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RNase L: Its Biological Roles and Regulation

Shu-Ling Liang . David Quirk and Aimin Zhou

Summary

2'-5'oligoadenylate-dependent ribonuclease L (RNase L) is one of the key enzymes involved in the function of interferons (IFNs), a family of cytokines participating in innate immunity against viruses and other microbial pathogens. Upon binding with its activator, 5'-phosphorylated, 2'-5' linked oligoadenylates (2-5A), RNase L degrades single-stranded viral and cellular RNAs and thus plays an important role in the antiviral and antiproliferative functions of IFNs. In recent years, evidence has revealed that RNase L displays a broad range of biological roles which are summarized in this review.

Keywords Interferon; RNase L; antiviral; apoptosis; prostate cancer.

INTRODUCTION

Interferons (IFNs) are a family of cytokines participating in innate immunity against a wide range of viruses and other microbial pathogens. IFNs also have anti-tumor activities due to their antiproliferative, immunoregulatory and apoptotic properties (1). The effects of IFNs are largely mediated through proteins encoded by IFN-stimulated genes (ISGs). One well-studied ISG is RNase L, which is one of the key enzymes in the IFN-induced 2-5A system. The 2-5A system consists of two types of enzymes: 2-5A synthetases and RNase L. IFNs induce a family of 2-5A synthetase genes. The 2-5A synthetases require double-stranded RNA (dsRNA) for their activities. After activation by dsRNA, which is frequently produced during viral infection, 2-5A synthetases convert ATP molecules to pyrophosphate and a series of unique, 5'-phosphorylated, 2'-5' linked oligoadenylates known as 2-5A with the general formula ppp(A2'p5')nA ($n \geq 2$). 2-5A binds RNase L with high affinity, converting it from its inactive,

monomeric state to a potent dimeric endoribonuclease, resulting in degradation of single-stranded viral and cellular RNAs (Fig. 1).

The cDNA of human 2-5A dependent RNase L gene encodes an 84 kDa protein with 741 amino acids. The structural and functional analysis of RNase L has revealed that this enzyme consists of two parts; the N-terminal half of RNase L functions as a repressor while the C-terminal half contains ribonuclease activity. The N-terminus contains 9 ankyrin repeats, typical protein-protein interaction domains, suggesting that RNase L may interact with other proteins. Previously, it has been reported that a duplicated P-loop like motif (GKT) in ankyrin repeats 7 and 8 is implicated in 2-5A binding (2). However, recent crystallographic data suggest that the 2-5A molecule directly interacts with ankyrin repeats 2-4. Reduction of 2-5A binding activity by disruption of two GKT motifs may be due to a conformational change (3). The C-terminal half of RNase L has a cysteine rich region with a protein kinase homology although kinase activity of RNase L has not been demonstrated to date. The kinase-like and ribonuclease domains at the C-terminus of RNase L are related to the Ire1 kinases/ribonucleases that function in the unfolded protein response in organisms from yeast to humans (Fig. 2). Recent evidence indicates that RNase L displays a broad range of biological functions including antiviral, apoptosis, antiproliferation, and pathogenesis of prostate cancer.

MECHANISM OF ACTION

RNase L is believed to display its biological function through regulating mRNA stability following IFN exposure. Ribosomal and viral RNAs are reportedly the first targets of RNase L. Recent evidence has shown that RNase L plays an important role in the stability of several gene products, including IFN-induced genes such as ISG₄₃, ISG₁₅ (4) and PKR (5) in RNase L null cells, MyoD mRNA in myocytes (6), mitochondrial mRNAs in H₉ lymphocytes (7) and mitochondrial DNA-encoded mRNA (mt-mRNA) in monensin-treated

