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Pathologic effects of RNase-L dysregulation in immunity and proliferative control

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1. ABSTRACT

The endoribonuclease RNase-L is the terminal component of an RNA cleavage pathway that mediates antiviral, antiproliferative and immunomodulatory activities. Inactivation or dysregulation of RNase-L is associated with a compromised immune response and increased risk of cancer, accordingly its activity is tightly controlled and requires an allosteric activator, 2',5'-linked oligoadenylates, for enzymatic activity. The biological activities of RNase-L are a result of direct and indirect effects of RNA cleavage and microarray analyses have revealed that RNase-L impacts the gene expression program at multiple levels. The identification of RNase-L-regulated RNAs has provided insights into potential mechanisms by which it exerts antiproliferative, proapoptotic, senescence-inducing and innate immune activities. RNase-L protein interactors have been identified that serve regulatory functions and are implicated as alternate mechanisms of its biologic functions. Thus while the molecular details are understood for only a subset of RNase-L activities, its regulation by small molecules and critical roles in host defense and as a candidate tumor suppressor make it a promising therapeutic target.

2. INTRODUCTION

Type 1 interferons (IFNs) are pleiotropic cytokines that mediate potent antiviral, antiproliferative and immunomodulatory activities (1). These activities are mediated, in large part, by the products of IFN-stimulated genes (ISGs) (2). Accordingly, efforts to dissect the mechanisms of IFN action and enhance its efficacy as an antiviral/antitumor therapeutic agent have focused on elucidating the activities of the several hundred ISGs identified to date (3). The 2-5A system is an RNA cleavage pathway that was among the first discovered mediators of IFN-induced antiviral activity. Seminal studies in the laboratory of Ian Kerr and others identified two enzymes that are the major components of the 2-5A system: the oligoadenylate synthetase (OAS) family of enzymes that produce 2',5'-linked oligoadenylates (2-5A: $p_x5'A(2'p5'A)_n$; x=1-2;n>2) for which the pathway is named (4), and RNase-L, the endoribonuclease that is activated by 2-5A to cleave single-stranded RNA (ssRNA) (5). Early investigations demonstrated an association of the 2-5A pathway with antiviral activity and extended its role to include antiproliferative activities. More recently, genetic studies have provided definitive evidence for these roles

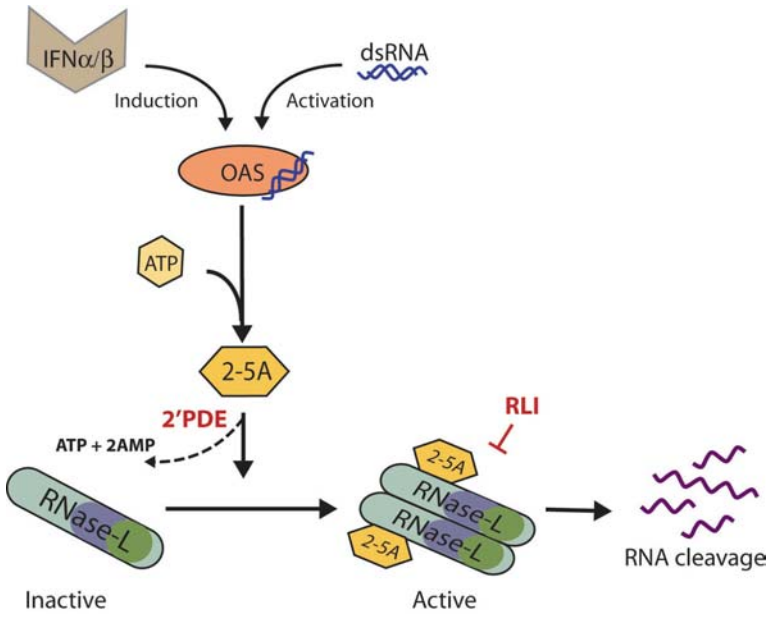

Figure 1. The 2-5A System. IFN treatment (shown) or antiproliferative or microbial stimuli induce the transcription of OAS genes; in the presence of dsRNA, OAS proteins are activated to produce 2-5A; 2-5A binds and activates latent RNase-L resulting in the cleavage of ssRNA. 2-5A system activity is attenuated by the 2'-phosphodiesterase-mediated degradation of 2-5A and inhibition of RNase-L by RLI.

and have identified additional functions for RNase-L the host response to exogenous pathogens and endogenous malignancies (6-9). Here we review the regulation, activities, and mechanism of action of the RNase-L and describe examples of how these functions are disrupted in pathologic conditions. Finally, we examine the potential for modulating the RNase-L as a strategy for therapeutic intervention.

3. THE 2-5A SYSTEM

3.1. Anatomy and regulation of the 2-5A system

The 2-5A system directs endonucleolytic cleavage of ssRNA through a series of tightly regulated steps (figure 1). This activity is initiated by a family of OAS enzymes that are transcriptionally induced by IFN and other microbial or antiproliferative stimuli. OAS proteins are encoded by multigene families in mice and humans (10). The specific isoforms occupy different subcellular compartments and are thought to mediate non-redundant activities. OAS enzymes require double-stranded RNA (dsRNA) for activity and thus function as pattern-recognition receptors for this class of pathogen-associated molecular pattern (11). In the presence of dsRNA, OAS polymerizes ATP into 2',5'-linked oligoadenylates. The only established function of these short, linear molecules is the activation of the latent endoribonuclease RNase-L (12-14). In the absence of 2-5A, intramolecular interactions between NH2-terminal ankyrin repeat domains and the COOH-terminal catalytic domain are thought to maintain monomeric RNase-L in an inactive state (14). This model further postulates that structural changes induced by 2-5A binding within ankyrin repeats 2-4 permits dimerization, RNA binding and ribonuclease activity (figure 2) (15). Activated RNase-L cleaves ssRNA to generate 3'-phosphorylated products with a preference for UU and UA doublets (16, 17). As dysregulated 2-5A pathway activity is deleterious to cells and can induce apoptosis (18), its activity is rapidly attenuated at multiple levels. 2-5A itself is shortlived in cells, being inactivated by nonspecific cellular phosphatases that remove a 5'-triphosphate that is required for optimal activity, and by a specific 2'-phosphodiesterase (2'PDE)(19). In addition, the RNase-L inhibitor RLI is induced by certain viruses and inhibits RNase-L activation by 2-5A (20). The expression of RNase-L mRNA and protein is low in most cell types and transcription is not markedly induced by IFN or other stimuli examined to date (21). In contrast, RNase-L is post-transcriptionally induced in response to cell stress via the binding of HuR to AU-rich elements (AREs) in the 3'UTR of RNase-L mRNA and stabilization of the transcript (22). However in resting cells, the effect of the 3'UTR is a reduction in RNase-L expression and this negative regulation is mediated in part by microRNAs (BAH unpublished). Finally, the proteasome-dependent posttranslational regulation of RNase-L has also been reported (23). The many levels of 2-5A pathway regulation thus permit its rapid activation to efficiently reprogram gene expression in response to innate immune and antiproliferative stimuli, and provide a means to shut down activity and prevent dysregulated RNA cleavage.

