 μl of serum, Ig fraction, or antibody solution.

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Immunoblotting—After transfer to nitrocellulose membranes (Protran®, Whatman), blocking was performed with 5% milk powder in TPBS. Nitrocellulose membranes were incubated with anti-ubiquitin antibody (clone 6C1, Sigma) diluted in TPBS (1:1000), Ro52 hybridoma supernatants diluted in TPBS (1:10), or anti-Myc (Santa Cruz Biotechnology) diluted 1:200 in TPBS followed by washing and incubation with anti-mouse or anti-rabbit IgH-HP (1:1000) (Dakopatts). Developing was performed with the ECL system (GE Healthcare). To detect IgGs in the Ig fractions or affinity-purified patient antibodies, the membranes were incubated with anti-human IgG-HRP antibodies (1:1000) (Dakopatts) for 30 min after blocking and then developed.

NMR Spectroscopy of the RING-RBL and UBE2E1—NMR spectra were acquired at temperatures ranging from 298 to 310 K at pH 6, 6.5, and 8, on Varian INOVA spectrometers at 600 and 800 MHz. All data sets were processed using NMRPipe (25) and analyzed using CARA. Sequential resonance assignments for RING-RBL were obtained from three-dimensional 1H,15N NOESY-HSQC (Tmix = 120 ms), three-dimensional 1H,15N TOCSY-HSQC (Tmix = 50 ms), and HCCTOCSY-NH, HNCA, HNCOCA, HNCO, HNCA, CBCA(CO)NH, and CBCANH experiments (26) with a water flip-back pulse for water suppression. Attempts at purifying the RING-RBL at pH 6 after NiNTA purification as well as screening of a wide range of different buffer systems failed. These samples produced only few and very broad NMR signals probably due to the formation of large complexes during the purification process. This agrees with our previous study where pH 8 and Tris buffer provided the best achievable conditions for biophysical studies of the RING-RBL (27). Although optimal solubility and stability of a monomeric RING-RBL were achieved at pH 8.0 and 25 °C (22), chemical exchange with solvent during these conditions resulted in only 57% of all residues providing observable NMR resonances. At pH 6, 22 additional peaks appeared in the 15N-HSQC (altogether 81% of all residues). During pH-titration, the pI (7.0) was passed and precipitation occurred quickly, leaving 0.1 to 0.15 mM protein in solution, but the more narrow line shape of the remaining protein due to the lower pH compensated for the loss of signal due to precipitation. Such a sample retained its NMR quality for 24 h and could only be used for the more sensitive triple resonance experiments, resulting in a final backbone resonance assignment of the RING-RBL for 71% of all residues, fairly well dispersed over the RING-RBL sequence.

RING-RBL and UBE2E1 binding was monitored by measuring NMR chemical shift perturbations (CSPs). A series of 1H,15N HSQC spectra were recorded for 15N-labeled RING-RBL with various amounts of unlabeled UBE2E1. CSPs were measured at the following protein ratios: 1:0, 1:0.25, 1:0.5, 1:0.8, and 1:1.4 of RING-RBL to UBE2E1, limited by the solubility of both components.

NMR titration measurements were performed at pH 8 because at pH 6 precipitation occurred within minutes.

The eluted proteins were eluted by boiling 3 min with 60 μl of SDS-PAGE sample buffer containing 20% glycerol, 4% (w/v) SDS, 125 mM Tris, 2-mercaptoethanol, and 250 μM imidazole. The eluted proteins were separated with SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting against Myc or ubiquitin.
UBE2E1 unlabeled NMR sample used for titration had pH 6.5 due to stability concerns at these high concentrations. Minor CSPs (<0.007 ppm) due to pH changes during addition of UBE2E1 to RING-RBL were accounted for by subtracting a buffer titration control experiment. Interacting residues were identified by assaying the fast-exchange contribution on ligand binding as judged by chemical shift changes for individual amino acids, which were evaluated by manually tracing peaks in the NMR spectra. Atom-specific chemical shift weighting was done according to Mulder et al. (28). To distinguish between interacting and noninteracting residues, a cutoff value of 0.059 was calculated as described (29).

