Ro52 is one of the major autoantigens targeted in the autoimmune disease Sjögren syndrome. By sequence similarity, Ro52 belongs to the RING-B-box-coiled-coil (RBCC) protein family. Disease-related antibodies bind Ro52 in a conformation-dependent way both in the coiled-coil region and in the Zn$^{2+}$-binding Ring-B-box region. Primarily associated with Sjögren syndrome, Ro52 autoantibodies directed to a specific, partially structured epitope in the coiled-coil region may also induce a congenital heart block in the fetus of pregnant Ro52-positive mothers. To improve our understanding of the pathogenic effects of autoantibody binding to the Zn$^{2+}$-binding region, a multianalytical mapping of its structural, biophysical, and antigenic properties is presented. Structure content and lipid binding of subregions, dissected by peptide synthesis and subcloning, were analyzed by fluorescence and circular dichroism spectroscopy. A novel matrix-assisted laser desorption ionization time-of-flight mass spectrometry strategy for time-resolved proteolysis experiments of large protein domains was developed to facilitate analysis and to help resolve the tertiary arrangement of the entire RBCC subregion. The linker region between the RING and B-box motifs is crucial for full folding, and Zn$^{2+}$ affinity of the RING-B-box region is further protected in the entire RBCC region and appears to interact with the coiled-coil region. Marine monoclonal antibodies raised toward the RING-B-box region were primarily directed toward the linker, further supporting a highly functional role for the linker in a cellular environment. Taken together with our previous analysis of autoantigenic epitopes in the coiled-coil region, localization of autoantigenic epitopes in Ro52 appears closely related to molecular functionalities.

Ro52 is one of the main autoantigens in Sjögren syndrome, but autoantibodies to this intracellular protein occur also in systemic lupus erythematosus and rheumatoid arthritis (1). The immunodominant epitopes in Ro52 are predominantly localized in the structurally stable regions (2). The Ro52 coiled-coil contains a putative leucine-zipper between residues 211 and 232. Patient-derived monoclonal antibodies that bind to a peptide representing residues 200–239 (p200) of the Ro52 protein cause accumulating intracellular calcium levels in neonatal cardiomyocytes and relate to the development of congenital heart block (3–5). Another antigenic epitope has been mapped to the N-terminal Zn$^{2+}$-binding region of Ro52 (6) and was also suggested to relate to development of congenital heart block (7). However, the detailed location of the epitope in this 15-kDa region is yet unknown. The epitope is presumably conformation-dependent, because antigenicity is only observed in the reduced state (6), and because cysteines are conserved in the suggested Zn$^{2+}$-binding sites.

The reason why Ro52 is targeted in autoimmune disease is not known to date. Ro52 belongs to the rapidly growing RING-B-box-coiled-coil (RBCC) family, also denoted as TRIM (tripartite motif) (8). RBCC proteins appear to perform highly diverse functions, and diseases that relate to malfunction in these proteins have little in common, as exemplified by Opitz syndrome type I (MID1; see Refs. 9 and 10) and promyelocytic leukemia (see Refs. 11 and 12). The Ro52 RBCC region consists of an N-terminal Zn$^{2+}$-binding region, containing a RING finger and a B-box, followed by a coiled-coil region. At the C terminus of the Ro52 RBCC region, a well conserved B30.2 domain is not yet associated with any defined function (13, 14). It has been suggested that Ro52 is involved in transcriptional regulation (15, 16), and mono-ubiquitination of Ro52 (17), as well as interaction with a deubiquitinating enzyme, has been shown (18). Still, the function of Ro52 remains to be defined and might shed light on its targeting in autoimmune disease.

Because the two main Ro52 epitopes in systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome are both conformation-dependent, understanding the structure of Ro52 could suggest molecular reasons for the erroneous recognition. However, the low solubility of native, intact Ro52, as well as of Ro52 RING-B-box and RBCC subcloned protein constructs (~50 μM; Ref. 2), makes three-dimensional structure determination of Ro52 difficult. Indeed, little is known about the molecular and structural organization of proteins in the entire RBCC family, perhaps due to experimental limitations also for other RBCC proteins. Previous bioinformatic analysis has identified high conservation and similarity between different RBCC proteins in general and their subdomains in particular, where RING and B-box domains show similarities above 40% (8). The conserved arrangement of the domains
in the RBCC region suggests that these could form a functional entity. In agreement with this, we have shown that the Ro52 RING-B-box region and the coiled-coil fold as independent units, whereas the C-terminal B30.2 region, which is not included in the RBCC motif, is unstable (2). Recent studies suggest that RBCC proteins may be involved in homoi-
and hetero-oligomerization (19–21). However, the role of Zn$^{2+}$ binding in governing the specificity of such RBCC interactions, and how different subdomains within the RBCC motif participate on a molecular level, is not yet known.

While keeping in mind that the RBCC region appears to be a func-
tional entity, structural information regarding its building blocks are available. The RING is a cysteine-rich Zn$^{2+}$-binding motif of the form C3HC4 (22), which binds two Zn$^{2+}$ ions in a tetrahedral manner (23), and the structural topology is very similar in the nine published RING finger structures (23–31). The RING is predominantly a protein-pro-
tein interaction motif, which also acts as a ubiquitin-protein isopeptide (E3) ligase in the ubiquitination pathway (32). In the RAG1 and BRCA1 proteins, the RING forms a homodimerization (25) and heterodimer-
ization (27) interface, respectively. A general biological role for RINGs was suggested to be the building of macromolecular scaffolds for enhancements of biochemical reactions such as ubiquitination (33, 34).

The second Zn$^{2+}$-binding motif of RBCC proteins, the B-box, is so far only identified in RBCC proteins (8) and has the form CHC3H2 (35). In the structure of the B-box in the RBCC protein XNF7, four of seven putative ligands bind to only one Zn$^{2+}$ ion in a tetrahedral manner, whereas the remaining three ligands are unoccupied (36). The coiled-coil motif occurs with high frequency in proteins (2–3%) and is responsible for the formation of supercoiled dimers, trimers, and tetramers depending on sequence constraints and biological context (37).

