

Reactivity With Dichotomous Determinants of Ro 60 Stratifies Autoantibody Responses in Lupus and Primary Sjögren's Syndrome

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Objective. Analysis of B cell determinants of Ro 60 exposed on the surface of apoptotic cells (apoptopes) or intracellular epitopes provides insight into the structural forms of the autoantigen that break immune tolerance. This study was initiated to compare anti-Ro 60 responses in systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (SS) against membrane-bound and intracellular forms of Ro 60.

Methods. The reactivity of autoantibodies from patients with SLE and primary SS to Ro 60 apoptopes and epitopes was assessed by multiparameter flow cytometry and solid-phase immunoassay. Anti-Ro 60 IgG was eluted from early apoptotic cells or recombinant Ro 60 immobilized on nitrocellulose, and binding to membrane-bound and intracellular forms of Ro 60 was quantitated by flow cytometry.

Results. An immunodominant apoptope, which was recognized by IgG from a subset of SLE patients with anti-Ro, but not anti-La, autoantibodies, was mapped to a region forming a helix-loop-helix at the apical tip of the Ro 60 molecule. Immobilization of this region to the solid phase exposed an epitope that was recognized by IgG from primary SS and SLE patients whose sera had both anti-Ro and anti-La autoantibodies. Autoantibodies eluted from either the surface of apoptotic cells or

the Ro 60 epitope on the solid phase were non-cross-reactive and specifically recognized membrane-bound or cytoplasmic forms of Ro 60.

Conclusion. This is the first example of a dichotomy of human autoantibody responses against mutually exclusive determinants linked to a single domain of a systemic autoantigen and supports a model in which tolerance is broken by different immunogenic forms of Ro 60.

The 60-kd Ro/SSA autoantigen is a conserved RNA binding protein that is a major target of humoral autoimmunity in systemic lupus erythematosus (SLE), primary Sjögren's syndrome (SS), and the neonatal lupus syndrome (1,2). Structural analysis of *Xenopus laevis* Ro 60 (78% identical to human Ro 60) has revealed that Ro 60 is shaped like a doughnut, with noncoding cytoplasmic (Y) RNAs binding to a region of α -helical repeats on the outside surface of the ring and misfolded single-stranded RNAs binding the inner hole (3). The regions involved in RNA binding appear to overlap with some autoepitopes mapped by recombinant fragments and linear peptides (4). However, delineation of B cell epitopes of Ro 60 by conventional immunoassays remains incomplete because the response is directed largely against conformational epitopes that are lost during the denaturation steps required to solubilize Ro 60 and not represented by peptide-based immunoassays (5,6).

We have recently developed a flow cytometric method that uses gated apoptotic and permeabilized live cells together with soluble Ro 60 fragments to map apoptopes (epitopes exposed on the surface of apoptotic cells) and intracellular epitopes expressed by native Ro 60 (7). An apoptope within the Ro 60 amino acid (aa) 82–244 region has been identified that is highly specific

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for a subset of anti-Ro-positive SLE patients without anti-La/SSB antibodies and not bound by IgG from patients with primary SS (8).

Subsequent RNase digestion experiments have revealed that the Ro 60 apotope is masked by RNA in the cytoplasm and presented as a neoepitope on the cell surface during apoptosis (9). Mapping of B cell responses to different immunogenic forms of Ro 60 by flow cytometry can therefore be used to identify new diagnostic markers as well as to provide insight into the mechanisms by which different cellular forms of a systemic autoantigen may break immune tolerance (10). Since maternal anti-Ro 60 antibodies are thought to initiate tissue damage in congenital heart block by binding to apotopes on the surface of apoptotic fetal cardiocytes, flow cytometric mapping of Ro 60 apotopes has the potential to identify pathogenic subpopulations of maternal anti-Ro 60 autoantibodies (11).

The current study was initiated to map apotopes of Ro 60 protein that are exposed on the surface of early apoptotic cells and to study their disease specificity. An immunodominant domain of Ro 60 was differentially recognized either as an apotope on the apoptotic cell surface or as an epitope on the intracellular Ro/La RNP complex. Reactivity with these 2 mutually exclusive determinants stratified anti-Ro 60 responses in SLE and primary SS. These findings reveal a previously unrecognized level of heterogeneity of anti-Ro 60 responses against distinct determinants contained within a single domain of the autoantigen and support the concept of disease-specific pathways of anti-Ro 60 autoimmunity.

PATIENTS AND METHODS

Patient and control sera. Sera characterized as positive for anti-Ro 60, with or without anti-La, by counterimmunoelectrophoresis and by enzyme-linked immunosorbent assay (ELISA) (RELISA; Immuno Concepts) were collected from 32 patients with SLE and from 26 patients with primary SS. Patients with SLE met the American College of Rheumatology 1982 revised criteria for SLE (12), and patients with primary SS fulfilled at least 4 of the 6 American-European Consensus Group classification criteria (13). Control sera were collected from 20 healthy donors and from a patient with high-titer anti-RNP antibodies. This study was approved by the Clinical Ethics Committee of the Flinders Medical Centre.

