

The Ro 60 kDa autoantigen comes into focus: Interpreting epitope mapping experiments on the basis of structure

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Abstract

A conserved RNA-binding protein, the Ro 60 kDa (Ro60) autoantigen, is a major target of autoantibodies in patients suffering from the rheumatic diseases Sjogren's syndrome, systemic lupus erythematosus, subacute cutaneous lupus erythematosus and neonatal lupus erythematosus. In both mice and certain bacteria, Ro60 is important for cell survival following ultraviolet irradiation. Although the function of Ro60 was mysterious for many years, recent experiments have demonstrated that this protein binds misfolded noncoding RNAs in vertebrate cells and likely functions in a pathway by which defective RNAs are recognized and targeted for degradation. Recent structural studies have revealed that Ro60 is shaped like a doughnut with an inner hole. Noncoding RNAs called Y RNAs bind on the outer surface of the ring, while the single-stranded ends of misfolded RNAs likely bind within the hole. Comparison of the Ro60 structure with the results of epitope-mapping studies reveals that many of the currently identified epitopes recognized by patient sera overlap regions of Ro60 that function in RNA binding. Moreover, in some patients with anti-Ro60 antibodies, the initial antigenic epitope corresponds to a loop involved in binding single-stranded RNA in the central cavity.

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1. Clinical associations of anti-Ro60 antibodies

An RNA-binding protein, the Ro 60 kDa protein (Ro60; also called 60 kDa SS-A), is a prominent and clinically important target of the autoimmune response in patients with certain rheumatic diseases. Depending on the study and the techniques used for detection, anti-Ro60 antibodies have been found in 38–90% of patients with Sjogren's syndrome [1–4]. Because of the high prevalence of anti-Ro60 antibodies in these patients, the presence of anti-Ro60 antibodies is one criterion used in the diagnosis of Sjogren's syndrome [5]. Anti-Ro60 antibodies are also present in 24–60% of patients with systemic lupus erythematosus and 70–100% of patients with subacute cutaneous lupus erythematosus [1–3]. Maternal anti-Ro60 antibodies are also highly associated with neonatal lupus, a syndrome in which mothers with these antibodies give birth to babies with one or more of the following: erythematous skin rashes, complete congenital block, hepatobiliary disease and thrombocytopenia [6,7].

2. Conservation and function of the Ro60 autoantigen

The Ro60 protein is found in many animal cells, including virtually all vertebrate cells and those of the nematode *Caenorhabditis elegans* [8]. Although Ro60 is not present in budding or fission yeast, potential orthologues have been described in the green algae *Chlamydomonas*, several eubacteria and one mycobacteriophage [8]. In all cells and species that have been studied, Ro60 is complexed with small noncoding RNAs of unknown function called Y RNAs. In vertebrate cells, a fraction of the Ro60/Y RNA complexes also contain the La protein, a nuclear phosphoprotein that binds many newly transcribed noncoding RNAs, including Y RNAs [9]. Approximately half of patients with anti-Ro60 antibodies possess antibodies against La, consistent with the hypothesis that the ribonucleoprotein particle is the immunogen [10,11]. Most patients with anti-Ro60 proteins also produce antibodies against a structurally unrelated protein, the Ro52 protein [12]. As several studies have failed to demonstrate that Ro52 is physically associated with the Ro60/Y RNA complex [13,14], it remains unclear why antibodies against both Ro52 and Ro60 are so frequently found in the same patients.

Although the function of Ro60 was elusive for many years, recent data indicate that the protein binds misfolded, defective noncoding RNAs that are eventually

degraded [15–17]. Thus, Ro60 is proposed to function in a quality control pathway in which incorrectly folded and otherwise imperfect noncoding RNAs are targeted for decay [8]. In addition, studies of Ro60 in both mammalian cells and bacteria have revealed that the protein is important for cell survival after ultraviolet irradiation [17,18]. Although it is presently unclear how these two functions of Ro60 relate to each other, ultraviolet light has long been known to result in RNA:protein and RNA:RNA crosslinks. Thus, one possibility is that Ro60 assists in the degradation of damaged RNA following UV irradiation. Most interestingly, mice lacking Ro60 develop a lupus-like syndrome consisting of autoantibodies against chromatin and ribosomes, glomerulonephritis, and photosensitivity [19]. Thus, the normal function of Ro60 may be important for the prevention of autoimmunity.

