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Characterization of Sequence-Specific Binding of LARP6 to the 5' Stem-Loop of Type I Collagen mRNAs and Implications for Rational Design of Antifibrotic Drugs

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Abstract

Excessive synthesis of type I collagen is a hallmark of fibrotic diseases. Binding of La-related protein 6 (LARP6) to the 5' stem-loop (5'SL) of collagen mRNAs regulates their translation leading to an unnaturally elevated rate of collagen biosynthesis in fibrosis. Previous work suggested that LARP6 needs two domains to form stable complex with 5'SL RNA, the La domain and the juxtaposed RNA recognition motif (RRM), jointly called the La-module. Here we describe that La domain of LARP6 is necessary and sufficient for recognition of 5'SL in RNA sequence specific manner. A three-amino-acid motif located in the flexible loop connecting the second α-helix to the β-sheet of the La domain, called the RNK-motif, is critical for binding. Mutation of any of these three amino acids abolishes the binding of the La domain to 5'SL. The major site of crosslinking of LARP6 to 5'SL RNA was mapped to this motif, as well. The RNK-motif is not found in other LARPs, which can not bind 5'SL. Presence of RRM increases the stability of complex between La domain and 5'SL RNA and RRM domain does not make extensive contacts with 5'SL RNA. We propose a model in which the initial recognition of 5'SL by LARP6 is mediated by the RNK epitope and further stabilized by the RRM domain. This discovery suggests that the interaction between LARP6 and collagen mRNAs can be blocked by small molecules that target the RNK epitope and will help rational design of the LARP6 binding inhibitors as specific antifibrotic drugs.

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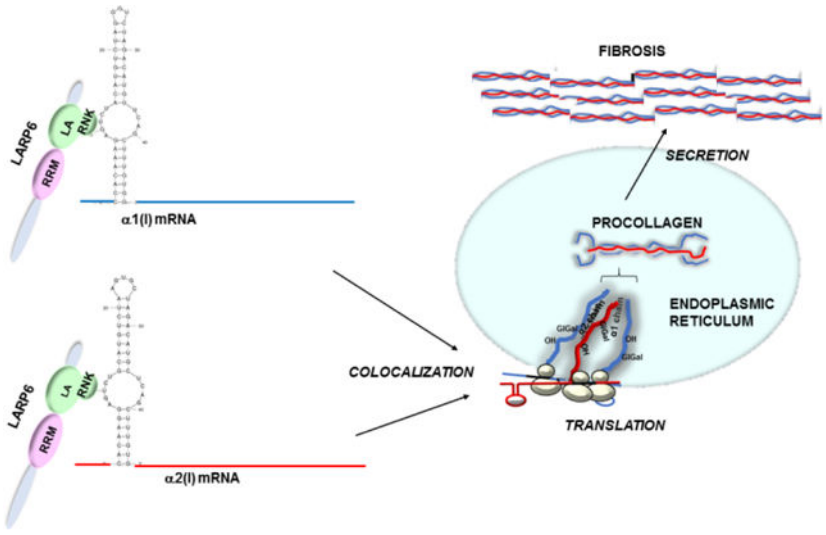
Graphical Abstract



Keywords

RNA/protein recognition; La-related protein 6; La-domain; fibrosis; binding affinity

Introduction

[1]LARP6 (La-related protein 6) is a member of the LARP superfamily of RNA binding proteins, which are post-transcriptional regulators [1–5]. While other members of the superfamily bind either homopolymers of RNA or show no strict sequence preference [6–8], LARP6 binds a unique sequence found in mRNAs encoding for collagen α1(I) and α2(I) polypeptides, which fold into type I collagen, and in collagen α1(III) mRNA which encodes for type III collagen [9]. This sequence was termed collagen 5’ stem-loop (5’SL) and is located in the 5’UTR of these mRNAs and includes the start codon (Fig 1A). The 5’SL is 48 and 46 nucleotides long in collagen α1(I) and α2(I) mRNAs, respectively, and folds into two stems flanking a central bulge of 9 nucleotides. Our previous work has identified 5 nucleotides within the bulge (circled in Fig 1A) which are critical for binding LARP6; mutation of any of these 5 nucleotides abolished the interaction of 5’SL with LARP6 in gel mobility shift experiments [10]. The stems are important only as dsRNA that flanks the bulge.

It has been reported that two domains of LARP6, the La domain and the RRM domain (together called the La-module, Fig 1A) are necessary for interaction with the 5’SL [11]. The involvement of two protein domains and five nucleotides of RNA suggested complex interactions between the 5’SL and LARP6. The K_d of 5’SL/La-module interaction was

[1]LARP6, La-related protein 6. 5’SL, 5’ stem-loop of collagen mRNAs. RRM, RNA recognition motif. LAM, La-module. LARP, La-related protein. ITC, isothermal titration calorimetry. FSR, fractional synthesis rate. EMSA, electrophoretic mobility shift assay. MST, microscale thermophoresis.

previously measured to be 0.4 nM using fluorescence polarization [10], 12 nM using gel mobility shift assay [12] and 40 nM using isothermal titration calorimetry (ITC) [11].

Collagens type I and III are fibrillar proteins expressed in bone, tendons and skin, with type I representing 90% of fibrillar collagen in these organs [13]. In the pathological state of fibrosis, type I collagen is expressed at high levels in other organs, such as liver, heart, kidney, blood vessels, joints or lungs, leading to their scarring and loss of function. Fibrosis affects 45% of the population in the USA [14] and represent a major medical problem, however, there is no cure. Type I collagen is composed of two α1(I) and one α2(I) polypeptides and its folding is highly dependent on local concentration of the individual polypeptides, because all three polypeptides have to register their C-terminal domains to initiate the nucleation of the heterotrimer [15]. It has been demonstrated that biogenesis of type I collagen takes place in highly organized structural bodies termed collagenosomes, and that binding of LARP6 is critical for their formation [16].

In normal tissues, type I collagen is one of the most stable proteins with half-life of 4–12 months and its fractional synthesis rate (FSR, defined as % synthesis per day) is about 2% in the skin [17], while in the liver it is only 0.19% [18]. This is significantly slower than the FSR of most intracellular and secreted proteins (18–140% per day) [19]. In fibrosis, however, the synthesis of type I collagen is significantly increased [20, 21], for example, the FSR in the liver increases to 0.6% [18]. Binding of LARP6 to collagen mRNAs is necessary for this acceleration of biosynthesis [22], because LARP6 recruits accessory translational factors to increase translational competency of collagen mRNAs and to couple translation of α1(I) polypeptide to that of α2(I) polypeptide [2, 23–27], resulting in formation of collagenosomes [16].

Experimental animals which synthesize type I collagen without LARP6 regulation were shown to be resistant to hepatic fibrosis. [28], indicating the importance of LARP6 dependent regulation in fibrosis. This resulted in a concept that inhibitors of LARP6 binding to 5’SL can be specific antifibrotic drugs. We have recently discovered and characterized one such inhibitor by high throughput screening [29] and developed a phenotypic screening method for additional drug screening efforts [30]. Here, we describe the molecular characterization of sequence specific interaction of the La-domain of LARP6 and 5’SL RNA which will help rational design of additional inhibitors.

Results

The La domain of human LARP6 is sufficient for the RNA sequence specific binding of 5’SL RNA.

Fig 1A shows schematic representation of the domains of human LARP6. Two domains, the La and RRM domains, are jointly called the La-module [4]. La-module, as well as full size LARP6, binds α1(I) 5’SL and α2(I) 5’SL with similar affinity (the sequence of these elements is shown in Fig 1A, bottom panel) [10]. In this work we have used both 5’SL RNA sequences, as indicated in the Figures. It has been reported before that the entire La-module is necessary for binding α1(I) 5’SL RNA [11]. However, using freshly prepared recombinant

proteins or cell extracts, we were able to show that La domain alone is sufficient to bind 5'SL in the RNA sequence specific manner.

To demonstrate the binding activity of La domain, we recombinantly expressed the La domain (residues 81–182) in *E. coli* and purified the protein by single step purification (Fig 1B). We observed that excessive purifications, as well as freezing and thawing of the La domain, inactivates its binding activity. If present, the contamination by bacterial proteins has no effect on binding of La domain to 5'SL RNA (supplementary figure 1). Fig 1C shows that when the recombinant La domain was added in increasing amounts to α2(I) 5'SL RNA, the RNA/protein complex was formed, which was stable to withstand gel electrophoresis through native acrylamide gels.

We also analyzed the binding of recombinant RRM domain (residues 183–298) to α2(I) 5'SL (Fig 1D). While the La domain showed binding activity to 5'SL RNA (Fig 1D, lane 2), the RRM domain only weakly interacted with 5'SL (Fig 1D, lane 3). When La and RRM domains were mixed, the binding was indistinguishable from that of La domain alone (compare lanes 4 and 2). This suggested that the RRM domain cannot complement the binding of La domain when present in trans. As control, we also analyzed the binding of the recombinant La-module (LAM, Fig 1D, lane 5), where both domains are present in cis. La-module showed strong binding with formation of two complexes; these complexes represent monomer and dimer of LAM bound to RNA, what has been occasionally seen with LAM preparations. Figure 1E shows the preparations of recombinant RRM and LAM.

