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The Ro 60 kDa autoantigen: insights into cellular function and role in autoimmunity

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Abstract An RNA-binding protein, the Ro 60 kDa autoantigen, is a major target of the immune response in patients suffering from two systemic rheumatic diseases, systemic lupus erythematosus and Sjogren's syndrome. In lupus patients, anti-Ro antibodies are associated with photosensitive skin lesions and with neonatal lupus, a

syndrome in which mothers with anti-Ro antibodies give birth to children with photosensitive skin lesions and a cardiac conduction defect, third degree heart block. In vertebrate cells, the Ro protein binds small RNAs of unknown function known as Y RNAs. Although the cellular function of Ro has long been mysterious, recent studies have implicated Ro in two distinct processes: small RNA quality control and the enhancement of cell survival following exposure to ultraviolet irradiation. Most interestingly, mice lacking the Ro protein develop an autoimmune syndrome that shares some features with systemic lupus erythematosus in patients, suggesting that the normal function of Ro may be important for the prevention of this autoimmune disease. In this review, we summarize recent progress towards understanding the role of the Ro 60 kDa protein and discuss whether the cellular function of Ro could be related to certain manifestations of lupus in patients.

Keywords Autoimmunity · Lupus · RNA · Ro 60 kDa protein · Ultraviolet irradiation

Abbreviations *RNP*: Ribonucleoprotein · *RRM*: RNA recognition motif · *SCLE*: Subacute cutaneous lupus erythematosus · *SLE*: Systemic lupus erythematosus · *snRNP*: Small nuclear ribonucleoprotein · *SS*: Sjogren's syndrome · *UV*: Ultraviolet · *VWFA*: von Willebrand factor A



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Introduction

A fascinating aspect of the systemic rheumatic diseases is that patients often produce antibodies against specific classes of highly conserved RNA-protein complexes. For example, patients with polymyositis and dermatomyositis frequently display antibodies against certain tRNA synthetases, while patients with mixed connective tissue disease often make antibodies against the U1 small nuclear ribonucleoprotein (snRNP), which functions in pre-mRNA splicing [1]. Although the reasons why

60 kDa protein/Y RNA complex in cells is not known. Some Ro protein/Y RNA complexes in mammalian cells contain La, a protein which binds newly synthesized RNA polymerase III transcripts, including Y RNAs [32]. Consistent with the hypothesis that the intact Ro ribonucleoprotein particle is the immunogen [33, 34], approximately half of patients with anti-Ro 60 kDa protein antibodies also have antibodies against La [35]. Most patients with antibodies against the Ro 60 kDa protein also produce antibodies against a structurally unrelated protein, the 52 kDa Ro protein. Although this protein has been proposed to be a component of the Ro RNP complex [36, 37], several groups have either failed to detect this protein in partially purified Ro RNPs [30, 38] or detected only very low levels [31], making its association with 60 kDa Ro controversial. Other proteins that have been reported to associate with the Ro protein/Y RNA complex include pp75/rip11, which functions in apical trafficking of endocytic vesicles [39, 40], RoBPI/Puf60/FIR, which functions in both transcriptional repression and pre-mRNA splicing [41, 42, 43], the polypyrimidine tract binding protein hnRNPI/PTB [44], and the nucleolar protein nucleolin [45]. For all these proteins, the functional significance of the interaction with the Ro protein/Y RNA complex is not understood.

Although the structure of Ro is not yet available, bioinformatic investigations have suggested that the protein contains two distinct modules. The N-terminal two thirds of Ro shares homology with the *Tetrahymena thermophila* p80 protein and its human homologue TEPI [46, 47, 48, 49]. TEPI is a component of a large cytoplasmic ribonucleoprotein particle of unknown function, known as the vault RNP [50]. TEPI/p80 may also be a component of telomerase, the enzyme that elongates the ends of chromosomes, although this is controversial [51, 52]. Like Ro, TEPI is an RNA-binding protein [52]. The C-terminus of Ro may contain a von Willebrand Factor A (VWFA) domain, a module found in many cell adhesion proteins that often functions in protein-protein interactions [48, 49, 53]. Both the N- and the C-terminus are required for RNA binding, as the affinity of Ro for RNA is eliminated by small deletions in the N-terminus (within the TEPI homologous region) or the C terminus (within the proposed VWFA domain) [25]. Although Ro was originally suggested to be a member of the family of RNA-binding proteins that contain an ~80 amino acid motif known as an RNA-recognition motif [54, 55, 56], this proposal has not been supported by recent bioinformatics studies [48, 49].