3. Structural studies reveal that Ro60 forms a ring with a central hole

Recently, the structure of the *Xenopus laevis* Ro60, which is 78% identical to human Ro60, was solved using X-ray crystallography. This revealed that Ro60 consists of two domains. One domain consists of a series of alpha-helical repeats, known as HEAT repeats, that are arranged to form a doughnut with an inner hole. The diameter of the hole is 10–15 Angstroms, wide enough to accommodate single-stranded, but not double-stranded, RNA. The ring of HEAT repeats is clasped shut by the second domain, which resembles the von Willebrand Factor A (vWFA) domain found in a number of extracellular matrix proteins and in proteins that function in cell adhesion. Within the vWFA domain is a motif known as a metal ion-dependent adhesion site (MIDAS), which in integrins functions as a cation-dependent ligand binding site [20].

The structure of Ro60 complexed with a fragment of Y RNAs was also determined. This revealed that Y RNAs bind to conserved residues on the outside of the doughnut. A combination of structural and biochemical experiments suggested that the 3' ends of misfolded RNA bind inside the central hole while helical portions of these RNAs bind to surfaces overlapping the Y RNA binding site [20]. Since Y RNAs and the helical parts of misfolded RNAs bind to overlapping sites, one function of Y RNAs may be to block access of the central cavity of Ro60 to other RNAs [20].

What does the structure of Ro60 tell us about the function of this protein? One possibility is that the central cavity, by forcing bound RNA to be single-

stranded, assists in destabilizing RNA helices [20]. Consistent with a role in destabilizing misfolded helices, a recent study in which human Ro60 was expressed in *Escherichia coli* revealed that the protein can promote correct folding of a misfolded RNA substrate in vivo [21]. An alternative, but not exclusive, possibility is that the Ro60 ring serves as a scaffold for the binding of other components of the RNA quality control pathway, such as helicases or ribonucleases.

4. Where do the epitopes recognized by patient anti-Ro60 sera reside?

The availability of the Ro60 structure provides an opportunity to determine where the epitopes recognized by patient sera reside on the three-dimensional structure. It has long been recognized that the major epitopes targeted by patient anti-Ro60 antibodies are discontinuous, or conformational, in that many sera react primarily with the native Ro60 protein [22–26]. Crystallographic studies of several antigen–Fab complexes have shown that such epitopes are formed by two or more separate segments of a protein that are brought together in the correctly folded structure [27]. Although the only definitive way to define such an epitope is through crystallization of the antigen–antibody complex, a number of investigators have attempted to identify the major epitopes of Ro60 using other methods.

One approach has involved generating truncated fragments of recombinant Ro60 protein and assaying whether epitopes recognized by patient antibodies are present on the truncated proteins, either under native conditions [26,28] or by Western blotting [29]. One assumption inherent in these experiments is that the truncated recombinant proteins are correctly folded. Moreover, as many anti-Ro60 sera react poorly with the denatured protein on Western blotting [22–24,26], the use of this assay can at best reveal only a subset of the epitopes recognized by the autoantibodies. Nonetheless, all these studies indicated that at least one major epitope exists within the middle third of the Ro60 protein, spanning roughly amino acids 140–325 (shown in Fig. 1). This portion of the protein contains all the residues that contact the Y RNA fragment in the crystal structure, as well as the residues in the inner hole that likely contact the 3' ends of misfolded RNAs [20]. However, these studies also revealed that the autoantibody response to Ro60 was heterogeneous, in that significant numbers of patient sera also recognized determinants outside this region [26,28,29].

Other investigators examined the reactivity of anti-Ro60 sera with a series of peptides, 8–22 amino acids in length, that spanned the entirety of the Ro60 sequence [29–32]. Since the major epitopes recognized by patient anti-Ro60 sera are widely considered to be conformational, this assay makes the assumption that at least part of the discontinuous epitope will be represented by one or more peptide sequences. Similar to the studies using large fragments of Ro60, most of these studies found that patient anti-Ro60 sera was heterogeneous, as many sera bind multiple peptides and no single peptide was recognized by all patient sera. However, several studies identified an epitope residing between amino acids 169 and 190 that was recognized by the majority of patient anti-Ro sera [31–33]. Most interestingly, McClain et al. [32] examined sera from 29 lupus patients who developed anti-Ro60 antibodies while under clinical observation. For nine of these patients, only a single epitope was detected in the earliest serum sample displaying anti-Ro reactivity. In all nine of these sera, the epitope resided between amino acids 169 and 180. On average, antibodies to amino acids 169–180 appeared in these patients nearly 4 years before the diagnosis of lupus. Because antibodies that were affinity purified against the 169–180 peptide cross-reacted with the Epstein–Barr virus protein EBNA-1, the authors proposed that autoimmunity to Ro60 was initiated through molecular mimicry following virus infection [32]. Over time, these patients developed responses to multiple peptide epitopes throughout the Ro60 protein, consistent with epitope spreading [32].