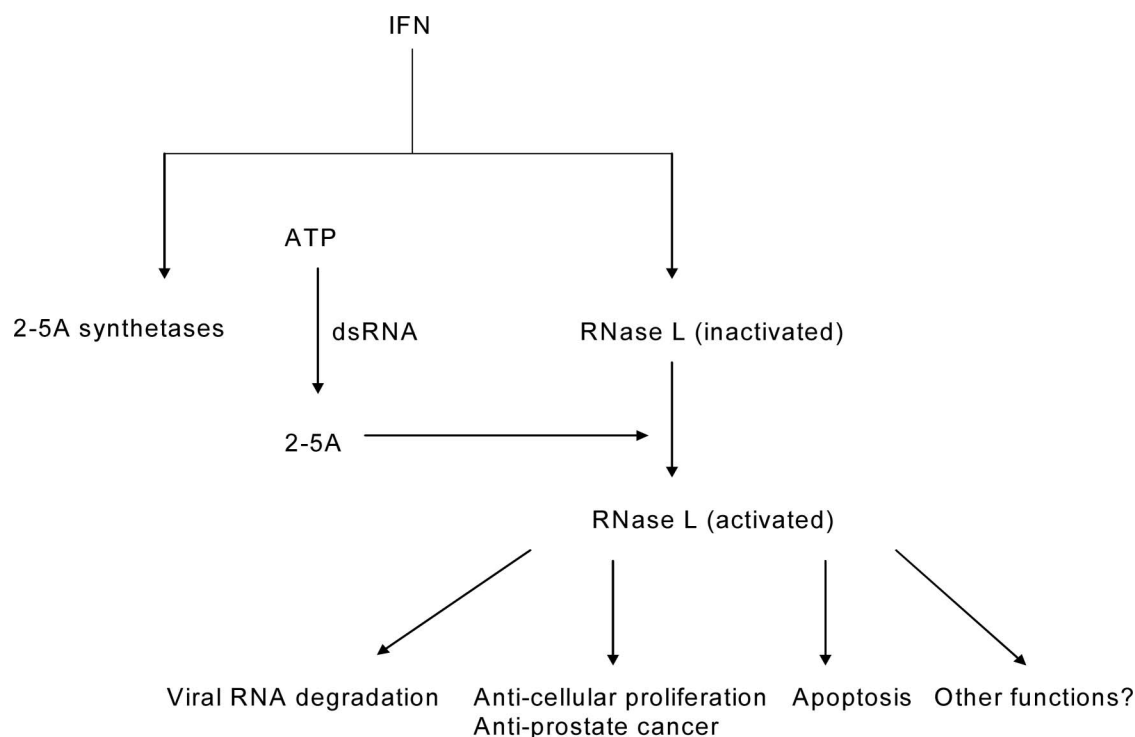


Figure 1. The 2-5A system.

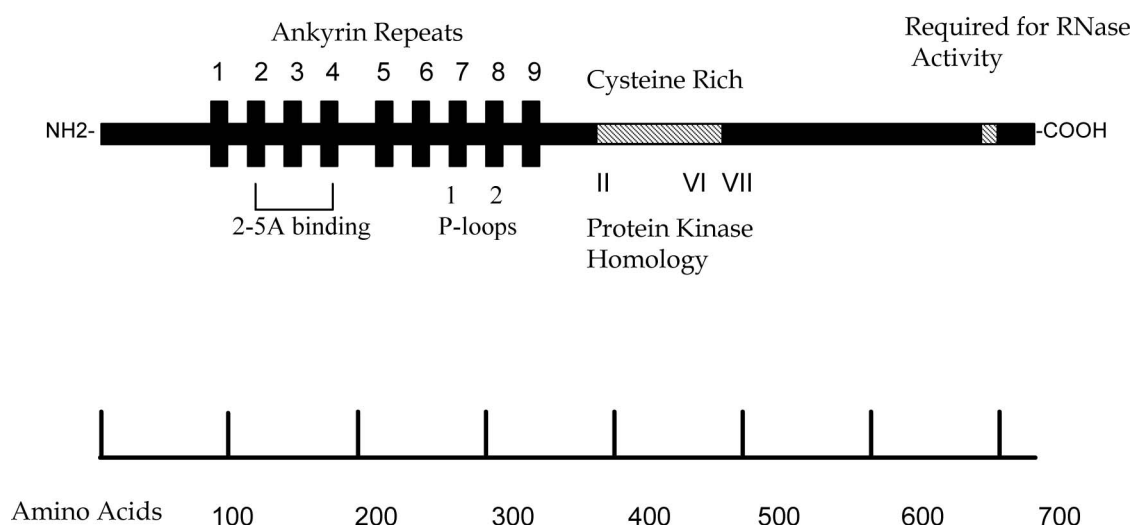


Figure 2. Functional domains of RNase L.