To verify these results with the La domain expressed in mammalian cells, we cloned the HA-tagged La domain and the HA-tagged RRM domain into mammalian expression vectors, expressed the domains in HEK293 cells and analyzed their binding activity to α2(I) 5'SL RNA in whole cell extracts using electrophoretic mobility shift assay (EMSA). We found that the La domain alone was able to interact with 5'SL RNA probe (Fig. 1F, lane 2), while the RRM domain was not (Fig. 1F, lane 3). When both domains were co-expressed in trans, the binding was indistinguishable to that of the La domain alone (Fig. 1F, lane 4). The expression level of the domains was analyzed by western blot using anti-HA antibody (Fig 1F, bottom panel). The RRM domain was expressed at higher level than the La domain, but no binding activity was evident. This suggested that the La domain is necessary and sufficient for recognition of 5'SL in mammalian cells and that RRM domain alone has no binding activity towards 5'SL RNA. When bound to 5'SL RNA, the electrophoretic mobility of La-domain expressed in mammalian cells was similar to that of recombinant La-domain, suggesting that no additional cellular proteins are present in the RNA/protein complex (supplementary fig 2).

La domain alone accumulated equally in the nucleus and in the cytoplasm of cells, as assessed by immunostaining (supplementary Fig 3). Full size LARP6 was predominantly cytoplasmic, with enhanced peri-nuclear accumulation, what was not seen with the La domain alone. We have reported previously that LARP6 associates with the ER membrane [31] and the peri-nuclear localization is consistent with its role in biosynthesis of type I collagen [16].

To analyze if binding of the La domain to 5'SL is RNA sequence specific, we competed the binding of recombinant La domain to labeled α1(I) 5'SL RNA with excess of unlabeled wt α1(I) 5'SL RNA (Fig 2A) or with excess of mutant α1(I) 5'SL RNA, which had the five nucleotides critical for binding of LARP6 changed (Fig 2B). In the presence of wt 5'SL competitor the complex started to diminish at ~10-fold molar excess of the competitor (Fig 2A, lane 4) and almost complete competition was seen with 100-fold excess of the competitor (Fig 2A, lane 7). The mutant 5'SL competitor had no effect on the La/5'SL complex even at >100-fold molar excess (Fig 2B).

Next, we assessed if the La domain alone can distinguish between the single nucleotide mutants of α1(I) 5'SL. We used the mutant 5'SL probes in which each of the nucleotides critical for binding of LARP6 (circled in Fig 1) was mutated [10] and analyzed binding of recombinant La domain by EMSA. Fig 2C shows that only wt α1(I) 5'SL RNA was recognized by the La domain (Fig 2C, lane 1), while all mutant α1(I) 5'SL RNAs failed to bind (Fig 2C, lanes 2–6). For comparison, Fig 2D shows the specificity of binding of recombinant La-module; La-module also recognized only the wt α1(I) 5'SL RNA (Fig 2D, lane 1). Thus, the recombinant La domain binds wt α1(I) 5'SL RNA with the same sequence specificity as the La-module, suggesting that the La domain contains all the information needed for the RNA sequence specific recognition of 5'SL.

RRM domain increases the affinity of binding to 5'SL RNA.

To assess the affinity of binding of La domain to α2(I) 5'SL RNA, we performed microscale thermophoresis (MST) measurements and compared the affinity to that of La-module (Fig. 3). These measurements showed that a stable complex between the α2(I) 5'SL RNA and the La domain was formed (Fig 3A), with a dissociation constant for the formation of the α2(I) 5'SL/La domain complex of ~634.1 nM, ranging from 533.6 nM to 753.9 nM according to the 95% confidence interval (Fig 3C). This value is comparable to the K_d of several other RNA binding proteins [32]. We also determined the dissociation constant for the formation of the α2(I) 5'SL A2/La-module complex, which was ~108.8 nM, ranging from 99.0 nM to 119.7 nM according to the 95% CI (Figs 3B and 3D). The K_d of 65 nM for binding of La-module to 5'SL-A1 RNA has been reported before [11], what is in excellent agreement with our result.

Thus, the α2(I) 5'SL RNA binds the La-module with 6-fold higher affinity than the La domain, suggesting that the role of RRM is to stabilize the complex. These experiments clearly indicated the formation of a 5'SL/La domain complex, as an independent verification of the gel mobility shift results shown in Figs 1 and 2.

Next, we directly tested the relative stability of 5'SL/LA and 5'SL/LAM complexes by performing cross-competition experiments. The difference in electrophoretic mobility between 5'SL RNA in complex with La domain and in complex with La-module allowed us to simultaneously analyze the formation of both complexes when the proteins compete for 5'SL RNA. In one experiment (Fig 4A) we prepared increasing amounts of recombinant La-module in absence (lanes 2–5) or presence (lanes 6–9) of fixed amount of La domain, added labeled 5'SL RNA and analyzed the equilibration of complexes by gel mobility assay. The increasing amounts of La-module bound increasing amounts of 5'SL RNA regardless of

the absence (lanes 2–5) or presence of La domain in the reaction (lanes 6–9), suggesting that La domain can not compete with La-module for 5'SL RNA. At the same time, the complex formed with La domain in presence of low amounts of La-module (lane 6) was competed out by increasing amounts of La-module (lanes 7–9).

In the opposite experiment (Fig 4B) we prepared increasing amounts of recombinant La domain in absence (lanes 2–5) or presence (lanes 6–9) of fixed amount of La-module and added labeled 5'SL RNA. The increasing amounts of La domain bound increasing amounts of 5'SL RNA in the absence of La-module (lanes 2–5), however, the fixed amount of La-module readily competed out the La domain complexes (lanes 6–9). These results clearly demonstrated that the La-module outcompetes the La domain for binding to 5'SL, consistent with its 6-fold higher binding affinity.

Crosslinking by UV light covalently attaches RNA to a protein if they are in close proximity [33]. We have previously reported a crosslink between La-module and α1(I) 5'SL RNA [2]. Here we compared the crosslinks of La domain and La-module to α2(I) 5'SL RNA. A weak UV crosslink was formed with La domain, while a stronger crosslink was seen with La-module (Fig 4C).

The C-terminus of La domain is relevant for activity.

The C-terminal 15 amino-acids of the La domain (R169-S183) have been reported to contribute to the synergistic activity of La/RRM in the La-module [11]. This sequence, commonly referred to as the linker between the La domain and the RRM domain, was shown in the NMR structure of apo La domain (PDB 2MTF) to be well defined between residues R269 and F176 with many side chains forming contacts to the "core" of La domain. However, the residues P177 to S183 of the linker seem to be more flexible [11].

To assess the role of the linker sequence for La domain activity, we deleted 8 or 16 amino acids from the C-terminus of La domain, named LAΔ8 (L81 to P174) and LAΔ16 (L81 to K166), respectively, expressed the truncated domains in HEK293 cells, and analyzed the binding in cell extracts by gel mobility shift (Fig 5A). Deletion of 8 or 16 amino acids at the C-terminus abolished the binding (lanes 3 and 5), suggesting that C-terminal amino acids are important for La domain activity.

To address if this is a sequence specific or length specific effect, we appended several random sequences to the truncated La domain. Fig 5B, lanes 2 and 3, show that two random sequences partially restored the binding of both truncated La domains LAΔ8 and LAΔ16. When we extended the full-length La domain with 26 random amino acids the binding was even further increased (lane 4).

With these experiments it was not possible to further ascribe the underlying mechanism of these observations. The C-terminal residues either affect the binding directly or indirectly, by helping the folding of active protein and their deletion may generate a misfolded, inactive La domain. However, from these experiments we can conclude that the C-terminus of La domain is needed and that appending unrelated sequences to the C-terminus can recover the activity.

The RNK epitope of the La domain is critical for 5’SL recognition.

Having established that La domain is involved in recognition of 5’SL, we performed site directed mutagenesis of the domain to identify if there is a short epitope involved in 5’SL recognition. We concentrated on three amino acids in the loop between the second helix and the first strand of β-sheet of La domain [11]. These amino acids, R121, N122 and K123, are unique to LARP6 and not found in the La domain of other LARPs (see below). We changed each of these amino acids into alanine, expressed the individual mutants in HEK293 cells and analyzed the binding in cell extracts by gel mobility shift. Fig 6A and 6B show that changing any of these three amino acids into alanine completely abolished the ability of La domain to recognize α2(I) 5’SL RNA and α1(I) 5’SL RNA.

Next, we tested if the same mutations have a detrimental effect in the context of full size LARP6. When the individual RNK mutations were tested in context of full size LARP6 a significant decrease in binding to 5’SL was observed, but the binding was not completely abolished (Fig 6C). In three independent experiments, we normalized the signal of 5’SL/LARP6 complex from gel shift experiment to the expression of the protein in same extract determined by western blot and compared the normalized binding between the mutants. This analysis indicated that each individual mutation reduced the binding of full size LARP6 by about 50% (Fig 6D).

Next, we created double mutants in the context of full size LARP6; RK, RN or NK were changed into alanine residues, creating RK/AA, RN/AA and NK/AA double mutants, and the analysis was repeated. Fig 7A and 7B show that the double mutations reduced LARP6 binding by 80–90%. To confirm this result, we made recombinant La-module with the RK/AA mutation and compared the binding to wt La-module. The binding of recombinant RK/AA mutant in the context of La-module was also drastically reduced (Fig 7C). The recombinant proteins used in this experiment are shown in the lower panel of Fig. 7C. From these experiments we concluded that the RNK epitope is critical for RNA sequence specific binding of LARP6 to 5’SL RNA. Because this epitope is found in a loop between the two secondary structures of La domain, it is unlikely that the alanine substitutions would cause gross structural alterations of the La domain.