**RESULTS**

**Ro52 E3 Activity Requires Its RING Domain and a Limited Subset of E2-conjugating Enzymes**—To identify domains required for E3 activity of human Ro52, several mutants of Ro52 were generated (Fig. 1). To define which E2s support the E3

![Figure 1. MaBP and His-tagged Ro52 mutants. Ro52-1 is the fusion of MaBP to full-length human Ro52 (residues 1–475). Ro52-2 lacks the B30.2 domain; Ro52-3 consists of the coiled-coil domain only, whereas Ro52-4 and Ro52-5 comprise the zinc binding and B30.2 domains, respectively. The deletion constructs Ro52β and Ro52βΔRING lack the leucine zipper or the RING and the leucine zipper, respectively. MaBP fused mouse Ro52, mouse Ro52ΔRING, and mouse Ro52 with point mutations (indicated by asterisks), and mRo52mut1 (C20A, C23A, and mRo52mut2 (C55A and C58A) were also used as well as His₆-tagged RING and B-box constructs.**
activity of Ro52, we screened a panel of 34 human E2 ubiquitin-conjugating enzymes. Of the analyzed E2s, only UBE2D1–4 (class I E2s) and UBE2E1 and UBE2E2 (class III E2s) supported Ro52-mediated polyubiquitination (Fig. 2A). UBE2E1 was chosen as a representative E2 for screening E3 activity for the various Ro52 constructs. Ro52 deletion mutants lacking the B30.2 domain (Ro52-2) or lacking the B30.2 and the coiled-coil domains (Ro52-4) retained the E3 activity, showing that the B30.2 or coiled-coil domains are not required for the E3 activity of Ro52 (Fig. 2B). Deletion mutants containing the B30.2 domain alone (Ro52-5) or the coiled-coil domain alone (Ro52-3) did not possess any E3 activity (Fig. 2B). In contrast, deletion mutants containing only the Zn²⁺ binding region (Ro52-4), i.e. the RING and the B-box domain, had E3 activity. The necessity of an intact RING domain was further confirmed because the E3 activity was abolished in Ro52 mutants where

**FIGURE 2.** Ro52 is a RING domain-dependent E3 that can form polyubiquitin chains both through Lys-48 and Lys-63. A, Ro52 was used in autoubiquitination reactions together with E1, ubiquitin, reaction buffer, and different E2 enzymes. Ro52 is autoubiquitinated in reactions with UBE2D1–4, UBE2E1, UBE2E2, and CDC34. The monoclonal antibody 7BC7 was used for anti-Ro52 immunoblotting. NEG, reaction without any E2. B, in vitro ubiquitination assays with different Ro52 mutants demonstrating that only mutants containing the RING domain have E3 activity. C, Ro52 without the RING domain or with point mutation in the RING domain lack E3 activity. mRo52RING, mRo52mut1 (C20A,C23A), and mRo52mut2 (C55A,C58A) were used for in vitro ubiquitination assays with UBE2E1. D, two isoforms Ro52 and Ro52β (lacking exon 4) both had E3 activity, whereas Ro52β lacking the RING domain had no E3 activity. E, 293T cells were transfected with His6-ubiquitin and Myc-Ro52, Myc-Ro52β, or empty Myc plasmid. After purifying ubiquitinated proteins with Ni-NTA resin, immunoblotting was made against the Myc epitope. Both Ro52α and Ro52β are ubiquitinated in vivo. F, ubiquitination assays using Ro52 with UBE2E1 and two ubiquitin mutants containing a single lysine residue only, Lys-48-only or Lys-63-only. Ro52 is autoubiquitinated with both Lys-48-only and Lys-63-only ubiquitin mutants.
Zn$^{2+}$ binding cysteine residues in the RING domain had been exchanged for alanine residues (Fig. 2C).

The alternative Ro52 isoform present in humans, Ro52β, also had robust E3 activity both in vitro (Fig. 2D) and in vivo, as shown in a ubiquitination assay using co-transfection with plasmids encoding Myc-Ro52 and His$_6$-ubiquitin in 293T cells (Fig. 2E). The RING domain was necessary also for Ro52β E3 activity, as the activity was lost when this domain was deleted (Fig. 2D).