mouse embryonic fibroblasts (MEFs) (8). Interestingly, activated RNase L selectively targets viral RNA in intact cells and the action is 2-5A level dependent (9). 2-5A activates RNase L and subsequently cleaves single-stranded RNA, resulting in inhibition of both viral replication and cellular proliferation. However, in the absence of viral infection, the sources of dsRNA and 2-5A necessary to activate 2-5A

synthetases and RNase-L remain obscure. Recently it has been reported that RNase L is able to regulate gene expression by interacting with another protein (10). Peltz's group observed that RNase L can interact with human translation termination factor eRF3/GSPT1 to modulate the translation termination process, resulting in regulation of gene expression. The results outline a novel role for RNase L as a regulator of translation.

Another report argues that interaction between the androgen receptor and RNase L mediates a cross-talk between the IFN and androgen signaling pathways (11).

Most recently, Silverman's group has demonstrated that 2-5A activation of RNase L remarkably stimulates the expression of genes that suppress virus replication and prostate cancer (12). These genes include IFN-inducible transcript 1/P56, IFN-inducible transcript 2/P54, IL-8, and ISG15. 2-5A treatment also significantly induced RNA transcription of the macrophage inhibitory cytokine-1/non-steroidal anti-inflammatory drug-activated gene-1, a TGF- β superfamily member implicated as an apoptotic suppressor of prostate cancer. However, the precise mechanism for the 2-5A transcriptional effects remains to be elucidated. It is possible that 2-5A activated RNase L suppresses the expression of inhibitory factors for certain genes, resulting in promoting a potent transcriptional response. The 2-5A stimulation of gene expression is apparently through the activation of JNK and ERK. These findings, no doubt, open a door for the further study of the 2-5A system.

REGULATION OF RNase L

2-5A is a series of oligoadenylates synthesized by 2-5A synthetases activated by dsRNA. To activate RNase L, 2-5A must be a trimer or higher with at least one (human) or two (mouse) 5'-phosphoryl groups. Natural 2-5A molecules are very unstable and are degraded by a combination of 5'-phosphatase and 2',5'-phosphodiesterase present in cells. RNase L inhibitor (RLI) is a cellular protein. The expression of RLI is not regulated by IFNs, but is induced by viruses and dsRNA. Overexpression of RLI in the cells strongly inhibits the 2-5A binding and nuclease activities of RNase L (13). RNase L activity can be regulated at its protein level (14). Phorbol-12-myristate-13-acetate (PMA) treatment of mouse L929 fibroblasts resulted in the rapid degradation of RNase L in a dose and time-dependent manner. Cellular RNase L levels were decreased to 40% of control levels after only 5 min of exposure to PMA. Following PMA treatment for 1 h, RNase L levels decreased to 18% of the pretreatment levels. Protein kinase C and selective proteasome inhibitors completely prevent PMA-induced degradation of RNase L, suggesting the involvement of protein kinase C and an active proteasome.

ANTIVIRAL ACTIVITY OF RNase L

Activation of the 2-5A system by IFN and virally produced dsRNA leads to the rapid destruction of RNA. Inhibition of viral replication in the IFN-treated cell could be the result of viral RNA destruction followed by death of the infected cell. The most striking evidence linking the 2-5A system to specific antiviral effects includes 2-5A accumulation and RNase L activation in virus-infected cells, antiviral effects in cells expressing 2-5A synthetase cDNA, and, through inhibition

of RNase L activity, enhanced virus production and reduction of the antiviral effects of IFN (2).

