

## Zinc Ion Dependent B-Cell Epitope, Associated with Primary Sjogren's Syndrome, Resides within the Putative Zinc Finger Domain of Ro60kD Autoantigen: Physical and Immunologic Properties

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The Ro/La ribonucleoprotein (RNP) complex is composed of the proteins Ro60kD, Ro52kD, and La48kD that are in association with one small cytoplasmic RNA (YRNA). Specific protein–RNA and protein–protein interactions are thought to occur through the RNP and zinc-finger secondary structure elements of the Ro60kD protein. The aim of our study was to investigate the antigenic properties of the zinc finger domain of the Ro60kD autoantigen and its contribution to the formation of Ro/La RNP complex. It was found that the peptide VSLVCEKLCNEKLLKKARIHPFHILIA (Zif-1), which corresponds to the natural sequence of the zinc finger domain (301–327), and the peptide C<sub>(Acm)</sub>NEKLLKKARIC<sub>(Acm)</sub>, analogous to the intermediate loop 310–319 (Zif-3) of the same domain of Ro60kD, are recognized by the majority of anti-Ro/SSA and anti-La/SSB positive sera (82.6% and 77.1%, respectively) in the absence of zinc ions. The same sera failed to react with Zif-1 peptide in the presence of Zn<sup>2+</sup>. In contrast, the addition of zinc ions was necessary for the binding of Zif-1 to recombinant Ro52kD as shown by direct binding experiments of the recombinant protein with synthetic peptides. Our data suggest the zinc finger domain of Ro60kD contains a B-cell epitope with high specificity for primary Sjogren's syndrome. Furthermore, depending on the presence of zinc ions, the zinc finger domain of the Ro60kD protein can exist in two different conformational states favoring either an interaction with the Ro52kD protein or binding with autoantibodies.

### Introduction

The Ro/SSA ribonucleoprotein complex (RNP) is composed of at least three proteins (Ro60kD, Ro52kD, and La48kD) in association with one small cytoplasmic RNA (YRNA). These proteins are of particular interest because they are targets of autoantibodies in patients with primary Sjogren's syndrome (pSS) and systemic lupus erythematosus (SLE).<sup>1</sup> In particular, the Ro60kD polypeptide holds a central role in the formation of the Ro RNP complex because it is associated with both YRNA and Ro52kD polypeptide.<sup>2–4</sup> It has been proposed that the specific protein–RNA and protein–protein interactions occur through the RNP and zinc finger secondary structure elements located on the 91–161 and 305–323 regions of the Ro60kD protein, respectively. The RNP motif has been well characterized as the YRNA binding site of the Ro60kD autoantigen.<sup>5</sup> On the other hand, the zinc finger motif, originally described as a DNA recognition sequence, has been previously found to serve also as an interface for protein–protein interactions, and it has been postulated that it plays a major role in the Ro60–Ro52 association.<sup>6–8</sup>

The clinical significance of the anti-Ro60kD antibodies prompted several investigators to define the antigenic determinants of the protein. A number of studies have clearly demonstrated that the autoantigen contains both

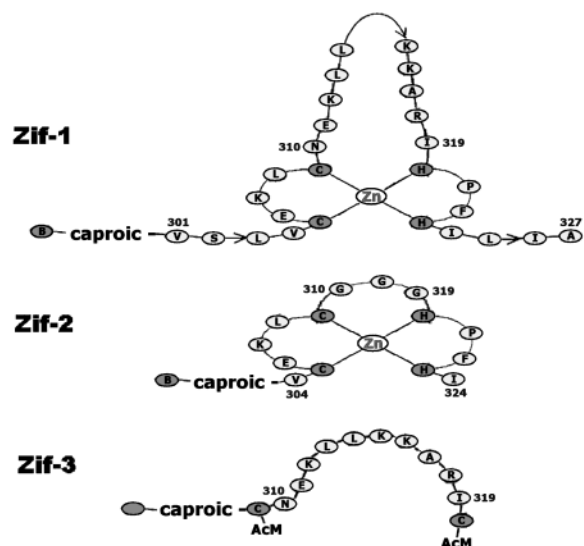
linear and conformational epitopes.<sup>9,10</sup> However, the majority of autoantibodies are directed against conformational epitopes.<sup>11,12</sup> The zinc finger domain of Ro60kD is a good candidate for a conformational epitope because both the secondary structure, affected by the binding of zinc ions, and the redox conditions can induce specific conformational changes favoring the recognition by antibodies.<sup>13,14</sup> In addition, the Zn<sup>2+</sup> binding domain of Ro52kD protein has been recently described as a target for conformation-dependent autoantibodies in the Ro60kD counterpart.<sup>15</sup>

In this report we have investigated the recognition of the Ro60kD zinc finger domain by autoantibodies and examined the role of the same region in the interaction with the Ro52kD protein using (i) a synthetic peptide corresponding to the sequence 301–327, which constitutes the full-length zinc finger motif in the Ro60kD protein (peptide VSLVCEKLCNEKLLKKARIHPFHILIA (Zif-1)), (ii) a truncated peptide containing the segments necessary for zinc binding but lacking the intermediate loop 310–319 of the zinc finger domain (Zif-2), and (iii) an intermediate loop peptide analogue without the zinc-binding segments (Zif-3). It was shown that Zif-1 and Zif-3 peptides are recognized by antibodies from the majority of the anti-Ro/SSA positive patients with primary Sjogren's syndrome, while the truncated peptide Zif-2 did not react against the same sera. The biotinylated Zif-1 and Zif-2 peptides were tested for their binding to Ro52kD. It was demonstrated that the presence of zinc ions was essential for the