Although the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* do not contain recognizable Ro proteins, several other unicellular eukaryotes, prokaryotes, and at least one mycobacteriophage contain potential orthologues of the Ro 60 kDa protein. These include the green algae *Chlamydomonas reinhardtii*, the radiation-resistant eubacterium *Deinococcus radiodurans*, the cyanobacteria *Nostoc punctiforme* and *Synechococcus*, the planctomycete *Pirellula* and a newly described mycobacteriophage, Bx1 gp220 ([11,

57, 58]; our unpublished observations). As only rare prokaryotes possess a Ro orthologue, these Ro proteins were most likely acquired by lateral transfer from a eukaryote [59]. Of these unicellular organisms, only the *D. radiodurans* Ro protein has been characterized. Remarkably, this protein binds a small RNA that resembles a Y RNA [11]. Genetic analyses have revealed that the *D. radiodurans* Ro protein contributes to the extreme resistance of this bacterium to ultraviolet light (see below). Although the functions of the other unicellular and mycobacteriophage Ro proteins are not understood, the finding that certain prokaryotes possess orthologues of the 60 kDa Ro protein raises the possibility that infection by these organisms plays a role in the development of anti-Ro autoantibodies in humans.

Evidence that the Ro 60 kDa protein participates in a quality control pathway for misfolded small RNAs

Recent experiments have revealed that the Ro 60 kDa protein binds misfolded small RNAs, suggesting the protein may function in a quality control pathway for small RNA biogenesis. First, in *X. laevis* oocyte nuclei, the Ro protein is complexed with a large class of variant 5S rRNAs [7]. In order to produce the large numbers of ribosomes needed for early development, *X. laevis* contains ~20,000 genes encoding 5S rRNA, a small RNA that is a component of the large subunit of the ribosome. Many of these genes contain changes from the consensus 5S rRNA sequence [60]. The 5S rRNA variants bound by the Ro protein contain extra nucleotides at the 3' end, most likely caused by read-through of the first site for transcription termination by RNA polymerase III. In addition to being longer, they contain nucleotide changes that cause them to misfold to form a helix that is not present in functional 5S rRNA [7, 8]. These misfolded pre-5S rRNAs are inefficiently processed to mature 5S rRNA and are eventually degraded. These observations suggested that the Ro protein may function in a quality control pathway for ribosome biogenesis [7, 8]. Consistent with this hypothesis, *C. elegans* worms lacking Ro have increased numbers of variant 5S rRNAs in their ribosomes, compared to wild-type worms [9]. However, introduction of a transgene encoding the Ro gene into the knockout worms failed to restore the levels of variant 5S rRNAs in ribosomes to that of wild-type worms. Thus, it was unclear whether the differences between the wild-type and Ro knockout worms were due to loss of the Ro protein or to an unrelated mutation [9].

More recently, the Ro protein was found to associate with a collection of largely variant U2 small nuclear RNAs in mouse embryonic stem cells [10]. U2 is one of several small nuclear RNAs that function in pre-mRNA splicing. Mammalian genomes contain numerous variant genes encoding U2 as well as the other spliceosomal small nuclear RNAs. Although the functional significance of these variant U RNA genes is unclear, they are often expressed in embryonic cells [61, 62]. Experiments in

which wild-type and variant U2 genes were injected into *Xenopus* oocytes revealed that Ro preferentially binds certain variant U2 RNAs. Moreover, similar to the finding for 5S rRNA variants, binding of Ro to a variant U2 snRNA occurred in the nucleus and required the formation of an abnormal RNA helix [10].