Picornaviridae is one family of viruses strongly inhibited by the 2-5A system. Encephalomyocarditis virus (EMCV), a representative of picornaviruses, is a single-stranded positive-sense RNA virus. The most cogent evidence that the 2-5A system mediates IFN function against viruses comes from studies of this virus. Upon infection, the mRNA of the virus is translated on cellular ribosomes into a polymerase which then generates the negative strand and forms a double-stranded RNA replication intermediate. dsRNA intermediates derived from EMCV bind to and activate 2-5A synthetase in interferon-treated cells. IFN-treated cells after EMCV infection showed an activated 2-5A system and rRNA cleavage products characteristic of RNase L activity (16). Another strategy to study the involvement of the 2-5A/RNase L system in the antiviral activity of IFN is to use the 2-5A analog, CH₃Sp(A₂p)₂A₂pp₃OCH₃, or a core 2-5A which lacks three phosphate groups at the 5' end. These bind to, but do not activate RNase L (2, 17). Transfection of the analog into IFN-treated, EMCV-infected murine L929 cells inhibited rRNA cleavage and increased virus production by up to 10 fold. Expression of a dominant negative truncated RNase L in murine SVT2 cells blocks the rRNA cleavage and reduces about 250-fold the anti-EMCV effects of IFN compared with the IFN-treated vector control cells (2). Interestingly, overexpression of human wild-type RNase L in NIH 3T3 cells displayed a weak inhibition of viral replication in the absence of IFN- α . However, protection against viral infection was dramatically increased after treatment with a low dose of IFN- α , indicating that a 2-5A synthetase is needed to synthesize 2-5A for the activation of RNase L (18). The RNase L knockout mice succumbed to EMCV infections more rapidly than infected wild-type mice (19). RNase L knockout mice treated with IFN prior to EMCV infection also died several days earlier than wild-type mice with the same treatment. However, IFN treatment extended survival to EMCV infection of both the RNase L wild-type and knockout mice, suggesting multiple and overlapping antiviral pathways of IFN. RNase L has been shown to have antiviral effects on other viruses such as vesicular stomatitis virus, vaccinia virus, HIV-1, West Nile virus, herpes simplex virus (HSV), and SV40 (15).

Several viruses have evolved strategies to counteract the antiviral activity of the 2-5A system. For example, vaccinia virus E3L proteins sequester the dsRNA from 2-5A synthetase (20) and reovirus S4 gene encodes a dsRNA-binding protein σ 3 with the same function (21). During HIV-1 infection, the level of RLI is increased in human T cells (22).

APOPTOTIC ACTIVITY OF RNase L

An infected cell undergoing apoptosis may represent an antiviral mechanism that acts by rapidly eliminating the

infected cell, thus preventing the release of viral progeny. Ribonuclease activity frequently has been associated with apoptosis. Increases in ribonuclease activity during metamorphosis, glucocorticoid treatment, irradiation, and viral infection have in some cases coincided with DNA fragmentation, indicating a correlation with classic biochemical markers of apoptosis. Many studies have associated the activation of the 2-5A system, RNA breakdown, or ribonuclease activation with cell death or tissue regression.

Indeed, activation of RNase L has been shown to induce apoptosis. Overexpression of RNase L by a recombinant vaccinia virus causes death of mammalian cells with morphological and biochemical characteristics of apoptosis. Coexpression of 2-5A synthetase augments the event (23). RNase L overexpression sensitizes cells to the induction of apoptosis by staurosporine: a general inducer of apoptosis although by unknown mechanisms. This finding suggests that RNase L is involved in multiple pathways of apoptosis. RNase L-null mice showed enlarged thymuses and reduced levels of spontaneous apoptosis in both the thymus and spleen, implicating RNase L in the development of immune cells. In addition, thymocytes and MEF isolated from RNase L-null mice were resistant to apoptosis induced by staurosporine and irradiation (19). Activation of RNase L by 2-5A transfection into the cells results in specific 18S rRNA cleavage and induction of apoptosis, as measured by TUNEL and annexin V binding assays. In contrast, the dimeric form of 2-5A, ppA₂p₅A, neither activates RNase L nor induces apoptosis. Apoptosis in response to RNase L activity is accompanied by cytochrome C release from mitochondria. Induction of apoptosis by 2-5A can be blocked with either the pancaspase inhibitor, Z-VAD-fmk, or with the caspase 3 inhibitor, DEVD-fmk, suggesting the involvement of caspase activation in RNase L-mediated apoptotic pathway. Evidence also shows that overexpression of Bcl-2 prevents apoptosis induced by RNase L activation (24).

