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Expression of mRNA and protein–protein interaction of the antiviral endoribonuclease RNase L in mouse spleen

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ABSTRACT

The interferon-inducible, 2′,5′-oligoadenylate (2-5A)-dependent endoribonuclease, RNase L is a unique antiviral RNA-degrading enzyme involved in RNA-metabolism, translational regulation, stress-response besides its anticancer/tumor-suppressor and antibacterial functions. RNase L represents complex cellular RNA-regulations in mammalian cells but diverse functions of RNase L are not completely explained by its 2-5A-regulated endoribonuclease activity. We hypothesized that RNase L has housekeeping function(s) through interaction with cellular proteins. We investigated RNase L mRNA expression in mouse tissues by RT-PCR and its protein–protein interaction in spleen by GST-pulldown and immunoprecipitation assays followed by proteomic analysis. RNase L mRNA is constitutively and differentially expressed in nine different mouse tissues, its level is maximum in immunological tissues (spleen, thymus and lungs), moderate in reproductive tissues (testis and prostate) and low in metabolic tissues (kidney, brain, liver and heart). Cellular proteins from mouse spleen [fibronectin precursor, β-actin, troponin I, myosin heavy chain 9 (non-muscle), growth-arrest specific protein 11, clathrin light chain B, a putative uncharacterized protein (Ricken cDNA 8030451F13) isoform (CRA_d) and alanyl tRNA synthetase] were identified as cellular RNase L-interacting proteins. Thus our results suggest for more general cellular functions of RNase L through protein–protein interactions in the spleen for immune response in mammals.

1. Introduction

The interferon-inducible 2′,5′-oligoadenylate-dependent ribonuclease L (RNase L) is a uniquely regulated endoribonuclease, a key player in the innate antiviral immune defense mechanisms of mammalian cells induced by interferons (IFNs) [1]. RNase L has been associated with a number of pathological conditions. Identification of RNase L gene (*RNASEL*) as a human prostate cancer (HPC1) susceptibility locus qualified it as a tumor suppressor against prostate cancer by virtue of its property to cause apoptosis of cells through RNA degradation [2]. Point mutations in RNase L (e.g., R462Q) decrease its RNase activity and possibly increase susceptibility to prostate cancer [3]. It has been reported that RNase L is not only a marker for hereditary prostate cancer but a single nucleotide polymorphism (SNP) in the 5′-untranslated region (5′-UTR) of the *RNASEL* gene predicts an increased risk of head and neck, uterine, cervix and breast cancer, thereby, suggesting its importance in maintaining homeostasis in normal cells against cancer [4]. Recently, the association of elevated 2-5A-dependent RNase L with lung cancer has been correlated with its deficient enzymatic activity and dimerization [5]. A deregulation in the 2-5A pathway has been reported in the immune cells from Chronic Fatigue Syndrome (CFS) patients, characterized by upregulated 2-5A synthetase and RNase L activities, as well as by the presence of a low molecular weight 2-5A-binding protein of 37-kDa related to RNase L [6].

Recent literature shows a wide variety of new and emerging functions of RNase L, e.g., antineoplastic [7], antibacterial [8], cell growth inhibition [9], stress response [10], small RNA-mediated immune regulation [11], RNA as well as translational regulations [12–14], senescence and longevity [15], apart from its established role in antiviral, antiproliferative, immunomodulatory and apoptotic functions [1]. However, it is not clear how RNase L is biochemically responsible for all these cellular functions.