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**Figure 1.** Schematic representation of the synthetic peptide analogues of the full-length zinc finger domain of Ro60kD protein (Zif-1), its truncated form without the intermediate loop 310–319aa (Zif-2), and the extended form of the intermediate loop 310–319aa (Zif-3) (Acm: acetamido group).

interaction between Zif-1 and Ro52kD. In contrast, the autoantibody binding to the zinc finger domain analogue (Zif-1) depended mainly on the absence of zinc cations. These findings suggest that the Ro60kD zinc finger domain may exist in different conformations favoring either the formation of the Ro-La RNP complex or the autoantibody recognition.

## Experimental Section

**Sera.** Sixty nine sera from patients with primary Sjogren's syndrome (pSS), 37 sera from patients with systemic lupus erythematosus (SLE), 15 sera from patients with rheumatoid arthritis, seronegative arthritis, and systemic vasculitis (disease controls), and 37 sera from normal individuals were tested. Patients with pSS were diagnosed according to the European–American classification criteria of primary Sjogren's syndrome, and patients with systemic lupus erythematosus were diagnosed according to the revised criteria of the American College of Rheumatology. Antibodies to Ro60 and La autoantigens were determined in all sera using counterimmunoelectrophoresis and immunoblot as previously described. It was found that 52 sera from pSS or SLE had anti-Ro60 autoantibodies while 46 sera had both anti-Ro60 and anti-La/SSB autoantibodies.<sup>18</sup>

**Peptide Synthesis and Purification.** The synthesis of H-V<sup>301</sup>SLVCEKLCNEKLLKKARIHPFHILIA<sup>327</sup>-NH<sub>2</sub> (Zif-1), H-VCEKLCGGGHPFHI-NH<sub>2</sub> (Zif-2), and H-C(Acm)NEKLLKKARIC(Acm)-NH<sub>2</sub> (Zif-3) (Acm = acetamido group) (Figure 1) was carried out with a stepwise solid-phase procedure on 4-methylbenzylamine resin (4-MBHA, substitution 0.36–0.38 mequiv/g), using the Boc-strategy.<sup>19</sup> Serine was introduced as Boc-Ser(Bzl)-OH, cysteine as Boc-Cys(Bzl)-OH or Boc-Cys(Acm)-OH, glutamic acid as Boc-Glu(OBzl)-OH, lysine as Boc-Lys(2Cl-Z)-OH, arginine as Boc-Arg(Tos)-OH, and histidine as Boc-His(Boc)-OH. The coupling reactions were performed using a molar ratio of amino acid/TBTU/HOBt/DIEA/resin 3:3:3:9:1. Biotinylated peptides were obtained after coupling of the N-terminus with aminocaproic acid as a spacer arm and biotin, TBTU, HOBt, and DIEA following the same procedure. When the synthesis of the peptides was accomplished, the peptides were cleaved from the resin with anhydrous hydrogen fluoride in the presence of anisole, *p*-cresol, and dimethyl sulfite (DMS) as scavengers. The crude peptides were purified by semipreparative HPLC and were identified by analytical HPLC and electrospray ionization mass spectrometry (ESI-MS). The

formation of the Zif-1/Zn<sup>2+</sup> and Zif-2/Zn<sup>2+</sup> complexes was assessed by ESI-MS (Figures 2 and 3).

**Recombinant Ro52kD and La48kD Proteins.** Recombinant Ro52kD protein was purchased from Immunovision (Immunovision, Springdale, AR). Recombinant La48kD protein was prepared from a La/SSB cDNA as previously described<sup>20</sup> and purified by poly(U)-Sepharose affinity chromatography.<sup>21</sup>

**ELISA for Antibodies to Zif-1, Zif-2, and Zif-3 in the Absence of Zinc Ions.** Anti-peptide antibodies were detected by a modified ELISA assay using Costar microtiter plates. Elisa microtiter plates were coated overnight at 4 °C with 5 μg/mL synthetic peptide in a carbonate buffer, pH 9.6. After blocking for 1 h at room temperature, with 5 mM 1,10-phenanthroline in 2% BSA/PBS solution, sera were added in a dilution 1/120 and incubated overnight at 4 °C. Plates were washed twice with PBS and incubated with anti-human IgG conjugated to alkaline phosphatase (1/1400) for 1 h at room temperature. After the mixture was washed three times with PBS, *p*-nitrophenol substrate was added to the plates and the optical density (OD) was measured at 410 nm at room temperature.