Ubiquitin contains seven lysines (residues 6, 27, 11, 29, 33, 4, and 63) through which several ubiquitin moieties can be linked to form a polyubiquitin chain (31). Depending on which lysine was used for building the polyubiquitination chain, the fate of the ubiquitinated protein differs. Polyubiquitination through degradation, whereas polyubiquitination through Lys-63 leads to functional alteration of the target protein (13). To investigate whether Ro52 can be polyubiquitinated by Lys-48 and/or Lys-63 chains, ubiquitination assays were performed using Lys-48-only and Lys-63-only ubiquitin mutants, where all lysine residues were mutated to arginine residues except Lys-48 or Lys-63, respectively. Ro52 could generate polyubiquitinated proteins with both mutants (Fig. 2F), suggesting that Ro52 is capable of building both Lys-48 and Lys-63 polyubiquitin chains and thus may both modify protein function and target proteins for degradation by the 26 S proteasome. 

Ro52 Autoubiquitination Activity Is Affected by N Termini in Class III E2s—Class I E2s are mainly cytoplasmic and class III E2s are nuclear substrates, and because Ro52 is active with E2s from both classes (Fig. 2A), Ro52 might have both cytoplasmic
and nuclear substrates. This is physiologically relevant as Ro52 translocates between these compartments (1). Interestingly, UBE2E3 did not support Ro52 ubiquitination, although, like UBE2E1 and UBE2E2, it is a nuclear class III E2 with an N-terminal extension (32). Because the E2 core domains of UBE2E1, UBE2E2, and UBE2E3 only differ at five positions (Fig. 3A), we performed in vitro ubiquitination assays with E2 mutants lacking the less conserved N-terminal extensions to understand whether core or N-terminal sequence differences were discriminating in E3 interactions. Indeed, E2 mutants comprising only class III core domains all supported Ro52 E3 activity (UBE2E1_H9004N, UBE2E2_H9004N, and UBE2E3_H9004N, see Fig. 3B). However, the presence of the N-terminal extension in UBE2E3 blocked autoubiquitination of Ro52, whereas intact UBE2E1 and UBE2E2 remained supportive (Fig. 3B). This suggests a regulatory role for the N-terminal extension of class III E2s in the ubiquitination process.

High Levels of Anti-Ro52 Autoantibodies Specific for the RING Domain Are Common in Patients with Sjögren’s Syndrome—To determine the frequency and levels of antibodies to different protein domains of Ro52 in patient sera, we screened sera from 75 Ro/SSA-positive patients with Sjögren’s syndrome or SLE with full-length recombinant Ro52 using ELISA. Anti-Ro52 antibodies were detected in 79% of Ro/SSA-positive patients with Sjögren’s syndrome and 82% of Ro/SSA-positive patients with SLE (Fig. 4A). The sera were further investigated for antibodies binding the Zn$^{2+}$ binding region (Ro52-4, including both the RING and B-box domains), the RING domain (RING-RBL), or the B-box (RB-linker-B-box) of Ro52 using a panel of Ro52 deletion mutants (Fig. 4, B–D). Antibodies to the Zn$^{2+}$ binding region were found in 57% of the patients with Sjögren’s syndrome and 55% of the patients with SLE. This reactivity was commonly generated by RING-binding antibodies as 52 and 49% of Ro52 positive sera from patients with Sjögren’s syndrome and SLE, respectively, were positive in ELISA with RING-RBL, whereas B-box-specific antibodies only were present in 21 and 9% of the same patient groups (Fig. 4B). The levels of the RING-specific antibodies were slightly higher in patients with SS than in SLE patients (p = 0.04) (Fig. 4, C and D). No patient had antibodies to the B-box without the presence of RING-specific antibodies.

Patient Autoantibodies Specific for the RING Domain Inhibit the E3 Activity of Ro52—Autoantibodies to Ro52 could potentially affect the function of the protein. To investigate if anti-Ro52 antibodies in patient sera inhibited the E3 activity of Ro52, we added sera from patients to in vitro ubiquitination reactions. Sera without anti-Ro52 autoantibodies were used as negative controls. Sera from some, but not all patients, clearly inhibited the Ro52 activity (Fig. 5A).

As our analysis of domains required for Ro52 E3 activity had shown that the RING structure was essential, we hypothesized that only anti-Ro52 antibodies to this domain inhibited the E3 activity. Categorizing the patient sera investigated by ELISA against the Ro52 deletion mutants (Table 1), three distinct autoantibody patterns were identified as follows: (i) sera with antibodies only to the coiled-coil domain, (ii) sera with antibodies to the RING and coiled-coil domains, and (iii) sera
with antibodies to both the RING, B-box and coiled-coil domains. We observed that only sera containing anti-Ro52 antibodies to the RING domain (groups ii and iii) could block E3 function but that sera from patients with autoantibodies only to the coiled-coil domain (group i) did not inhibit Ro52 E3 activity. Representative sera from each group of pattern were used for further investigation (Table 1).