Increased understanding of the basis for autoimmune targeting of Ro52 requires knowledge of molecular connections between targeting by patient antibodies and structural properties. We have already suc-
cessfully dissected such connections for an autoimmune epitope in the Ro52 coiled-coil region (2, 5, 38). However, the recognition of the con-
formation-dependent autoantigenic epitope in the RING-B-box region of Ro52 is far less understood. Such knowledge is crucial both for under-
standing autoimmune disease development and for designing novel bio-
therapies and diagnostics in this field (39, 40).

The aim of the present study was to resolve in molecular detail the antigenic properties and structural organization of the Ro52 RING-B-
box motif, and how these are affected by Zn$^{2+}$ binding. To this end, biophysical and immunological methods, tailored to the properties of the protein, were used jointly in a complementary way. We found that the same region that houses a conserved, dominant autoimmune epitope in the RING-B-box region is also of fundamental importance to Ro52 folding and Zn$^{2+}$ binding. Thus, the current investigation contrib-
utes to the understanding of the coupling between structural and bio-
logical properties of Ro52 in particular and in RBCC proteins in general.

**MATERIALS AND METHODS**

**Expression and Purification of Ro52 Constructs**—Ro52 RING-B-box (residues His$_n$–(1–128)) and RBCC (His$_n$–(1–258)) proteins were expressed, purified, and refolded as described elsewhere (2). Protein concentrations were estimated by UV spectroscopy at 280 nm. Experi-
mentally determined extinction coefficients (41) were 32669 cm$^{-1}$ M$^{-1}$ for the RBCC and 13366 cm$^{-1}$ M$^{-1}$ for the RING-B-box region.

**Peptide Synthesis**—All peptides were synthesized by Fmoc chemistry using solid-phase (Fmoc-PAL-PEG-PS, Applied Biosystems) on an Applied Biosystems Pioneer Peptide Synthesis System. Protected amino-
acids were supplied from Novabiochem, and trifluoroacetic acid was used to cleave the peptide from the resin and remove side-chain protec-
tion groups. Cysteine side chains were protected with acetalimidomethyl protection groups subsequently removed with Hg(II)Ac (42). An excess of dithiothreitol was used in order to ensure reduced cysteines in the resulting peptide as well as efficient removal of Hg$^{2+}$, which otherwise bound strongly to the peptides. High pressure liquid chromatography and MALDI-TOF mass spectrometry were used for peptide identification and purification (>95%), and mass spectra for the RING finger peptide, B-box peptide, and the RB linker peptide showed peaks with 5403, 4076, and 3862 Da, respectively, in agreement with the theoretical masses. Experimental extinction coefficients (41), 18,714 cm$^{-1}$ M$^{-1}$ for the RING finger and 6759 cm$^{-1}$ M$^{-1}$ for the B-box, were used for concentration determination by UV light. The concentration of the RB linker peptide was estimated by weight/volume because of the absence of strongly absorbing groups. Purified peptides were lyophilized and stored at −20 °C.

**Limited Proteolysis, Collection, and Analysis of MALDI-TOF Mass Spectrometry Data**—Purified Ro52 RING-B-box and Ro52 RBCC in 50 mm Tris, 100 mm KCl, and 10 mm β-mercaptoethanol, pH 9 and 8, respectively, were quantified and used directly in limited proteolysis experiments with trypsin (Sigma, T-4665). Range-finding experiments were done to optimize protease:protein ratios for following the degra-
dation of protein and accumulation of subsequent protein fragments (43). In the time course experiment, reactions were stopped after 1, 5, 10, 20, 50, 100, and 200 min by adding 0.2% trifluoroacetic acid solution in a 1:1 ratio, followed by trichloroacetic acid precipitation (44), and reconstituted in 20 μl of 50% acetonitrile (ACN), 0.1% trifluoroacetic acid solution. C18 ZipTips (Millipore) were used for concentration and purification of the samples before MALDI analysis. The ZipTips were washed three times with 50% ACN, 0.2% trifluoroacetic acid and acidified with 5× 10 μl of 0.1% trifluoroacetic acid solution before the sample solution was applied (20 times). After washing three times with 0.1% trifluoroacetic acid, the sample was eluted with 20 μl of 50% ACN, 0.1% trifluoroacetic acid solution. A saturated matrix solution was prepared by dissolving ~50 mg of α-cyanoaninamic acid in 50% ACN, 0.1% trifluoroacetic acid solution. Matrix was mixed with samples in a 1:1 ratio and spotted immediately on a MALDI sample plate in duplicates. Mass spectra were acquired in duplicates on a PE Biosystems Voyager MALDI-TOF mass spectrometer equipped with a nitrogen laser. To ensure that the ion yield and ion distribution are representative of the entire sample spot, the laser was rastered across the sample spot, aver-
aging 500 laser shots per spectrum. Base-line correction and integration of peaks were performed by using the Applied Biosystems Data Explorer program, version 4.0.0.0. Identification of peptide fragments was per-
formed from the mass of the fragments and the known specificity of trypsin with assistance of the ProteinProspector (see Ref. 45; prospec-
tor.ucsf.edu).

**Circular Dichroism Spectroscopy Studies**—Circular dichroism spec-
troscopy was performed on a Jobin Yvon-SpeX CD 6. For Zn$^{2+}$ binding experiments, all synthesized peptides were reconstituted from the lyophilized powder with 10 mM sodium phosphate buffer, pH 6.5. Spectra were acquired immediately and at 5 °C to reduce the risk of disulfide formation. Peptide concentrations ranged from 5 to 10 μM and with corresponding equivalent metal ion concentrations. Spectra were recorded between 190 and 260 nm with an increment of 0.5 nm. The RING-B-box was measured in 20 mM phosphate buffer, pH 9 (2), whereas the RING and the B-box peptides were assayed at pH 6.5 to avoid Zn$^{2+}$-hydroxide precipitation. Spectral evaluation and calcu-
lation of secondary structure content were performed using DICHROWEB (46, 47), which provides among others the algorithms CDSSTR (48–50), Contin (51, 52), and Selcon3 (53, 54). For the evalu-
ation, the protein reference set (4 or 7, see Refs. 50 and 55), which gave
the lower normalized root mean square deviation value as defined in Ref. 56, was chosen.