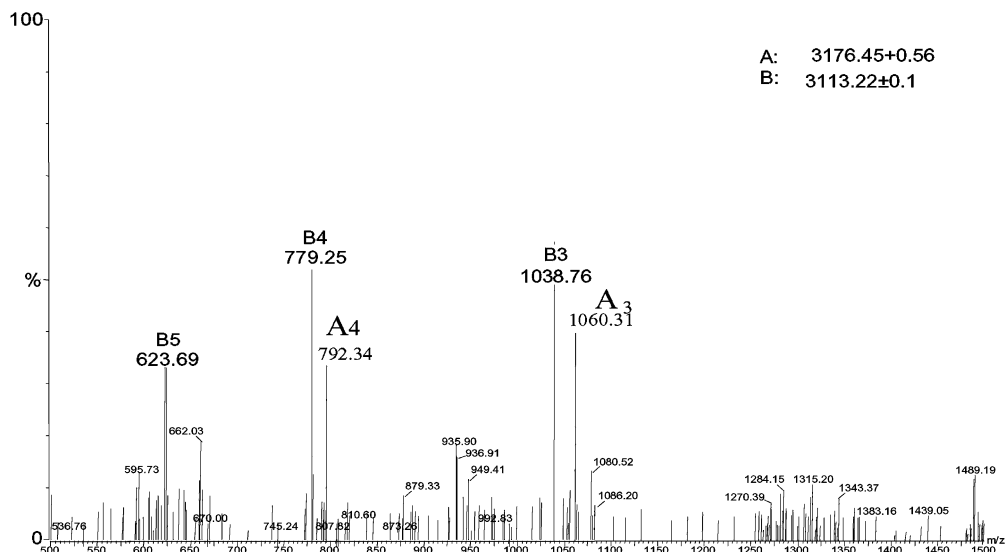
**ELISA for Antibodies to Zif-1, Zif-2, and Zif-3 in the Presence of Zinc ions.** ELISA microtiter plates (Costar) were coated overnight at 4 °C with 5 μg/mL synthetic peptide in phosphate buffer, pH 6.3, containing 35 mM DL-dithiothreitol and 5 mM ZnCl<sub>2</sub>. After blocking for 1 h at room temperature with 5 mM zinc chloride in 2% BSA/PBS solution, sera were incubated overnight at 4 °C in a 1/120 dilution. Plates were washed twice with PBS and incubated with anti-human IgG conjugated to alkaline phosphatase (1/1400) for 1 h at room temperature. After washing the plates with PBS, the color was developed using *p*-nitrophenol substrate at room temperature.

**Binding of Ro52kD to Zif-1 and Zif-2 Peptides.** The binding of recombinant human Ro52 to Zif-1 and Zif-2 peptides was tested by ELISA. The recombinant human Ro52 antigen in a concentration of 3.3 μg/mL in carbonate buffer, pH 9.6, was added into the wells of a Costar high-binding microtiter plate and incubated overnight at 4 °C. After blocking for 1 h at room temperature with either 5 mM 1,10-phenanthroline or 5 mM zinc chloride in 2% BSA/PBS solutions, Zif-1 and Zif-2, (15 μg/mL) peptides were incubated overnight at 4 °C either in the presence or in the absence of zinc ions. Plates were washed twice with PBS, and streptavidin/peroxidase (1/1000) in 2% BSA/PBS was added and incubated for 1 h at room temperature. After the plates were washed in a similar manner, the color was developed by adding a peroxidase substrate solution. As controls, recombinant La/SSB and albumin were tested for their interaction against Zif 1 and Zif 2 peptides, using the same procedure