To demonstrate that the E3 activity was indeed inhibited by antibodies and not some other factor in the sera, the immunoglobulin (Ig) fractions from patient and control sera were purified by protein-A-Sepharose for use in ubiquitination assays. Ig from patients with anti-RING, anti-B-box, and anti-coiled-coil antibodies as well as Ig from patients with anti-RING and anti-coiled-coil antibodies clearly inhibited Ro52 activity, whereas Ig from Ro52 patients without antibodies binding the RING domain did not inhibit Ro52 (Fig. 5B). Anti-Ro52-negative Ig also did not affect the E3 ligase activity of Ro52. The IgG content in the Ig fractions was analyzed by ELISA using Ro52. E, Ig fraction and affinity-purified antibodies of patient serum P2 were tested in ELISA for retained activity to the RING domain using the RING-RBL construct in ELISA. F, no antibodies were detected against ubiquitin, UBE2E1, UBE2E2, or E1 using patient sera P1–P6 in ELISA. (Pos, positive control antibodies.)

Autoantibodies Sterically Inhibit the E3 Activity of Ro52

FIGURE 5. Patient sera reactive to the Ro52 RING domain inhibit the E3 activity of Ro52. A, Ro52 was used with UBE2E1 for in vitro ubiquitination reactions. Serum P1, not containing anti-Ro52 antibodies, did not inhibit the E3 activity of Ro52. In contrast, serum P2 containing anti-RING antibodies markedly inhibited the ubiquitination reaction. B, Ig fractions of the sera P2, P3, and P6 all inhibited ubiquitination, whereas Ig fractions of the anti-RING-negative sera P1, P4, and P5 did not inhibit ubiquitination. A monoclonal antibody toward the linker peptide of the zinc binding domain (7.12E11) did not inhibit Ro52 E3 activity. An anti-FLAG monoclonal antibody was used as a negative control. HC, heavy chain. C, immunoblotting of Ig-purified sera and affinity-purified serum P2 to detect the IgG content. The affinity-purified serum is shown in the 3rd lane and is denoted by P2 aff. D, Ro52 binding activity in patient sera and corresponding Ig fractions was analyzed by ELISA using Ro52. E, Ig fraction and affinity-purified antibodies of patient serum P2 were tested in ELISA for retained activity to the RING domain using the RING-RBL construct in ELISA. F, no antibodies were detected against ubiquitin, UBE2E1, UBE2E2, or E1 using patient sera P1–P6 in ELISA. (Pos, positive control antibodies.)

from patients with anti-RING, anti-B-box, and anti-coiled-coil antibodies as well as Ig from patients with anti-RING and anti-coiled-coil antibodies clearly inhibited Ro52 activity, whereas Ig from Ro52 patients without antibodies binding the RING domain did not inhibit Ro52 (Fig. 5B). Anti-Ro52-negative Ig also did not affect the E3 ligase activity of Ro52. The IgG content in the Ig fractions was confirmed by immunoblotting and ELISA (Fig. 5, C and D), and sustained Ro52 binding after purification of the Ig was confirmed by ELISA (Fig. 5E).

Because no serum and thus no Ig fraction contained solely antibodies to the RING domain (Table 1), this raised the possi-
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<table>
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<td>Constructs bound by anti-Ro52 monoclonal antibodies and patient sera</td>
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<tr>
<td>RING-RBlinker</td>
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<tr>
<td>Monoclonal antibody</td>
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<tr>
<td>7.12E11</td>
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<td>7.8C7</td>
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<td>7.2F4</td>
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<td>Mixa</td>
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<td>Anti-RING-RBl</td>
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a A mixture of monoclonal antibodies 7.12E11, 7.8C7, and 7.2F4 was used.
b Anti-FLAG antibodies bind the FLAG epitope and were used as a negative control.

bility that antibodies with other specificities might be responsible for the inhibition of Ro52. To confirm that it was indeed only anti-RING-RBl antibodies that inhibited the function of Ro52, antibodies specific for the RING domain were affinity-purified from sera using recombinant RING protein (RING-RBl clone, Fig. 1). Affinity-purified anti-RING-RBl antibodies inhibited the Ro52 E3 activity (Fig. 5B), confirming that anti-RING-RBl antibodies present in the patient sera are necessary and sufficient to block Ro52-mediated polyubiquitination.

