REVIEW

The RNA chaperon activity of the human La protein (LARP3)

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Single-stranded RNA molecules fold intensively into secondary and tertiary structures and are often trapped in non-functional configurations. To adapt a functional configuration, structural changes have to be achieved. RNA helicases and RNA chaperones are proteins able to assist those structural rearrangements in an ATP-dependent or ATP-independent manner, respectively. The cancer-associated RNA-binding protein La (LARP3) is an RNA chaperone involved in various aspects of the RNA metabolism. Recently the RNA chaperone domain within the human La protein has been mapped and demonstrated that its activity is required to stimulate cyclin D1-internal ribosome entry site (IRES)-dependent protein synthesis. Furthermore, it has been shown that the La protein can be phosphorylated by serine/threonine kinase AKT *in vitro*. Taken together, we suggest a model in which the RNA chaperone La stimulates translation of specific target mRNAs by assisting structural changes in their translation start site surrounding RNA region.

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The RNA-binding protein La (LARP3, La autoantigen, La/SSB) [1] mainly localizes to the nucleus but shuttles to the cytoplasm [2-4]. The La protein (Fig. 1) contains three RNA-binding surfaces: the La motif (LAM) and two RNA recognition motifs (RRMs). The N-terminally located and conserved LAM is found in a variety of proteins which are classified as LA Related Proteins (LARPs; http://www.larp-society.com; [5]). The first RRM is located in the N-terminal domain, whereas the second RRM is located in the C-terminal domain and is unique to the mammalian version - the genuine La protein [1]. La interacts with the precursors of a variety of different RNA molecules e.g. tRNAs, mircoRNAs, snoRNAs [6-8] [9-11] and binds to long viral and cellular mRNAs. Besides its LAM:RRM1-mediated interaction with the oligo(U) 3'-termini common to nascent RNA polymerase III transcripts such as pre-tRNAs[12], human La can bind to the 5'-terminus of mRNAs referred to as terminal *polypyrimidine tract* (TOP) mRNAs[13-15], and internal RNA elements in RNAs such as MDM2[16], cyclin D1 [17], XIAP [18], *human immunodeficiency virus*[19], hepatitis C [20] and A virus [21] and polio virus [22].

In recent years it became clear that the La protein is aberrantly regulated in cancerous cells. First it has been shown that La expression is elevated in different cancer cell lines as well as in chronic myelogenous leukemia, and more recently it has been revealed that La is overexpressed in various types of solid tumor tissue when compared to normal adjacent tissue[16, 17, 23, 24]. This finding sparked the interesting question whether the La protein promotes tumor pathogenesis when overexpressed. Recent studies have shown that the human La protein supports proliferation, migration, and invasion of cancer cells [17, 23]. The murine La protein has a small amino acid insertion in the C-terminal domain and is 7 amino acids longer than the human La. Experiments showed that murine La facilitates internal ribosome entry site (IRES)-dependent Laminin B1 mRNA translation and supports tumorigenesis in xenograft mouse models [25-27].

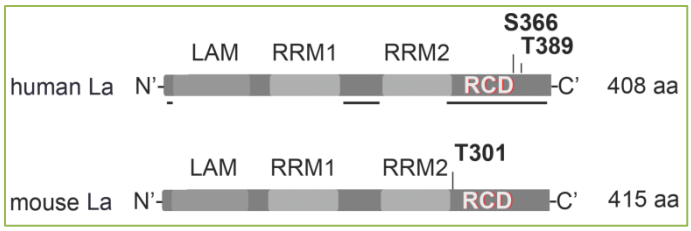

Fig 1. Comparison of human and murine La protein. LAM = La motif [1]; RRM = RNA Recognition motif [1]; RCD = RNA Chaperone domain [36]; S366 = Casein Kinase II phosphorylation site [42]; T389 = AKT phosphorylation site [36]; T301 = AKT phosphorylation site in murine La [43]. Black lines = intrinsic disordered regions [39].