3.2. Molecular mechanisms of RNase-L action

3.2.1. RNA targets and consequences of RNase-L cleavage

The 2-5A pathway regulates the endonucleolytic activity of RNase-L, therefore its biologic functions are predicted to be mediated by the effects of RNase-L-directed

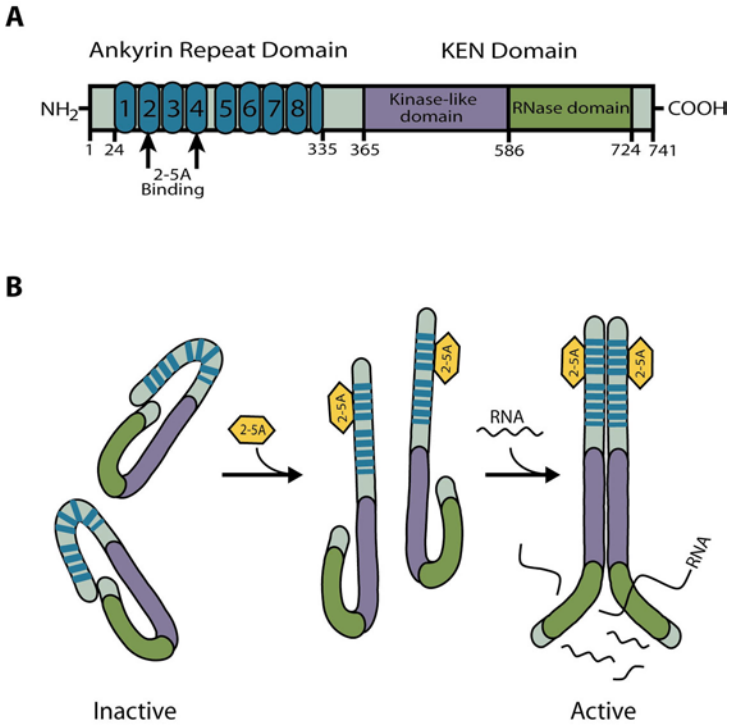

Figure 2. RNase-L domain structure and model of activation. A. The functional domains of RNase-L protein and corresponding amino acid numbers are shown. B. Model of 2-5A-induced conformational changes that result in RNase-L dimerization and activation of enzymatic activity.

substrate cleavage. Accordingly, understanding the mechanism of RNase-L action requires identification of its RNA substrates. Towards this goal, several groups have utilized microarray analysis to identify RNase-L-regulated transcripts (9, 24-27). These studies have identified diverse mRNAs and suggest that RNase-L regulates a distinct set of substrates in different physiologic settings. Using the criteria that authentic RNase-L substrates exhibit increased expression in the absence of RNase-L, display a RNase-L-dependent decrease in RNA stability and can be detected in a physical complex with RNase-L, only a few of these RNAs have been validated as true substrates (11). Direct cleavage of substrate RNAs may also result in secondary effects that also contribute to the biologic activities of RNase-L. For example, RNase-L cleavage of a transcription factor-encoding mRNA may impact the downstream expression of its target genes. In fact, RNase-L activation was shown to induce transcriptional signaling in prostate cancer cells (24). RNase-L can thus impact gene expression at multiple levels to mediate an extensive reprogramming of gene regulatory networks (figure 3); validation of additional RNase-L substrates will permit a better understanding of the relationship between RNase-L-regulated RNAs and their roles in its biologic activities.

The paucity of validated RNase-L substrates has precluded identification of a sequence or structure that may act as a cis determinant of RNase-L recognition. RNase-L preferentially cleaves at UU and UA doublets and structural features can direct RNase-L to cleave ssRNA loops in Hepatitis C virus (HCV) RNA , however contribution of these elements to RNase-L target recognition in cells is not known (16, 17, 28). Relatedly, it has been proposed that RNAs with sufficient double-stranded character to activate OAS enzymes may produce 2-5A and activate RNase-L in a localized manner, thereby providing a mechanism of target selectivity that links OAS activation to RNase-L-mediated cleavage (29). Synthetic RNAs have provided support for this model but it remains to be tested with an endogenous substrate (29). In most studies of selective mRNA degradation to date, target recognition is accomplished via RNA binding proteins (RNAbps) that, in turn, recruit or occlude the decay enzymes (30). By analogy, RNase-L may interact with RNAbps to selectively cleave its target RNAs. In this regard, RNase-L is reported to interact with three RNAbps to date: the translation termination factor eRF3 (31), the mitochondrial translation initiation factor IF2mt (32) and the ARE-binding protein tristetraprolin (TTP) (BAH unpublished and see review by Sanduja *et al.*, this series). Among these, eRF3 is associated with all translating mRNAs and IF2mt is restricted to the mitochondrion suggesting that additional factors are required for selective RNA interaction (also see the section on RNase-L protein interactors). In contrast, our recent finding that TTP interacts with RNase-L provides a mechanism to direct RNase-L cleavage to specific mRNAs. In support of this model, we observed the RNase-L-dependent regulation of a subset of TTP targets (BAH unpublished). Interestingly, this study also demonstrated the association of RNase-L with Decapping enzyme-1 in processing bodies (P-bodies)(33), subcellular sites of mRNA decay, providing the first link between RNase-L