RNK epitope is not found in other LARPs, but is conserved in LARP6 of vertebrates.

Other members of LARP superfamily have the La-module [4], but can not bind 5’SL. When the sequence of La domains of human LARP superfamily members is compared, the RNK epitope is not found in other LARPs (Fig 7D). This explains why only LARP6 can bind 5’SL and is a compelling evidence that RNK epitope is not necessary for the folding of La domain, but solely for the interaction with 5’SL.

Because collagen 5’SL is found in collagen α1(I) and α2(I) mRNA of all vertebrates, LARP6 of all vertebrates must have the RNK epitope. Sequence comparison of LARP6 of distant vertebrates confirmed that the RNK epitope is conserved in vertebrates; only in birds the epitope is KNK instead of RNK (Fig 7D). Figure 7E displays the 20 lowest energy structures of the La domain as determined by solution NMR spectroscopy [11]. While the residue-specific RMSD is low for the majority of the La domain, particularly the

loop region harboring the RNK epitope was found to be very dynamic. As a visual aid, the position of the Cα atoms of residues R121 (green spheres). N122 (purple spheres) and K123 (orange spheres) are shown, revealing that all three residues of RNK epitope are structurally heterogeneous.

One site of crosslinking between the La-module and 5'SL RNA involves the N122.

To map the amino acid of LARP6 which forms a covalent crosslink to 5'SL RNA upon UV irradiation we took advantage of the fact that the single amino acid mutants of the RNK epitope bind 5'SL when analyzed in the context of full size LARP6 (Fig 6C). We surmised that a mutant that can bind 5'SL but can not be UV crosslinked has the amino acid involved in the crosslink formation changed. Therefore, we made R121A, N122A or K123A mutations in the context of La-module, prepared the recombinant proteins and compared their binding and crosslinking to α2(I) 5'SL RNA. In this experiment we adjusted the amount of mutant proteins to give similar binding signal in gel mobility shift experiment (Fig 8A), and then we compared their UV crosslinking efficiency (Fig 8B). While the R121A and K123A mutants crosslinked to 5'SL with the efficiency slightly less than wt La-module, the N122A mutant showed greatly reduced UV crosslinking signal (compare lanes 2 and 4). Thus, for similar binding activity, the UV induced crosslink formation between the N122A mutant and RNA was greatly reduced, strongly suggesting that the side chain of N122 participates in the formation of the crosslink. The fact that the crosslinking was not completely abolished, suggests that R121, K123 or some other amino-acid may also participate in the crosslinking.

Mapping the interactions on 5'SL RNA.

To assess which regions of 5'SL RNA are protected from nuclease digestion by binding the La domain or La-module we performed RNA footprinting experiments. The 5' end fluorescently labeled α1(I) 5'SL RNA or α2(I) 5'SL RNA were bound to recombinant La domain or La-module and subjected to partial nuclease digestion. The regions shielded by the protein were then displayed as the attenuated or absent nuclease cleavage products. In Fig 9A, α2(I) 5'SL RNA was bound by recombinant La domain and the bound (lanes 4, 5 and 6) and free RNA (lanes 2, 3 and 7) was partially digested with Benzonase. Benzonase is a nuclease which digests RNA irrespectively of the sequence and secondary structure [34]. The fact that the free RNA was not uniformly cleaved by Benzonse (lanes 2, 3 and 7), suggests that it may have some tertiary structures that were poorly digested, what should be considered when interpreting the footprinting results. As a marker, free RNA was partially digested with RNase A which preferentially cleaves after pyrimidines in the single stranded regions (lane 1). This lane allowed us to assign the sizes of cleavage products. Binding of La domain protected the B1 portion of the bulge up to nucleotide 14 (indicated as FP1 in Fig 9A), it also protected nucleotides 32–34 just 5' to the B2 portion of the bulge (FP2), and nucleotides close to the 3' end, which correspond to the descending part of stem 1 (FP3). The location of these footprints relative to the RNA sequence and secondary structure is shown in Fig 9E.

Binding of La-module (Fig 9B) showed similar protection of the B1 part of the bulge of α2(I) 5'SL up to the nucleotide 14 (FP1), and from nucleotides 31 to 35 adjacent to the B2

portion of the bulge (FP2). A protection close to the 3’ end is also seen (FP3). All footprints were stronger with La-module than the footprints seen with La domain, but the extent of the protected regions was not significantly increased. This suggested that RRM increases the strength of binding, but does not protect sizable regions of 5’SL RNA (Fig 9E).

Footprinting of α1(I) 5’SL RNA by La domain is shown in Fig 9C. There was weak protection of the B1 segment of the bulge (FP1) up to the nucleotide 16, protection of nucleotides 33–35 adjacent to the B2 segment (FP2) and protection of nucleotides close to the 3’ end, which correspond to the descending part of stem 1 (FP3). This pattern was similar to that seen with the α2(I) 5’SL (Fig 9E).

Fig 9D shows the protection of α1(I) 5’SL RNA by La-module. La-module strongly protected the B1 segment of the bulge and part of stem 2 up to the nucleotide 16 (FP1). La-module also showed strong protection of nucleotides 32–34, which are adjacent to the B2 segment of the bulge (FP2) and of nucleotides close to the 3’ end, which correspond to the descending part of stem 1 (FP3). Again, the footprints were stronger, but not significantly larger than with La domain (Fig 9E).

Overall, these results indicate that the footprints on α1(I) and α2(I) 5’SL cover the equivalent parts of the RNA and that they are stronger with La-module, but the area covered by the footprints is not enlarged compared to the footprints with La domain (Fig 9E).

Discussion

LARP6 is the specific regulator of translation of type I collagen mRNAs and recognition of 5’SL of collagen mRNAs by LARP6 is critical for fibrosis development [28, 29]. Therefore, LARP6 binding inhibitors show promise as antifibrotic compounds [29], because they overcome the main drawback of antifibrotic drugs currently in development; the lack of specificity towards type I collagen. This work provides insight into the molecular recognition between LARP6 and 5’SL, as the first step to help rational design of such inhibitors. The main findings are: 1. La domain of LARP6 is necessary and sufficient to bind 5’SL in RNA sequence specific manner (Figs 1 and 2), 2. RRM domain serves to stabilize the interaction of La domain with 5’SL RNA (Fig 3), but does not make extensive contacts with the RNA (Fig 9). 3. A short epitope of La domain, the RNK, is critical for binding (Figs 6 and 7), suggesting that limited number of amino-acids convey the sequence specific recognition. 4. The direct contact between LARP6 and 5’SL RNA, as mapped by UV crosslinking, is between N122 of the RNK epitope and one of the nucleotides within the B2 portion of the bulge (Figs 8 and 9). 5. The sequences at the C-terminus of La domain, which form a linker with the RRM, also participate in binding, but not in an amino-acid sequence specific manner (Fig 5).

It has been reported previously that La domain alone can not bind 5’SL [11]. However, that work used isothermal titration calorimetry (ITC) to measure the binding, while we employed gel mobility shift assays (EMSA) and microscale thermophoresis (MST). During the course of our experiments, we noted that the binding activity of La domain rapidly deteriorates upon manipulations and storage of the protein, what is not the case with

La-module. Therefore, in our experiments we always used freshly prepared samples of La domain. We were able to show that the recombinant La domain or La domain expressed in mammalian cells can bind 5'SL RNA in the RNA sequence specific manner and can discriminate between the single nucleotide mutants of 5'SL (Figs 1 and 2).

The identification of RNK motif as critical sequence for recognition of 5'SL RNA is an important new finding. All LARP family members have the highly conserved La domain, but their La domain lacks the RNK sequence, what can explain why they can not bind collagen 5'SL. Mutations of individual RNK amino acids completely abolish the interaction of La domain with 5'SL RNA. In the context of full size LARP6, the individual mutations are less detrimental and reduce binding by ~50%. However, simultaneous mutations of two amino acids of the epitope reduce binding by ~90% (Figs 6 and 7), indicating the importance of all three amino acids. The RNK epitope resides in the loop that connects the second α-helix to the first β-sheet strand of La domain. Thus, the epitope is not part of the secondary structure and is exposed on the surface of the protein. In the NMR spectra of La domain the resonances of atoms in this loop are broad [11], suggesting an inherently flexible structure; Fig 7E depicts the structure and flexibility of this epitope.

Besides the RNK epitope, La domain requires amino acids at the C-terminus, which connect La domain to RRM (La/RRM linker). Truncation of 8 amino acids at the C-terminus is detrimental to La domain binding, but the binding can be restored by substituting random amino acids. We have found one amino acid sequence which increased the binding (Fig 5B), what suggested that the sequences appended to the C-terminus can modulate the strength of interaction of the La domain. Previous work indicated that the La/RRM linker is important for binding of La-module, as well [11]. The linker has a flexible structure and together with the RNK loop forms a highly dynamic cavity (PDP ID: 2MTF).

The crystal structure of La-module of LARP3 [35] and LARP7 [36] bound to short poly-U RNA have been solved. In both cases the RNA is sandwiched in the binding pocket between La domain and RRM with both domains making contacts with the RNA. The NMR structure of La domain of human LARP6 indicates that a cavity is formed between the C-terminus of La-domain and the loop containing the RNK epitope (PDP ID: 2MTF) [11]. Therefore, it is possible that the interaction between LARP6 and 5'SL RNA involves the structure formed by these two parts of La domain.