Those findings raised the question by which mechanism La facilitates tumor-promoting processes. Although the interaction between La and small RNA molecules, like pre-tRNAs, has been studied intensively, it remains to be demonstrated that the potential aberrant regulation of small RNAs by elevated La levels in cancerous cells contributes to tumor pathogenesis. However, La is not only implicated in facilitating the processing of precursor RNAs but is also implicated to play a role in protein synthesis. Initially, it has been shown that the La protein supports cap-independent internal ribosome entry site (IRES)-mediated translation of polio virus [22, 28]. Subsequently, it has been demonstrated that La also contributes to IRES-mediated mRNA translation of, hepatitis C virus [29], but on the other hand impairs IRES-mediated translation of hepatitis A virus [30]. Moreover, a growing number of reports demonstrate that the La protein stimulates translation of mRNAs coding for cellular proteins - specifically of tumor-promoting factors - allowing us to speculate that La, when aberrantly regulated in cancerous cells, contributes to tumor pathogenesis by misregulating the protein synthesis of critical factors such as MDM2, cyclin D1, XIAP, Lamimin B. However, not much is known about the molecular mechanism by which La facilitates or impairs protein synthesis. Previous work suggested that the La protein might facilitate 48S complex formation during translation initiation of polio virus and hepatitis C virus translation and that La might bind in close proximity to translation start sites [31-34]. Furthermore, earlier work proposed that the C-terminal domain of La is required to promote polio virus IRES-mediated translation [35]. Those studies pointed out that La might play a role in translation initiation of specific mRNAs. However, it is critical to understand the molecular mechanism in more detail to elucidate how La acts during translation. We started out to investigate whether the RNA chaperone activity of La is required to facilitate protein synthesis.

It has been shown recently that La protein depletion causes a massive drop in cyclin D1 protein levels and stimulates cyclin D1 IRES-mediated translation [17]. Mapping the La binding site in cyclin D1 mRNA revealed that La binds in close proximity to the translation start site [36]. It has been shown earlier that the two RRMs of La are both required and sufficient for binding to hepatitis B virus RNA [21] and both RRMs are also sufficient to bind cyclin D1 RNA[36]. Interestingly, computer calculated (mfold, [37]) structure of the La binding site in cyclin D1 RNA predicts a stem-loop structure in which the translation start site (AUG) is buried. We consider that the stem might impair efficient recognition of the AUG by the scanning 43S subunits and that it would beneficial for translation initiation to unwind the helical region. This notion triggered experiments aiming to demonstrate that La is actually able to assist structural changes in cyclin D1 mRNA-derived RNA molecules. We proposed that the RNA chaperone activity of La destabilizes the helical region and thereby easing the access of the 43S subunit to the AUG. To test the first part of the assumption an RNA chaperone assay has been developed using the La binding site in cyclin D1 RNA as molecular beacon (D1-AUG-MB). This molecular beacon has a fluorescence group at the 5'-end and a quencher at the 3'-end [36]. Based on the structural prediction of the D1-AUG-MB the 5'- and 3'-end should be located next to each other closing the stem. The RNA chaperone assay bases on the assumption that if the La protein destabilizes the stem the fluorescence molecule (5'-end) would be spatially separated from the quencher (3'-end), allowing fluorescence light emission after excitation. Using the RNA chaperone assay it has recently been shown that recombinant human La protein can destabilize the helical region of the D1-AUG-MB in an ATP-independent manner [36]. However, the question remains how La is able to assist structural changes in an ATP-independent manner. According to a model for RNA chaperones [38] structural changes are first induced in the RNA chaperone upon RNA-binding. This structural rearrangement in the protein might allow additional binding of the RNA substrate via an auxiliary RNA-binding surface in the RNA chaperone. Eventually the transfer of entropy would facilitate structural rearrangements in the bound RNA [38]. The C-terminal domain of the La protein is intrinsically disordered (Fig. 1) and might undergo structural rearrangements upon binding of La to an RNA substrate [39]. Previous work suggested that a basic stretch of amino acids in the C-terminal domain might be involved in RNA-binding and additional work implied that the C-terminal domain is required for polio virus IRES-mediated translation [35, 40]. By introducing two sets of mutations in the C-terminal domain focusing on aromatic and positively charged amino acids known to preferentially interact with RNA, it has been demonstrated recently that the mutated amino acids, referred to as RNA chaperone domain (RCD), were critical for La to assist structural changes in D1-AUG-MB [36]. This finding suggests that RRM1 and RRM2 are required for binding of the D1-AUG-MB and that the RNA chaperone domain of La

is required to assist structural changes in the RNA. Of note, this region, which is unique to mammalian La, overlaps with the nucleolar localization signal [41] and lays in close proximity to the well-established CK2 phosphorylation site at serine 366 [42].