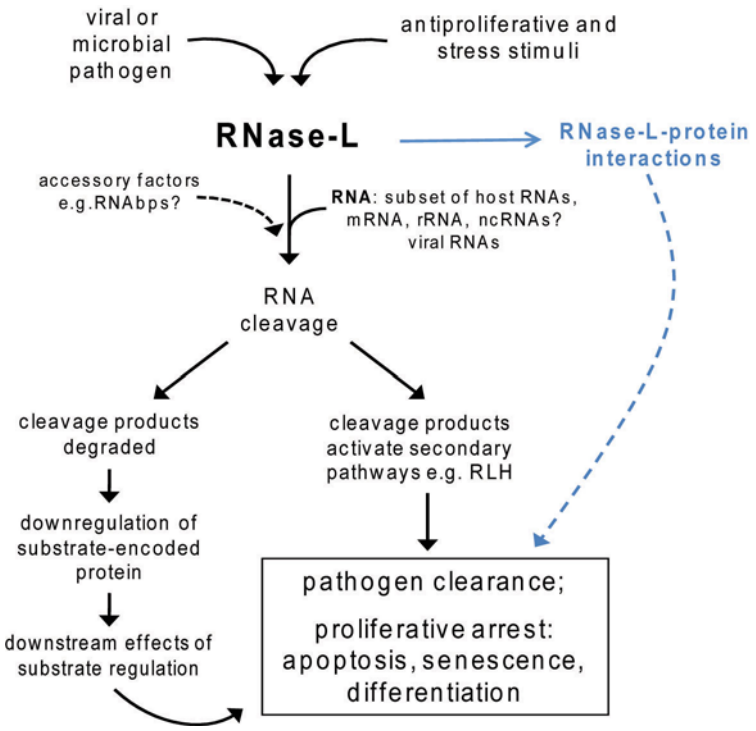

Figure 3. Working model depicting the mechanisms of RNase-L action in host defense and proliferative control. As discussed in the text, direct and indirect effects of RNA cleavage mediate the biological activities of RNase-L. RNase-L-protein interactions may also contribute to these functions (dotted line). Disruption of RNase-L expression or activity is associated with pathologic conditions including increased susceptibility to pathogens, dysregulated immune responses, and an altered proliferative phenotype. Steps leading to 2-5A production are not included in the diagram and RNase-L is shown in its activated form.

and established mRNA decay machinery. The extent to which RNase-L interacts with other decay enzymes and RNAbps to mediate selective mRNA cleavage in distinct physiologic contexts remains to be determined.

The RNase-L-dependent regulation of mRNA targets is associated with a decrease in their half-life; moreover, the products of RNase-L cleavage are not readily detected leading to the idea that cleavage products are rapidly degraded by cellular exonucleases. In contrast to this model, RNase-L cleaves rRNA into discrete products that are easily measured and serve as an index of RNase-L activity in cells (34). The stability of these products may reflect the high degree of secondary structure and abundance of the rRNA substrate. The role of rRNA cleavage in the biological activities of RNase-L is not clear as rRNA cleavage has primarily been measured in conditions of high cellular 2-5A that are associated with cell death (e.g. following IFN treatment and virus infection). Low levels of rRNA cleavage that elude detection by current assays may have a profound impact on protein synthesis and warrants further analysis. Evidence of a functional role for RNase-L cleavage products was provided by a recent study that demonstrated the activation of the cytosolic RNA sensor, RIGI, by small RNase-L-generated cellular RNAs (35). The identities of these RNAs remains to be determined, however this work raises the possibility that RNase-L may function in a RNA processing capacity in which cleavage products go on to mediate some of its biologic activities. In light of the rapidly expanding functions for small non-coding RNAs, the application of RNA-protein crosslinking and deep sequencing technologies (36) to identify cellular products of RNase-L cleavage will provide important insights into its mechanism of action.

3.2.2. Protein-Protein Interactions

While the endonucleolytic activity of RNase-L is clearly a major mechanism by which it elicits its biologic activities, several proteins have been shown to interact with RNase-L providing an alternate mechanism of action. Though the functional repercussions and mechanisms are still being elucidated, these interactions clearly impact the activities of both RNase-L and its interacting partners.

3.2.2.1. RLI

RNase-L inhibitor (RLI; ABCE1) is the best characterized protein modulator of RNase-L activity and can be found as part of a complex composed of RNase-L, RLI, and eRF3 (see below) (20). Since RLI does not readily bind 2-5A and therefore does not compete for it, the mechanism for this inhibition is likely to be through direct interaction. While RLI has not been shown to directly bind to RNase-L independent of eRF3, it does co-sediment with RNase-L in fractionation experiments and forms a high molecular weight complex that would suggest an RLI-RNase-L heterodimer (37). Regardless of whether the interaction is direct or indirect, RLI is a potent inhibitor of

RNase-L activity. For example, RLI overexpression enhances and antisense knockdown of RLI blocks its ability to inhibit RNase-L binding to 2-5A, rRNA cleavage, destabilization of mitochondrial mRNA, and antiviral activity against HIV and EMCV (20, 38-40).

Unlike RNase-L, RLI is conserved from archaea to humans, implicating a role beyond its namesake. It contains two ATP binding cassette (ABC)-type nucleotide binding domains (NMD), an N-terminal iron sulfur cluster, and two P-loop motifs. The crystal structure of yeast RLI has revealed that the two NMDs are arranged head-to-tail with a hinge doma*in situ*ated along the NMD1:NMD2 interface that may allow it to undergo a clamp-like motion seen in ABC transporters (41). Transcription of RLI is induced by dsRNA or virus infection, however it is not regulated by IFN-alpha. (20, 42). The RLI gene encodes two transcripts (3.5 kb and 2.8 kb) that differ in their 3'-untranslated regions (3'UTR) through use of alternate polyadenylation signals. The 3.5 kb transcript is more abundant suggesting that elements in the extra 3'UTR sequence serve to stabilize the mRNA (20, 43, 44). Although they are ubiquitously expressed, the levels of these transcripts and the ratio of their expression varies in different tissues.