To exclude the possibility that patient sera were blocking ubiquitin, E1, or UBE2E1 in the ubiquitination reaction, sera were tested in ELISA with ubiquitin, E1, and UBE2E1, respectively. No reactivity of sera to any of these components was observed (Fig. 5F). Three different anti-Ro52-specific monoclonal antibodies, targeting the linker region (RBl) between the RING and B-box domains (7.12E11), the leucine zipper (7.8C7), or the B30.2 domain (7.2F4), were also tested for their ability to inhibit Ro52. Each of them recognized a different epitope in Ro52 (Table 1), but none inhibited Ro52 activity supporting the observation that only RING-specific antibodies inhibit Ro52 E3 activity. A mixture of all the monoclonal antibodies also did not inhibit ubiquitination (Fig. 5B).

In all, antibodies to the RING domain inhibit Ro52 E3 activity, whereas neither control Ig nor Ig with anti-Ro52 antibodies to domains other than the RING domain affected Ro52 activity. These data show that only antibodies specific for the RING domain inhibit the activity of Ro52.

Mapping the Binding Surface on RING-RBl to UBE2E1 by NMR and Limited Proteolysis—The ubiquitination process depends on an E2/E3 interaction, which for RING-containing E3s involves direct contact between the E2 and the E3 RING domain (33, 34). To investigate whether inhibiting antibodies physically blocked this interaction, we started by molecularly characterizing the interaction between the RING-RBl and UBE2E1, which we had found consistently supported Ro52-mediated ubiquitination.

Ligand titration of unlabeled UBE2E1 into labeled RING-RBl was pursued by NMR, where resonances displaying single N-NH correlations in a two-dimensional HSQC experiment were traced. To perform these experiments, the RING-RBl resonances first had to be assigned. Despite limitations in solubility and NMR signal loss for the RING-RBl at both pH 8 and pH 6, a backbone assignment coverage of 71% of the RING-RBl residues was obtained using triple-resonance methodology. Highly resolved and assigned HSQC's thus formed the basis for titration experiments with UBE2E1 as well as with patient sera (Fig. 6A).

On binding to UBE2E1, CSPs were observed in four regions of the RING-RBl (Fig. 6, A and B). Altogether, the CSPs were small, indicating little structural changes upon complex formation. The strongest CSP was observed for isoleucine 18 located between the Zn2+ ligands 1 and 2 (Cys-16 and Cys-19) and constitutes part of loop 1 (L1). The second region (Val-50–Phe-58) includes Zn2+ ligands 7 and 8 (Cys-51 and Cys-54) and part of loop 2 (L2). These two loop regions have previously been identified to take part in E2/E3 interactions with RING- and RING-like containing E3s (33–35). The third region with CSPS, Cys-39–Gln-42, is part of a helix in homologous RING structures. In a possible interacting fourth region, the CSP of Asn-74 is slightly below the cutoff for significance. Several signals exhibiting CSPs also showed line broadening (Fig. 6A), suggesting intermediate exchange contributions to the line shape. The small size of the CSPs, the magnitude of line broadening effects, and the unsaturable binding in the titration experiment are all consistent with previous observations of high micromolar $K_d$ values for RING-E2 interactions (36–38).