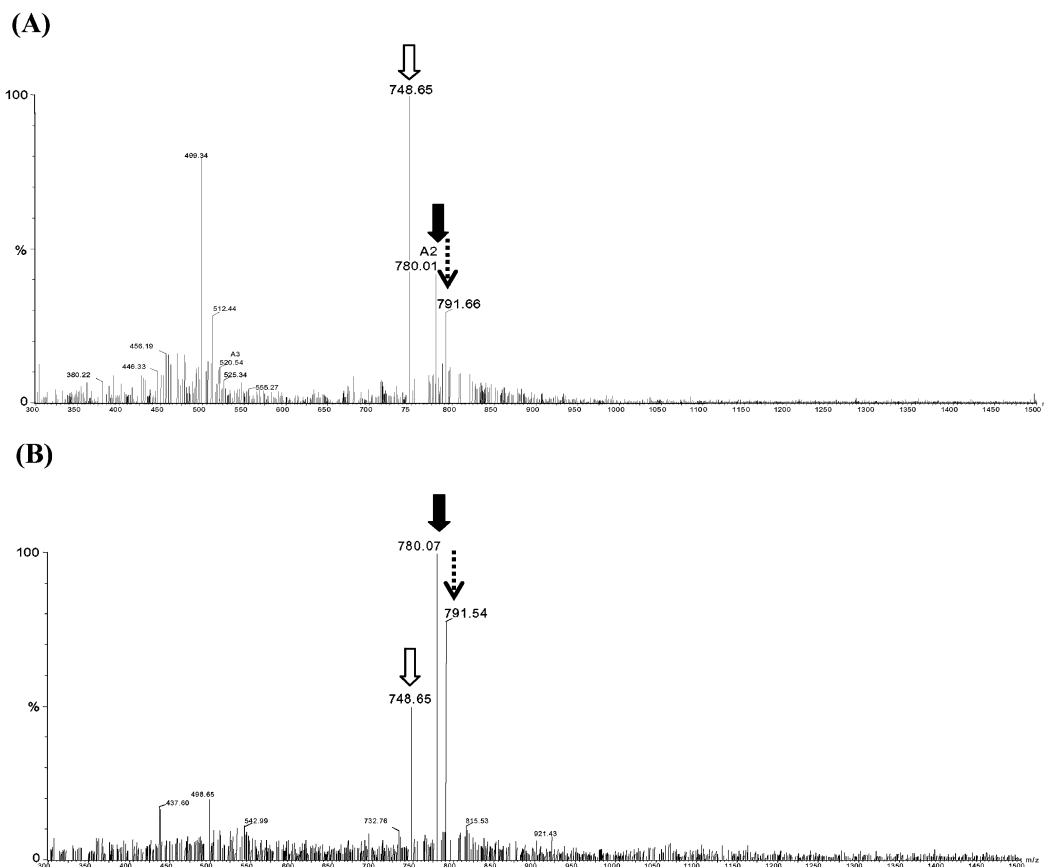
## Results

**Zif-1 and Zif-2 peptides directly coordinate zinc ions.** Studies with electrospray ionization mass spectrometry (ESI-MS) demonstrated that the peptides Zif-1 and Zif-2 readily formed complexes with zinc at pH 6.5. The Zif-1/Zn<sup>2+</sup> and Zif-2/Zn<sup>2+</sup> ratio was 1/1 as suggested by the molecular mass of the ESI-MS ions (Zif-1/Zn<sup>2+</sup> complex, (M + H<sup>+</sup>)<sub>calculated</sub> = 3177, (M + H<sup>+</sup>)<sub>found</sub> = 3176.45; Zif-2/Zn<sup>2+</sup> complex, (M + H<sup>+</sup>)<sub>calculated</sub> = 1558, (M + H<sup>+</sup>)<sub>found</sub> = 1559.49) (Figures 2 and 3). As expected, the Zif-3 peptide, which lacks the zinc-binding segments, did not coordinate zinc ions. The molecular mass of the detected Zif-3 ion corresponded to its calculated neat molecular weight.

We also aimed to study the kinetics of the interaction between zinc finger peptides Zif-1 and Zif-2 and zinc ions. Figure 3 indicates that the ratio of zinc-bound to free Zif-2 increased from 0.4:1 to 2:1 (5-fold) with an increase of the incubation time of the peptide with Zn<sup>2+</sup> from 3 to 15 h (5-fold increase). Thus, prolonged coating of the ELISA plates (overnight coating in a buffer



**Figure 2.** Electrospray ionization mass spectrometry reveals the interaction of the intact zinc finger domain of Ro6KD (Zif-1) with zinc ions in a ratio of 1:1. Molecular ions 1060.31 (A<sub>3</sub>) and 792.54 (A<sub>4</sub>) correspond to the complex Zif-1/Zn<sup>2+</sup> with  $(M + H^+) = 3176.45 \pm 0.56$ , while molecular ions 1038.76 (B<sub>3</sub>), 779.25 (B<sub>4</sub>), and 623.69 (B<sub>5</sub>) correspond to the peptide Zif-1 alone with  $(M + H^+) = 3113.22 \pm 0.10$ .

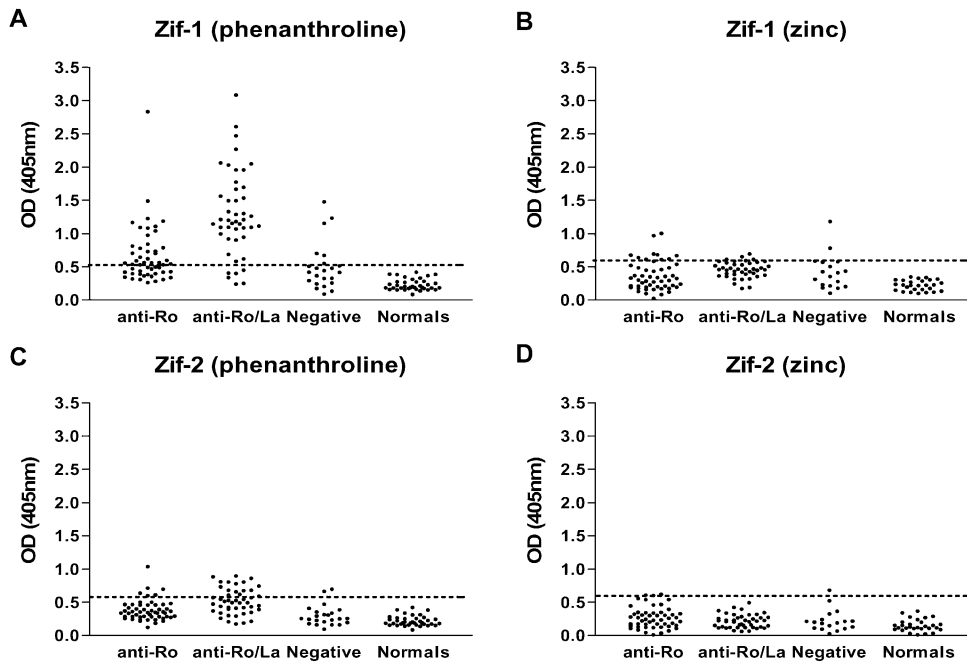


**Figure 3.** ESI-MS kinetic analysis of the Zif-2/Zn<sup>2+</sup> complex formation (in 1:1 ratio) after 3 h (A) and after 15 h (B). The open arrow corresponds to the peptide Zif-2 alone with  $(M + H^+)_{\text{found}} = 1495.89 \pm 0.43$  and  $(M + H^+)_{\text{calculated}} = 1495.71$ . The filled arrow indicates the formation of the complex Zif-2/Zn<sup>2+</sup> with  $(M + H^+)_{\text{found}} = 1558.55 \pm 0.60$  and  $(M + H^+)_{\text{calculated}} = 1559.10$ . The hatched arrow points to the complex Zif-2/Zn<sup>2+</sup> with the additional coordination of one molecule of H<sub>2</sub>O with  $(M + H^+)_{\text{found}} = 1581.06 \pm 4.09$  and  $(M + H^+)_{\text{calculated}} = 1577.1$ . The ratio of zinc bound Zif-2 (filled or hatched arrows) to free Zif-2 peptide (open arrows) rose 5-fold after an increase of the incubation time, of free Zif-2 with Zn<sup>2+</sup>, from 3 to 15 h.