A new role in translation has been emerging for RLI since its original characterization. RLI has been shown to bind to Hcr1, eRF3, eRF1, and eIF3, all of which are associated with translation and/or ribosomal RNA processing. In yeast, suppressed expression of RLI is lethal to the cell, underscoring its importance. Several publications have demonstrated its involvement in precursor rRNA processing, export of the subunits to the cytoplasm, assembly of the preinitiation complex, and transcription termination (45-47). A role for RNase-L in translation termination in conjunction with eRF3, and perhaps RLI, has been reported ((31) and see eRF3 below), however the full extent to which RNase-L is involved in the translation regulatory activities of RLI remains to be determined.

As a natural attenuator of RNase-L activity, RLI may contribute to RNase-L dysregulation in human diseases. RLI is induced by several viruses and may serve as a mechanism to evade the antiviral activity of RNase-L (39, 42). In the case of HIV infection, subversion of RLI may serve dual roles by inhibiting RNase-L, which has been implicated in anti-HIV activity (48), and by interacting with the gag protein to mediate capsid assembly (49, 50). RLI is downregulated in Chronic Fatigue Syndrome (CFS, see below) where it is thought to contribute to the dysregulated increase in RNase-L activity associated with this disease (51-53). Knockdown of RLI expression in small cell lung cancer resulted in reduced proliferation and invasiveness consistent with an oncogenic function; the extent to which these activities are mediated through the inhibition of RNase-L tumor suppressor activity remains to be determined (54). RLI functions in ribosome recycling (55) in conjunction with eRF1/eRF3-mediated translation termination which is also thought to be influenced by RNase-L (31). RNase-L may influence ribosomal recycling through targeting the RNA component of these complexes, however mechanistic insights into this process, and how it may be altered in human cancers, await further investigation. Finally, RLI is coordinately upregulated with other ABC transporters in cancer (56, 57) yet its role as a classical transporter and mediator of drug resistance, and whether RNase-L impacts this function, has not been extensively examined. Importantly, RNase-L may function in only a subset of RLI activities as RLI is evolutionarily conserved whereas RNase-L is only present in vertebrates. Nonetheless in light of their antagonistic roles in cell proliferation and host defense, it is essential to include analysis of both RNase-L and RLI to fully assess the role of the 2-5A system in physiologic and pathologic conditions.

3.2.2.2. eRF3

In 1993, Salehzada *et al* identified a component of an RNase-L complex that was isolated by a monoclonal antibody, mAb3, generated to what was believed to be purified RNase-L (58). This protein, originally called RNABP (RNA binding protein) was able to bind to RNase-L as well as RNA, but not to 2-5A (59). Its interaction with RNase-L is enhanced in the presence of 2-5A and this association is believed to be important to RNase-L function as treatment with mAb3, and presumed inhibition of RNABP, can block RNase-L-mediated rRNA cleavage and antiviral activity. In 2005, the true identity of RNABP was discovered to be the translation termination release factor eRF3/GSPT1 (31).

eRF3 binds to numerous proteins. Primarily, eRF3 functions as a GTPase in the eRF1/eRF3 eukaryotic translation termination complex. eRF1 is responsible for recognizing stop codons in the mRNA and then the peptidyl-tRNA bond is hydrolyzed in an eRF3/GTPase-mediated reaction to release the polypeptide chain from the ribosome. In a related activity, eRF3 interacts with RLI and poly(A)-binding protein (PABP). PABP can bind the translation initiation complex component eIF4G, which facilitates ribosome recycling by reinitiating translation after termination. An important role for eRF3 is its function in cellular RNA surveillance pathways. eRF3 is implicated in nonsense mediated decay of mRNAs with premature stop codons through its interaction with the Upf1-3 complex that targets these transcripts for degradation (60). Most recently, the peptide release function of eRF1/eRF3 was shown to be critical for the degradation of mRNA and rRNA in stalled translation complexes (61-63).

Upon identifying eRF3 as the RNABP known to bind to RNase-L, Le Roy *et al* sought to decipher the impact of this interaction on eRF3 function. Previous reports have shown that inhibition or depletion of eRF3 can lead to inefficient translation termination resulting in readthrough of the stop codon, and the production of proteins with extended C-termini. Similarly, RNase-L activation or overexpression of RNase-L led to increased readthrough in a reporter system suggesting that RNase-L inhibits eRF3 activity. The presence of RNase-L also facilitated a translational +1 frameshift stimulated by a stop codon. Interestingly, RNase-L and PABP competitively

bind eRF3, which favors a model in which binding of active RNase-L to eRF3 inhibits reinitiation, increases readthrough and frameshifting, and may target RNase-L to mRNAs for degradation. Conversely, when PABP preferentially binds eRF3 (perhaps in the absence of the RNase-L activator 2-5A) reinitiation occurs normally (31).

eRF3 is present at low basal levels in resting cells and its expression is induced by mitogens prior to the initiation of DNA synthesis (64) which may correspond with an increased requirement for surveillance and degradation of defective RNAs that are produced at higher levels in rapidly proliferating cells. As a proliferation-associated gene, eRF3 mRNA is upregulated in gastric and breast cancers (65, 66). Furthermore, the eRF3 N-terminus contains a poly-glycine expansion consisting of 5 alleles, the longest of which encodes 12 glycine residues and is strongly correlated with susceptibility to gastric and breast cancers (65, 66). The poly-glycine stretch falls within the PABP binding domain which is deduced from eRF3 competition binding studies to overlap with the site of RNase-L interaction (31). The poly-glycine extension may disrupt the interaction between eRF3 and PABP or RNase-L thereby impacting RNA turnover and the cellular RNA profile. This scenario provides a potential mechanism by which the poly-glycine expansion is linked to altered gene expression in human malignancies but awaits direct experimental testing. Another mechanism by which eRF3 may impact oncogenesis is through the induction of apoptosis, an important tumor suppressor mechanism. Specifically eRF3 is proteolytically cleaved to produce a peptide that binds inhibitor of apoptosis (IAP) proteins to stimulate apoptosis (67). More recently eRF3 was shown to interact with apoptosis signal-regulating kinase-1 (ASK1) which also led to increased caspase-3 activation and apoptosis (68). Interestingly proteolytic cleavage of eRF3 occurs in the N-terminus where RNase-L and PABP bind, whereas ASK1 binds to the eRF1 interaction domain in the eRF3 C-terminus which may also be influenced N-terminal interactors (69). Thus, the presence of RNase-L, PABP or a poly-glycine stretch, may influence eRF3 cleavage or ASK1 binding and, in turn, its capacity to induce apoptosis. Finally, the apparent preference of eRF3 for interaction with activated RNase-L suggests that this interaction may occur in conditions in which 2-5A is elevated such as following exposure to virus. A role for eRF3 in host defense has not been reported, however it will be of interest to determine how eRF3 mutants that lack the RNase-L interaction domain or contain the poly-glycine expansion impact RNase-L antiviral activity.