To extend the interaction analysis, time-resolved limited proteolysis was employed (Fig. 6C). In this experiment, proteolytic relative cleavage propensities are compared between digestions of RING-RBl alone and in complex with UBE2E1, where lower cleavage propensities are to be expected at the binding interface due to protection against proteolytic cleavage (22). Overall, proteolysis of the RING-RBl suggests a well protected RING domain with the main cleavage propensities located in the regions flanking the Zn2+ binding core region (Fig. 6C). On adding UBE2E1, cleavage propensities at RING-RBl residues Arg-57, Lys-77, and Glu-78 are drastically reduced, whereas cleavage at other sites is essentially unaffected (Fig. 6C). The proteolysis experiment does not shed light on E2 binding to RING-RBl regions L1 or Cys-39–Gln-42, because these are already protected in the absence of E2. However, the proteolysis results confirm the involvement of the L2 region, where Arg-57 appears more protected from proteolytic cleavage in presence of the E2, although the CSP was weak. Furthermore, this experiment clearly suggests the involvement of a fourth region in this E2/E3 interaction, because there are clear differences in relative cleavage propensities of residues Lys-77 and Glu-78 between the RING-RBl and in complex with UBE2E1. This result is in agreement with the fair CSP of residue Asn-74 (Fig. 6, A and B). Residue Lys-77 could not be assigned in the NMR experiment, and Glu-78 shows only a weak CSP. It thus appears that both Arg-57 and Glu-78 experience little change in environment or structure upon addition of UBE2E1 but that they are still in close vicinity of the interacting residues to become less accessible to proteases. Taken together, NMR and limited proteolysis suggest that UBE2E1 interacts with RING-RBl in four locations as follows: the L1 and L2 loops, the helix comprising residues 39–42, and an RBl region, including residues 74 and 78.
Mapping of the Binding Epitope of Patient and Monoclonal Anti-RING-RBL Antibodies—To investigate where and how antibodies bind onto the RING-RBL, and why RING-specific patient autoantibodies inhibit ubiquitination whereas the RBL-binding monoclonal antibody 12E11 does not, we measured CSPs of RING-RBL resonances upon binding to autoantibodies derived from patient sera and to the monoclonal antibody 12E11. These experiments indicated that patient sera containing autoantibodies that inhibited ubiquitination perturbed chemical shifts of residues Val-50, Leu-59, as well as Ala-83 and Arg-84, whereas the 12E11 monoclonals perturbed only the chemical shifts of residues Ala-83 and Arg-84 in the RBL region (data not shown).

To further analyze whether these CSPs represented true interactions between antibodies and protein, we generated two RING-RBL mutants aiming to destroy the antibody-binding
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FIGURE 7. Mutational analysis of Ro52/autoantibody interaction in the RING domain. A, 35 anti-Ro52 RING-RBL antibodies containing human patient sera were tested in ELISA to assess binding to the RING-RBL, as well as a mutant designed to disrupt the L2 epitope (RING-RBL V50D,L59D) and a mutant designed to disrupt the RBL epitope (RING-RBL A83D,R84A). B, RING-RBL binding monoclonal 7.12E11, which does not affect ubiquitination, was tested in ELISA with the same mutants as in A. C, E3 ligase activity of the mutants in A assessed by in vitro ubiquitination. All lanes were derived from the same blot with the same exposure but were nonadjacent in the original image. w/o, without.

epitopes in the L2 and RBL regions. The Val-50 and Leu-59 residues in the RING-RBL L2 loop, which showed significant shift changes in both antibody and E2 titrations, were exchanged for glutamic acid (V50D,L59D). Furthermore, a mutant of Ala-83 and Arg-84 (A83D,R84A), targeting a C-terminal epitope in the linker region jointly recognized by patient sera and the 12E11 monoclonal antibody, was generated. The V50D,L59D mutation targeting the L2 loop led to significantly lower binding of patient sera ($p < 0.001$) compared with nonmutated RING-RBL as determined by ELISA (Fig. 7A), whereas binding of the monoclonal 12E11 was not affected (Fig. 7B). In contrast, mutation of Ala-83 and Arg-84 (A83D,R84A) resulted in a near-complete loss of monoclonal 12E11 binding to RING-RBL (Fig. 7B), as well as significantly decreased binding of human sera (Fig. 7A). This suggests that although the monoclonal 12E11 only binds to the Ala-83–Arg-84-containing epitope outside of the E2 binding interface, human patient sera also targets the E2 interface which involves the L2 loop. A functional analysis of the two mutants in ubiquitination assays revealed that both the V50D,L59D and A83D,R84A constructs had retained E3 ligase activity and thus can be expected to maintain major folding properties (Fig. 7C).

Taken together, the loop regions that are actively interacting with the E2-conjugating enzyme are bound only by patient-derived autoantibodies that recognize epitopes in the folded RING-RBL, suggesting that the inhibition of Ro52 E3 activity and ubiquitination by patient autoantibodies is mediated via steric hindrance disrupting E2/E3 interaction after autoantibody binding to the sites vital for E2/E3 contact. Antibodies that only bind the RBL at residues 83–85, which are not involved in the E2/E3 interaction, also do not inhibit ubiquitination.