La domain binds 5'SL RNA with 6-fold lower affinity than La-module (Fig 3). When they compete for the 5'SL RNA, the La-module easily outcompetes binding of La domain (Fig 4B and 4C), suggesting that the complexes formed with La-module are more stable. This indicates the distinctive roles of the two domains: the RNA sequence specific recognition conveyed by the La domain and stabilization of the RNA/protein complex executed by the RRM. RRM alone can not bind 5'SL RNA and in trans can not augment the binding of La domain (Fig 1), therefore, it has to be tethered to 5'SL by the La domain. The RNA footprinting experiments corroborate this notion. The area of 5'SL RNA that is protected from nuclease digestion by La domain includes the bulge and stem 1, however, this area is not significantly larger when La-module is bound, just a stronger protection is seen (Fig 9).

How RRM increases the affinity of La domain for 5'SL RNA can not be discerned from this study and must await determination of the structure of La-module.

It has always been surmised that protein-RNA binding involves large surface area and that it can not be dissociated by small molecules [37]. However, we showed that the relatively small RNK epitope is critical for recognition (Figs 6 and 7) and that a major crosslink between the LARP6 and 5'SL RNA is formed by the N122 (Fig 8). This suggests that the initial recognition is driven by small number of contacts and that blocking of RNK docking to 5'SL by small molecules would inactivate LARP6 in collagen biosynthesis. Finding LARP6 binding inhibitors is of utmost importance, because the RNK epitope is not found in other LARPs and 5'SL RNA is found only in type I and type III collagen mRNAs, thus, such inhibitors may be specific for excessive expression of type I collagen in fibrosis [29, 38].

Material and methods

Preparation of recombinant proteins.

For expression of La domain of human LARP6 in E. coli the sequence of human LARP6 cDNA encoding for amino acids 81–182 was cloned in pET15b vector, while for expression of RRM domain the sequence encoding amino acids 183 to 298 was cloned. The construct expressing recombinant La-module was described in [10]. Site directed mutagenesis was performed with QuickChange II kit (Stratagene) according to manufacturer instructions and the identity of mutants was verified by sequencing. pET15b vectors were transformed into Rosetta cells and protein expression was induced with 1mM IPTG for 3h. Cells were sonicated, and recombinant proteins purified in a single step on Ni-agarose column according to manufacturer instructions (Invitrogen). Protein samples were dialyzed against PBS with 5 mM $MgCl_2$ and 0.1% glycerol for 2h and used immediately in experiments. The purity of preparations was analyzed by SDS-PAGE and Coomassie staining.

Expression in mammalian cells.

La domain and RRM described above were cloned into pCDNA3 vector containing 2HA tags at the N-terminus for La domain and 2HA tags at the C-terminus for RRM domain. Full size LARP6 construct was described in [10]. Site directed mutagenesis was performed with QuickChange II kit (Stratagene) according to manufacturer instructions and the identity of mutants was verified by sequencing. The constructs were transfected into HEK293 cells and two days after transfection cells were lysed in PBS containing 5 mM $MgCl_2$, 0.5% NP-40. 0.5% glycerol and protease inhibitors and cell extracts were used in gel mobility shift experiments and western blots immediately after preparation.

Gel mobility shift experiments.

The binding reactions contained 5–40 μL of recombinant proteins or HEK293 cell extract, 20 nM of 5'SL RNA probe fluorescently labeled at the 5' end (Dharmacon) and 10 μg of yeast t-RNA in total volume of 50 μl. The probe RNAs had sequence of human collagen α1(I) and α2(I) 5'SL and were 48 and 46 nucleotides long, respectively. Reactions were incubated at RT for 15 min and resolved on 6% native acrylamide gels, as described [10]. For cross-competition experiments recombinant proteins were mixed in the indicated

amounts prior to addition of 5'SL RNA probe. Imaging of the gels was done by Biorad ChemiDoc MP imaging system and intensity of bands was quantified by Image J program.

Microscale Thermophoresis (MST).

Cy5-labeled 5'SL-A2 (A2-Cy5) was generated by enzymatic addition of pCp-Cy5 (Jena Bioscience, Germany) to the 3'-OH of 5'SL-A2 using T4 RNA Ligase 1 (New England Biolabs) [39]. The RNA concentration was determined by UV/VIS spectrophotometry at 95°C, using the extinction coefficient of ε_{260} = 443,000 $M^{-1}cm^{-1}$ for single-stranded RNA [40]. Size-exclusion chromatography (SEC) was used to exchange A2-Cy5, the La-module of LARP6 (hLARP6(73–303)), and the La domain of LARP6 (hLARP6(73–183)) into the assay buffer containing 20 mM sodium phosphate pH 7.25, 100 mM KCl, 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 0.4 mg/mL BSA. Protein samples were prepared by serial dilution, after which 19.7 µl of each protein sample (La domain or La-module) was added to 0.3 µl of RNA sample (A2-Cy5). The final protein concentrations were 15.88 µM, 7.94 µM, 3.97 µM, 1.99 µM, 992.50 nM, 496.25 nM, 248.13 nM, 124.06 nM, 62.03 nM, 31.02 nM, 15.51 nM, 7.75 nM, 3.88 nM, 1.94 nM, and 969.24 pM for the La domain, and 8.02 µM, 4.01 µM, 2.00 µM, 1.00 µM, 501.00 nM, 250.50 nM, 125.25 nM, 62.63 nM, 31.31 nM, 15.66 nM, 7.83 nM, 3.91 nM, 1.96 nM, 978.52 pM, and 489.26 pM for the La-module.. The final concentration of A2-Cy5 was kept constant at 10 nM. Microscale thermophoresis (MST) measurements were performed in triplicates on the Monolith NT.115 system using Monolith NT.115 capillaries (Nanotemper Technologies, Germany). Dose-response values were calculated by dividing the average normalized fluorescence between seconds 4 and 5 after IR-Laser activation (F_{HOT}) by the average normalized fluorescence between seconds −1 and 0 prior to IR-Laser activation (F_{COLD}) [41] and were fitted globally using Prism 9.0.0 (GraphPad) applying equation (1) describing single-site binding [42].

$$Y = U + \frac{(B - U) \cdot X}{K_d + X} \quad (1)$$

where U and B are the unbound and bound fractions, respectively, X is the protein concentration, Y is the dose-response value at that protein concentration, and K_d is the dissociation constant. The fitting was performed once to determine the values for U, B, and K_d. The normalized dose-response curve was generated by dividing dose-response values by (B-U). The error in K_d was estimated using a 95% asymmetrical (profile-likelihood) confidence interval (95% CI).

Western blots.

The same cell extracts used in gel mobility shift experiments were analyzed by western blots. 30 µl of extract was resolved on 10 or 12% SDS-PAGE gels and after transfer the membranes were probed with 1:1000 dilution of anti-HA antibody (Sigma). Imaging of blots was done by Biorad ChemiDoc, 2.3.0.07 imagining system and intensity of bands was quantified by Image J program.

UV crosslinking.

For analysis of crosslinks the reactions used for gel mobility shift were irradiated with UV light of 254 nM at distance of 5 cm for 15 min. After crosslinking, samples were resolved on 10 or 12% SDS-PAGE gels and the gels were imaged as above. For preparative crosslinking, ds-oligonucleotide containing the sequence of human collagen α1(I) 5'SL was cloned into BamHI site of pGEM3 vector, the construct was linearized by HindIII and used as template for in vitro transcription. The in vitro transcription reaction was supplemented with 4-thio uridines (Sienna Bioresearch) to incorporate 4-thio uridines at 50% of total uridines and the RNA was 5' end labeled with ^{32}P. Crosslinking was performed with 30 μM recombinant La-module in 200 uL reaction and resolved on 10% preparative SDS-PAGE gel. After radiography, the crosslinked band was excised, eluted and subjected to partial NaOH hydrolysis. 50 μl of sample was hydrolyzed with 25 μl of 50 mM bicarbonate buffer, pH 9.2 for 4 min at 95C and fragments were resolved on 8% sequencing gel and visualized by autoradiography. As a marker, the same RNA was partially digested with RNAse A and digestion products were run on the same gel.

RNA footprinting.

The 5'SL RNAs used in gel mobility shift experiments and labeled at the 5' end by fluorescein were used free or bound to recombinant La domain or La-module and were digested in 50 μl PBS, 5 mM MgCl2 with 2.5U of Benzonase (AMD Millipore) for 6 min at RT, phenol/chlorophorm extracted and analyzed on 8% acrylamide/8M urea gel. For RNase A generated ladder, free RNAs were digested with 100 ng of RNAse A for 8 min at RT and processed as above. The gels were imaged with Biorad ChemiDoc MP imaging system.