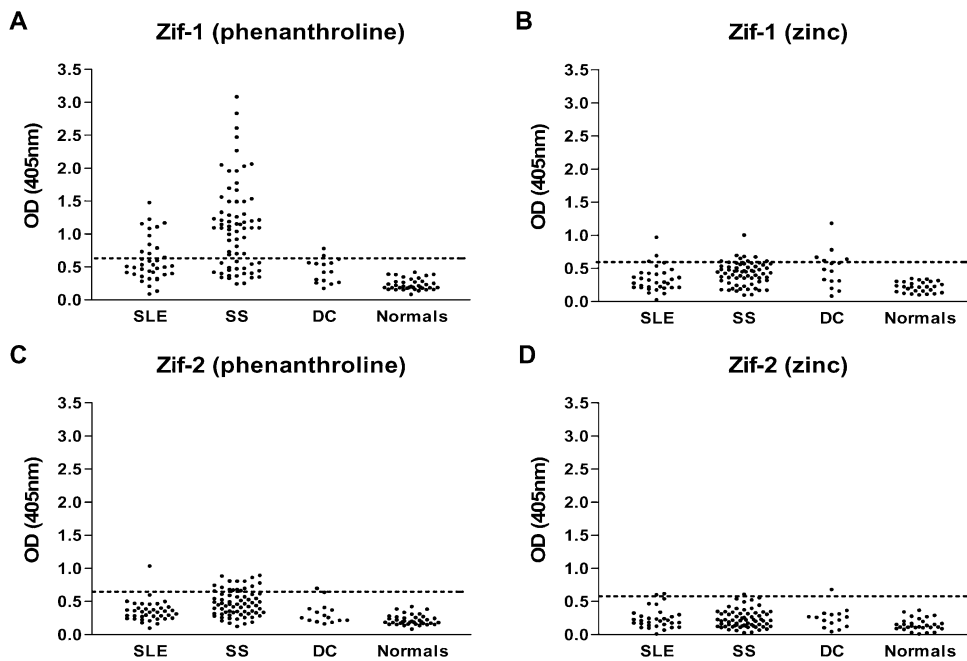
containing Zn<sup>2+</sup> ions) was adopted to achieve the optimum zinc/peptide formation for the interaction with human autoantibodies.

**Zinc ions negatively affect the recognition of the Ro60kD zinc finger domain of Ro60kD protein by autoantibodies.** By use of the ELISA assay described

above, 69 sera of patients with primary Sjogren's syndrome and 37 sera from SLE patients were tested against the zinc finger peptide analogue (Zif-1). In the presence of zinc ions, the Zif-1 peptide reacted with only 4.8% of anti-Ro60/La and 15.4% of anti-Ro60 positive sera. When the zinc cations were removed, using the



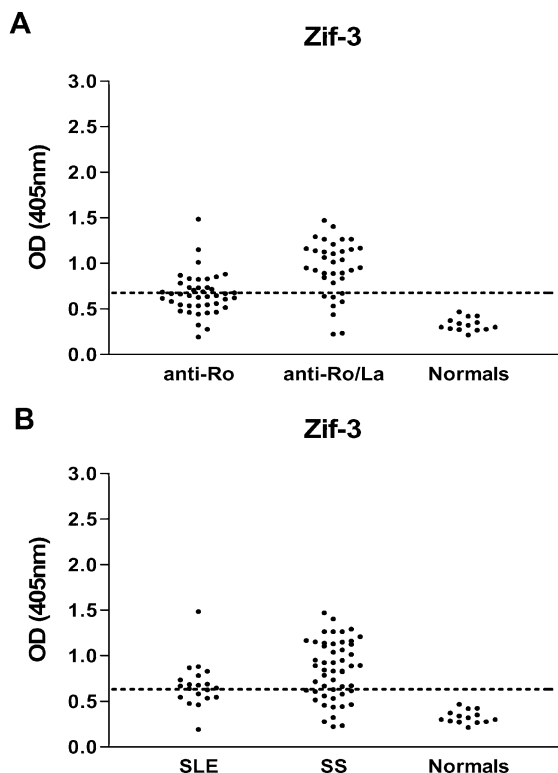
**Figure 4.** Prevalence of serum reactivity according to autoantibody specificity to Zif-1 and Zif-2 peptides in the presence and the absence of zinc ions. (A) The majority of anti-Ro and anti-La positive sera (82.6%) and 34.6% of anti-Ro positive sera bound on the synthetic peptide analogue of the zinc finger domain of Ro60kD (Zif-1) after removal of zinc ions. Patients' sera without anti-Ro and/or anti-La autoantibodies gave only a limited reaction, while sera from normal individuals were all negative. (B) Zif-1 in the presence of zinc ions did not significantly react with all sera tested. (C) The truncated form of the zinc finger domain (Zif-2) presented a very low reactivity with some sera with anti-Ro and anti-La or anti-Ro antibodies alone in the absence of zinc ions. (D) No reactivity against Zif-2 was observed in the presence of zinc ions.