Mapping of Interaction Surfaces onto the Structure Model of the RING—To obtain a structural understanding of how patient antibodies could block ubiquitination, and in the absence of a complete experimental RING-RBL structure, we evaluated the results on a previously derived homology model of the RING domain (27). The E2 interaction with Ro52 RING involves the L1 and L2 loops, which meet across the $\beta$-strands sandwiched between the two zinc-binding sites (Fig. 8A). E2 binding also affects residues Cys-39–Glu-42, which form part of the structural scaffold on which the L1 and L2 loops are anchored. Thus, these residues may not be directly involved in E2 binding but still experience slight structural changes upon E2-E3 complex formation due to the direct involvement of the L1 and L2 loops. The third E2 interaction site involving residues Asn-74, Lys-77, and Glu-78 is located in a region outside of the RING motif, which could either fold back adjacent to the zinc-binding E2-binding site or form complementary interactions with other parts of the E2.

Mutational data suggest that patient antibody binding involves residue Val-50 in the L2 loop and residue Leu-59, which is located on the same face of the RING domain (Fig. 8A). Both of these residues are close to the L1 loop, which is also involved in E2 binding. Thus, if patient antibodies bind the Ro52 RING domain, the E2 would not be able to dock. In contrast, monoclonal noninhibiting antibodies can physically be bound to Ro52 without disturbing the E2/E3 interaction (Fig. 8, B and C). This structural interpretation of the interaction data suggest that patient antibodies inhibit ubiquitination of Ro52 by physically hindering the formation of an E2-E3 complex, whereas noninhibiting monoclonal antibody binding only to a C-terminal RING-RBL epitope distant from the E2 binding interface is compatible with E2-E3 complex formation and thus allows for ubiquitination to proceed.

DISCUSSION

The basis for autoimmune and rheumatic diseases is becoming clearer as large scale genetics continuously identify polymorphisms linked to autoimmune disease development (39). Many of the identified polymorphisms are located in immune genes, but data pinpointing the functional consequence of these polymorphisms are still scarce. Furthermore, the mechanisms by which an established autoreactive immune response mediates tissue damage and the role of autoantibodies in systemic autoimmune disease are not well understood. In this study, we
addressed the question whether patient-derived autoantibodies can inhibit the function of their corresponding antigen, as well as the mechanism for such inhibition. For this investigation, we studied the effect of autoantibodies on the autoantigen and immunomodulatory protein, Ro52, which we recently identified as an E3 ubiquitin ligase (7).

To investigate the effects of anti-Ro52 autoantibodies on the E3 activity of Ro52, the requirements for Ro52 E3 activity were first analyzed in detail. Scanning a panel of E2s, we found that UBE1–4 and UBE2E1–2 supported the E3 activity of Ro52. The UBED1–4 class I E2s are cytoplasmic, whereas UBE2E1–2 class III E2s are localized to the cell nucleus (32, 40). This cellular compartmentalization of E2s may contribute to both temporal and substrate specificity of the ubiquitination process, as Ro52 is predominantly cytoplasmic (1, 7), but translocates into the nucleus upon interferon stimulation (1).

By our observation that UBE2E1–2 but not the homologous UBE2E3 supported Ro52 E3 activity, we identified an additional novel factor conferring specificity in the E2/E3 interaction. The core domains of the three class III E2s are 97% identical, and deletion constructs of UBE2E1–3 containing only the core motif all supported Ro52-mediated ubiquitination. However, only full-length protein with N-terminal extensions of UBE2E1 and UBE2E2 supported Ro52-mediated ubiquitination, whereas no polyubiquitination was observed with the full-length N-terminal containing UBE2E3. The N-terminal extensions of UBE2E2E2s show only weak homology and differ in size between 37 and 52 amino acid residues and thus may well harbor discriminative qualities. Our findings indeed suggest that the N-terminal extensions in the class III E2 family may have a specificity-regulating role in interactions with E3s. Our observation is also supported by another study, where the N terminus of UBE2C controls the threshold for the anaphase-promoting complex activation (41). The N terminus of UBE2C does not, however, share any homology with the N-terminal extension of class III E2s and is about 30 amino acid residues shorter. Further support for a role of N-terminal extension in mediating specificity stems from the fact that class I E2s, lacking N- or C-terminal extensions, are highly promiscuous supporting activity of most of the E3s studied to date (42).