RNase L is possibly the only known ankyrin repeat containing protein, which also has an enzymatic (ribonuclease) activity. Also, ankyrin repeat containing proteins such as NF-κB/IκB, yeast Swi6p, Cdc10p and Notch are mostly part of cellular multi-protein complexes with several interacting partners [16]. Till date, only three RNase L-interacting proteins are known from studies involving cell lines. These interacting proteins are ribonuclease L inhibitor (RLI) [17], eukaryotic release factor 3 (eRF 3) [12,13] and androgen

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receptor (AR) [18]. Interestingly, RNase L with a higher number of ankyrin repeats (nine in human RNase L and eight in mouse RNase L), in comparison to IκB-α and Notch, has rather limited interacting partners reported so far. Also, the multiple and emerging functions of RNase L cannot be fully explained only on the basis of its 2-5A-regulated endoribonuclease activity [19]. Involvement of RNase L in several pathological situations such as CFS, colorectal carcinogenesis [20], glutamate toxicity [21] indicates its importance in normal physiological functions. RNase L is highly expressed in mouse spleen [22] and RNase L-knock-out ($^{-/-}$) mice showed enlarged spleen and thymus indicating its physiological role in these two tissues [23]. We hypothesized that RNase L may be expressed in mammalian tissues under normal physiological conditions for housekeeping function(s) and it may interact with cellular proteins possibly through its ankyrin repeats. We have studied RNase L mRNA expression in mouse tissues and its protein–protein interaction in the mouse spleen. We report tissue-specific expression of RNase L mRNA in mouse and a number of cellular RNase L-interacting proteins in the mouse spleen.

2. Materials and methods

2.1. Animals, reagents, plasmids, oligonucleotides, E. coli strains

Swiss albino male mice and male rabbits were obtained from the Animal House of Jawaharlal Nehru University and the study was carried out as per the guidelines of the Institutional Animal Ethics Committee (IAEC). *Escherichia coli* DH5-α and *E. coli* BL21 strains were used as host cells for cloning and expression of the recombinant glutathione-S-transferase (GST) fusion dominant negative (DN) mouse RNase L, respectively. The LB-medium and LB-agar plates were supplemented with 100 μg/ml of ampicillin. The pGEX 2TK vector (Amersham, U.S.A.) was used for the protein expression. Oligonucleotides were commercially synthesized (Microsynth, Switzerland) and *Pfu* DNA polymerase (Biotools) was used for PCR-amplification of the RNase L cDNA. Glutathione-agarose beads, isopropyl thiogalactoside (IPTG), TRI reagent, anti-rabbit IgG (whole molecule)-HRP conjugated (Cat No. A9169) were purchased from Sigma–Aldrich (U.S.A.). Other biochemicals and molecular biology reagents were from Sigma–Aldrich (U.S.A.), Merck (Germany), Qualigens (India) and Spectrochem (India). The partial mouse RNase L cDNA plasmid (pZB1) [24] was a generous gift from Prof. R. H. Silverman, Cleveland Clinic Foundation, OH, U.S.A. Most of the common molecular biology methods were adopted from the protocol book, *Molecular Cloning: a laboratory manual* [25] with certain modifications.

2.2. Recombinant DN-mouse RNase L protein and bead-binding

Since Bam HI site is present in the mouse RNase L cDNA, in order to subclone it in pGEX2TK expression vector (at Bam HI site), a Bam HI site was constructed by addition of the last '**C**' to GGATC on either ends of the DN-mouse RNase L (DN-mRNase L) cDNA by PCR and by end-filling of Bam HI-digested pGEX2TK by Klenow polymerase. The fragment encoding DN-mRNase L (corresponding to 1–646 amino acids of mouse RNase L) was PCR-amplified by *pfu* DNA polymerase from pZB1 plasmid. The blunt-ended PCR-product was 5′-phosphorylated by T4 polynucleotide kinase followed by ligation into blunt-ended pGEX2TK vector by T4 DNA ligase. This generated the pGEX-DNmRNL expression plasmid, which was transformed into *E. coli* DH5-α cells to prepare the plasmid DNA and *E. coli* BL-21 cells to express the GST-DNmRNase L recombinant protein.