Immunostaining.

pCDNA3 vectors expressing HA-tagged full size LARP6 or La domain were transfected into HEK293 cells and 24h after transfection the cells were fixed and incubated with anti-HA primary antibody (Sigma), followed by incubation with fluorescent secondary antibody. Non-transfected cells served as negative control. The images were taken with Keyence BZ-X710 microscope at 60x magnification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

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- Binding of LARP6 to collagen mRNAs regulates type I collagen production in fibrosis
- La domain of LARP6 determines the sequence specific recognition of collagen mRNAs
- A short epitope present only in the La domain of LARP6 is critical for binding
- RRM domain of LARP6 only increases the affinity of binding
- New antifibrotic drugs can be developed by targeting the epitope of La domain

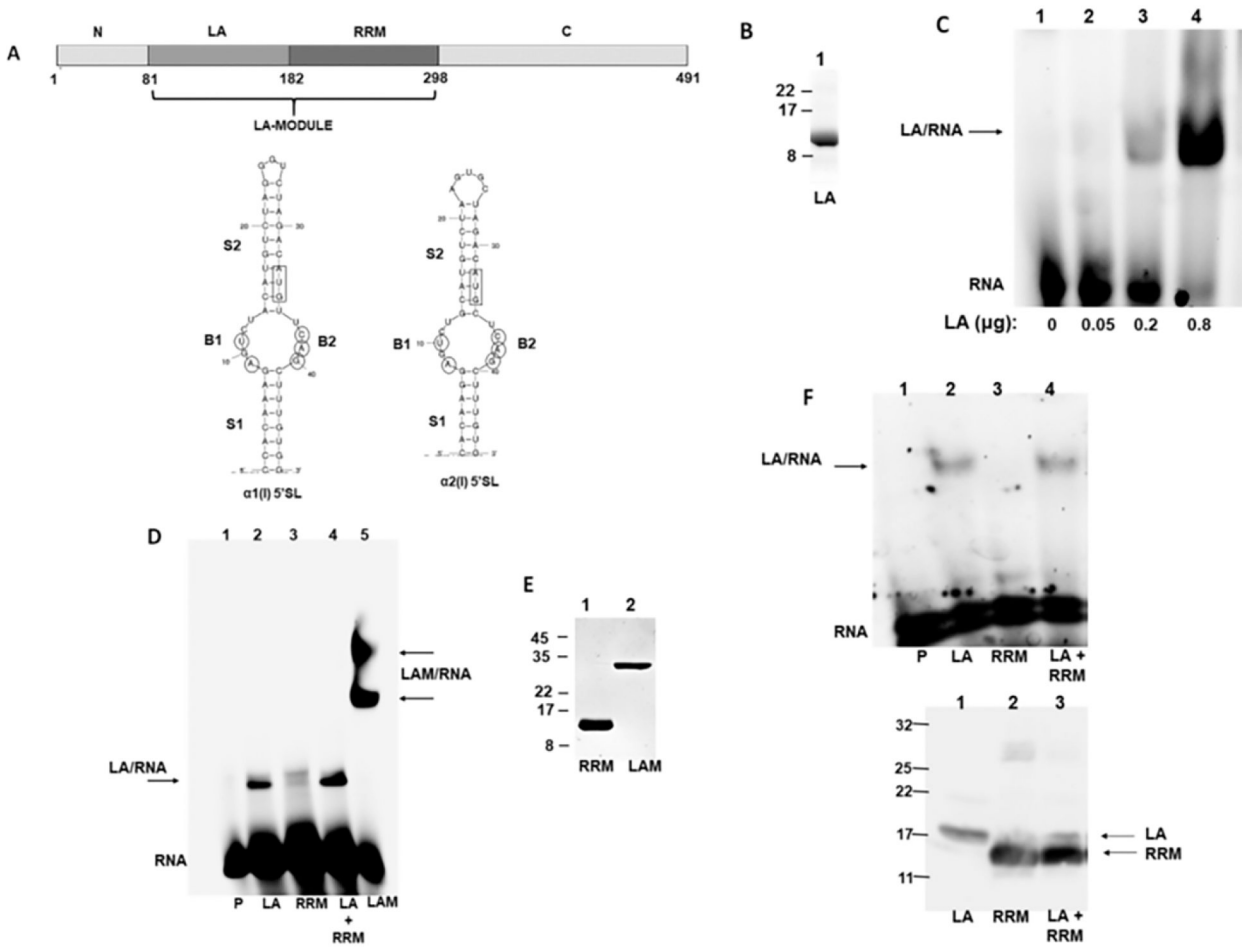

Figure 1.

The La domain of LARP6 recognizes and binds the 5'SL RNA motif. (A) Schematic representation of the domains of LARP6. La-module is indicated. LA, La-domain, RRM, RNA recognition motif, N and C, N-terminal and C-terminal domains. Lower panel: sequences of human collagen 5'SL of collagen α1(I) and α2(I) mRNAs. The nucleotides important for binding LARP6 are circled. B1 and B2, single stranded parts of the central bulge. S1 and S2, stems. The translational initiation codon is boxed. B. Recombinant La domain used in experiments (Coomassie staining). C. Binding of recombinant La-domain to α2(I) 5'SL RNA. La domain was added at increasing amounts to α2(I) 5'SL RNA probe (RNA) and complexes were resolved by gel mobility shift assay. Arrow indicates migration of La/5'SL complex. D. Binding of recombinant RRM to α2(I) 5'SL RNA. Recombinant La domain (lane 2), recombinant RRM (lane 3), an equimolar mix of La and RRM (lane 4) or recombinant La-motif (LAM, lane 5) were added to α2(I) 5'SL RNA and the complexes resolved by gel mobility shift assay. Lane 1, 5'SL RNA alone (P). E. Recombinant RRM and LAM visualized by Coomassie staining. F. Binding of La domain expressed in mammalian cells. Upper panel: gel mobility shift with α2(I) 5'SL RNA probe and extracts of cells expressing La-domain (lane 2, LA) or RRM (lane 3, RRM) or co-expressing both domains in trans (lane 4). Lane 1 is RNA probe alone. Arrow indicates La/5'SL complex. Bottom panel: expression of transfected proteins in the same extract.

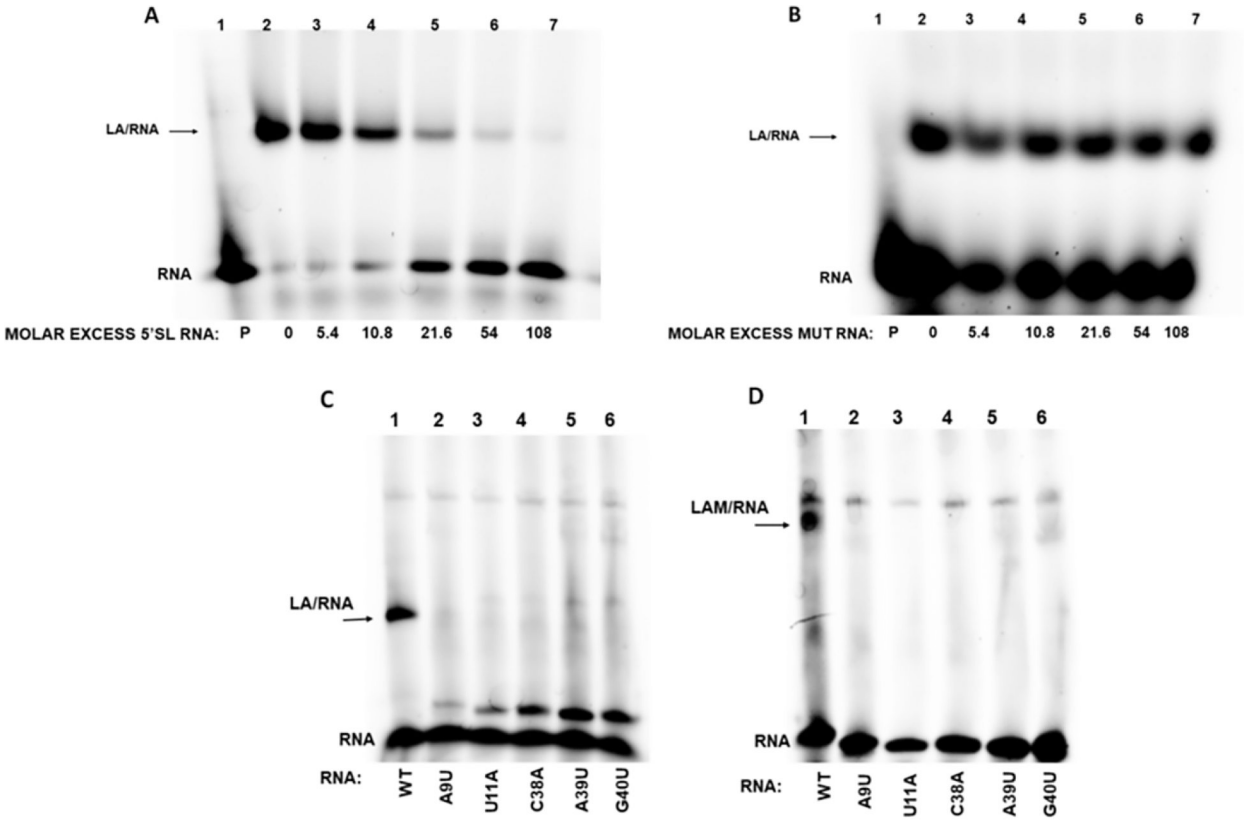

Figure 2.

Sequence specific binding of La-domain to 5'SL RNA. A. Competition of La-domain binding by specific competitor RNA. Binding of recombinant La-domain to α2(I) 5'SL RNA probe was analyzed in the presence of indicated molar excess of unlabeled α2(I) 5'SL RNA by gel mobility shift. P, probe alone (lane 1). Arrow indicates La/5'SL complex. B. Competition by the mutant RNA with five nucleotides circled in Fig 1 changed. Same experiment as in D, except molar excess of the mutant 5'SL RNA was used. C. Binding of recombinant La-domain to the single mutants of 5'SL. WT α1(I) 5'SL RNA probe (lane 1) or mutant probes in which the individual bulge nucleotides had been changed (lanes 2–6) were incubated with recombinant La-domain and complexes analyzed by gel mobility shift. Arrow indicates La/5'SL complex. D. Experiment as in C, except recombinant La-module (LAM) was used. Arrow indicates LAM/5'SL complex.