3.2.2.3. IF2mt

A number of studies have demonstrated a role for RNase-L in the destabilization of mitochondrial (mt) RNA. Though generally found in the cytoplasm, RNase-L and its regulators, OAS, RLI, and 2'PDE have all been observed in the mitochondria (38, 70, 71). Several mt mRNAs, such as cytochrome b, ATPase 6, ND5, and cytochrome oxidase subunit II are downregulated or upregulated in the presence or absence respectively of RNase-L in multiple cell systems (32, 38, 72). Consistent with a role for the 2-5A system in regulating mt mRNAs, modulation of the RNase-L inhibitor RLI or the 2'PDE which degrades 2-5A also impacted the mt mRNA profile (38, 71). Functionally, modulation of mitochondrial gene expression is an important mechanism to regulate cellular energy production in response to cell stress or proliferative arrest. Accordingly, the RNase-L-dependent regulation of mt mRNAs was observed following treatment with the excitatory amino acid glutamate or the sodium-ionophore monensin to mimic ischemic injury (72, 73), and was required for the antiproliferative and proapoptotic activities of IFN-alpha (32).

Another piece to the puzzle of a role for RNase-L in the mitochondria was revealed by the identification of the mitochondrial translation initiation factor (IF2mt) as a protein binding partner for RNase-L (32). IF2mt is a nuclear-encoded translation factor that delivers N-formyl methionyl-tRNA to the P-site of the mitochondrial ribosome during initiation (74). IF2mt was isolated from a yeast two-hybrid screen using RNase-L as bait and the interaction was confirmed using IF2mt translated from rabbit reticulocyte lysate. Using human H9 T cell lymphoma cells in which IFN induces RNase-L-dependent mt mRNA degradation, antiproliferative effects and cell death, it was demonstrated that translation was necessary for a decrease in mt mRNA. When IF2mt was overexpressed to outcompete RNase-L-IF2mt binding in the translation initiation complex, the degradation of mt mRNA degradation was suppressed resulting in an increase in proliferation and decrease in apoptotic signaling (32). This observation demonstrated the functional significance of the RNase-L-IF2mt interaction and suggested that the mechanism of RNase-L-target recognition may involve mRNA translation status and ribosome association mediated in part by IF2mt. Thus mt mRNAs comprise an important target of RNase-L regulation that may contribute to its antiproliferative activities. In this regard, it is well established that mitochondrial dysfunction is involved in diverse human pathologies including cancer and ischemia (75); the extent to which RNase-L-dependent activities are altered in these diseases remains to be determined. In addition, fundamental information about how the subcellular distribution of RNase-L between the mitochondrion and cytosol is regulated in physiologic and pathologic conditions will shed light on this question.

3.2.2.4. Androgen receptor

The androgen receptor (AR) is a member of a family of nuclear ligand activated transcription factors. Normally found in the cytoplasm, AR activation by the hormone testosterone or its more active hydrolyzed variant 5-alpha-dihydrotestosterone (DHT), causes AR to interact with coregulators, become phosphorylated, and translocate into the nucleus. Nuclear AR interacts with promoter elements to drive the transcription of genes involved in the normal development and maintenance of the prostate, some of which may also contribute to cancer progression. Upon diagnosis, 80-90% of prostate tumors are androgen-dependent, and over 80% of those patients who receive androgen ablation therapy respond initially. However, many patients progress to a hormone refractory state where

AR may continue to signal independently of androgen, possibly in response to other growth factors (76).

IFN-gamma exerts antitumor activity in prostate cancer and antagonizes the effects of DHT on a subset of cellular genes. A search for AR-interacting proteins that may mediate crosstalk between DHT and IFN-gamma identified RNase-L as a novel AR binding partner (77). Given that AR had previously been shown to inhibit apoptosis and promote cell proliferation, this interaction provided a potential mechanism for the tumor suppressive role of RNase-L in familial prostate cancer (see section 4.2)(78). The interaction between RNase-L and AR was enhanced by treatment with DHT and despite the presence of ankyrin repeats in the RNase-L N-terminus, the critical AR binding region mapped to the C-terminal 170 aa nuclease domain. Functionally, overexpression of RNase-L in conjunction with IFN-gamma treatment suppressed expression from a DHT-responsive promoter. In contrast, expression of catalytically inactive R462Q RNase-L mutant or the D541E missense mutation failed to inhibit DHT signaling suggesting that RNase-L enzymatic activity is required to block AR-mediated gene expression (77). Of further relevance is the finding that XMRV expression is stimulated by androgen ((79) and see section 4.2 below), thus RNase-L-mediated inhibition of AR activity may impact prostate tumorigenesis by downregulating both cellular and viral AR targets. While the mechanisms by which RNase-L influences AR require further study, the potential of RNase-L to modulate androgen sensitivity presents intriguing therapeutic opportunities.