**Figure 5.** Distribution of serum reactivity according to diagnosis of patients. (A): The majority of sera from primary Sjogren syndrome patients (SS) (69.6%) and 29.7% of SLE sera reacted with the synthetic peptide analogue of zinc-finger domain of Ro60kD (Zif-1) after removal of zinc ions. (B): Zif-1 in the presence of zinc ions did not significantly react with all sera tested. (C): minimal reaction was observed with SS and SLE sera against the truncated form of zinc finger domain (Zif-2) in the absence of zinc ions and (D): no reactivity against Zif-2 was observed in the presence of zinc ions.

chelating agent 1,10 phenanthroline, 82.6% of anti-Ro60/La and 34.6% of anti-Ro60 positive sera bound strongly to the Zif-1 peptide analogue. Sera with anti-Ro60/La exhibited a statistically significant higher binding on Zif-1, compared to anti-Ro60 positive sera alone ( $\chi^2 = 22.96$ ,  $p < 0.001$ ) (Figure 4). When the statistical analysis was performed according to diagnosis

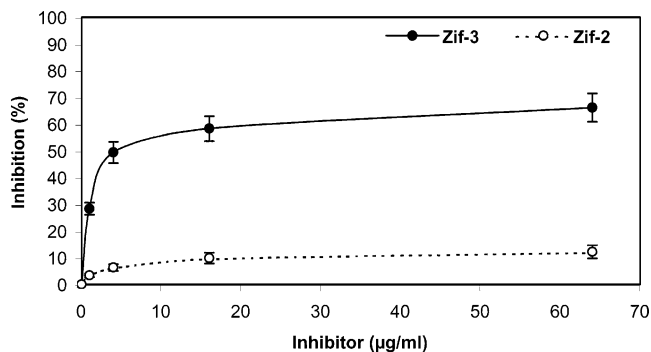
of diseases, it was found that patients with pSS had statistically significant higher proportion of positivity, compared to patients with SLE (pSS, 69.6%; SLE, 29.7%,  $\chi^2 = 15.49$ ,  $p < 0.001$ ). Normal sera remained negative, even after the 1,10-phenanthroline addition. A minor reaction was observed in 13.3% of sera from disease controls (Figure 5). Although a minority of the



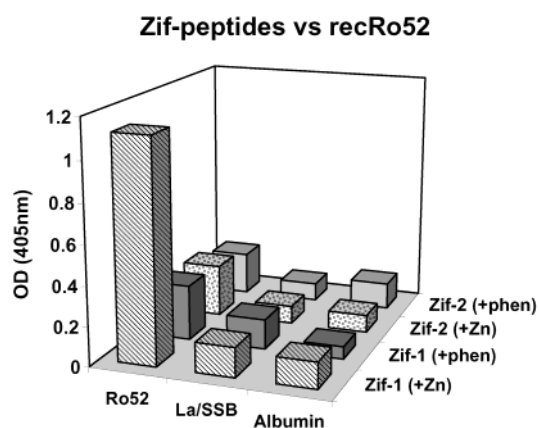
**Figure 6.** Prevalence of reactivity of different sera against the synthetic peptide analogue corresponding to the intermediate loop of the Ro60kD zinc finger domain 310–319aa. Most of the anti-Ro/La positive sera (71.1%) (A) and primary Sjogren's syndrome sera (62.7%) (B) reacted with this region, indicating that the major B-cell epitope of the Ro60kD zinc finger domain is located in the intermediate loop of the zinc finger secondary structure element.

autoimmune sera recognized the Zif-1 analogue in the presence of zinc ions, their mean OD values (0.356 and 0.448 for anti-Ro60 and anti-Ro60/La sera, respectively) were significantly lower than the mean ODs obtained from the evaluation of the same sera against the Zif-1 peptide in the absence of zinc ions (0.648 and 1.329 for anti-Ro60 and anti-Ro60/La sera, respectively). These results can be interpreted in two ways. First, a specific extended conformation of the Zif-1 peptide, disrupted by zinc binding, is required for recognition by autoantibodies. Second, the antibody binding depends on interaction with cysteine and histidine residues, released only after the removal of the zinc ions. To distinguish between these alternative interpretations, Zif-2 truncated peptide was tested against the same set of sera.

**The 310–319 region of the zinc finger domain of the Ro60kD autoantigen is essential for the interaction with autoantibodies.** The Zif-2 peptide, which possess the zinc binding segments but lacks the intermediate loop 310–319, gave only a minor reaction with all the sera tested (Figures 4 and 5). The removal of zinc ions did not significantly affect the recognition by autoantibodies, indicating that Cys and His residues are not directly involved in the autoantibody binding. This observation was further confirmed by testing the intermediate loop 310–319 analogue, peptide Zif-3, against the same set of sera. As expected, the majority (77.1%) of anti-Ro60/La and 39.5% of anti-Ro60 positive sera recognized the Zif-3 peptide analogue in contrast to normal sera, which did not give any positive reaction



**Figure 7.** Dose-dependent inhibition of antibody binding on the full-length zinc finger domain of the Ro60kD protein (Zif-1) by the intermediate loop (310–319aa) peptide analogue (Zif-3) or its truncated peptide lacking the intermediate loop (Zif-2). As depicted, Zif-3 produced an inhibition of 66.2% in the antibody binding on the intact zinc finger domain analogue. Zif-2 had no inhibitory effect.