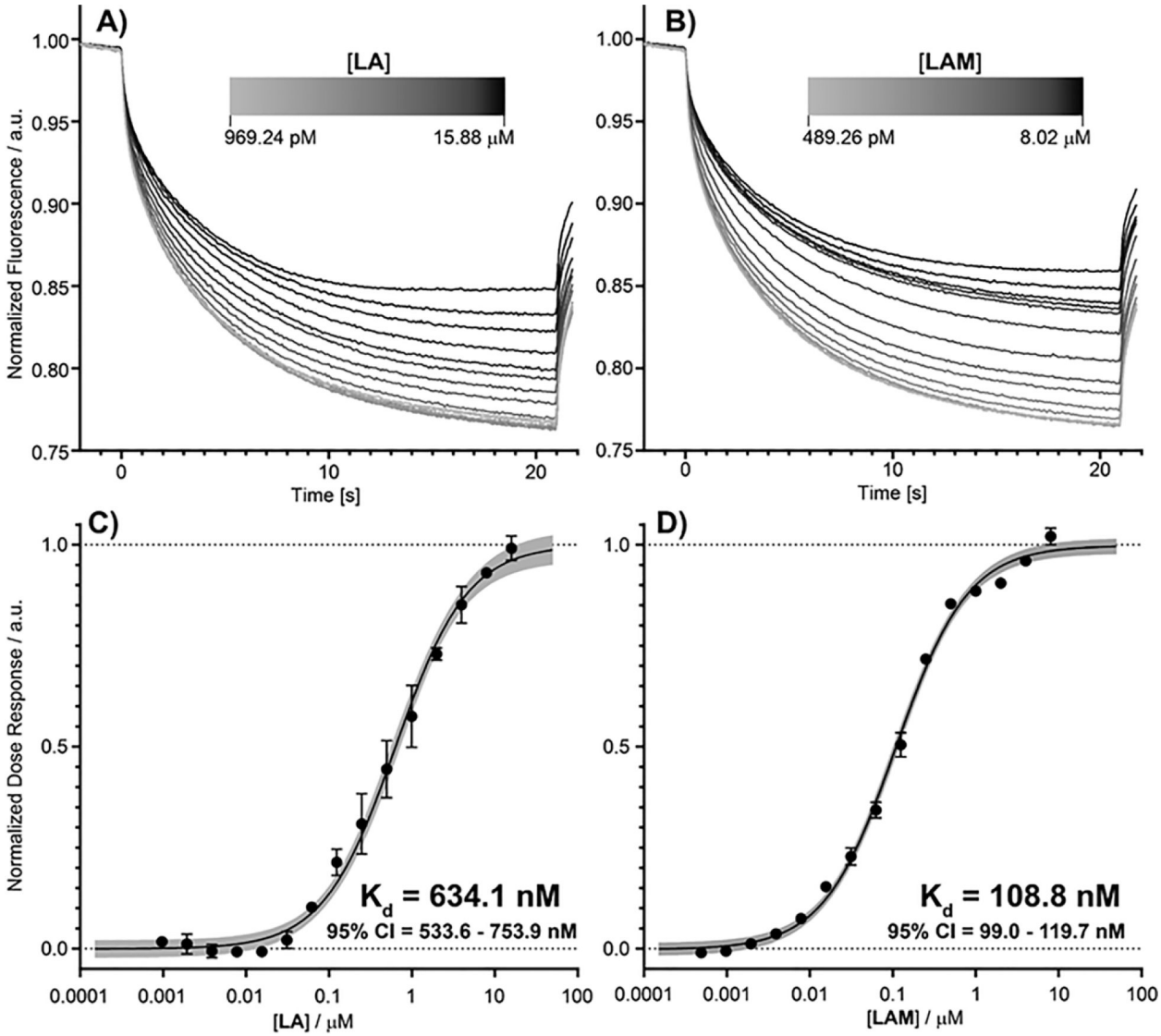

Figure 3:

Affinity of binding of La-domain and La-module of LARP6 to 5’SL RNA. A. Normalized fluorescence traces of microscale thermophoresis measurements of α2(I) 5’SL RNA binding to La-domain (LA). B. Normalized fluorescence traces of microscale thermophoresis measurements of La-module (LAM) binding. The varying protein concentrations are visualized as a gradient from light gray (low concentration) to black (high concentration). C and D. Normalized dose-response curves for LA (C) and LAM (D) are shown as average from triplicate measurements. Error bars: +−1SD (some error bars are not visible because they are smaller than the data point). 95% confidence interval (95% CI) is shown as a gray area. The calculated Kd and the Kd ranges are indicated.

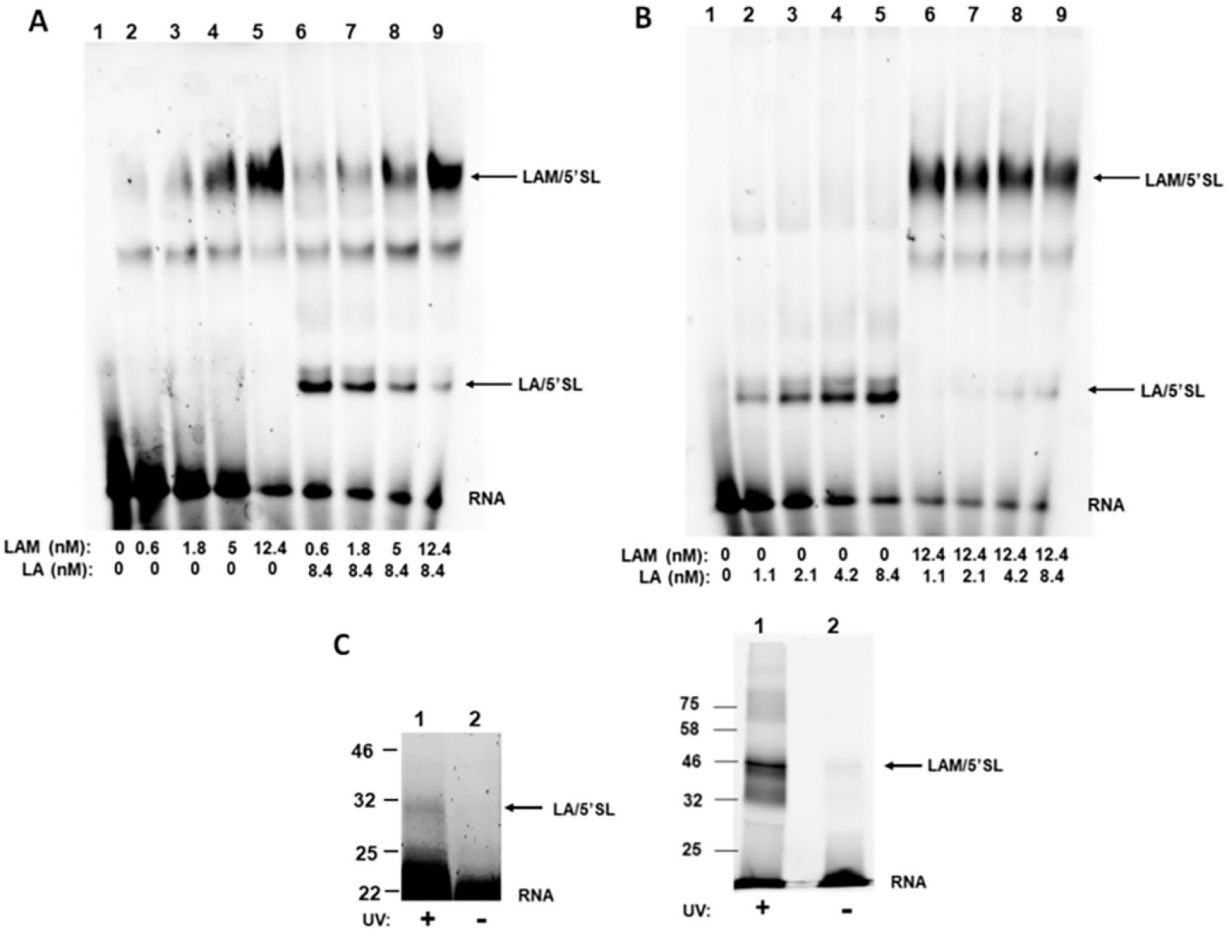

Figure 4.

Cross-competition between La-domain and La-module for 5'SL RNA. A. Competition of La-module (LAM) binding by La-domain (LA). Increasing amounts of recombinant LAM were incubated with α2(I) 5'SL RNA probe in absence (lanes 2–5) or in presence (lanes 6–9) of fixed amount of LA and complexes were resolved by gel electrophoresis. Lane 1, probe alone. Arrows indicate migration of the LA/5'SL and LAM/5'SL complexes. B. Competition of La-domain (LA) binding by La-module (LAM). Experiment as in A, except increasing amounts of LA were incubated with α2(I) 5'SL RNA probe in absence (lanes 2–5) or in presence (lanes 6–9) of fixed amount of LAM. C. UV crosslinking of La-domain (LA) and La-module (LAM) to 5'SL RNA. Recombinant LA (left panel) and LAM (right panel) were bound to α2(I) 5'SL RNA probe and separated on denaturing SDS-PAGE gel after UV crosslinking (lane 1) or without UV crosslinking (lane 2) and visualized by imaging of RNA fluorescence. Arrows indicate protein/RNA complex.