3.2.2.5. IQGAP1

The most recent addition to the list of RNase-L interactors is IQGAP1 (IQ motif-containing Ras GTPase-activating-like protein 1). IQGAP1 is a large, ubiquitously expressed scaffold protein that functions in cell-cell adhesion, migration, actin reorganization, cell polarization, proliferation, and differentiation. It contains four Ile-Gln (I-Q) motifs, a Ras GTPase-related domain, and a domain homologous to calponin's calcium-binding domain. Atypical of most GTPase-activating proteins (GAPs), IQGAP1 stabilizes the Rho GTPases Rac1 and Cdc42 in the GTP-bound state, rather than catalyzing the switch from GTP to GDP. In addition, IQGAP1 is known to have a role in both MAPK and Wnt signaling. One mechanism by which IQGAP1 may become active is through the binding of calmodulin, a calcium binding protein involved in multiple cellular functions, which causes a conformational change that alters protein interactions (80). IQGAP1 is also phosphorylated, possibly by protein kinase C, which may contribute to its activation (81).

IQGAP1 was identified as an RNase-L interactor through a screen searching for proteins that preferentially bound to RNase-L during 1-(3-C-ethynyl-beta-D-ribo-pentofuanosyl) cytosine (ECyd) induced cell death (82). A previous report demonstrated that RNase-L is a key mediator of ECyd induced apoptosis, possibly through the activation of c-jun N-terminal kinase (JNK) and mitochondrial membrane damage (83). IQGAP1 immunoprecipitated with RNase-L and this binding was enhanced during ECyd treatment. Though both proteins are necessary for ECyd induced cell death, no direct consequence of this interaction was demonstrated. A potential role for RNase-L as the kinase for IQGAP1 phosphorylation in response to ECyd was suggested, however kinase activity has not been shown for RNase-L (82). In addition, interaction between RNase-L and two IQGAP1 homologues, IQGAP2 and IQGAP3, has not been explored.

4. BIOLOGIC ROLES FOR RNASE-L AND DISEASE-ASSOCIATED ALTERATIONS IN ITS ACTIVITY

RNase-L functions in diverse cellular processes via direct and indirect effects that, in turn, contribute to its physiologic functions. Disruption of one or more of these activities is associated with human diseases in the context of microbial challenge and cancer. In the examples below, we present the evidence of a role for RNase-L in specific diseases and discuss potential mechanisms involved.

4.1 Antiviral functions

RNase-L was originally characterized as an antiviral effector of IFN action. In the decades since its discovery, it has been shown to have activity against numerous RNA viruses (*Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae, and Retroviridae*) as well as several DNA viruses (*Poxviridae, Herpesviridae, and Polyomaviridae*) which is reviewed more thoroughly in (84). In most cases, it has been demonstrated or presumed that RNase-L directly cleaves the viral genome or transcripts, however, the increasing roles for RNase-L in immunomodulatory activities may provide an alternative or additive mechanism for its antiviral function. In parallel with this activity, many viruses have adapted ways to inhibit RNase-L or OAS as a means to evade their antiviral activities. Here we discuss several clear cases in which RNase-L mediates antiviral activity against infections that still plague the worldwide population.

4.1.1. Picornaviruses

The prototypical virus used to study the antiviral activity of RNase-L is encephalomyocarditis virus (EMCV). It typically infects rodents but can crossover into humans and cause febrile illness (85). EMCV is a member of the very large and diverse family Picornaviridae, which also includes hepatitis A virus, other cardioviruses (Theiler's virus and Mengovirus), enteroviruses (poliovirus and coxsackievirus), rhinoviruses, and several other non-human viral genera. Picornaviruses are small, positive-stranded RNA viruses that encode 3-4 structural proteins and 7-8 non-structural proteins which are translated as a single polyprotein using an internal ribosome entry site (IRES) in the 5'UTR. Collectively, this virus family can cause myocarditis, meningitis, diabetes, poliomyelitis, hepatitis, and the common cold (86).

Early studies have shown that the OAS/RNase-L pathway possesses potent antiviral activity against EMCV, as well as other members of the Picornaviridae family.

Upon infection, OAS has been isolated while bound to both positive and negative strands of the EMCV genome (a replicative-intermediate dsRNA), leading to its activation and synthesis of 2-5A (87, 88). Production of 2-5A then activates RNase-L resulting in cleavage of both cellular RNAs and viral genomic RNA (89). As expected, *in vitro* overexpression of RNase-L can lead to suppression of EMCV while expression of a dominant-negative RNase-L mutant prevents this inhibition (6, 89). Consistent with these findings, RNase-$L^{-/-}$ mice not only died from EMCV infection at a higher rate than wild type mice, but also with faster kinetics, particularly when mice were pretreated with IFN. These studies demonstrated that RNase-L was critical for mediating IFN-induced anti-EMCV activity and that it also possessed antiviral activity in the absence of IFN treatment (7). Though RNase-L is clearly an important mediator of antiviral activity, mice deficient in three major antiviral effectors, RNase-L PKR, and MxA, were capable of mounting an antiviral response (e.g. as compared to IFN-alpha receptor deficient mice) indicating that additional cellular factors are required for complete IFN-induced antiviral activity (90).

In addition to EMCV, RNase-L mediates antiviral activity against other picornaviruses such as coxsackievirus and poliovirus. Experiments with coxsackievirus B4 indicate that RNase-L is required for an optimal IFN response in murine pancreatic islet cells and that RNase-$L^{-/-}$ mice are extremely sensitive to this virus. Although infection studies of RNase-$L^{-/-}$ mice have not been conducted, poliovirus has been shown to encode a RNA structure capable of inhibiting RNase-L. The structure is composed of two segments which are located in the region of the genome that encodes the 3C protease (91). Using a bioinformatics and mutagenesis approach, the RNAs are believed to form a loop E motif and an H-H kissing loop which interact to inhibit the endoribonuclease domain of RNase-L (92, 93). Surprisingly, overexpression of either wildtype or a dominant-negative mutant of RNase-L has little effect on viral yield in culture. In the early stages of infection, RNase-L activity is not detected, probably due to the inhibitory RNA, and the virus is able to replicate. Upon completion of replication and assembly, RNase-L becomes activated and mediates poliovirus induced apoptosis, enabling viral release and spread. This may be evidenced by the formation of larger plaques when RNase-L is overexpressed (18, 84).