IgG was purified from patient and control sera on protein A-Sepharose columns according to the manufacturer's recommendations (Pharmacia).

Cell culture and induction of apoptosis. Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamate, penicillin, and streptomycin. Early apoptosis was induced with 1 μ g/ml of staurosporine for 3 hours at 37°C as described elsewhere (7) and

verified by microscopic observation of cell size and morphology and by flow cytometric analysis of phosphatidylserine exposure (annexin V binding). To hinder the progression of early apoptotic cells into late apoptosis, all washing and IgG incubation steps were performed at 4°C.

Mapping Ro 60 apotopes by multiparameter flow cytometry. Soluble fragments of mouse Ro 60 aa 82–146, aa 82–192, aa 149–244, and aa 193–236 expressed as maltose binding protein (MaBP) fusion proteins were prepared from pMAL complementary DNA vectors and purified by maltose-affinity chromatography according to the manufacturer's recommendations (New England BioLabs). The specificity of anti-Ro 60 antibody binding to early apoptotic cells was determined by inhibition experiments using the MaBP-Ro 60 fragments as described previously (7,8).

Briefly, 0.5 mg/ml of IgG from SLE patients previously shown to bind Ro 60 aa 82–244 on the surface of apoptotic cells (8) was incubated for 1 hour at room temperature with 0.2 mg/ml of each of the MaBP-Ro 60 fragments or MaBP control. IgG-fusion protein mixtures were then added to apoptotic cells for 1 hour at 4°C. After washing, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (DakoCytomation) for 30 minutes at 4°C. Early and late apoptotic cell populations were distinguished by annexin V-allophycocyanin and propidium iodide (PI) staining as described previously (14). IgG binding to early apoptotic cells was analyzed on a FACSCanto instrument (BD) and expressed as the mean fluorescence intensity (MFI). The binding of anti-Ro 60 IgG to early apoptotic cells in the presence or absence of each MaBP-Ro 60 fragment was compared with that of MaBP and was expressed as the percentage inhibition. Patient IgG were considered positive for Ro 60 apotope reactivity if the IgG binding was 3 SD above the mean IgG binding obtained in a group of 12 healthy controls and an MaBP-Ro 60 fragment reduced anti-Ro 60 IgG binding by >20% as compared with an MaBP control. Data were assessed by Fisher's exact test.

ELISA. Soluble recombinant MaBP-Ro 60 aa 193–236 or MaBP control (5 μ g/ml in 0.1M sodium bicarbonate buffer, pH 9.6) were immobilized onto hydrophilic microtiter plates (MaxiSorp; Nunc) for 1 hour at 37°C. Nonspecific sites were blocked for 1 hour at 37°C with 3% skim milk powder in phosphate buffered saline (PBS), washed with PBS–0.5% Tween 20 (PBST), and incubated with 0.1 mg/ml of IgG from either healthy controls, SLE patients, or primary SS patients. Bound IgG was detected with an anti-human IgG alkaline phosphatase-conjugated antibody followed by *p*-nitrophenyl phosphate substrate. Patient IgG was considered to be reactive with the MaBP-Ro 60 aa 193–236 fusion protein if the optical density at 405 nm was 4 SD above the mean value obtained in IgG from a group of 20 healthy controls.

Immunoblots. Recombinant MaBP-Ro 60 aa 193–236 or MaBP control (1 μ g/lane) was electrotransferred to nitrocellulose and blocked for 1 hour in 5% nonfat powdered milk in PBS. Filters were washed 5 times in PBST and incubated for 1 hour at room temperature with 25 μ g/ml of IgG from patients with primary SS or SLE in 1% nonfat powdered milk in PBS. Following further washing, nitrocellulose filters were probed with horseradish peroxidase-conjugated anti-human IgG antibody. Reactivity with the MaBP-Ro 60 aa 193–236 was detected by chemiluminescence.