The murine La protein is phosphorylated by AKT at position T301 [43], which would be upstream of the potential RCD in murine La (Fig. 1). A genome-wide identification of AKT-phosphorylated proteins suggested that the La protein is phosphorylated by AKT at threonine 389 [44] located downstream of the RCD. To confirm that threonine 389 can be phosphorylated by AKT a La-T389A mutant has been created. As shown by in vitro AKT kinase assays recombinant La-wildtype (La-WT) but not La-T389A mutant protein can be phosphorylated by AKT at threonine 389 [36]. Next the question was asked whether phosphorylation at T389 might affect the RNA chaperone activity of La. Interestingly, native La-WT and La-T389A protein had comparable RNA chaperone activity. However, the RNA chaperone activity of phosphorylated La-WT was strongly impaired by AKT phosphorylation suggesting that the RNA chaperone activity can be regulated by phosphorylation[36]. Since the CK2 phosphorylation site is also located in very close proximity of the RCD it will be interesting to test in future experiments whether CK2 phosphorylation of the La protein has a similar effect on the RNA chaperone activity as AKT phosphorylation. Although phosphor-mimetic mutations of T389 were tested, those mutants did not display impaired RNA chaperone activity [36].

According to the concept that translation start sites might be buried in structural elements are inefficiently recognized during translation initiation it has been test whether the RNA chaperone activity of La is critical to support translation. Since La regulates the cyclin D1-IRES a bi-cistronic cyclin D1 IRES reporter was used to test whether the RNA chaperone activity is required for cyclin D1-IRES-mediated translation in cell-based assays [36]. Interestingly, after transfection of capped and poly-adenylated biscitronic cyclin D1-IRES reporter RNA into cells expressing gfp-tagged La-WT or La-ΔRCD (deletion mutant of RCD), it was found that the RNA chaperone activity is not only required to stimulate cyclin D1-IRES-mediated translation (firefly luciferase) but also cap-dependent translation of the renilla luciferase open reading frame [36]. Those experiments suggested that the RNA chaperone activity might contribute to the translation of several groups of mRNAs such as IRES-containing mRNAs or mRNAs with specific structural features in their 5'-UTR such as stem loops (e.g. MDM2). The finding that the RNA chaperone activity of La is required to stimulate both IRES- as well as cap-dependent translation raises the question about the location and the nature of RNA elements/structures recognized by La. It is possible that the chaperone activity of La has an indirect effect on translation by contributing to the processing of RNA molecules required for protein synthesis such as pre-tRNAs [6, 7, 45] or due to effects on microRNA processing [9, 10]. However, ^{35}S-metabolic-labeling and 2D gel electrophoresis analysis does not suggest that La depletion has a global effect on protein synthesis in HeLa cells [17].

By testing the effect of the La-T389A mutant on translation of capped and poly-adenylated biscitronic cyclin D1-IRES reporter RNA in cell-based assays it has been found that threonine 389 is important to stimulate IRES-mediated but not cap-dependent translation [36]. This interesting finding suggests that AKT phosphorylation preferentially affects La's ability to facilitate IRES-mediated translation and is not affecting to the same extend cap-dependent translation. However, because AKT phosphorylation of recombinant La impaired its RNA chaperone activity it was expected that La-T389A has a stimulating effect on the translation of transfected RNA and not a repressing effect. Future work will focus on solving this puzzling observation. Moreover, it is not known to which extend cellular La is phosphorylated by AKT and more work is required to dissect the regulation of cellular La functionality by AKT phosphorylation.