**Figure 8.** Zif peptides vs recRo52, showing the determination of human recRo52kD, recLa/SSB, and human albumin binding on different forms of the zinc finger region of Ro60kD, using biotinylated peptide analogues. RecRo52 was reacted only with the biotinylated intact zinc finger domain of the Ro60kD protein (Zif-1) in the presence of zinc ions. Removal of zinc ions from Zif-1 or elimination of the intermediate loop (Zif-2) abolished Ro52–peptide interaction. Recombinant La/SSB or human albumin did not interact with any form of the peptides.

(Figure 6). The mean OD values of anti-Ro60/La sera were also higher (0.931) in comparison with the OD values from the evaluation of anti-Ro60 positive sera (0.657) when tested against the Zif-3 peptide.

**Inhibition experiments demonstrate that autoantibodies specifically recognize the 310–319 region within the zinc finger domain.** To explore the specificity of the reaction between autoantibodies and the zinc finger domain of Ro60kD (Zif-1), we performed inhibition experiments, using the Zif-2 and Zif-3 truncated peptide analogues as inhibitors. It was found that the Zif-3 peptide (310–319 region analogue) was able to inhibit 66.2% of the binding of autoantibodies to the full zinc finger domain. In contrast, the Zif-2 peptide (which lacks the 310–319 region) only inhibited 12% of the binding (Figure 7). Given that small peptide moieties in solution cannot efficiently compete with surface-immobilized peptides for interaction with antibodies (monovalent vs bivalent binding),<sup>22,23</sup> our data suggest that the main target of autoantibodies for the zinc finger domain of Ro60kD is located within the 310–319 intermediate loop.

**The intact zinc finger domain of the Ro60kD interacts with the Ro52kD autoantigen in the presence of zinc ions.** To investigate the role of the zinc finger domain of Ro60kD in the Ro RNP complex formation, we examined the interaction of the biotinylated Zif-1 and Zif-2 peptides with recombinant Ro52kD, using a specific ELISA assay. Recombinant Ro52kD reacted with the Zif-1 peptide (analogue of the complete zinc finger region) in the presence of zinc ions, while the same reaction was not observed with truncated Zif-2 peptide (Figure 8). Removal of zinc ions with 1,10-phenanthroline abolished the binding of Zif-1 peptide to Ro52kD, indicating that the full-length zinc finger folded structure is required for Ro60kD–Ro52kD interaction. Control proteins La/SSB and albumin did not interact with Zif-1 and Zif-2 peptides either in the presence or in the absence of zinc ions (Figure 8).

## Discussion

Little is known about the functional structure of the Ro ribonucleoprotein complex, the mechanisms involved in the breakdown of the immune tolerance, and the establishment of an autoimmune response against the Ro autoantigen in systemic lupus erythematosus and Sjogren's syndrome. Ro60kD plays a central role in the assembly of the Ro RNP complex, interacting with both YRNA and the Ro52kD protein,<sup>2–4</sup> as well as in the antigenicity of the complex because it has been described as the main target of conformation-dependent autoantibodies.<sup>11,12</sup> Previous data suggest that the immune response against the Ro60kD antigen can easily expand to the other protein components of the complex through intermolecular epitope spreading.<sup>24–26</sup>

The zinc finger domain of the Ro60kD antigen is of particular interest because it can potentially serve as an interface for protein–protein interactions with the Ro52kD protein,<sup>6–8</sup> as well as a conformation-dependent target for autoantibodies.<sup>15</sup> In addition, the antibody binding capacity of this region may also be modulated by zinc binding and the redox conditions. Thus, in some cases zinc binding is essential for the antibody recognition,<sup>13</sup> while in other cases reducing conditions, without the presence of zinc, are required.<sup>14</sup>

The analogue of the zinc finger motif of the Ro60kD antigen, Zif-1 peptide, was found to coordinate zinc ions in a ratio of 1:1 (peptide/Zn<sup>2+</sup>) under slightly acidic conditions (to avoid oxidation of –SH groups of Cys residues). We first evaluated the autoantibody binding to the Zif-1 peptide in the presence or absence of zinc ions. Autoantibodies readily recognized the Zif-1 peptide in the absence of zinc ions but failed to react with it in the presence of Zn<sup>2+</sup>. The interaction of autoantibodies with the Zif-1 peptide via its cysteine<sup>305</sup>, cysteine<sup>309</sup>, histidine<sup>320</sup>, and histidine<sup>323</sup> residues, which are available in the absence of Zn<sup>2+</sup>, seems unlikely because (i) the same residues, present in the Zif-2 peptide, did not induce any antibody binding activity to Zif-2 and (ii) the Zif-3 peptide possessing Acn-protected cysteine residues in positions 309 and 320 and lacking the cysteine and histidine residues in positions 305 and 323 (Figure 1) was specifically recognized by anti-Ro positive sera. An alternative possibility is that in addition to the cysteine and histidine residues, which directly coordinate zinc ions, other residues of the intermediate 310–319 loop may interact inter se in the folded, hairpin-like struc-

ture of the 310–319 region, induced by zinc binding (Figure 1). Such interactions between non-zinc-binding residues have been found in other known zinc finger structures and are thought to play a stabilization role.<sup>27</sup> If this is the case, the removal of zinc ions by the addition of a chelating agent or the deletion/protection of cysteine/histidine side chain groups would disrupt the folded structure and release these amino acids, making them available for antibody binding. Thus, residues without zinc-binding capacity may be exposed after unfolding/different folding of the 310–319 loop, increasing the antigenicity of the peptide. This sequence of events could explain well the observed antigenicity of our zinc finger peptides.