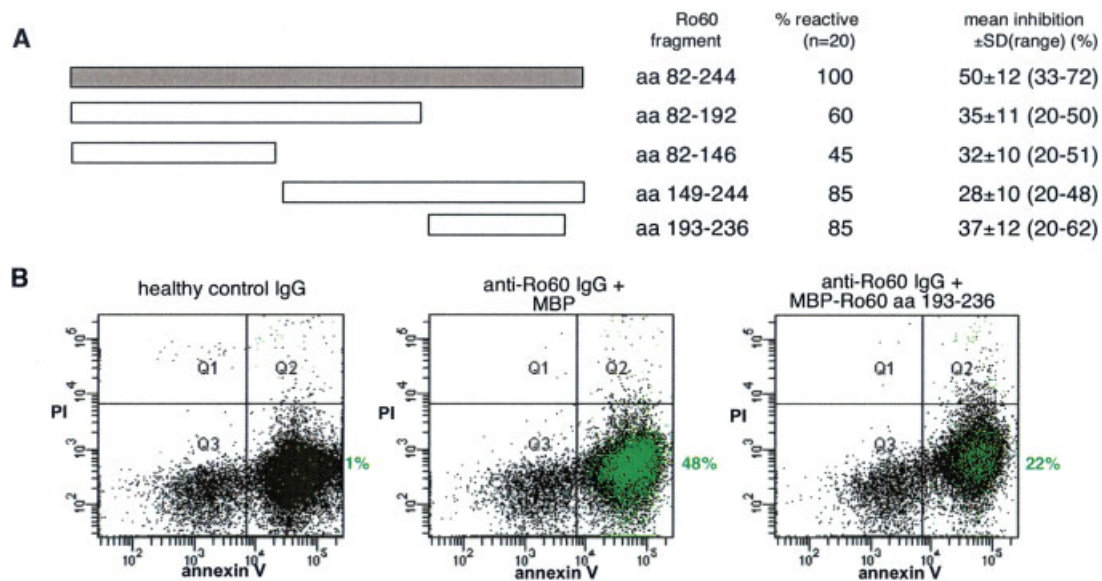


Figure 1. Mapping Ro 60 epitopes on the surface of early apoptotic cells by multiparameter flow cytometry. **A**, Ro 60 epitopes within amino acids (aa) 82–244 region were mapped using soluble recombinant maltose binding protein (MaBP [MBP])–Ro 60 subfragments prepared from the pMAL vector. Shown are the region of protein encoded by each subfragment (indicated by the aa numbers), the percentage of systemic lupus erythematosus (SLE) anti–Ro 60 IgG reactive with each subfragment, and the mean \pm SD (range) of the percentage inhibition for each fragment. **B**, Representative flow cytometry dot plots depicting early apoptotic cells (annexin V–positive, propidium iodide [PI]–negative), with specific binding of anti–Ro 60 IgG (percentages and dots shown in green), as compared with healthy control IgG. The presence of MaBP–Ro 60 aa 193–236 reduced the binding of SLE anti–Ro 60 IgG as compared with the MaBP control. The quadrants (Q1–4) represent different cell populations based on annexin V and PI positivity.

Antibody elution. IgG specific for the Ro 60 epitope residing between aa 193 and 236 (Ro 60 aa 193–236 epitope) was eluted from the surface of apoptotic cells incubated with IgG from 3 patients positive for the Ro 60 aa 193–236 epitope as described above. Briefly, 10^7 early apoptotic (annexin V+, PI–) Jurkat cells were incubated with 0.5 mg/ml of patient IgG for 1 hour at 4°C. Cells were washed extensively, and bound IgG was eluted with 0.1M glycine, 0.5M sodium chloride, pH 2.3. Eluted IgG fractions were neutralized in 1M Tris HCl, pH 8.0, and dialyzed against PBS, pH 7.4.

To purify antibodies specific for the Ro 60 solid-phase epitope residing between aa 193 and 236 (Ro 60 aa 193–236 epitope), IgG from 3 patients with primary SS positive on the epitope and negative on the apoptote (as described above) were affinity-purified on the fusion protein that was bound to nitrocellulose. One hundred micrograms of recombinant protein was electrotransferred to nitrocellulose, followed by blocking with 5% skim milk powder. The filter was incubated with 0.1 mg/ml of patient IgG in PBS, and after washing, the bound IgG was eluted with 3M potassium thiocyanate and 0.05M Tris HCl, pH 8, for 5 minutes. The eluate was concentrated and dialyzed overnight at 4°C against PBS.

Multiparameter flow cytometry for evaluating intracellular and surface-bound IgG. To assess anti–Ro 60 IgG surface binding, apoptotic cells were incubated for 1 hour at 4°C with 1 μ g/ml of affinity-purified IgG from patients with SLE, patients with primary SS, healthy controls, or an anti-RNP control. Binding of IgG to selected early apoptotic cells

was measured by flow cytometry (7). To assess the binding of affinity-purified anti–Ro 60 IgG to intracellular epitopes, viable cells were permeabilized with 0.1% saponin and 4% paraformaldehyde in PBS for 10 minutes at 4°C. Permeabilized cells were washed with PBS, incubated with IgG as described above, and assessed by flow cytometry.