The findings that Ro binds misfolded 5S rRNA precursors in *Xenopus* oocytes and variant, apparently misfolded U2 snRNAs in mouse ES cells suggest that Ro may participate in a general quality control pathway for defective small RNAs. It is well accepted that cells possess mechanisms to recognize defective mRNAs as well as misfolded and mutant proteins [63]. Targeting of defective mRNAs for degradation requires that the mRNA be translated [63], making this pathway inaccessible to small noncoding RNAs. As the mouse genome encodes many variant U2 snRNAs [64], while *Xenopus* contains many variant oocyte-specific 5S rRNA genes [60], the particular RNA detected bound to Ro could reflect the abundance of the misfolded transcripts in the cell type and species under study.

Many questions remain about the putative role of Ro in small RNA quality control. For example, how does Ro recognize misfolded RNAs? Are the misfolded RNAs folding or assembly intermediates, or does Ro binding target them for degradation? What role do the Y RNAs play in this pathway? What are the other protein components? Much experimentation will be required to address these issues.

The Ro 60 kDa protein assists cell survival following ultraviolet irradiation

A conserved role for the Ro 60 kDa protein in facilitating cell survival after ultraviolet irradiation has recently emerged from studies of Ro in bacteria and mammalian cells. The first clue came from the observation that the enormously radiation-resistant eubacterium *D. radiodurans* contains orthologues of both the Ro protein and a Y RNA [11]. Experiments in which the Ro orthologue was deleted revealed that Ro contributed to survival of this bacterium after ultraviolet irradiation [11]. Moreover, following ultraviolet irradiation, both the Ro protein and the Y RNA are upregulated [11]. Recent findings in mice and mammalian cells have confirmed that conferring UV resistance is a conserved role for the Ro protein. First, in one strain of mice lacking the Ro 60 kDa protein, the number of apoptotic keratinocytes increases two-fold following irradiation with ultraviolet light [12]. Second, mouse embryonic stem cells lacking Ro have a lower survival rate following exposure to ultraviolet irradiation [10]. No differences were detected in the survival rate of *Ro*^{-/-} cells following ionizing radiation [10]. Following ultraviolet irradiation, both the Ro protein and a Y RNA, which are both largely cytoplasmic in unirradiated cells, accumulate in nuclei [10] (Fig. 2).

These experiments with mammalian and bacterial cells lacking Ro suggest that the Ro protein has a conserved

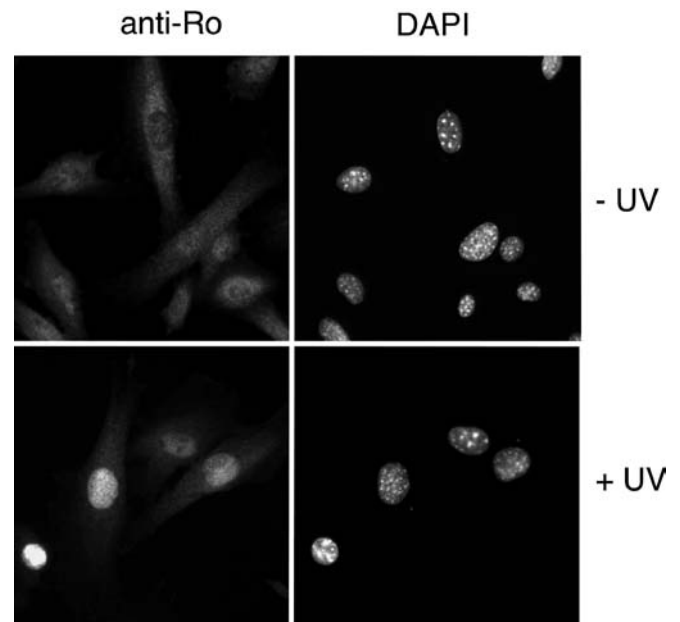


Fig. 2 The Ro 60 kDa protein accumulates in the nucleus following ultraviolet irradiation. Mouse embryonic fibroblasts were either untreated (*top*) or irradiated (*bottom*) with 5 J/m² UV-C light. After 20 h, the cells were fixed and the subcellular distribution of the Ro protein determined by performing immunofluorescence with an antibody against the mouse protein (see reference [10])

role in the recognition or repair of cellular damage. An alternative but not exclusive possibility is that Ro is part of a more general pathway by which cells and organisms survive certain types of environmental stress. Interestingly, *C. elegans* worms lacking Ro are unable to form dauer larvae, a developmental stage formed when worms are starved, overcrowded or subjected to elevated temperatures [65]. Although the ultraviolet irradiation sensitivity of these worms has not been reported, it has been hypothesized that Ro is part of a mechanism by which internal cellular damage is sensed and relayed to components of the dauer formation pathway [65].