Fluorescence Spectrophotometry Studies—Fluorescence emission spectra were conducted on a Hitachi F-4500 fluorescence spectrophotometer for the B-box peptide, and on a Jobin Yvon-SpeX FluoroMax-2 for the RING finger peptide and RING-B-box region. The buffer was 10 mM phosphate buffer, pH 6.5, and 1 mM dithiothreitol, with 5% glycerol added for the RING finger and the RING-B-box. Spectra were acquired at 5 °C in a 1-cm path length quartz cell. Emission spectra were excited at 290 nm. Each scan was baseline-corrected and volume-corrected prior to tabulating the data. In Zn\(^{2+}\) titration experiments, the concentrations for the RING finger, B-box peptide, and the RING-B-box region were 15, 15, and 19 μM, respectively. B-box titrations were carried out manually, and emission intensities at maximum emission wavelength (350 nm) were recorded. Titrations of the RING finger and RING-B-box region were done automatically with a Hamilton Microlab 1000 titrator, adding 1 μl of a 3 mM ZnCl\(_2\) solution into 2 ml of sample solution every 4 s until saturation was reached. Emission was measured every second at maximum emission wavelength (350 nm for the RB region and 357 nm for the RING finger peptide). Binding constant determinations and binding curve fits were calculated using the CALIGATOR program (www.bpc.lu.se/research/caligator, see Ref. 57).

Sequence Analysis—Similarity searches on the consensus Ro52 RING-B-box region (residues 1–123) were made by BLAST (58). Multiple sequence alignments of mouse and human Ro52 together with 19 other RBCC/TRIM proteins were done with ClustalW (59). Secondary structure prediction of the Ro52 RING-B-box region was calculated by a consensus prediction program provided by PBIL-NPSA (npsa-pbil.ibcp.fr, see Ref. 60).

Antibodies—Human serum was obtained from patients with Sjögren syndrome as classified by Ref. 61. Monoclonal antibodies to Ro52 were developed by standard protocols by fusing spleen cells from mice immunized with recombinant Ro52 (38) fused with SP 2/0 myeloma cells to generate a hybridoma producing monoclonal antibodies (62). Resulting hybridoma were subcloned and characterized by Western blot and enzyme-linked immunosorbent assay (ELISA) using Ro52 deletion constructs (63).

ELISA of Ro52 Peptides and Fragments—ELISA was performed essentially according to standard procedures. In short, high binding 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 1 μg of peptide or recombinant protein in 100 μl of carbonate buffer, pH 9.6, per well. All subsequent steps were performed at room temperature. Plates were washed three times with phosphate-buffered saline, 0.05% Tween 20 (TBPS) and blocked with 200 μl of TBPS, 5% milk powder per well for 45 min. After three washings with TBPS, 100 μl of patient sera diluted 1:1,000 or mouse anti-human Ro52 hybridoma supernatant in TBPS, 1% milk powder was added, and the plates were incubated for at least 1 h. After washing three times with TBPS, the secondary antibody, alkaline phosphatase-conjugated rabbit anti-human IgG or goat anti-mouse IgG antibody (Dako) diluted 1:1,000 in TBPS, 1% milk powder was added, and the plates were incubated for 1 h. Plates were washed three times, and phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.6, was added. Absorbance was measured at 405 nm after 20 min. For ELISA with reduced antigens, the coated plates were incubated with reducing buffer (100 mM Tris, 50 mM NaCl, 10 mM dithiothreitol, pH 6.8) for 1 h prior to blocking as described previously (6).

RESULTS

Sequence Analysis of the Zn\(^{2+}\)-binding Region in Ro52—The Ro52 protein contains the RING finger, B-box, and coiled-coil domains of TRIM/RBCC proteins. A similarity search with the RING-B-box region revealed more than 20 human RBCC proteins with a similarity above 40% to human Ro52, in particular within the RING and B-box motifs (Fig. 1A). Furthermore, more than 15 RBCC proteins show high similarities in the region comprising residues 55–91 (RB linker; Fig. 1, A and B), possibly related to the degree of conservation of the RING and B-box motifs. In particular, residues 62–78 of the RB linker show an even higher conservation with five hydrophobic residues in defined positions. Secondary structure prediction predicts a high random coil propensity in the RING region, a high amount of β-sheet and random coil in the B-box, and high helix propensity in the RB linker (Fig. 1B). No prospective Zn\(^{2+}\) ligands are present in the RB linker region.

Peptide Synthesis of Tentative Building Blocks in the RING-B-box Region—To analyze the molecular basis for folding and Zn\(^{2+}\) binding in the RING-B-box region (2), peptide synthesis was employed as a tool to synthesize functional building blocks. Primary targets for synthesis were the RING, the B-box, and the RB linker region. The extension of all synthesized peptide sequences is shown in Fig. 1B. The peptide design of the Zn\(^{2+}\)-binding motifs was based on established sequence consensus in RING- and B-box domains (8) but was slightly extended to improve solubility, to include a native tryptophan for fluorescence measurements (Trp-11), and to include another putative Zn\(^{2+}\) ligand (His-123, see Fig. 1B). The RB linker peptide ranges from the end of the synthesized RING finger to the start of the consensus sequence of the B-box. The synthesis was performed on solid phase as described under “Materials and Methods.” All peptides were obtained in high yield and purity (>95%).