To determine whether intracellular epitopes of Ro 60 were masked in the Ro/La RNP complex, permeabilized cells were treated with bovine pancreatic RNase A (Roche) as described previously (9). Briefly, RNase A dissolved in 10 mM Tris HCl, pH 7.5, 15 mM NaCl was boiled for 15 minutes and frozen to remove DNase and proteinase contaminants. Thawed RNase A was added to permeabilized cells (final concentration 0.2 mg/ml) for 30 minutes at 37°C in the presence or absence of 10 units/ μ l of SUPERase-In RNase inhibitor (Ambion). Cells were washed, and affinity-purified IgG was added. Data are presented as the MFI of cells bound by affinity-purified anti–Ro 60 IgG minus the MFI of FITC-conjugated anti-human IgG–treated cells.

Molecular modeling of human Ro 60 aa 193–236. A homology model of the 3-dimensional structure of human Ro 60 was constructed based on the crystal structure of *X laevis* Ro 60 using the SwissModel program (15). The aa 193–236 region of human Ro 60 was further evaluated in vacuo by energy minimization using the GROMOS96 biomolecular simulation software (16). Molecular visualization of the structures was performed using Swiss PdbViewer (17).

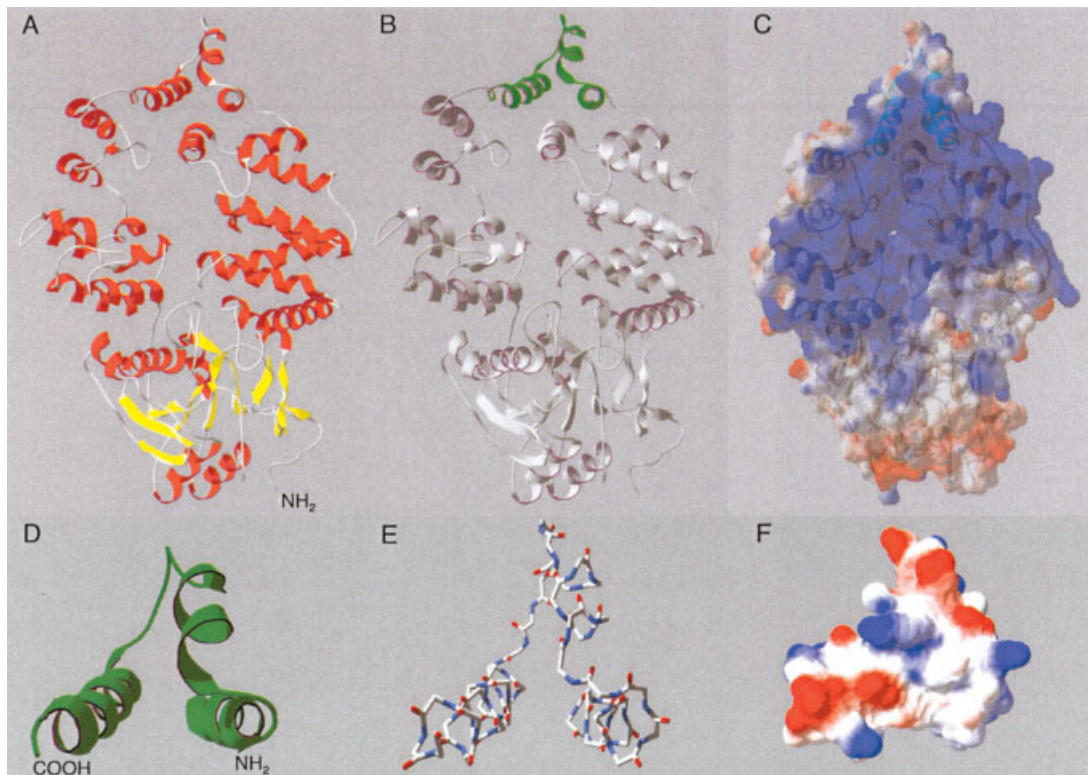


Figure 2. The predicted structure of human Ro 60 amino acids (aa) 193–236. **A**, Ribbon representation of human Ro 60, which was constructed by homology modeling using the *Xenopus laevis* Ro 60 structure (Swiss-PdbViewer ID PDB 1YVP) as a template. Secondary structural elements are shown as helices (red) and β -sheet structures (yellow). **B**, The Ro 60 ribbon structure, with amino acids (aa) 193–236 highlighted (green). **C**, A transparent surface representation of the human Ro 60 model, highlighting aa 193–236 as a relatively acidic/neutral and prominent structure situated above a large central basic cavity where RNA is known to bind. The images in **D–F** are representations of Ro 60 aa 193–236 shown in the same orientation as in the intact structures in **A–C**. **D**, Ribbon diagram of the structure, highlighting the basic helix-loop-helix structure, with a small segment of helix on the right-hand face of the “peg” structure. **E**, A ball-and-stick representation of the same region as in **B**, color-coded in CPK format. **F**, Surface representation of the apotope, with the surface electrostatic charge mapped onto the surface (red = negative, blue = positive, and white = neutral).