How might Ro enhance cell survival after ultraviolet irradiation or environmental stress? In addition to inducing pyrimidine crosslinks in DNA, ultraviolet light also causes RNA:RNA and RNA-protein crosslinks [66]. Newly synthesized, incompletely assembled RNAs may be especially vulnerable to radiation-induced crosslinks. Since Ro binds misfolded pre-5S and U2 RNAs, one possibility is that following irradiation, Ro sequesters nascent RNAs that misfold or fail to assemble into RNPs. Such a role would be consistent with a general role for Ro in resistance to environmental stress, as many stress conditions may perturb nascent RNA folding and/or RNP assembly. Alternatively, Ro may function in a separate process following irradiation, such as transcription or DNA repair. More work is clearly needed to elucidate the mechanism(s) by which the Ro protein enhances the survival of irradiated cells and to determine whether the Y RNAs contribute to this function.

The Ro 60 kDa protein may be important for the prevention of autoimmunity

Studies of mice lacking the Ro 60 kDa protein suggest that the normal function of Ro may be important for the prevention of autoimmune disease [12]. In these studies, mice lacking Ro were found to develop autoantibodies and membrano-proliferative glomerulonephritis. Analyses of the autoantibodies revealed that they were largely directed against chromatin and ribosomes, although one mouse developed antibodies against the U1 snRNP [12]. Moreover, after three backcrosses into the C57/BL6 background, but not in the original mixed (129/Sv×C57BL/6) background, *Ro*^{-/-} mice exhibit a two-fold increase in apoptotic keratinocytes upon exposure to physiologic doses of UV-B irradiation as compared to wild-type mice. Thus, in at least one genetic background, lack of the Ro protein also results in photosensitivity [12], a common manifestation of human lupus.

How might the absence of the Ro 60 kDa protein result in autoimmunity? Mouse mutations that result in systemic autoimmunity fall into several classes [67]. Mutations in cytokines and other signaling molecules that alter immune system function can cause autoimmunity, as can mutations that affect apoptosis of lymphocytes [67]. However, T and B cell populations and function in *Ro*^{-/-} mice appeared normal [12], suggesting that the autoimmunity in these mice may not be caused by a primary defect in immune system function. However, a second class of mutations that result in murine autoimmunity affects components of the machinery involved in clearing extracellular debris. These include mutations in the complement protein C1q and the receptor tyrosine kinase Mer, both of which are involved in removing apoptotic cell debris, as well as mutations in DNase I, the enzyme that digests DNA released during cell death [68, 69, 70]. Thus, one possibility is that in *Ro*^{-/-} mice, a small population of incorrectly assembled ribosomes containing misfolded 5S rRNAs causes a breach of tolerance by exposing normally cryptic determinants to the immune system [12].

Possible clinical implications

Do the very basic studies that have been carried out on the Ro 60 kDa protein in mice, frogs, worms and bacteria have clinical implications? One obvious question is whether loss of Ro function plays a role in the development or perpetuation of autoimmunity in lupus patients. Interestingly, the human Ro gene, which contains nine exons spanning 18 kb [71], maps to chromosome 1q31 [72], which has been linked to lupus in several studies [73, 74, 75]. Thus, some patients could have mutations that ablate Ro function. To date, one study has examined whether patients with cutaneous lupus erythematosus possess exonic polymorphisms in the Ro gene [76]. Although no significant differences were detected between patients and control groups, patients with concom-

itant SLE were excluded from the study [76]. Since mice lacking Ro exhibit a systemic manifestation, glomerulonephritis [12], it will be important to also examine SLE patients for mutations that alter or eliminate Ro function. An alternative possibility is that human anti-Ro antibodies could enter cells and interfere with their function, resulting in a failure of small RNA quality control mechanisms and the presentation of abnormal RNPs to the immune system. In support of this idea, anti-Ro and anti-La antibodies are often detected in patients years before the onset of clinical symptoms [77]. However, while there are many reports that antibodies enter cells e.g. [78, 79, 80, 81, 82, 83, 84], it is unclear that antibodies can access the cytosol in quantities sufficient to inactivate an abundant intracellular target [85].