Interestingly, amino acids 169–180 reside on a loop involved in binding single-stranded RNA in the Ro60 central cavity [20] (Fig. 1). Three conserved residues within this sequence make hydrogen bonds to the phosphate backbone of the RNA. Mutagenesis confirmed that two of these residues, K170 and R174, were important for binding of misfolded pre-5S rRNA [20]. Thus, as has been described for several other autoantigens [34], anti-Ro60 antibodies may target a functionally important part of the protein. One prediction of this hypothesis is that anti-Ro60 antibodies that have been affinity-purified using the 169–180 peptide should block binding of misfolded pre-5S rRNA to Ro60.

Several groups have described other peptide epitopes that are recognized by subsets of patient anti-Ro sera. In one set of studies, a peptide spanning amino acids 216–232 was found to react with 10–53% of anti-Ro60 sera [33,35,36]. Another group found that a peptide containing amino acids 300–320 reacted with ~50% of tested sera [29]. Interestingly, both of these

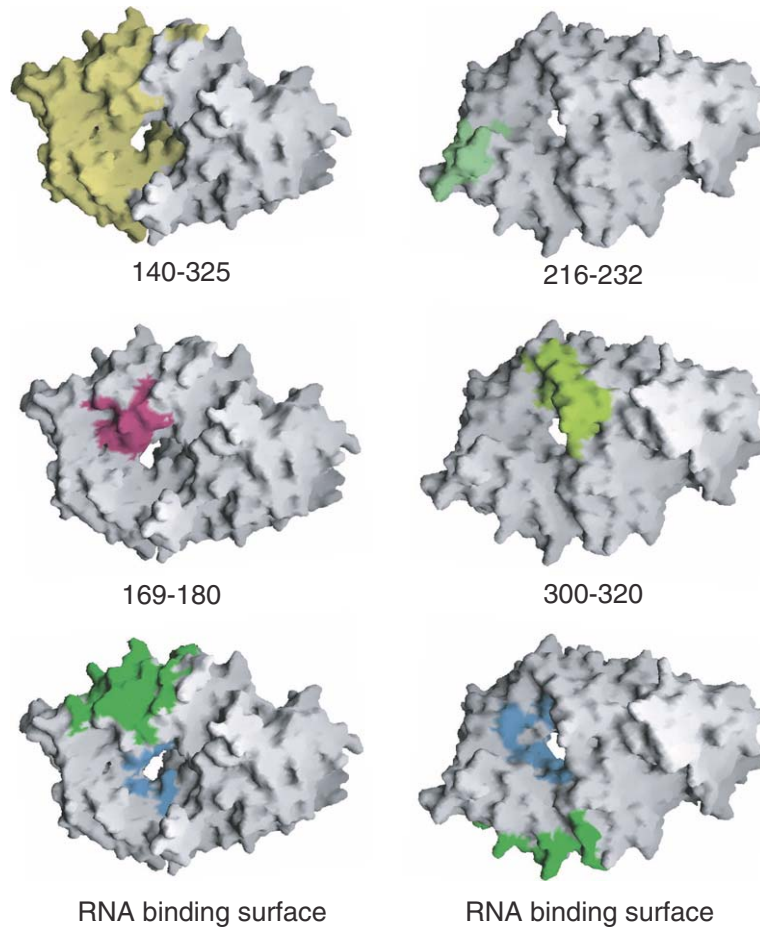


Fig. 1. Previously defined epitopes recognized by anti-Ro60 autoantibodies overlap with portion of Ro60 involved in RNA binding. Molecular surface representations of *Xenopus laevis* Ro60 are shown. The three views on the right are related to those on the left by a 180° rotation about a vertical axis. Residues corresponding to human amino acids 140–325 are colored yellow, amino acids 169–180 are pink, while 216–232 and 300–320 are light green. In the two bottom views, surfaces that interact with Y RNAs are dark green, while surfaces that interact with single-stranded RNA are blue [20]. As the crystal structure contains a fragment of Y RNA complexed with Ro60, there may be additional surfaces that contact the full-length Y RNA, or that contact misfolded RNAs, that have not yet been identified.

epitopes, like 169–180, are in the HEAT repeat portion of Ro60 that interacts with RNA, and all may be targeting regions of Ro60 that are important for RNA binding (Fig. 1).