Recently, we have described the expression, purification and characterization of the interferon-inducible, antiviral and tumor suppressor protein, human RNase L [26]. For expression of the recombinant dominant negative mouse RNase L protein, a different method was optimized and followed. Briefly, a freshly-transformed colony of pGEX-DNmRNL/BL-21 cells was grown overnight on LB-agar^{Amp} plate at 37 °C and inoculated into a primary culture of 10 ml LB^{Amp} and again grown overnight at 37 °C, 220 rpm. Then a secondary culture of 100 ml LB^{Amp} was inoculated with 1% (v/v) inoculum from the primary culture. The secondary culture was incubated at 37 °C, 220 rpm until the O.D. at 600 nm reached ~0.6–0.8. The culture was then incubated at 18 °C, 220 rpm for 30–40 min until the culture temperature reached ~18 °C. Then GST-DNmRNase L protein was induced by adding IPTG up to 25 μM and continuing the culture for 5 h at 200 rpm. Then the cells were collected by low speed centrifugation and washed once with 10 ml Buffer A [Phosphate buffered saline (PBS), 10% (v/v) glycerol, 1 mM EDTA, 0.1 mM ATP, 5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 2 μg/ml leupeptin and 2 mM PMSF]. The cell pellet was resuspended in 10 ml Buffer A supplemented with lysozyme (100 μg/ml) and incubated at 4 °C for 30 min on a rocking platform. The cell suspension was lysed on ice by sonication at 18 micron for 15 s for five times. Triton X-100 was added to a final concentration of 1% (v/v), and the cell lysate was again incubated on a rocking platform at 4 °C for 30 min. The supernatant was collected after centrifugation at 20,000 × *g* for 15 min at 4 °C. Purification of the fusion protein was performed by batch-affinity by using Glutathione-agarose beads. Glutathione-agarose [500 μl of a 50% (v/v) slurry was pre-equilibrated with buffer A] and added to the clarified cell lysate and incubated on ice on a rocking platform at 4 °C for 1 h. After washing the bead-bound-protein mixture with 10 ml of Buffer A for three times, it was used for the GST-pulldown assay.

The GST-DNmRNase L recombinant protein was also characterized by MALDI-TOF analysis.

2.3. RNase L mRNA expression by RT-PCR analysis

Healthy male Swiss albino mice of 8–10 weeks of age were sacrificed by euthanization using chloroform and total RNA was isolated from the nine tissues (liver, kidney, brain, heart, prostate, testis, spleen, thymus and lungs) by using the TRI-reagent (Sigma–Aldrich) method. For the synthesis of first strand cDNAs, 2 μg RNA (1 μg/μl) from each tissue was mixed with 500 ng of oligo-dT primer (100 ng/μl) in a total volume of 15 μl containing deionized DEPC-treated water and then subjected to heat-denaturation at 70 °C for 5 min in a thermal cycler followed by quick-chill on ice for 5 min. A 10 μl RT-mix (1× RT buffer, 0.5 mM dNTPs, 20 U RNasin RNase-inhibitor and 200 U MMLV-RT (Promega) was added to the denatured RNA + oligo-dT mix and the 25 μl reaction mixture was incubated at 42 °C for 1 h in a thermal cycler. The first strand cDNAs was used for subsequent PCR with multi-exonic RNase L gene-specific primers (forward primer = 5′CTGCAACCACAAAACATCTTAATA3′ and reverse primer = 5′AGATCTGGAAATGTCTTCTGAAAATA3′) in a 35 cycle (95 °C × 45 s, 60 °C × 1 min and 72 °C × 1 min) reaction, which generated an amplified product of 644 bp from the mouse RNase L mRNA. Similarly, RT-PCR reactions were performed for GAPDH for the nine tissues using the following primers (forward primer = 5′ACCACAGTCCATGCCATCAC3′ and reverse primer = 5′TCCACCACCCTGTTGCTGTA3′) in a 35 cycle (95 °C × 45 s, 60 °C × 45 s and 72 °C × 1 min) reaction, which generated an amplified product of 452 bp. RT-PCR products for RNase L and GAPDH from the nine tissues were resolved by electrophoresis on 1.5% (w/v) agarose-TBE gel at 30–50 mA. Quantitation of the RT-PCR products by densitometry was carried out by AlphaEase FC™ software and Alpha imager 3400 (Alphainnotech corporation).