RESULTS

A major apotope of Ro 60 resides between aa 193 and 236. We previously identified a region of Ro 60 encompassing aa 82–244 that is exposed on the surface of early apoptotic cells and is specific for a subset of patients with SLE with isolated anti-Ro responses (8). We next sought to map more precisely the apotope(s) contained with the Ro 60 aa 82–244 region, using soluble recombinant MaBP–Ro 60 fusion proteins. Flow cytometry inhibition experiments identified at least 3 distinct aptopes of Ro 60. A major apotope of Ro 60 residing between aa 193 and 236 was identified in 17 of 20 SLE patients with isolated anti-Ro (85%). Patient IgG samples that were inhibited by MaBP–Ro 60 aa 193–236 were also inhibited by a larger Ro 60 aa 149–244 fusion

protein containing this region (Figure 1). IgG from 9 of 20 patients with SLE (45%) reacted with a minor apotope within Ro 60 aa 82–146, and 12 of 20 (60%) were also positive for Ro 60 aa 82–192 (Figure 1). Three of the latter (15% of samples) were negative for the shorter Ro 60 aa 82–146 fragment, consistent with the presence of a minor apotope within the Ro 60 aa 147–192 region (Figure 1).

Predicted structure of Ro 60 aa 193–236. A homology model of human Ro 60 was constructed based on the previously determined structure of intracellular Ro 60 from *X laevis* (3). Figure 2A displays a ribbon structure of the human Ro 60 model color coded for secondary structural elements. The region containing an apotope, which is highlighted in green (Figure 2B),

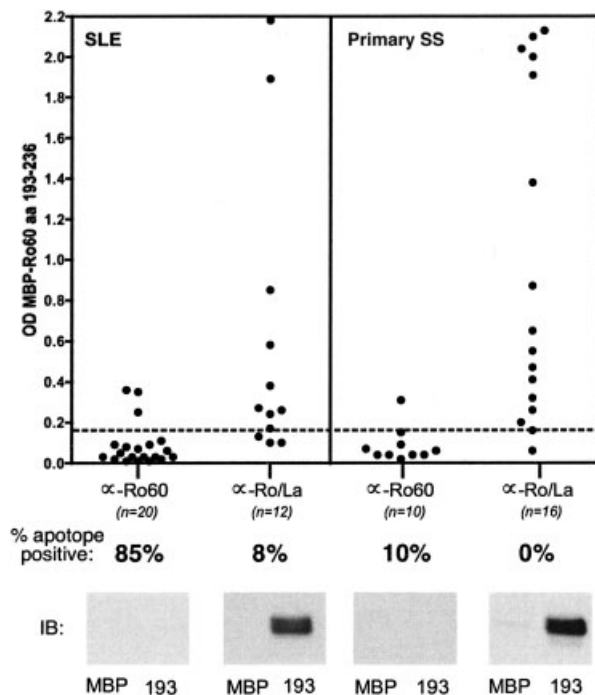


Figure 3. Reactivity of IgG from patients with systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (SS) to Ro 60 amino acids (aa) 193–236 by enzyme-linked immunosorbent assay (ELISA) (top), flow cytometry (middle), and immunoblotting (IB) (bottom). Patient IgG samples were considered positive for binding to Ro 60 aa 193–236 immobilized on ELISA plates if the optical density (OD) value was 4 SD above the mean OD value in 20 healthy controls (broken line). The corresponding findings of flow cytometric analyses are presented as the percentages of patients positive for the Ro 60 aa 193–236 apotope. A representative immunoblot of Ro 60 aa 193–236 immobilized on nitrocellulose is shown for each of the 4 groups. MBP = maltose binding protein.

forms an apical tip of the Ro 60 molecule above the basic central cavity region known to bind RNA (Figure 2C). As such, this region is not altered in its conformation in the RNA bound and free states. Figures 2D–F show representations of the isolated aa 193–236 region, which is predicted to form a helix-loop-helix type structure peaked by Glu²¹⁵ at the tip of the apex. A partial helical structure exists between Glu²⁰⁸ and Leu²¹² in the loop region, joining the well-defined α -helices between Glu¹⁹³ and Tyr²⁰¹ and between Thr²²² and Lys²³⁶.