Zn\(^{2+}\)-dependent Folding and Secondary Structure Determination of the Ro52 RING-B-box Subregions by Circular Dichroism Spectroscopy—The CD spectra show Zn\(^{2+}\)-dependent structure formation in both the RING (Fig. 2A) and B-box peptides (Fig. 2B). The increase in ellipticity at 222 nm suggests helix formation in the RING peptide, whereas spectral changes in the B-box are more subtle. The RB linker region appears randomly structured in solution with no spectral changes on Zn\(^{2+}\) addition (Fig. 2C). Numerical analysis using spectral decomposition (TABLE ONE) confirms the increase in RING helix content in the RING with Zn\(^{2+}\) binding, which is consistent with the structural change observed for the RING finger of BRCA1 (64). The B-box contains β-sheet secondary structure with a high random coil contribution, in agreement with the published NMR solution structure of the XNF7 B-box (TABLE ONE) (36). The RB linker peptide consists mainly of random coil with a small β-sheet contribution (TABLE ONE), in contrast to the helical structure content predicted from sequence alone (Fig. 1B).

Theoretical spectra of the RING-B-box region with and without Zn\(^{2+}\) were calculated from the sum of the RING, B-box, and RB linker CD spectra with and without Zn\(^{2+}\) (Fig. 2D). Comparison of these theoretical spectra with the experimental RING-B-box region spectrum in the absence of Zn\(^{2+}\) shows a significantly lower ellipticity at 222 nm in the entire RING-B-box region. Thus, the RING-B-box region without Zn\(^{2+}\) appears more structured than the sum of structure content in its constituting subregions. No comparison with the Zn\(^{2+}\)-bound RING-B-box region could be made, because CD spectra with Zn\(^{2+}\) of this region were unobtainable due to precipitation (2).

Determination of Zn\(^{2+}\) Binding Constants by Fluorescence Spectrophotometry—Fluorescence spectroscopy was employed to study the changes on Zn\(^{2+}\) binding in the local environment of the single tryptophans in the RING and B-box peptides and of the two corresponding tryptophans in the entire RING-B-box region. The intensity of the fluorescence emission in the RING and B-box peptides increases on Zn\(^{2+}\) binding, with no wavelength shift (Fig. 3, A and B). In contrast, the
fluorescence emission intensity in the RING-B-box region is strongly reduced on Zn\textsuperscript{2+} binding, suggesting an altered structural effect compared with that in the Zn\textsuperscript{2+}-binding motifs, when studied separately. Furthermore, the RING-B-box emission maximum is shifted to lower wavelength, indicating tryptophan burial in a hydrophobic environment (Fig. 3C).

Zn\textsuperscript{2+} binding constants for the RING and B-box peptides and for the entire RING-B-box region were obtained from nonlinear fitting of the intensity change to appropriate binding equations using CALIGATOR (Fig. 3, D–F, and TABLE TWO) (57). The data of the RING-B-box region were fitted with a custom-made version of the CALIGATOR software introducing a three-binding site model, which will be published elsewhere. The general procedure has been outlined previously (57). Despite the presence of eight putative Zn\textsuperscript{2+} ligands in the B-box of Ro52, the fit is excellent to a one-site binding model with micromolar affinity (Fig. 3E and TABLE TWO). A single Zn\textsuperscript{2+}-binding site is also found in the B-box of XNF7 with seven tentative ligands but with higher affinity (6.1 \times 10\textsuperscript{-8} M, see Ref. 12). For the RING finger, a three-binding site model gave an optimal fit (Fig. 3D), where the high affinity site is most likely occupied first (TABLE TWO). This is similar to the RING finger of the breast and ovarian cancer-associated protein BRCA1, where sequential and anticooperative Zn\textsuperscript{2+} binding to the two sites has
been reported \( K_d = 2.6 \times 10^{-8} \) M and \( 7.9 \times 10^{-6} \) M, see Ref. 64), and similar results were obtained for the promyelocytic leukemia protein (24).

The fit of the RING-B-box fluorescence required a three-binding site model (Fig. 3F), where two of the sites have high affinity (TABLE TWO). Thus, the RING-B-box region binds \( \text{Zn}^{2+} \) much more efficiently than the RING and B-box peptides alone. The increase in affinity could be related to increased structure content in the RING-B-box region compared with that in the sum of its parts. This would be in agreement with the magnitude of maximum emission wavelength shift on \( \text{Zn}^{2+} \) binding, suggesting increased burial of the RING and B-box tryptophans when linked together in the RING-B-box region. Extrapolating from the single \( \text{Zn}^{2+} \)-binding motifs, it is likely that one of the high affinity sites is located in the RING, whereas the second high affinity site can be assigned either to the RING or to the B-box. Although the RING-B-box region fit did not improve by using a four-binding site model, a much weaker site \((\sim 10^{-3} \) M) was detected in a separate experiment titrated to higher \( \text{Zn}^{2+} \) equivalents (data not shown). This could indicate that the third binding site of the RING, which was weak already in the peptide, was even weaker in the entire RING-B-box region and possibly unspecific.

Development of a New Strategy for Analyzing MALDI Data Over a Large Mass Range in Limited Proteolysis Experiments of Ro52—To map structural organization within the RBCC and the RING-B-box regions and further effects of \( \text{Zn}^{2+} \) binding, time-resolved proteolysis was employed (43), surveyed by MALDI mass spectrometry (65–67) over the entire fragment mass range. Trypsin was chosen to assay the presence of unfolded and/or exposed regions, because 36 well distributed cleavage sites (Lys and Arg residues) are present in the RBCC region (Fig. 1).