4.1.2. HCV

Hepatitis C virus (HCV) currently infects approximately 3% of the world's population and there is no vaccine or completely effective cure. Of those infected with the virus, 80% develop chronic disease and are at higher risk for liver failure and hepatocellular carcinoma. HCV is a positive-stranded RNA virus with a 9.6 kb genome that encodes a polyprotein which is cleaved into three structural and seven nonstructural proteins. Due to the poor fidelity of the virus-encoded RNA-dependent RNA polymerase, HCV has evolved into at least six genotypes and many more subtypes. Front line therapy for the virus is IFN-alpha in combination with the nucleoside analogue ribavirin. This treatment is only 50% effective, with some genotypes characteristically more resistant than others (94).

RNase-L impacts HCV at multiple steps of infection, the most well characterized being the cleavage of the viral genome. OAS becomes activated in response to the HCV ssRNA genome, possibly due to a replicative intermediate or the secondary structures at both its 3' and 5'UTRs. Production of 2-5A results in RNase-L activation and cleavage of the HCV genome at UU and UA dinucleotides into 200-500 bp fragments. Upon discovering the sensitivity of HCV RNA to RNase-L, Han *et al* explored whether this IFN-regulated anti-viral protein could mediate the differential responsiveness of HCV to IFN-alpha therapy (95). The 1a and 1b genotypes of HCV are known to be more resistant to IFN than genotypes 2 and 3. Analysis of genome sequences from each genotype revealed that (1) irrespective of genotype, given the number of A and U nucleotides in the genome, UU and UA dinucleotides occur at a less than predicted frequency, and (2) IFN-resistant genotypes have fewer UU and UA dinucleotides than IFN-sensitive genotypes. Clearly, minimization of cleavage sites could be an effective RNase-L evasion strategy. Indeed, *in vitro* assays demonstrate that a HCV genotype 2 RNA containing more cleavage sites is more readily degraded than a 1a genotype RNA (95, 96). The possible mutation of UU and UA dinucleotides during the course of IFN-alpha treatment regimens has also been explored as a mechanism of resistance. Though specific UU and UA sites may become mutated, new ones also emerge so that the overall number remains the same (95, 97). Further analysis of the RNase-L cleavage sites in HCV has revealed preferential characteristics regarding the UU and UA dinucleotides. First, a 3' proximal C or G is unfavored. Second, UU and UA sequences within a dsRNA structure do not get cleaved, and third, multiple single stranded UU and UA sequences are preferred (98). Most recently, characterization of the cleavage fragments led to the identification of a small RNA product capable of activating RIG-I to amplify type 1 IFN production as an additional antiviral strategy (28).

Since HCV seems to be targeted by RNase-L activity, it is logical that the virus would have mechanisms for inhibiting its activity. One possibility is the HCV protein NS5A. Full length and the N-terminus (1-148 aa) of NS5A binds to OAS and presumably inhibits its ability to generate 2-5A. Overexpression of either NS5A or NS5A(1-148) can inhibit IFN-induced protection against EMCV infection, and a point mutant at amino acid 37, which is unable to bind OAS, is no longer able to diminish this protection (99). This mediation of IFN activity is independent of the controversial role of NS5A inhibition of PKR, as the NS5A (1-148) construct does not contain the PKR binding domain or ISDR sequence (100-102). In addition, another study reported that the HCV NS4A and NS4B proteins may inhibit the expression level of RNase-L (103). Collectively, HCV may have adapted several mechanisms for RNase-L evasion.

4.1.3. West Nile Virus

Like HCV, West Nile virus (WNV) is a member of the *Flaviviridae* family. It is a positive stranded RNA virus that infects <3% of the US population and can cause encephalitis, meningitis, poliomyelitis or even death in the

immune compromised or people of early or advanced age. The virus, originally isolated in 1937 in Uganda, is transmitted to humans from birds through infected mosquitoes. Upon infection, the virus is believed to replicate in Langerhans dendritic cells, migrate to the lymph nodes, enter the bloodstream, and eventually cross into the central nervous system (CNS)(104).

The first association of RNase-L with WNV was through its relationship with OAS. It was discovered that the locus that conferred virus susceptibility to a congenic mouse strain was the gene for Oas1b. The WNV susceptible strain (C3H.PR.Flv^{R}) encoded a truncated Oas1b gene that resulted from a premature stop codon (105). Mouse embryonic fibroblasts from these mice, as compared to wild type, resistant, C3H/HeJ mice, produce higher viral titres upon WNV infection. Overexpression of dominant-negative RNase-L in the resistant cells renders them more permissive to infection, indicating a role for RNase-L in anti-WNV immunity. Indeed, RNase-L is capable of becoming activated by WNV as well as cleaving the viral genome. Similar to HCV, WNV has a lower than predicted frequency of UA dinucleotides in its genome, possibly as an evasion mechanism (106). Infection of RNase-L$^{+/+}$ and RNase-L$^{-/-}$ mice show increased lethality in knockout mice as well as higher viral titres in the CNS, lymph node, CD11b^{+} splenocytes, and the spinal chord. Infection of mice defective in both RNase-L and the IFN-induced, dsRNA-activated protein kinase, PKR, demonstrate a more pronounced pathology and viral susceptibility, indicating that PKR, and likely other antiviral proteins, are also involved (107).

4.1.4. HIV

In 2009, there were 2.6 million new cases of human immunodeficiency virus (HIV) reported globally, a 19% drop from the epidemic's peak in 1999. Despite the decrease in new cases, the total number of worldwide cases, 33.3 million, continues to grow due to increased survival from antiretroviral therapies (108). Along with vaccine development, therapeutic strategies and insights into pathogenesis continue to be aggressively pursued in hopes for a cure. In the mid 1980's, RNase-L was identified as a potential therapeutic target and possible diagnostic for HIV infection (109). Early studies indicated that the OAS/RNase-L pathway was inactive in peripheral blood mononuclear cells in AIDS patients and that the 2-5A binding activity of RNase-L was diminished by 65% in the lymphocytes isolated from AIDS patients (110). These clinical observations imply that RNase-L has antiviral activity against HIV. In fact, *in vitro* experiments show that HIV infection stimulates 2-5A production and that the ssRNA genome is cleaved by activated RNase-L (111). Overexpression of RNase-L resulted in lower HIV titres and increased apoptosis; furthermore expression of antisense RNase-L or increased expression of RLI yielded higher levels of HIV mRNA and reverse transcriptase activity, indicating that RNase-L can be a potent inhibitor of HIV (39, 112). As indicated by the clinical data however, HIV has adapted mechanisms to inhibit this antiviral activity. For example, subsequent to infection, HIV has been found to induce the expression of RLI, likely resulting in the reduced 2-5A binding activity seen in patients (39, 110).