A second determinant of Ro 60 aa 193–236 expressed on the solid phase. We next sought to determine whether autoantibodies directed against the major Ro 60 aa 193–236 apotope could be detected by direct-binding ELISA using the MaBP–Ro 60 aa 193–236 fusion protein. Patients with primary SS and SLE with anti-Ro/La who were previously shown to be Ro 60

apotope–negative were included in this analysis. Three of the 20 SLE patients with isolated anti-Ro who were positive for the Ro 60 aa 193–236 apotope on flow cytometry also showed reactivity with the fusion protein by ELISA (15%), as compared with 9 of the 12 SLE patients with both anti-Ro and anti-La (75%) ($P = 0.0017$ by Fisher's exact test). Similarly, 1 of 10 patients with primary SS and anti-Ro were positive by ELISA (10%), as compared with 14 of 16 patients with primary SS with anti-Ro/La (88%) ($P = 0.0002$ by Fisher's exact test). Irrespective of disease, the high prevalence of Ro 60 aa 193–236 epitope reactivity in patients with both anti-Ro and anti-La antibodies (23 of 28 [82%]) as compared with patients with isolated anti-Ro (4 of 30 [13%]) ($P < 0.0001$ by Fisher's exact test) indicates that reactivity with the Ro 60 epitope is a specific biomarker for intermolecular epitope spreading to La antibodies in SLE and primary SS.

The ELISA results showed a strong discordance with the flow cytometry data, in that IgG samples positive on the Ro 60 aa 193–236 ELISA generally failed to react with the apotope contained within the same region of Ro 60 (Figure 3). This suggests that there are 2 distinct B cell determinants contained within Ro 60 aa 193–236 that stratify anti-Ro 60 responses in SLE and primary SS: an apotope (Ro 60 apotope) expressed on the surface of early apoptotic cells that can be competed for binding with recombinant Ro 60 aa 193–236, and an epitope (Ro 60 epitope) that is exposed upon binding to the solid phase in ELISAs. Recognition of these determinants by anti-Ro 60 IgG is virtually mutually exclusive, with only 7% of patient samples (4 of 58) recognizing both apotope and epitope.

Immunoblot experiments performed with the MaBP–Ro 60 aa 193–236 and the MaBP control showed the same pattern of reactivity with anti-Ro/La IgG as the ELISA, indicating that the Ro 60 epitope is exposed by immobilization to either plastic or nitrocellulose (Figure 3).

Recognition of the Ro 60 aa 193–236 apotope and solid-phase epitope by distinct, non-cross-reactive populations of autoantibodies. To confirm whether the Ro 60 apotope and epitope are immunologically distinct determinants that are expressed differentially on the cell surface or cytoplasm, flow cytometry experiments were performed using Ro 60 apotope-specific and Ro 60 epitope-specific antibodies eluted from apoptotic cells or MaBP–Ro 60 aa 193–236 bound to nitrocellulose, respectively. The reactivity of the apotope- and epitope-specific IgG samples were assessed by flow cytometry

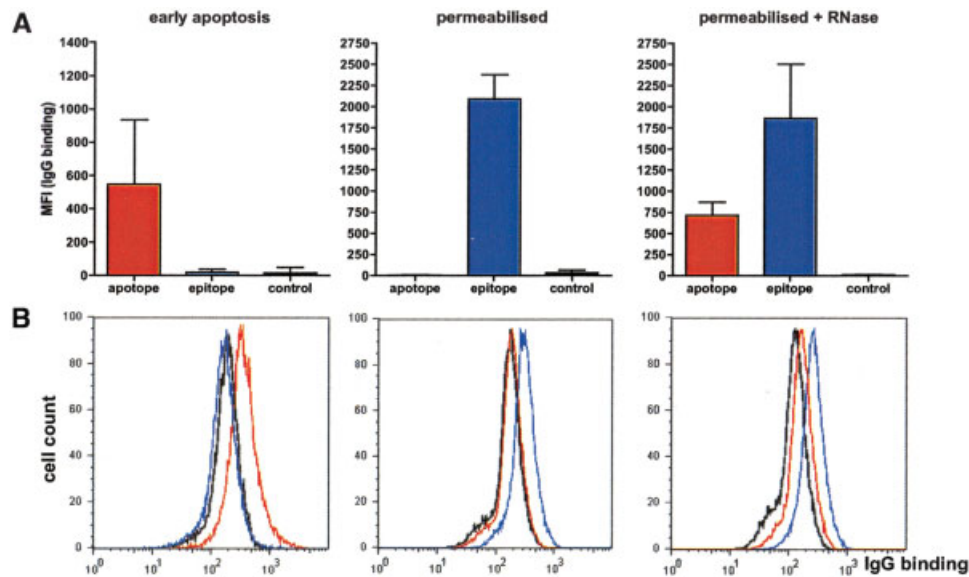


Figure 4. Distinct binding profiles of autoantibodies eluted from apoptotic cells (Ro 60 apotope-specific) or maltose binding protein–Ro 60 amino acids (aa) 193–236 immobilized on nitrocellulose (Ro 60 epitope-specific) to early apoptotic, permeabilized, and permeabilized plus RNase-treated Jurkat cells by flow cytometry. **A**, Ro 60 apotope-specific and Ro 60 epitope-specific IgG binding to cell populations as compared with IgG from 3 healthy control subjects. Values are mean and SEM of apotope-specific and epitope-specific IgG, each of which was prepared from 3 separate patients. MFI = mean fluorescence intensity. **B**, Representative flow cytometry traces depicting the binding of IgG specific for the Ro 60 apotope (red line) and the Ro 60 epitope (blue line) to early apoptotic, permeabilized, and permeabilized plus RNase-treated cells as compared with healthy control IgG (black line).

using early apoptotic cells and permeabilized viable cells in the presence or absence of RNase (9).