Several steps were taken to ensure maximal information gain from the time-resolved proteolysis experiment. Previous studies have shown

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**TABLE ONE**

| Peptide | \( \alpha \)-Helix | \( \beta \)-Sheet | Turns | Random coil |
|---------|------------------|-----------------|-------|-------------|
| \( \text{Zn}^{2+} \) | \( +\text{Zn}^{2+} \) | \( \text{Zn}^{2+} \) | \( +\text{Zn}^{2+} \) |
| RING    | 7                | 15              | 10    | 67          |
|         | 13               | 11              | 12    | 64          |
|         | 11               | 11              | 14    | 56          |
| B-box   | 16               | 25              | 16    | 43          |
|         | 4                | 11              | 11    | 34          |
|         | 11               | 34              | 21    | 34          |
| RB linker | 2               | 19              | 13    | 65          |
|         | 6                | 16              | 13    | 65          |

---

**FIGURE 2.** CD spectra of Ro52 subregions. CD spectra of the RING peptide (A), B-box peptide (B), and RB linker (C) are shown in the absence (solid line) and presence (dashed and dotted line) of \( \text{Zn}^{2+} \). CD spectra of the recombinant RING-B-box construct (D) are shown as calculated from CD data of single peptides in the presence and absence of \( \text{Zn}^{2+} \) (dashed and solid line, respectively) and as experimental data without \( \text{Zn}^{2+} \) (dotted line).
Structural and Functional Mapping of the Ro52 RBCC Region

![Diagram](image)

**TABLE TWO**

| Construct          | \( K_{d1} \)  | \( K_{d2} \)  | \( K_{d3} \)  |
|--------------------|---------------|---------------|---------------|
| B-box peptide      | \( 3.5 \times 10^{-5} \) M | \( 9.2 \times 10^{-5} \) M | \( 6.1 \times 10^{-5} \) M |
| RING finger        | \( 2.2 \times 10^{-5} \) M | \( 1.8 \times 10^{-6} \) M | \( 3.4 \times 10^{-6} \) M |
| RB region          | \( 3.1 \times 10^{-10} \) M | \( 6.1 \times 10^{-5} \) M | \( 3.4 \times 10^{-6} \) M |

The calibrated normalized relative peak intensities were used for all further evaluations.

To obtain a gauge for how often a cleavage site is cleaved relative to other cleavage sites, the calibrated relative peak intensities of all fragments containing the cleavage site at either end, and at a specific time point, were scaled by the sum of all calibrated relative intensities in the same time point according to Equation 2,

\[
\bar{C}_c = \frac{\sum I_i}{2 - \sum I_i - 2I_{FL}} \quad (\text{Eq. 2})
\]

where \( C_c \) is defined as the cleavage propensity and is a site-specific and time-specific measure. \( I_i \) is the relative intensity of a peak corresponding to a fragment where either end has been cleaved C-terminal to residue \( i \); \( \bar{I}_i \) is the relative intensity of a peak corresponding to a fragment cleaved only N- or C-terminally, meaning this fragment belongs to the N or C terminus of the full-length protein; and \( I_{FL} \) is the relative intensity of the full-length protein. In the nominator, the sum of all calibrated relative intensities of every peak in one time point is two, because every fragment is taken into account twice (the N- and C-terminal cleavage site) and must be subtracted by the relative intensities of fragments containing the N or C terminus of the full-length protein, because they are taken into the numerator only once, and by the doubled relative intensity of the peak corresponding to the full-length protein if present, because it never occurs in the numerator.

Together, the calibration of relative peak intensities and the definition of cleavage propensities simplify evaluation and visualization of time-resolved proteolysis data. Factors limiting the resolution of the analysis include that only the major factors that affect peak intensities have been calibrated and that differences may relate to the relative scaling in the definition of cleavage propensities. Furthermore, a decrease of relative cleavage propensity with time is likely due to subsequent cleavage into smaller fragments that are not included in the cleavage propensity, because the lower limit of the observation range was set to 1500 Da.

that it is possible to visualize the difference in cleavage patterns from MALDI-TOF data by using normalized relative peak intensities, providing the data are reproducible with acceptable deviations (68, 69). Trichloroacetic acid precipitation of the Ro52 RBCC (see "Materials and Methods") gave high quality MALDI spectra (Fig. 4), and duplicate data sets were highly consistent over the entire observed fragment mass range (1500–31,000 Da). Acquired mass spectra of the time course experiment of the Ro52 RBCC region are exemplified in Fig. 4, where the entire Ro52 RBCC region is detected both after 20 and 50 min of digestion in the presence of Zn\(^{2+}\), whereas the corresponding peak disappears after 20 min in the absence of Zn\(^{2+}\). Starting from a set of unambiguously assigned fragments, correlated fragments with higher and lower masses were consequentially identified. Out of 77 detected MALDI peaks, 70 peaks were unambiguously assigned to Ro52 RBCC fragments (supplemental TABLE ONE). The assigned fragment peaks cover the whole original sequence of the RBCC construct.

To calibrate relative peak intensities \( \bar{I}_i \) versus different laser intensities, delay times, and fragment sizes over the wide mass range addressed, they were multiplied by a mass factor, taken into account the corresponding mass of each peak, according to Equation 1,

\[
\bar{I}_i = I_i (1 + \varepsilon(x)) \quad (\text{Eq. 1})
\]

where \( \varepsilon(x) \) is a mass-dependent linear equation (supplemental Fig. 1). This equation was empirically determined by monitoring the change of relative peak intensities corresponding to different masses through a change in delay time at different laser intensities (supplemental Fig. 1).