Furthermore, it remains to be studied whether La assists structural changes in bound mRNAs in cell-based assays and it has to be addressed whether La, if assisting structural changes, would be released from the RNA. In the scenario that La has reduced RNA chaperone activity, due to AKT phosphorylation, it raises the question whether the on/off rates of La are affected by phosphorylation and whether La remains bound to an RNA substrate while unable to unwind the RNA structure. These are important mechanistic questions because if La remains bound to the RNA, another mechanism must exist to remove La and to facilitate translation initiation. Furthermore, if La releases the restructured RNA it is questionable whether the RNA folds back into its former structure or stays in the new conformation. The potential regulation of La's RNA chaperone activity by phosphorylation might affect certain subsets of mRNAs, which are dependent on La-mediated restructuring of RNA and might slow down translation of the very same RNAs when the RNA chaperone activity is low and La is bound more tightly.

The La protein is overexpressed in various types of tumor cells. AKT and CK2 kinases are both implicated in supporting tumor pathogenesis by affecting many cellular processes. Mutation of the CK2 site (S366) in human La revealed that rpl37 TOP mRNA was associated with fewer

ribosomes in LaS366A expressing cells suggesting that rpL37 proteins synthesis was reduced [15], Furthermore, mutation of the AKT site in human La impairs IRES-dependent cyclin D1 translation in cell-based assays, suggesting that both kinases might have an effect of La facilitated protein synthesis of specific subsets of mRNAs. The regulation of La's activity by posttranslational modifications is probably a key aspect to understand its important cellular function.

Both murine and human La is phosphorylated by AKT in the C-terminal domain (mouse: T301; human: T389, Fig. 1). Furthermore, the RNA chaperone domain and its subdomains (RCD1 and RCD2) are well conserved[36], suggesting that murine La might have a similar RNA chaperone activity which is regulated by AKT phosphorylation. Recently, it has been shown that AKT phosphorylation triggers the nuclear-cytoplasmic shuttling of murine La to the cytoplasm [43]. Mutagenesis of the murine AKT site prevented the nuclear export and affected translation of a number of mRNAs [43]. It remains to be studied whether AKT phosphorylation of human La is also affecting its subcellular distribution.

In summary, in recent years the role of mammalian La protein in translation of specific mRNAs has been well documented. Data are accumulating that La's activity in translation is regulated by phosphorylation suggesting that posttranslational modifications regulate La-dependent protein synthesis in response to activation of specific cellular signaling pathways. How those posttranslational modifications are affecting the RNA-binding activity, the RNA chaperone activity, and the cellular localization of human La remains to be determined in future studies.