Activation of RNase L in cells results in cleavage of rRNA, leading a cellular stress response and activation of the Jun N-terminal kinases (JNK) pathway. 2-5A treatment activates JNK as determined by Western blot assay for phospho-JNK and phospho-c-Jun, a downstream target of JNK. Various inhibitors of MAP kinase, such as p38 and ERK, do not affect apoptosis in response to RNase L activation. SP600125, a competitive inhibitor of ATP binding to JNK and some other kinases, is able to significantly attenuate apoptosis induced by 2-5A. Furthermore, JNK1^{-/-}/JNK2^{-/-} MEFs are resistant to 2-5A induced apoptosis, suggesting that activation of RNase L requires JNK for efficient induction of apoptosis (25). dsRNA, an intermediate product of viral infection, is able to activate JNK. Activation of JNK by dsRNA is greatly reduced in cells lacking RNaseL (26). However, how RNase L mediates the activation of JNK remains to be fully understood.

ROLE OF RNase L IN THE REGULATION OF CELL PROLIFERATION

Introduction of 2-5A into cells results in an inhibition of growth rates, suggesting a role of RNase L in anticellular proliferation (27). Furthermore, RNase L and 2-5A synthetase levels were reported to be elevated in growth arrested or differentiated cells and reduced in rapidly dividing cells, indicating RNase L is involved in the fundamental control of cell proliferation and differentiation (28, 29). Cells expressing a dominant negative RNase L are resistant to the antiproliferative activity of IFN- α . Similar to the role of RNase L in antiviral activity, overexpression of wild-type RNase L has limited impact on cell growth in the absence of IFN- α . However, the cells are exquisitely sensitive to the antiproliferative effect of IFN- α (18). Interestingly, without IFN- α treatment, RNase L^{-/-} MEF cells grew 1.6-fold faster when compared to RNase L^{+/+} cells. RNase L regulation of cell proliferation independent of IFN- α was not limited to a specific cell type. The growth rate of bone marrow cells isolated from RNase L^{-/-} mice was 1.73-fold faster than that from RNase L^{+/+} in the presence of granulate macrophage colony stimulating factor (GM-CSF) (Zhou et al., 2006, unpublished results), suggesting that RNase L may also mediate unknown pathways to regulate cell growth.

RNase L IN PROSTATE CANCER

RNase L has also been shown to have an impact on the pathogenesis of prostate cancer. Recent findings from several laboratories have supported the identification of the RNase L gene, *RNASEL*, as a strong candidate for the long sought after hereditary prostate cancer 1 (*HPC1*) allele. Carcinoma of the prostate is the second leading cause of cancer deaths in men > 50 years of age and the most frequent visceral cancer in males. Indeed, the American Cancer Society estimates that in the US there will be 234,460 new cases and 27,350 deaths from prostate cancer in 2006 (www.cancer.org).

The linkage of *HPC1* to *RNASEL* implies that RNase L directly or indirectly suppresses one of more steps in prostate pathogenesis and/or metastasis. RNase L was initially proposed to be a candidate tumor suppressor on the basis of its involvement in the antiproliferative activity of IFN and on the location of *RNASEL* at chromosome 1q25, a region deleted or rearranged in some cancers (30). In addition, RNase L was shown to be deficient in a human hepatoma, HEPG₂ cell line (31). However, the first *in vivo* evidence implicating RNase L as a tumor suppressor was the identification of *RNASEL* as the candidate for *HPC1* (24).