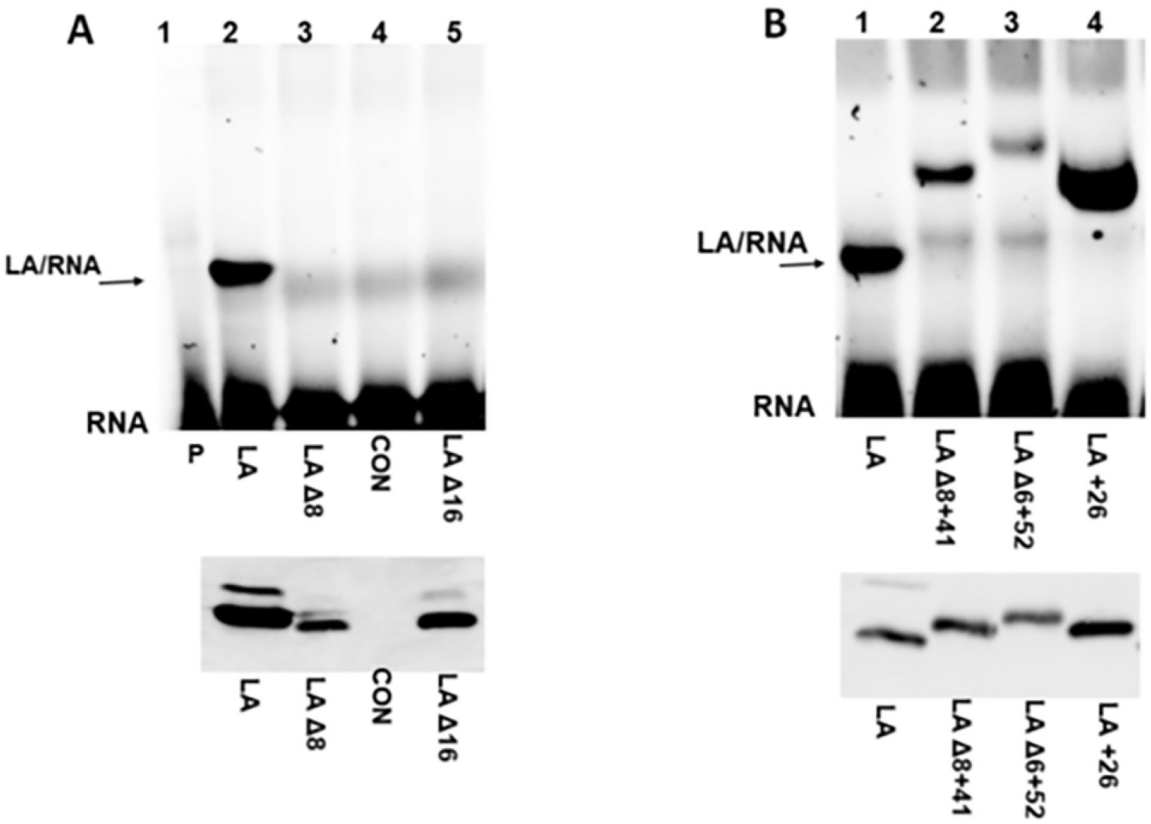

Figure 5.

The C-terminus of La-domain modulates binding. A. Shortening of C-terminus abolishes binding. Top panel: La-domain (LA, amino acids 81–182, lane 2), La-domain with eight C-terminal amino acids deleted (LAΔ8, amino acids 81–174, lane 3) or La-domain with sixteen C-terminal amino acids deleted (LAΔ16, amino acids 81–166, lane 5) were expressed in HEK293 cells and cell extract analyzed for binding to α2(I) 5'SL probe by gel mobility shift. Lane 1, probe alone, lane 4, control untransfected cells (CON). Bottom panel: expression of proteins in the same extract measured by western blot. B. Extension of La-domain restores binding. La-domain (LA, amino acids 81–182, lane 1) or LA with various random sequences appended at the C-terminus (lanes 2–4) were analyzed as in A.

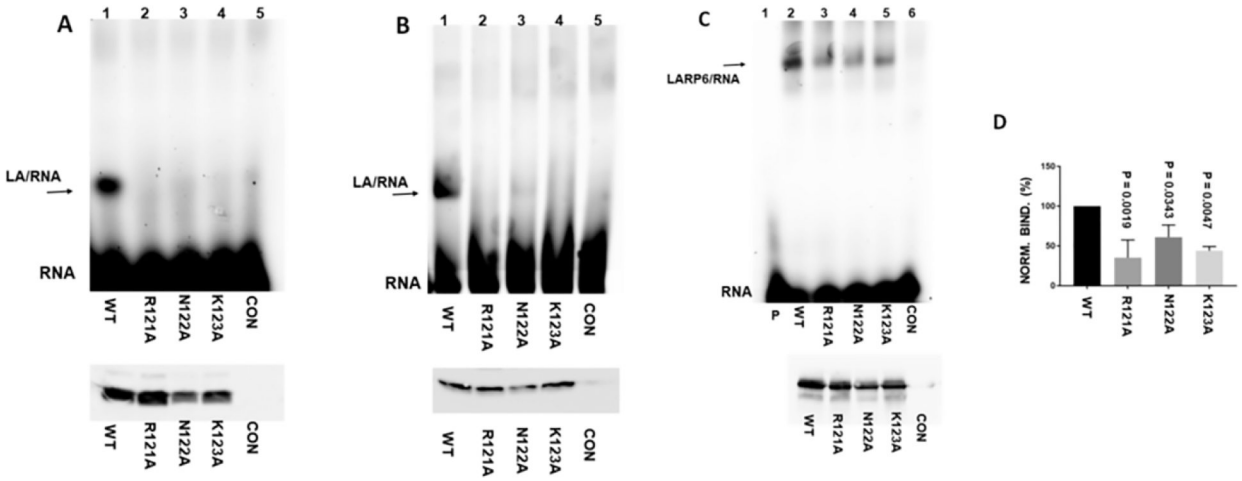

Figure 6.

RNK epitope of La-domain is critical for binding 5’SL. A. Mutation of single amino acids of the RNK epitope abolishes binding of La-domain to 5’SL RNA. WT La-domain (lane 1) or the single amino acid mutants of RNK epitope (lanes 2–4) were expressed in HEK293 cells and cell lysates were analyzed for binding α2(I) 5’SL RNA by gel mobility shift. Lane 5, untransfected cells (CON). Bottom panel, expression of proteins in the same extract measured by western blot. B. The same experiment, but the binding to α1(I) 5’SL was analyzed. C. Mutation of individual amino acids of the RNK epitope reduces binding of full size LARP6. Experiment as in A, except the mutations were made in full size LARP6. D. Quantification of binding of the individual mutants of full size LARP6. Intensity of LARP6/5’SL RNA complex in gel shift experiment was normalized to expression of transfected LARP6 in the same extract and arbitrarily set as 100% for WT LARP6. N=3, error bars, +−1SD.

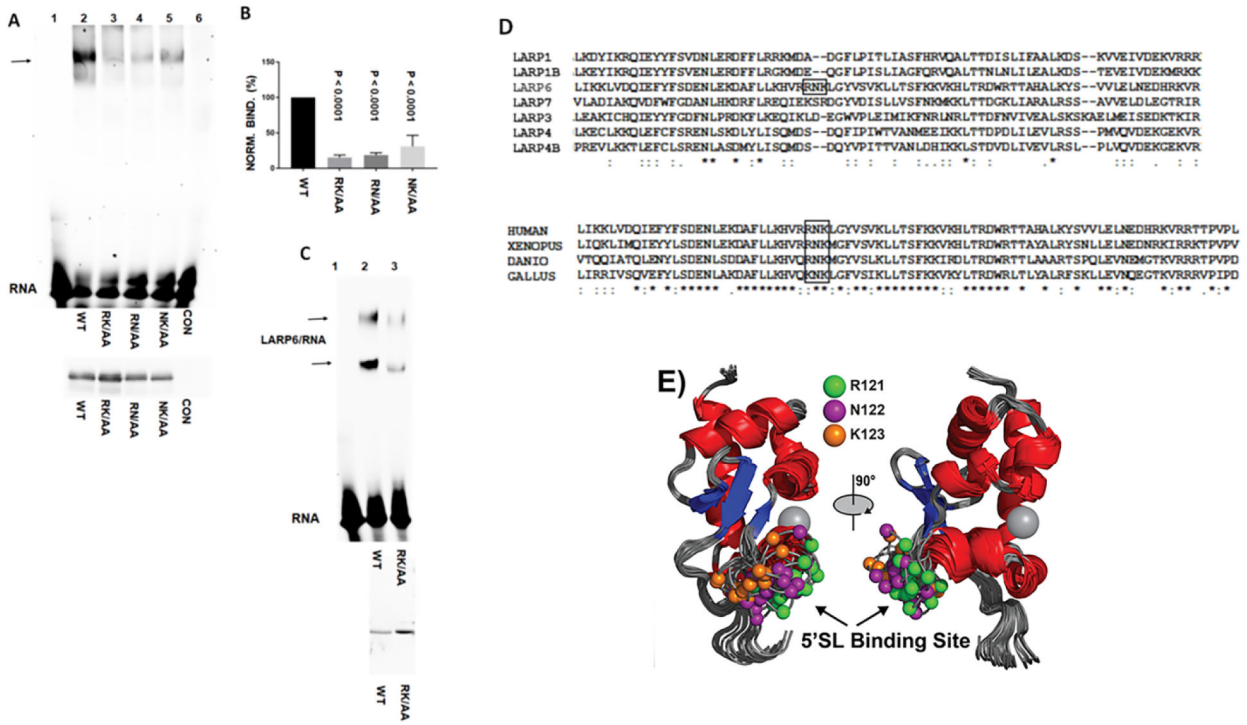

Figure 7.