Analysis of Ro52 domain requirements for E3 activity demonstrated that the RING domain is necessary and sufficient for
the E3 activity of Ro52, as the Zn$^{2+}$ binding domain itself could mediate polyubiquitination, whereas disruption of amino acid residues critical for RING folding abolished any E3 activity of Ro52.

The other domains of Ro52 were dispensable for the E3 activity and presumably mediate other functions of Ro52. The RING domain is necessary for recruiting the ubiquitin-charged E2, whereas the B30.2 domain mediates the binding to the target protein (9, 15). Data from both NMR analysis and limited proteolysis in our study support a role for the RING domain in interaction with E2 ubiquitin-conjugating enzymes, and the L1 and L2 loops of the RING were confirmed to interact with the E2 in agreement with earlier studies (33–35). Two additional regions were involved in E2/E3 interactions as follows: the putative helix region between both loops, as well as residues Lys-77 and Glu-78 in the RBL. This intriguing linker region, conserved throughout TRIM proteins (27), has also been shown to take part in inhibiting HIV infection restriction in TRIM5 (43). Further studies will show if these novel E2 interaction patches are conserved throughout the TRIM protein family. The low RING-RBL/UBE2E1 affinity is consistent with that in other RING/E2 interactions (36, 44–46). However, it has recently been shown that allosteric activation of the E2 by regions of the E3 further apart in sequence from the RING domain can decrease the E2/E3 dissociation constant substantially (46), which may also be the case for Ro52 and other TRIM proteins.

While investigating the ability of anti-Ro52 antibodies to bind different domains of Ro52 and inhibit the E3 activity of the protein, we found that antibodies in patients against the RING domain blocked the function of Ro52 and disrupted Ro52-mediated polyubiquitination. Mutational analysis confirmed antibody binding to two epitopes as follows: one located in the L2 loop of the RING, binding to which was crucial for inhibition of the E3 activity, and one containing residues Ala-83–Arg-84 in the RBL. Although close to the E2-binding site at Lys-77–Glu-78, antibody binding to the latter epitope does not suffice to inhibit Ro52 activity as monoclonal antibodies raised against the RBL only bind this region and do not inhibit autoubiquitination. Patient antibodies against other Ro52 domains also did not interfere with the E3 activity of Ro52. Antibodies against the substrate binding B30.2 domain (15) could theoretically also inhibit the ubiquitination of Ro52 target proteins such as IRF transcription factors by blocking the E3/substrate interaction; however, antibodies against the B30.2 domain are not common in patients with SS (47).

Our data indicate that the molecular basis for ubiquitination inhibition by anti-RING antibodies is mediated by steric hindrance (Fig. 8). The patient autoantibody-binding sites in the L2 loop of the RING domain partly overlapped with the contact areas for the UBE2E1 with Ro52, and the bulky IgG antibodies may thus block the access for the E2 to interact with Ro52 and thereby disrupt ubiquitination. In contrast, monoclonal binding to the alternative noninhibiting epitope does not hinder formation of the E2-E3 complex, and ubiquitination can therefore proceed.

In summary, we demonstrate here that patients with Sjögren’s syndrome have autoantibodies specific for the RING domain of Ro52 and that these antibodies disrupt the ubiquitin ligase activity of Ro52 by competing for the same binding site as required for proper E2 interaction. Our data indicate that the anti-Ro52 autoantibodies may have a direct pathogenic role in disease development and are consistent with the observation that the diminished Ro52 activity in Ro52-deficient mice leads to increased production of proinflammatory cytokines transcriptionally regulated by IRFs, as is also observed in Ro52 autoantibody-positive patients with Sjögren’s syndrome and SLE. The possibility of a pathogenic outcome when interfering with RING-E2 interactions is vividly demonstrated by RING domain L1 and L2 mutations in breast and ovarian cancer patients that disrupt BRCA1-mediated ubiquitination activity (48). However, it remains to be determined whether Ro52 RING-specific patient antibodies are actively involved in the pathogenesis of rheumatic autoimmune disease by inhibiting Ro52-mediated ubiquitination in vivo in patients during the disease process.

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REFERENCES