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References

1. Wolin SL; Cedervall T. The La protein. Annu Rev Biochem 2002; 71:375-403.
2. Fok V, Friend K, Steitz JA. Epstein-Barr virus noncoding RNAs are confined to the nucleus, whereas their partner, the human La protein, undergoes nucleocytoplasmic shuttling. J Cell Biol 2006; 173:319-325.
3. Bachmann M, Chang S, Slor H, Kukulies J, Muller WE. Shuttling of the autoantigen La between nucleus and cell surface after uv irradiation of human keratinocytes. Exp Cell Res 1990; 191:171-180.
4. Bachmann M, Pfeifer K, Schroder HC, Muller WE. The La antigen shuttles between the nucleus and the cytoplasm in CV-1 cells. Mol Cell Biochem 1989; 85:103-114.
5. Bousquet-Antonelli C, Deragon JM. A comprehensive analysis of the La-motif protein superfamily. RNA 2009; 15:750-764.
6. Chakshusmathi G, Kim SD, Rubinson DA, Wolin SL. A La protein requirement for efficient pre-tRNA folding. EMBO J 2003; 22:6562-6572.
7. Yoo CJ, Wolin SL. The yeast La protein is required for the 3' endonucleolytic cleavage that matures tRNA precursors. Cell 1997; 89:393-402.
8. Fan H, Goodier JL, Chamberlain JR, Engelke DR, Maraia RJ. 5' processing of tRNA precursors can be modulated by the human La antigen phosphoprotein. Mol Cell Biol 1998; 18:3201-3211.
9. Liang C, Xiong K, Szulwach KE, Zhang Y, Wang Z, Peng J, *et al*. Sjogren syndrome antigen B (SSB)/La promotes global microRNA expression by binding microRNA precursors through stem-loop recognition. J Biol Chem 2013; 288:723-736.
10. Liu Y, Tan H, Tian H, Liang C, Chen S, Liu Q. Autoantigen La promotes efficient RNAi, antiviral response, and transposon silencing by facilitating multiple-turnover RISC catalysis. Mol Cell 2011; 44:502-508.
11. Kufel J, Allmang C, Chanfreau G, Petfalski E, Lafontaine DL, Tollervey D. Precursors to the U3 small nucleolar RNA lack small nucleolar RNP proteins but are stabilized by La binding. Mol Cell Biol 2000; 20:5415-5424.
12. Teplova M, Yuan YR, Phan AT, Malinina L, Ilin S, Teplov A, *et al*. Structural basis for recognition and sequestration of UUU(OH) 3' temini of nascent RNA polymerase III transcripts by La, a rheumatic disease autoantigen. Mol Cell 2006; 21:75-85.
13. Cardinali B, Carissimi C, Gravina P, Pierandrei-Amaldi P. La Protein Is Associated with Terminal Oligopyrimidine mRNAs in Actively Translating Polysomes. J Biol Chem 2003; 278:35145-35151.
14. Crosio C, Boyl PP, Loreni F, Pierandrei-Amaldi P, Amaldi F. La protein has a positive effect on the translation of TOP mRNAs in vivo. Nucl Acids Res 2000; 28:2927-2934.
15. Schwartz EI, Intine RV, Maraia RJ. CK2 Is Responsible for Phosphorylation of Human La Protein Serine-366 and Can Modulate rpL37 5'-Terminal Oligopyrimidine mRNA Metabolism. Mol Cell Biol 2004; 24:9580-9591.
16. Trotta R, Vignudelli T, Candini O, Intine RV, Pecorari L, Guerzoni C, *et al*. BCR/ABL activates mdm2 mRNA translation via the La antigen. Cancer Cell 2003; 3:145-160.
17. Sommer G, Dittmann J, Kuehnert J, Reumann K, Schwartz PE, Will H, *et al*. The RNA-binding protein La contributes to cell proliferation and CCND1 expression. Oncogene 2011; 30:434-444.
18. Holcik M, Korneluk RG. Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry

site element: role of La autoantigen in XIAP translation. Mol Cell Biol 2000; 20:4648-4657.