FIGURE 3. Zn\(^{2+}\) dependence of fluorescence emission wavelength scans in Ro52 subregions. A, RING finger (0–4 eq ZnCl); B, B-box (0–4 eq ZnCl); C, entire Ro52 RING-B-box region (0–10 eq ZnCl). Each scan resembles one Zn\(^{2+}\) concentration, shown in different line styles, where the lowest line indicates the value at 0 added Zn\(^{2+}\) for the RING and the B-box peptides at maximum intensity wavelength, and the uppermost line for the RING-B-box region. D–F show the fluorescence emission recorded at the maximum emission wavelength as a function of Zn\(^{2+}\) concentration (open circles), and the corresponding MALDI fits (solid line) that give the binding constants in Table I. D, the RING finger; E, B-box; and F, the entire Ro52 RING-B-box region. Data from A, D, or C, and F, respectively, do not correspond to each other and are taken from different experiments.
FIGURE 4. MALDI-TOF mass spectrometry spectra of Ro52 RBCC region digested with trypsin in the presence and absence of Zn$^{2+}$. Spectra recorded after 20 and 50 min of digestion are shown. Prior to addition of protease, only the full-length Ro52 RBCC region was present (data not shown). Spectra are divided into two different mass ranges for better visualization. Numbers above the peaks correspond to the position of the fragment in the native Ro52 sequence. Numbers in the upper corners of each panel resemble the absolute intensity of the base peak.

Modular Organization of the Ro52 RBCC and RING-B-box Regions and Alterations on Zn$^{2+}$ Binding—To obtain qualitative information about the tertiary structure organization of the Ro52 RBCC region in the absence and presence of Zn$^{2+}$, relative cleavage propensities of the trypsin cleavage sites during a time-resolved proteolysis experiment were determined (Fig. 5A). Cleavage C-terminal to residues 6, 148, 177, and 235 is observed early and to a high degree in the experiment, which indicates that these residues are exposed and/or not part of a folded region. In contrast, the RB linker appears well protected from proteolytic digestion in the presence and absence of Zn$^{2+}$, as shown by low cleavage propensities at residues 55, 61, 67, 77, and 90. Although RING finger peptides are known to be stabilized by Zn$^{2+}$ binding, the proteolysis data of the RBCC region suggests a high protection from enzymatic digestion even in the absence of Zn$^{2+}$. The reason for this is the lower number of cleavage sites present in the RING region (only residue 45). Range-finding experiments were used to determine the optimal enzyme concentration over the time range addressed. These experiments were designed to find conditions that produce fragments that are small, but larger than the minimal fragments corresponding to complete digestion of the protein. To prove the effect of Zn$^{2+}$ on the proteolytic pattern of the RING region, subsequent proteolysis experiments on the RING-B-box construct at different enzyme concentrations were performed (see below and Fig. 6). A stabilizing effect of Zn$^{2+}$ was clearly visible at higher enzyme concentrations. Cleavage propensities in the B-box are higher, especially in absence of Zn$^{2+}$. The suggested coiled-coil region includes two stable regions, comprising residues 150–175 and 186–217, where cleavage occurred hardly or not at all at residues 164, 168, 169, 195, 202, and 205. The high cleavage propensity at residue 177 suggests local exposure and/or flexibility and could indicate a structural subdivision of the coiled-coil region. Differences in the coiled-coil region between samples with and without Zn$^{2+}$ are small but interesting: sites 155 and 163 appear slightly less cleaved in the presence of Zn$^{2+}$, although there are no Zn$^{2+}$-binding sites in this part of the protein.

By analyzing the time dependence of peak intensities of stable fragments, further information on folded subregions in the Ro52 RBCC region and their Zn$^{2+}$ dependence was obtained (Fig. 5, B–D). Stable fragments include 7–57, 99–121, 122–147, 178–258, 217–258, and 235–258 (Fig. 5, B–D) and are reflected in the increasing cleavage propensity at their end points (Fig. 5A). Fragments comprising the entire coiled-coil region include 119–258, 120–258, and 122–258 (Fig. 5B), where the N-terminal cleavage sites are part of the C-terminal region of the B-box. Although these fragments appear more stable with higher relative intensities in the absence of Zn$^{2+}$, it is more likely that their N-terminal cleavage sites are more protected when Zn$^{2+}$ is bound to the B-box, thus reducing their relative amount. This is supported by the fact that fragments His$_6$-(1–164) and His$_6$-(1–148), covering the entire RING-B-box region and cleavage sites 118, 119, and 121, are significantly more stable in the presence of Zn$^{2+}$ (Fig. 5C), most likely because cleavage sites 118, 119, and 121 are cleaved to a lesser extent. Among the smaller stable fragments (Fig. 5D), fragment 7–57 covering most of the RING finger is much more stable in the presence of Zn$^{2+}$, because there are no fragments presently cleaved at site 45 and only a few with low intensity cleaved at site 55. Fragment 178–258 is fairly stable, because it is accumulated to a high degree after 20 min of digestion. The accentuated decrease in relative intensity in comparison with fragment 7–57 may be due to the presence of a higher number of cleavage sites in fragment 178–258. The fragment 99–121 gives further support for the higher protection of B-box cleavage sites 98, 118, and 119 in the presence of Zn$^{2+}$, because this fragment has higher relative intensities at earlier time points (Fig. 5D).

The same experiment was performed with the RING-B-box region alone. Stable fragments were obtained for both the RING and the B-box region. During mild reaction conditions, with a protease: protein ratio of 1:225, the B-box part is more stable to proteolytic cleavage in the presence of Zn$^{2+}$ (Fig. 6A). As can be followed in the time course (Fig. 6B), the fragments 92–118, 85–118, and 85–128 remain for longer time in the presence of Zn$^{2+}$, whereas the fragment 109–118 is produced rapidly in the absence of Zn$^{2+}$, most likely due to Zn$^{2+}$-dependent protection of residue 108. In particular, fragment 85–128, covering the entire B-box, is still present with a strong peak after 200 min of digestion in the presence of Zn$^{2+}$, whereas the fragment 109–118 is produced rapidly in the absence of Zn$^{2+}$, because it has vanished already after 20 min in the absence of Zn$^{2+}$. To obtain fragments containing the RING finger region or parts of it, the protease:protein ratio had to be increased 10-fold. Under these conditions, and in the absence of Zn$^{2+}$, a strong peak corresponding to fragment 7–45 and a weaker peak corresponding to 7–55 appear after 30 min of digestion (Fig. 6C). In the presence of Zn$^{2+}$, the peaks are still visible but in a lower relative intensity compared with peaks corresponding to parts of the B-box or the RB linker. Thus the RING finger sites 45 and 55 seem to be protected from protease activity and are highly affected by Zn$^{2+}$ binding. Cleavage at residues 61, 67, 77, and 84 in the RB linker occurs frequently in the RING-B-box region alone, both in the absence and presence of Zn$^{2+}$, producing fragments 46–77, 62–77, 68–91, 68–77, 68–91, and 68–84. Taken together, the results of the proteolysis of the RING-B-box region alone, and of the entire RBCC region, show that a stabilizing
The effect of Zn$^{2+}$ binding is primarily localized to the RING and B-box motifs. In addition, two stable regions were identified in the coiled-coil region, one of which is affected by Zn$^{2+}$ binding to the RING-B-box (approximately residue 163). A most interesting finding is that although cleavage in the RB linker is quite pronounced in the RING-B-box region alone, it is essentially abolished in the entire RBCC region. This suggests that the RB linker is involved in further interactions in the entire RBCC region.