On clinical grounds, this study demonstrates that the Ro60 kD zinc finger domain holds a conformational epitope recognized preferentially by sera with anti-Ro60 and anti-La/SSB antibodies (82.6%). In addition, antibodies to this region are more frequently found in sera of primary Sjogren's syndrome patients (70% prevalence) than in sera of SLE patients (30% prevalence). Providing that anti-Ro60 antibodies have a low disease specificity, the presence of antibodies against this region appears to have a discriminatory effect between pSS and SLE and is potentially a useful diagnostic tool between anti-Ro60 positive sera.

We next examined the capacity of the zinc finger motif of Ro60kD (Zif-1 peptide) to interact with the recombinant Ro52kD protein. The Zif-1 peptide–Ro52kD interaction occurred only in the presence of zinc ions, emphasizing the functional role of the putative zinc finger motif of the Ro60 protein for Ro52kD binding. The truncated control peptide Zif-2 did not react with Ro52kD protein either in the presence or in the absence of zinc ions. Furthermore, La/SSB recombinant protein, a member of the RoRNP complex that interacts with hyRNA, and an irrelevant protein such as albumin did not possess any binding capacity on the zinc finger domain of Ro60kD. These findings suggest the high specificity of the reaction between the Ro60kD zinc finger domain and Ro52kD, and they are in agreement with previous studies reporting that only the full-length functional zinc finger structure can form the suitable interface for protein–protein interactions.<sup>6–8</sup>

Taking these together, our data suggest that the zinc finger domain of Ro60kD can exist in two different conformational states, depending on the binding of zinc ions: one is the denatured (extended in conformation) form, which is recognized by autoantibodies (in the absence of zinc), and one is the native folded form, which interacts with the Ro52kD protein in the presence of zinc ions. Previous epitope-mapping experiments from our laboratory revealed the existence of linear epitopes within or near the RNA-binding domains of the La and Ro60kD autoantigens, indicating that the denatured form of the antigens are the targets of autoantibodies.<sup>28</sup> However, the native forms of autoantigens are required for the Ro RNP complex assembly, and the intact Ro RNP particle is mandatory for the intermolecular spreading of the autoimmune response within the Ro RNP complex.<sup>24,29</sup> One possible explanation for this paradox is that besides the intermolecular spreading, which requires the intact Ro RNP particle, intramolecular spreading involving the denatured/isolated forms

of the autoantigens also occurs. Both intermolecular and intramolecular spreading have been reported for all the protein components of Ro RNP.<sup>24–26,30</sup> The two different states of the zinc finger domain of Ro60kD may reflect the existence of the autoantigen in two different redox environments, one in the interior of the cell (reductive conditions) where the cysteine residues are available for zinc binding (as thiol groups) and one in the cell membrane or in the extracellular space where the conditions are oxidative and the cysteine residues form disulfide bonds abrogating their ability to bind zinc ions.<sup>31</sup> This hypothesis is further supported by previous studies showing that the Ro60kD autoantigen is translocated onto the cell membrane in vitro after UV irradiation or viral infection of keratinocytes.<sup>32,33</sup> In addition, these stimuli can function in a final common pathway, generating reactive oxygen species (ROS). ROS such as H<sub>2</sub>O<sub>2</sub>, hydroxyl radicals, and nitric oxide can serve as agents for both oxidative damage of the zinc-coordinating cysteine centers<sup>34</sup> and induction of apoptosis.<sup>35,36</sup> In this regard, previous studies have shown that Ro52 and Ro60 autoantigens are localized in discrete cell surface blebs during apoptosis: Ro60 in the larger structures called “apoptotic bodies” and Ro52 in the smaller blebs,<sup>37</sup> suggesting that disruption of the native structure and disassembly of the Ro RNP particle had occurred. In the final stage, progression of apoptosis to secondary necrosis can be associated with presentation of the modified (oxidized) Ro autoantigens to the immune system.<sup>38</sup>

In summary, this study demonstrated the existence of a B-cell epitope within the zinc-finger domain of Ro60kD with high specificity for primary Sjogren's syndrome. This epitope can be potentially used for the development of new diagnostic methods. Furthermore, the identification of two distinct functional conformational states of the Ro60kD zinc finger domain, involved either in the interaction with Ro52kD or in antibody binding, provides new insights into the understanding of mechanisms related to autoimmunity of ribonucleoprotein complexes and creates a new field for therapeutic intervention. Thus, peptide analogues of secondary structures, such as the zinc finger motifs, can be designed with the potential to specifically block Ro RNP complex formation and to prevent intermolecular spreading of autoimmune response, without the danger of being neutralized by the anti-Ro60kD autoantibodies.

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