19. Svitkin YV, Pause A, Sonenberg N. La autoantigen alleviates translational repression by the 5' leader sequence of the human immunodeficiency virus type 1 mRNA. J Virol 1994; 68:7001-7007.
20. Ali N, Siddiqui A. The La antigen binds 5' noncoding region of the hepatitis C virus RNA in the context of the initiator AUG codon and stimulates internal ribosome entry site-mediated translation. Proc Natl Acad Sci U S A 1997; 94:2249-2254.
21. Horke S, Reumann K, Rang A, Heise T. Molecular characterization of the human La protein.hepatitis B virus RNA.B interaction in vitro. J Biol Chem 2002; 277:34949-34958.
22. Meerovitch K, Pelletier J, Sonenberg N. A cellular protein that binds to the 5'-noncoding region of poliovirus RNA: implications for internal translation initiation. Genes Dev 1989; 3:1026-1034.
23. Sommer G, Rossa C, Chi AC, Neville BW, Heise T. Implication of RNA-Binding Protein La in Proliferation, Migration and Invasion of Lymph Node-Metastasized Hypopharyngeal SCC Cells. PLoS One 2011; 6:e25402.
24. Al-Ejeh F, Darby JM, Brown MP. The La autoantigen is a malignancy-associated cell death target that is induced by DNA-damaging drugs. Clin Cancer Res 2007; 13:5509s-5518s.
25. Petz M, Them N, Huber H, Beug H, Mikulits W. La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. Nucleic Acids Res 2012; 40:290-302.
26. Petz M, Them NC, Huber H, Mikulits W. PDGF enhances IRES-mediated translation of Laminin B1 by cytoplasmic accumulation of La during epithelial to mesenchymal transition. Nucleic Acids Res 2012; 40:9738-9749.
27. Petz M, Kozina D, Huber H, Siwiec T, Seipelt J, Sommergruber W, *et al*. The leader region of Laminin B1 mRNA confers cap-independent translation. Nucleic Acids Res 2007; 35:2473-2482.
28. Meerovitch K, Svitkin YV, Lee HS, Lejbkowicz F, Kenan DJ, Chan EK, *et al*. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. J Virol 1993; 67:3798-3807.
29. Ali N, Pruijn GJ, Kenan DJ, Keene JD, Siddiqui A. Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation. J Biol Chem 2000; 275:27531-27540.
30. Cordes S, Kusov Y, Heise T, Gauss-Muller V. La autoantigen suppresses IRES-dependent translation of the hepatitis A virus. Biochem Biophys Res Commun 2008; 368:1014-1019.
31. Costa-Mattioli M, Svitkin Y, Sonenberg N. La Autoantigen Is Necessary for Optimal Function of the Poliovirus and Hepatitis C Virus Internal Ribosome Entry Site In Vivo and In Vitro. Mol Cell Biol 2004; 24:6861-6870.
32. Pudi R, Abhiman S, Srinivasan N, Das S. Hepatitis C virus internal ribosome entry site-mediated translation is stimulated by specific interaction of independent regions of human La autoantigen. J Biol Chem 2003; 278:12231-12240.
33. Pudi R, Srinivasan P, Das S. La protein binding at the GCAC site near the initiator AUG facilitates the ribosomal assembly on the hepatitis C virus RNA to influence internal ribosome entry site-mediated translation. J Biol Chem 2004; 279:29879-29888.
34. McBratney S, Sarnow P. Evidence for involvement of trans-acting factors in selection of the AUG start codon during eukaryotic translational initiation. Mol Cell Biol 1996; 6:3523-3534.
35. Svitkin YV, Meerovitch K, Lee HS, Dholakia JN, Kenan DJ, Agol VI, *et al*. Internal translation initiation on poliovirus RNA: further characterization of La function in poliovirus translation in vitro. J Virol 1994; 68:1544-1550.
36. Kuehnert J, Sommer G, Zierk AW, Fedarovich A, Brock A, Fedarovich D, *et al*. Novel RNA chaperone domain of RNA-binding protein La is regulated by AKT phosphorylation. Nucleic Acids Res 2015; 43:581-594.
37. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003; 31:3406-3415.
38. Tompa P, Csermely P. The role of structural disorder in the function of RNA and protein chaperones. FASEB J 2004; 18:1169-1175.
39. Kucera NJ, Hodsdon ME, Wolin SL. An intrinsically disordered C terminus allows the La protein to assist the biogenesis of diverse noncoding RNA precursors. Proc Natl Acad Sci U S A 2011; 108:1308-1313.
40. Goodier JL, Fan H, Maraia RJ. A carboxy-terminal basic region controls RNA polymerase III transcription factor activity of human La protein. Mol Cell Biol 1997; 17:5823-5832.
41. Horke S, Reumann K, Schweizer M, Will H, Heise T. Nuclear trafficking of La protein depends on a newly identified nucleolar localization signal and the ability to bind RNA. J Biol Chem 2004; 279:26563-26570.
42. Fan H, Sakulich AL, Goodier JL, Zhang X, Qin J, Maraia RJ. Phosphorylation of the human La antigen on serine 366 can regulate recycling of RNA polymerase III transcription complexes. Cell 1997; 88:707-715.
43. Brenet F, Socci ND, Sonenberg N, Holland EC. Akt phosphorylation of La regulates specific mRNA translation in glial progenitors. Oncogene 2009; 28:128-139.
44. Moritz A, Li Y, Guo A, Villen J, Wang Y, MacNeill J, *et al*. Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. Science signaling 2010; 3: ra64.
45. Intine RV, Sakulich AL, Koduru SB, Huang Y, Pierstorff E, Goodier JL, *et al*. Control of transfer RNA maturation by phosphorylation of the human La antigen on serine 366. Mol Cell 2000; 6:339-348.