Because anti-Ro60 antibodies are associated with Sjogren's syndrome, systemic lupus erythematosus, subacute cutaneous lupus erythematosus and neonatal lupus, there has been interest in determining whether certain epitopes are specifically associated with one of these diseases. While there are several reports of peptide epitopes that are more strongly associated with Sjogren's syndrome than with systemic lupus erythematosus [31,37,38], others have failed to confirm these reports [39] and none of the reported differences have yet been demonstrated to have diagnostic value. Nonetheless, the prospect of using autoantibody epitopes to

better differentiate the various rheumatic syndromes associated with anti-Ro60 antibodies remains an exciting possibility.

5. Conclusions

Recent experiments are at long last beginning to uncover the cellular and physiological function of the Ro60 autoantigen. These experiments have implicated Ro60 in the decay of defective noncoding RNAs while also revealing that Ro60 is important for cell survival following ultraviolet irradiation. Structural analyses have revealed that Ro60 is shaped like a doughnut, with an inner hole that binds single-stranded RNA. Future experiments will likely focus on determining how the doughnut-shaped structure of Ro60 contributes

to its functions and on identifying additional components of the proposed RNA quality control pathway involving Ro60.

The determination of the Ro60 structure has also made possible the evaluation of epitope data collected over the last decade. The structure has revealed that at least some epitopes recognized by patient anti-Ro60 antibodies overlap with portions of the protein involved in RNA-binding. Further structural studies of Ro in complex with patient autoantibodies may elucidate more precisely the three-dimensional epitopes recognized and may assist in determining whether specific epitopes are associated with distinct clinical outcomes. Lastly, as neonatal lupus is nearly always associated with maternal anti-Ro60 antibodies [40], anti-Ro antibodies have long been proposed to play a role in pathogenesis. Knowledge of the Ro60 structure may eventually allow the design of peptide mimetics that block these autoantibodies.

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Take-home messages

- The Ro60 protein is an important autoantigen in Sjogren's syndrome, neonatal lupus erythematosus, systemic lupus erythematosus and subacute cutaneous lupus erythematosus.
- Ro60 is normally complexed with noncoding RNAs known as Y RNAs.
- Ro60 also binds misfolded noncoding RNAs and may function in RNA quality control.
- Structural studies reveal that Ro60 forms a ring with an inner hole that binds single-stranded RNA. Y RNAs bind on the outer surface of the ring. The ends of misfolded RNAs likely insert into the hole.
- Most of the currently described major epitopes recognized by patient sera correspond to regions of Ro60 implicated in RNA binding.
- In at least some patients, the initial antigenic epitope resides on a loop involved in recognizing single-stranded RNA.

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IgG-antiphospholipid antibodies in follicular fluid of IVF-ET patients are related to low fertilization rate of their oocytes

Patients undergoing in vitro fertilization and embryo transfer (IVF-ET) failures show an increased incidence of antiphospholipid antibodies (aPL) in their blood. Pathological effects of aPL on embryos in vitro have been documented. Thus, Matsubayashi H. et al. (*Am J Reprod Immunol* 2006; 55:341-8) questioned whether aPL is found in follicular fluids (FFs) could result in embryonic damage. Blood from 44 patients with three or more IVF-ET failures were tested for the presence of IgG, IgM, and IgA aPL. Both the 29 aPL-positive and 15 aPL-negative patients gave permission for FF collection during their next IVF-ET attempt for additional aPL determinations. Patients with no aPL in their blood, had no aPL in their FFs. Patients with IgG and/or IgM aPL in their blood had IgG but not IgM in their respective FFs. The presence of IgG aPL in FFs and increased infertility length were significantly related to lower fertilization rates, independently. Follicular fluid IgG aPL appears as a risk factor in association with successful IVF-ET outcomes.