As the normal function of Ro is important for cell survival after exposure to ultraviolet irradiation, it is intriguing that anti-Ro antibodies have long been associated with photosensitive skin lesions in lupus patients, particularly the lesions of SCLE [2, 86]. Although many studies documenting a correlation between anti-Ro antibodies and SCLE have not distinguished between antibodies against the Ro 60 kDa protein and the structurally unrelated 52 kDa Ro protein, one group has reported that 100% of SCLE patients examined (17/17) had antibodies to 60 kDa Ro, while the association with anti-52 kDa Ro antibodies, while also high, was not as striking [87]. Others have not found as strong a correlation with 60 kDa Ro [88], possibly because these studies used immunoblotting to distinguish the 52 and 60 kDa Ro autoantigens. While antibodies against the Ro 52 kDa protein are easily detected by immunoblotting, most patient anti-60 kDa Ro antibodies recognize only the native form of the 60 kDa Ro protein, leading to a high false-negative rate in immunoblotting assays [21, 29, 89].

More evidence for a role for anti-Ro antibodies in the pathogenesis of photosensitive skin lesions comes from studies of neonatal lupus erythematosus. This syndrome, which is strongly associated with maternal anti-Ro antibodies, is characterized by neonatal skin lesions indistinguishable from those of SCLE and by a specific cardiac conduction defect, third degree heart block [4, 90]. The duration of the skin disease is similar to the length of time that maternal autoantibodies persist in the infants' circulation, with the lesions resolving when the antibodies are no longer detected. While these findings implicate the autoantibodies in pathogenesis, it remains controversial whether antibodies against the 52 kDa Ro protein or the 60 kDa Ro protein are more closely associated with the various disease manifestations [4, 90]. Given the role of the Ro 60 kDa protein in facilitating mammalian cell survival after exposure to UV irradiation, it is tempting to speculate that antibodies against this protein trigger the photosensitive skin lesions. However, it remains a conundrum how antibodies could enter cells and access cytosolic and nuclear proteins. One possibility is that exposure to UVB triggers transient wounding of keratinocyte plasma membranes, allowing entry of antibodies into a fraction of cells. Alternatively, there are

several reports that following UV-irradiation of keratinocytes, anti-Ro antibodies bind to the surface of a fraction of keratinocytes, implying that the Ro autoantigen somehow becomes accessible on the cell surface [91, 92, 93]. However, a mechanism by which a cytosolic or nucleoplasmic protein could reach the cell surface, other than through cell lysis, has not been described. Thus, despite significant circumstantial evidence implicating anti-Ro antibodies in the pathogenesis of SCLÉ and neonatal lupus skin lesions, the mechanism(s) by which the antibodies contribute to the development of photosensitive skin lesions remains to be determined.

Conclusions and perspectives

Recent genetic and biochemical studies have revealed that the Ro 60 kDa protein and its associated Y RNAs are important components of vertebrate cells. Ro has now been implicated in two conserved processes, the recognition of misfolded small RNAs and the survival of cells after ultraviolet irradiation. Additional findings that mice lacking Ro develop autoantibodies and glomerulonephritis have further raised the tantalizing possibility that the normal function of Ro is important for preventing autoimmune disease in humans.

While these observations have begun to place the long mysterious Ro protein within a cellular and molecular context, the challenge now is to obtain a mechanistic understanding of Ro function. Nothing is yet known of how Ro binding to misfolded RNAs affects the fate of these RNAs, or how the nuclear accumulation of Ro aids cell survival after irradiation. The role played by Y RNAs in these processes has also yet to be addressed. Future advances will likely involve structural analyses of Ro and its associated RNAs, as well as the continued identification and functional dissection of interacting proteins. A better understanding of the roles played by Ro RNPs in cells will likely give new insights into basic cellular mechanisms, and may also be helpful in determining whether diminished Ro function contributes to autoimmunity and/or photosensitivity in patients.

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