Characterization of Autoantigenic Epitopes in the RING-B-box Region and Its Subregions by ELISA—The antigenicity of the RING-B-box region, the RING finger, the B-box, and the RB linker was analyzed by ELISA using anti-Ro52-positive patient sera (Fig. 7A). Mouse anti-hu-

FIGURE 5. Cleavage propensities in the RBCC region as detected by MALDI-TOF-MS in the time-resolved proteolysis experiment. A, three-dimensional views of relative cleavage propensity at all cleavage sites of the Ro52 RBCC region (annotated on x axis) over time (y axis). Peak heights are proportional to the relative cleavage propensity at each cleavage site. Results in the presence (upper panel) and absence (lower panel) of Zn$^{2+}$ are displayed separately. The large three-dimensional views show only the cleavage sites, whereas the small three-dimensional view includes the entire RBCC region sequence (including the numbering of cleavage sites) in the same scale as the schematic overview of the Ro52 RBCC region (bottom). B, time course profiles from Ro52 RBCC tryptic digestion of significantly stable and large C-terminal fragments. C, large N-terminal fragments. D, smaller stable fragments, where (119–149) means that all fragments in the range of residues 119–149 are summed for each individual time point. All profiles are shown in the presence (upper panel) and absence (lower panel) of Zn$^{2+}$. A fragment is defined as stable if present at several time points during the proteolysis.
man Ro52 monoclonal antibodies from hybridoma supernatants were analyzed with the same constructs and to the RBCC region (Fig. 7, B–D). In previous studies, patient immunoglobulins were reported to bind the entire RING-B-box region under reducing conditions only (6), which we could confirm. No binding was detected for the RING finger or the B-box in the presence or absence of Zn\(^{2+}\)/H\(_{11001}\), neither with patient sera nor with the monoclonal antibodies. However, the monoclonal antibodies bound the RB linker, which was not recognized by patient sera (Fig. 7, A and B). Furthermore, all monoclonal antibodies recognized the RING-B-box as well as the RBCC region (Fig. 7C). Reducing conditions did not affect monoclonal antibody binding to the RING-B-box region compared with the reduced RING-B-box region was, however, observed for all monoclonal antibodies (Fig. 7D), suggesting that the coiled-coil region interferes with the binding site of the antibodies. Even larger differences between the RING-B-box and the RBCC region were observed in the unreduced state, with particularly low monoclonal antibody responses to the RBCC region (Fig. 7C). The monoclonal antibody recognition profiles in reducing and nonreducing conditions (Fig. 7C) suggest that monoclonal antibodies 7.1E8, 7.7G2, and 7.12E11 bind to similar epitopes.

DISCUSSION

Our main focus has been to investigate how Zn\(^{2+}\) binding affects the structural organization of the RBCC motif. We have shown previously (2) that the Ro52 RING-B-box region and the entire coiled-coil form intact folding units in the absence of Zn\(^{2+}\), suggesting a structural interaction between the two Zn\(^{2+}\)-binding motifs. Furthermore, it has been shown that antibody binding to the RING-B-box region is affected by reducing conditions, thereby suggesting antibody binding to structure-dependent epitopes (6). We have also identified recently a disease-related native epitope in the coiled-coil region (5). From these studies, questions arose regarding how Zn\(^{2+}\) binding affects the RING-B-box region as a whole, and whether Zn\(^{2+}\) binding results in structural and/or functional changes in the coiled-coil region.

Limitations in solubility of the Ro52 RING-B-box and RBCC regions in the presence and absence of Zn\(^{2+}\) required a careful choice of methods that could provide information at reasonably low concentrations (\(~10\ \mu\text{M}\)). CD and fluorescence were used to map changes in local and secondary structure for the smaller subregions, and MALDI-MS was employed to estimate tertiary structure organization and its possible Zn\(^{2+}\)-dependent changes within the entire RBCC region. The choice of antibody binding analysis for bifunctional analysis was obvious due to its molecular resolution and is, for Ro52, both structure- and disease-related.

MALDI-TOF-MS has proved itself to be an invaluable detection tool for analysis of enzymatic digestion patterns of protein fragments (2, 38, 65, 68, 69). Because of its high sensitivity and formidable resolution, the results provide more information than the conventional gel detection method, where fragments below 10 kDa of mass are hardly separable. This study is the first where the MALDI-MS analysis covers the entire mass range, from the uncleaved full-length protein (30.7 kDa) to its fingerprint region (500–3000 Da), which increases the information yield. Our novel evaluation method facilitates access to this large body of information, including the degree of digestion at each cleavage site.
which allows for increased structural interpretation compared with looking at stable fragments only.