Double mutations of RNK epitope have additive effect on binding of full size LARP6. Two amino acids of RNK were simultaneously mutated as indicted (lanes 3–5) and the mutants were expressed in HEK293 cells and analyzed for binding α2(I) 5'SL RNA by gel mobility shift. Lane 1, RNA alone, lane 2, binding of wt LARP6, lane 6, non-transfected cells (CON). B. Quantification of binding of double mutants of full size LARP6 as in Fig 5. C. Binding of recombinant full size LARP6 with the double mutation RK/AA. The binding of recombinant RK/AA mutant (lane 3) was compared to that of wt LARP6 (lane 2). Lane 1, RNA alone. Bottom panel: recombinant proteins used in analysis. D. Sequence comparison of La-domains of human LARP family members (upper panel) and of La-domain of LARP6 of distant vertebrates (lower panel). The RNK epitope is boxed. E. NMR structure of LaM (PDB ID: 2MTF) [11]. The RNK epitope loop is flexible and the location of the Cα atoms of R121/N122/K123 are shown as green, purple, and orange spheres, respectively. Gray spheres show the location of the previously suggested binding site. Structures were generated using the PyMOL software package.

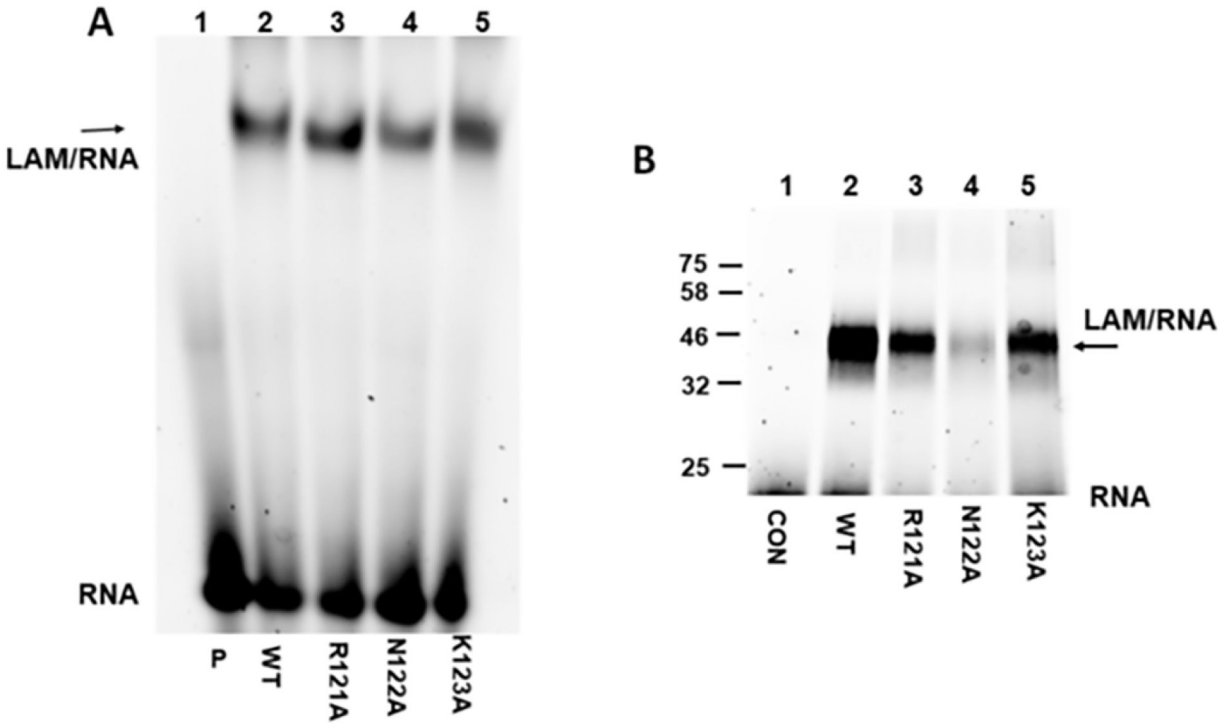

Figure 8.

UV crosslinking of LARP6 to 5'SL RNA by RNK epitope. A. Binding activity of recombinant proteins. Gel mobility shift with the amount of recombinant wt La-module (WT, lane 2) and the indicated mutants (lanes 3–5) adjusted to give similar extent of binding to α2(I) 5'SL. LA/5'SL complex is indicated by arrow. Lane 1, RNA alone (P). B. UV crosslinking of the same samples. After UV irradiation the RNA/protein complexes were resolved on SDS-PAGE and visualized by imaging of RNA fluorescence. Lane 1, RNA without protein (CON). Arrow, LA/5'SL complex.

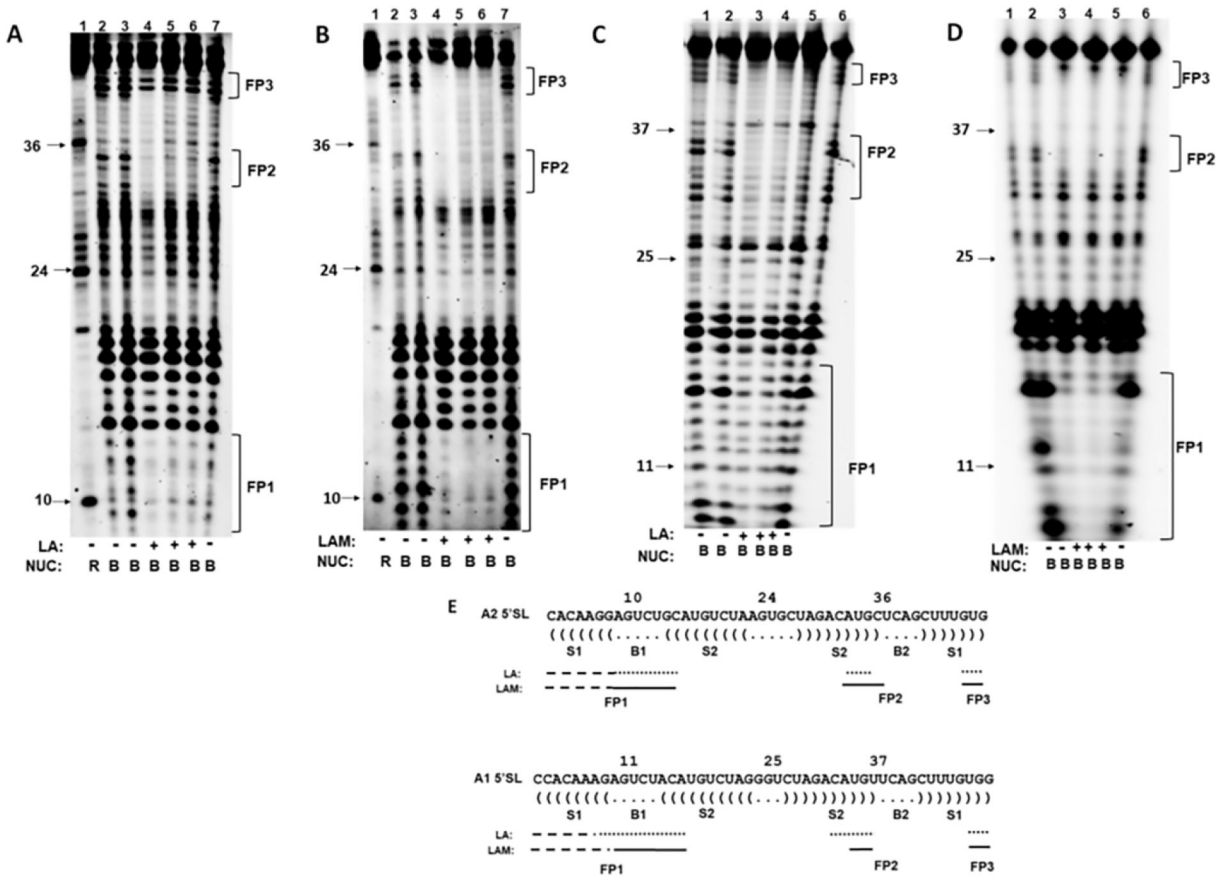

Figure 9.

Mapping of footprints of La-domain and La-module on 5’SL RNA. A. Footprint of recombinant La-domain (LA) on α2(I) 5’SL RNA. The 5’ end labeled 5’SL RNA, free (lanes 2, 3 and 7) or bound to LA (lanes 4–6) was partially digested with benzonase (B) and the fragments resolved on 8% denaturing acrylamide gel. The areas protected from benzonase cleavage are indicated as FP1, FP2 and FP3. Lane 1, partial digestion with RNase A (R), with the sizes of cleavage fragments indicated. B. The same experiment using recombinant La-module (LAM). C. Footprint of La-domain (LA) on α1(I) 5’SL. D. Footprint of La-module (LAM) on α1(I) 5’SL. E. Schematic representation of footprints in the sequence of α2(I) 5’SL (A2 5’SL, upper panel) and α1(I) 5’SL (A1 5’SL, lower panel). Nucleotide numbers are on the top of the sequence and secondary structures are indicated under the sequence. Dotted line; footprints of La-domain, solid line; footprints of La-module, dashed line; region close to the 5’ end where footprints could not be determined. S1, lower stem, B1 and B2, bulge, S2, upper stem.