Mutation screening and an association study of *RNASEL* as a prostate cancer susceptible gene have identified three major *RNASEL* germline variants: E265X, M1L and R462Q. E265X is associated with poor prognosis in clinical features. This mutation terminates translation within the 2-5A binding

domain of RNase L, thus abolishing 2-5A binding ability (32). The second mutation, M1L, occurred in the translational start codon: a methionine to isoleucine missense mutation. E265X or M1L carriers had half the level of RNase L activity as compared with homozygous wildtype *RNASEL* family members suggesting that both mutations were inactivating. However, R462Q variant is implicated in up to 13% of prostate cancer cases. Carrying one copy of R462Q variant gene increased the risk of prostate cancer by about 1.5 fold, while having two variant alleles doubled the risk (33). An additional mutation in *RNASEL*, 471ΔAAAG, causing a frame shift at codon 157 and a translation stop after 7 additional codons, was discovered by Rennert et al. in Ashkenazi Jews at a relatively high frequency (4%) and in the model human prostate cancer cell line, LNCaP (34).

Although a relationship between the *RNASEL* gene and prostate cancer is established, much more work needs to be done to show how commonly it causes or modifies the clinical course of the disease. A larger population, both with and without strong family histories, and with and without prostate cancer need to be studied to see how often mutations in the gene are associated with the disease and to find out how often mutations occur in men without the disease. Furthermore, how RNase L functions as a tumor suppressor in prostate cancer tumorigenesis and/or metastasis remain largely unknown. To the best of our knowledge, there exist several possible mechanisms by which RNase L could function as an anti-tumor protein.

First, the prostate has been reported to be a host organ for multiple viral infections, including the human polyoma JC virus and HPV (35). Recent identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q *RNASEL* variant provides a possibility that there may be a close relationship between exogenous infection and cancer development in genetically susceptible individuals (36). RNase L is an established antiviral gene. Involvement of a viral pathogen in prostate cancer development remains a possibility.

Second, RNase L has antiproliferative and proapoptotic activities, which suggest that RNase L may have a tumor suppressor function. The 2-5A system is involved in cellular responses to several external stimuli that result in apoptosis. After knock-down of RNase L by siRNA in DU145 cells, a prostate cancer cell line, cells were resistant to TRAIL and camptothecin induced apoptosis (37). These findings indicated that RNase L is involved in an apoptotic signaling pathway in prostate cancer cells.

ROLE OF RNase L IN OTHER CELLULAR FUNCTIONS

RNase L has been linked to chronic fatigue syndrome (CFS). CFS is a debilitating and complex disorder, characterized by extreme fatigue, which is not improved by bed rest and which may be aggravated by physical or mental activity. A number of studies have revealed that the 2-5A system is impaired in CFS patients. Full length RNase L is degraded in

peripheral blood mononuclear cells (PBMC) by unknown proteases, probably by an elastase. The proteolysis of RNase L produces two major fragments with molecular masses of 37 and 30 kDa. The 37 kDa fragment is from the N-terminus of RNase L with the 2-5A binding site whereas the 30 kDa fragment contains the ribonuclease site in the C-terminal end (38). Intriguingly, pre-incubation of RNase L with active 2-5A results in a significant protection of the native 84 kDa RNase L against cleavage by endogenous and purified proteases, suggesting that dimerization of RNase L induced by active 2-5A may produce a conformation that covers the proteolytic sites (39). However, the clinical significance of these truncated forms of RNase L in CFS needs to be further investigated. More interestingly, RNase L^{-/-} mice showed a delayed rejection of skin allograft, arguing that RNase L may also contribute to tumor rejection through the immune system (40).

CONCLUSION

The unique mode of regulation and its involvement in the mechanism of IFN action have been the focus of RNase L study in the past three decades. Studies on prostate cancer genetics and pathogenesis are providing a new direction for future studies on this intriguing, regulated nuclease. Furthermore, the roles of RNase L in the regulation of gene expression, signaling transduction and apoptosis offer a possibility for RNase L as a therapeutic target.

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