The current results can be put together in a schematic structural model of the functional and molecular properties of the Ro52 RING-B-box and RBCC regions (Fig. 8). The RBCC region of Ro52 was studied at three levels as follows: A, the separate subregions of the RING-B-box region; B, the entire RING-B-box region; and C, the RBCC entity (Fig. 8). Both previous proteolysis experiments (2) and current MALDI analysis, including smaller mass fragments, consistently show that the RBCC part is organized in two main parts, the RING-B-box region and the coiled-coil domain. Subdivision of the RING-B-box region into smaller entities was made based on sequence analysis.

As separate subregions, the RING finger binds Zn\(^{2+}\) most strongly, with three binding sites, and is structurally most affected by ion binding, whereas the B-box binds one Zn\(^{2+}\) only at intermediate affinity and with less structural effects. The RB linker between the two zinc fingers is unstructured in isolation and does not bind Zn\(^{2+}\) but is a major antigenic target (Fig. 8A).

The entire RING-B-box region (Fig. 8B) appears more structured as an entity than would be predicted from the single motifs. The RING-B-box region has two high affinity Zn\(^{2+}\)-binding sites, one of which is most likely located in the RING, and the second could be assigned to either motif (drawn in the B-box in Fig. 8B). The total Zn\(^{2+}\) binding affinity to the entire RING-B-box region is ~1000-fold higher than would be predicted from its parts (TABLE ONE). The fluorescence quenching and blue shift upon Zn\(^{2+}\) binding suggests tryptophan burial, which is not observed in the RING or B-box alone, and B-box residues in the vicinity of the tryptophan become proteolytically protected on Zn\(^{2+}\) binding in the RING-B-box region. Taken together, it is highly suggestive that upon Zn\(^{2+}\) binding to the entire RING-B-box region, the RING and the B-box both contribute to a jointly folded RING-B-box core, which may also involve the RB linker (Fig. 8, A and B).

The MALDI-MS detected proteolysis experiments give hints both toward the identity and stability of folded entities within the RBCC region, as well as to their relative tertiary organization (Fig. 8C). Increased folding of both the RING and the B-box with Zn\(^{2+}\) is observed in both the RING-B-box and in the RBCC region. Furthermore, the RB linker is significantly more proteolytically protected in the RBCC region than in the RING-B-box region. The RB linker also seems to be a structural unit in the RBCC region even in the absence of Zn\(^{2+}\). Notably, cleavage at residue 163 in the coiled-coil region is reduced in the presence of Zn\(^{2+}\), which indicates interactions with the Zn\(^{2+}\)-dependent RING-B-box region. Indeed, the reduced cleavage of the RB linker in the presence of the coiled-coil could thus result from reciprocal interactions. Together, this makes it possible to outline the tertiary structure organization of the RBCC region of the Ro52 protein in the presence of Zn\(^{2+}\) (Fig. 8C).

The proteolysis data are consistent with previous immunological mapping, which suggests that the Ro52 regions 2–11 and 107–122 are surface-exposed in the absence of Zn\(^{2+}\) (70). This is mirrored in our
proteolysis experiment, where residue 6 is highly accessible independent of Zn\(^{2+}\) presence, and the mentioned B-box cleavage sites are prone to tryptic cleavage if no Zn\(^{2+}\) is present. Notably, the putative coiled-coil region of Ro52 is cleaved early at residue 177 and to a high extent, suggesting a loose coiled-coil. Although it has been reported that the predicted leucine zipper motif in Ro52 promotes dimer formation (16), analytical ultracentrifugation and analysis in a mammalian two-hybrid system show only weak dimer formation for the Ro52 coiled-coil (2). Indeed, the high accessibility of residue 177 to enzymatic cleavage is consistent with locally low probability of coiled-coil formation in residues 170–180 as predicted by three different programs (71–73). In this respect, it is interesting to note that the immune response in Ro52-positive mothers where the children are not affected by congenital heart block have their main immune response directed to the region between amino acids 176 and 196 (63). Autoimmune responses toward exposed loops have also been described previously in autoantigens targeted in rheumatic diseases (74).

A dominant antigenic epitope in the RING-B-box region seems to be located in the RB linker, which bound with high affinity to monomolecular antibodies in ELISA, whereas no immune response was detected to the RING or B-box with patient sera or monoclonal antibodies. Previous studies report patient autoantibody responses to two constructs ranging from residues 1 to 69 and 68 to 120, both including parts of the RB linker (6), and to the entire RING-B-box region 1 to 128 (2). In this perspective, it is confusing that patient sera do not give a response to any of the subregions that form the RING-B-box region. However, our results suggest that the RB linker folds in the context of the protein and could participate in a range of interactions as follows: with the RING, the coiled-coil, and possibly also with the B-box, whereas in isolation, the RB linker is unstructured. Notably, the monomolecular antigenic response was directed toward the isolated RB linker. All monomolecular antibodies also bound the RING-B-box and RBCC regions, although the binding was clearly reduced when the coiled-coil region was present. This could suggest that the monomolecular antibodies recognize mainly linear epitopes in the RB linker, which are less accessible in the folded molecular contexts of the larger Ro52 fragments. In particular, the lower monoclonal antibody recognition of the RBCC compared with the RING-B-box (Fig. 7D) supports our data indicating an interaction between the RB-linker and the coiled-coil domain. The particularly low RBCC responses for some monoclonal antibodies could suggest that cysteine oxidation under nonreducing conditions stabilizes interacting conformations that further conceal the epitope. In contrast, the patient antibodies appear to recognize a native epitope that involves part of the N-terminal flanking residues, will be required to determine their specific contribution to folding and interactions in Ro52.

By taking all experiments into account, the region between the RING Zn\(^{2+}\)-binding motifs referred to as the RB linker seems to be of major importance for determining the overall fold, both of the RING-B-box region and for the entire RBCC region. It has a remarkable conservation, seems to be a stable entity in a protein context, and creates strong antigenic response in mice. The present results give improved molecular understanding of Ro52 molecular functionalities in a disease-related context and opens up for further investigations of other RBCC motifs.

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