Ro 60 apotope-specific IgG bound to the surface of apoptotic Jurkat cells as predicted, but it did not bind to intracellular Ro 60 in the permeabilized cell preparations. Conversely, the Ro 60 epitope was exposed on the intracellular autoantigen but not on the apoptotic cell surface. Treatment of permeabilized cells with RNase to dissociate the Ro/La RNP complex revealed binding of the Ro 60 apotope-specific IgG, but it did not alter the binding of the Ro 60 epitope-specific IgG (Figures 4A and B).

The specificity of the RNase experiment was verified by incubating permeabilized cells with both RNase and RNase inhibitor prior to the IgG binding step. The RNase inhibitor reversed the RNase-mediated enhancement of Ro 60 apotope-specific IgG binding, reducing the MFI from 598 (RNase alone) to 117 (RNase plus RNase inhibitor). Furthermore, the binding of Ro 60 epitope-specific IgG to permeabilized cells was not altered by RNase inhibitor.

The differential binding characteristics and distinctive subcellular locations of apotope versus epitope,

both of which are contained within the Ro 60 aa 193–236 domain, signify the presence of 2 immunogenic forms of Ro 60 autoantigen: a membrane-bound form on the surface of apoptotic cells, and a cytoplasmic form on the Ro/La RNP complex. These data derived from analyses of human cells indicate that the apotope and epitope are conserved on human Ro 60 and imply that the amino acid substitutions that distinguish mouse and human Ro 60 (glutamic acid to leucine at position 212 and alanine to threonine at position 222) are not critical for autoantibody recognition.

IgG subclass analysis of Ro 60 apotope-specific and Ro 60 epitope-specific IgG with subclass-specific antibodies revealed that both autoantibody populations were IgG1 κ and λ (data not shown). This is consistent with previous studies showing that anti-Ro 60 antibodies are confined to IgG1, a subclass capable of mediating tissue injury via complement or other cellular effector pathways (18,19).

DISCUSSION

This study defines an immunodominant domain of Ro 60 that can form either an apotope or intracellular

epitope, depending upon the subcellular location of the autoantigen. This dichotomy of determinant expression translates to distinct human anti-Ro 60 autoantibody subsets. Based on their differential expression, we conclude that the Ro 60 apotope and Ro 60 epitope are mutually exclusive B cell determinants that are linked to a common domain of the Ro 60 protein. Although distinctions between paired sets of autoantibodies against either Ro/La or U1 RNP/Sm in SLE are well recognized (20), our findings represent the first example of a dichotomy of human autoantibody responses against mutually exclusive determinants contained within a single domain of a systemic autoantigen.

Anti-Ro 60 responses in patients with SLE and with primary SS can now be stratified into 4 categories based on the findings of flow cytometry and ELISAs: SLE with anti-Ro 60 (apotope-positive, epitope-negative); SLE with anti-Ro 60 and anti-La (apotope-negative, epitope-positive); primary SS with anti-Ro 60 and anti-La (apotope-negative, epitope-positive); and primary SS with anti-Ro 60 (apotope-negative, epitope-negative). As reported previously, reactivity with the Ro 60 apotope may be of diagnostic utility in distinguishing between patients with SLE and primary SS who have isolated anti-Ro 60 responses, particularly in patients with overlapping clinical and serologic features (8). Furthermore, a recent study has confirmed that SLE patients with isolated anti-Ro 60 antibodies (i.e., Ro 60 apotope-positive) have a higher risk of nephritis and seizures than do patients with anti-La antibodies (15). A new finding from this study is that the Ro 60 epitope is a highly specific biomarker for intermolecular epitope spreading to La protein, irrespective of whether the patient has SLE or primary SS. Although the Ro 60 epitope ELISA is simple to perform and accurately distinguishes patients with anti-Ro/La from those with anti-Ro 60 alone, the lack of disease specificity suggests that it will not be of any additional value beyond that of routine anti-Ro/La antibody testing.

Ro 60, as noted for other systemic intracellular autoantigens (21), appears to be an inherently unstable protein that is thought to undergo a conformational change upon translocation to the apoptotic cell surface (22). During apoptosis, La dissociates from the Ro/La RNP complex; the RNA components are degraded, except for a highly conserved Y RNA stem that remains bound to Ro 60 (23). The mechanism by which Ro 60 protein is transported to the cell surface and traverses the cell membrane of early apoptotic cells is unknown, but chaperones such as calreticulin and Grp78 have been

implicated in the process and are themselves linked with anti-Ro 60 responses (24–28).

How can 2 determinants localized to the same domain be expressed in a mutually exclusive manner on intracellular and membrane-bound forms of an autoantigen? The simplest explanation is that the Ro 60 aa 193–236 domain undergoes a conformational change upon translocation to the cell surface, which exposes the apotope and conceals the intracellular epitope. The molecular basis of the proposed structural shift remains unknown, but it might involve domain inversion, with exposure of alternative determinants on membrane-bound and cytoplasmic forms of Ro 60. Another possibility is that the apotope may be masked in live cells by binding partners on the Ro/La RNP complex, such as La protein or Y RNA; the loss of these molecules from the complex during apoptosis would lead to exposure of the apotope as a neoepitope on the cell surface. This idea is supported by the findings of experiments in which an RNase digestion step on permeabilized cells enhanced the binding of apotope-specific IgG. RNase digestion is likely to dissociate the Ro/La RNP complex, leaving the Ro 60 bound to the Y RNA stem and a portion of pre-5S RNA, which are protected from degradation (29,30); the removal of La or other RNA components from the complex may reveal the Ro 60 apotope in these experiments. The Ro 60 epitope, on the other hand, may be masked on the apoptotic cell surface by molecules that complex with Ro 60, such as calreticulin (25,26,31).

The generation of dichotomous sets of autoantibodies that are linked to different anti-Ro 60 subsets suggests the presence of at least 2 immunogenic forms of Ro 60 that can elicit anti-Ro 60 autoimmunity. Isolated anti-Ro 60 responses in SLE that are specific for the Ro 60 apotope arise presumably from interactions between the Ig receptor on Ro 60-specific B cells and membrane-bound Ro 60 displayed on early apoptotic cells. Potential accessory molecules in this context include calreticulin and the heat-shock protein Grp78, which may provide additional signals for B cell activation (24). Pathologic conditions of defective apoptotic cell clearance, as reported in human SLE but not in primary SS, would favor an immune response against the Ro 60 apotope (32,33). Because Ro 60 translocates to the apoptotic cell surface independently of La (7), the immune response is unlikely to spread to La, consistent with our finding that virtually no anti-Ro 60 apotope-positive sera contained anti-La.

Reactivity with the intracellular Ro 60 epitope is highly specific for anti-Ro 60 with anti-La in both SLE and primary SS, consistent with a model of T cell-

dependent Ro/La intermolecular epitope spreading that is thought to be driven by Ro/La RNPs released by cell lysis or necrosis (34,35). HLA-DR3/DQ2 is associated with anti-Ro/La-linked antibody sets both in patients with SLE and in patients with primary SS (36,37), raising the possibility that the Ro 60 aa 193–236 domain may also harbor an HLA-DR3/DQ2-restricted T cell determinant. This could be tested in recently generated DR3/DQ2-transgenic mice, which have been used to map T cell determinants involved in Ro/La epitope spreading (38). Patients with primary SS with isolated anti-Ro demonstrate a third type of response that spares both Ro 60 apotope and epitope. Sera from these patients are negative on recombinant Ro 60 ELISA but react with native Ro 60 on ELISA and line blots (data not shown). The autoimmune background underlying this anti-Ro 60 autoantibody response is unknown.

Structural analysis based on the crystal structure of *X laevis* Ro 60 predicts that the Ro 60 aa 193–236 domain defined in the present study is located on the outside surface of the doughnut-shaped Ro 60 molecule. That this domain can present in 2 distinct immunogenic forms implies that the Ro 60 protein undergoes extensive structural remodeling during apoptosis and translocation to the cell surface. Further studies will be required to unravel the molecular characteristics of these mutually exclusive Ro 60 determinants, beginning with a detailed mutational analysis to identify the key amino acids involved in autoantibody binding. However, structural studies of Ro 60 autoantibody complexes will ultimately be required to elucidate the 3-dimensional epitopes (4). Since human autoantibodies often react with functional domain regions on their cognate autoantigens (39), it will be interesting to investigate whether the Ro 60 aa 193–236 domain is involved in interactions of surface-exposed Ro 60 with β_2 -glycoprotein I and the recently proposed Ro 60 receptor (11,40).

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gordon had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Reed, Kaufman, Purcell, Gordon.

Acquisition of data. Reed, Dudek, Osborne, Kaufman, Purcell, Gordon.

Analysis and interpretation of data. Reed, Dudek, Jackson, Purcell, Gordon.

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