4.1.5. Influenza

Influenza A is a negative stranded, segmented, RNA virus. Due to its high mutation rate, it continually alters its viral glycoproteins creating seasonal epidemics that annually result in 250,000 to 500,000 deaths worldwide (113). There have also been three pandemics in the last century, the 1918 Spanish flu, the 1957 Asian flu, and the 1968 Hong Kong flu, which resulted in the deaths of tens of millions of people. One of the key antagonists of the immune response to influenza is the NS1 protein. It has been shown to inhibit IFN's antiviral activity, regulate viral RNA synthesis, splicing, and translation, suppress host cell apoptosis, and activate PI3K signaling (114).

In order to suppress the IFN response to influenza infection, NS1 prevents IFN-alpha induction through RIG-I, binds PKR to inhibit its activation, and impedes RNase-L activity. In 2006, Min and Krug demonstrated a role for RNase-L in influenza infection by generating an influenza A/Udorn/72 virus with a point mutant in NS1 (R38A) that abrogated its ability to bind to dsRNA. This mutated virus was extremely sensitive to the antiviral effects of IFN, indicating that the NS1-dsRNA interaction is important for virus survival. In the absence of RNase-L expression, the R38A virus was resistant to IFN and showed similar virus replication to wild type virus (115). This indicated that the dsRNA binding activity of NS1 is a mechanism for inhibiting RNase-L-mediated antiviral activity, which may include direct cleavage of viral RNA or the generation of small RNAs that activate RIG-I and induce IFN.

4.2. A role for RNase-L in cancer

Although the 2-5A system was discovered as a mediator of IFN-induced antiviral activity, analysis of its components in the absence of virus infection revealed an inverse correlation between 2-5A pathway activity and cell proliferation. For example, OAS and RNase-L activities were elevated in confluence-associated proliferative arrest (116), following the induction of differentiation (117, 118) and in response to antiproliferative agents (119, 120). Consistent with this role as an endogenous constraint on cell proliferation, ectopic expression of OAS or RNase-L resulted in quiescence, senescence or apoptosis depending on the cell type examined, and the induction of apoptosis and senescence was reduced in RNase-L-deficient cells and tissues (6, 7, 18, 121, 122). Apoptosis and senescence are important tumor suppressor mechanisms thus a role for RNase-L in these processes suggested that RNase-L may function as a tumor suppressor *in vivo*. Moreover, replicative senescence is a cellular model for *in vivo* ageing and the incidence of most human malignancies is known to increase with age. Consistent with this model, RNase-L-/- mice exhibited an increased lifespan (122) and the impact of RNase-L on mouse models of tumorigenesis is currently under investigation. The first evidence of a link between RNase-L and human cancers came from the finding that the human *RNASEL* gene mapped to the Hereditary Prostate Cancer-1 (*HPC1*) locus on chromosome 1q25 (8). Sequence analysis of the *RNASEL* gene in hereditary

prostate cancer patients revealed several germline mutations; among these, the missense mutation R462Q was implicated in up to 13% of prostate cancer cases and resulted in a doubling of prostate cancer risk in homozygous individuals (78). The R462Q mutation decreased RNase-L activity by three-fold in transfected cells thereby correlating reduced RNase-L activity with increased prostate cancer risk as predicted for a tumor suppressor (123). Subsequent studies both confirmed and refuted a relationship between *RNASEL* mutations and prostate or other cancers suggesting that additional factors or study parameters contribute to the impact of *RNASEL* on tumorigenesis (124, 125). For example, a recent study found an association of R462Q homozygosity with prostate cancer risk only in cases prior to the use of PSA as a biomarker when detection may have occurred at a more advanced stage of the disease (126). In addition to the originally identified variants in the *RNASEL* protein coding region, a SNP in the 5'UTR was associated with increased risk of head and neck, cervix and breast cancer and a SNP downstream from the *RNASEL* gene was correlated with overall risk for prostate cancer and advanced disease (126, 127). These SNPs may act through a regulatory mechanism to modulate RNase-L expression; alternatively they may be in linkage disequilibrium with undetected mutations that impact RNase-L activity or expression. Further studies are thus required to determine the impact of these SNPs on RNase-L protein levels.

In light of the dual antiviral and prostate cancer suppressor functions of RNase-L, Silverman and colleagues conducted an elegant study to search for viruses that may be associated with the R462Q RNase-L mutation in prostate cancer. This work identified the first authentic human gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), that was detected at high frequency in patients homozygous for the RNase-L mutation but was found at significantly reduced rates in heterozygous or wild type individuals (128). Consistent with a functional role for RNase-L in the anti-XMRV activity, IFN reduced XMRV replication and this antiviral activity was diminished following knockdown of RNase-L (129). Like other retroviruses, XMRV may promote tumorigenesis by integrating adjacent to cellular genes and altering their expression; in fact, mapping of XMRV integration sites in prostate cancer revealed a preference for cancer-associated genes and regions (130). In addition, XMRV contains a glucocorticoid response element which may explain viral tropism for androgen-responsive prostate tissue and confer androgen-inducible transcription to genes adjacent to sites of virus integration (79). Thus a model in which XMRV promotes oncogenic transformation and is countered by the anti-XMRV activity of RNase-L to mediate prostate cancer tumor suppressor activity has been supported by some studies. However, conflicting reports preclude a consensus on the role of XMRV in the etiology of prostate cancer and its association with RNase-L (124, 125,131). These studies highlight the need for further investigations into the relationships between these parameters as risk factors and as potential therapeutic targets.

In addition to XMRV-associated prostate cancer, RNase-L functions in established tumor suppressor mechanisms that may mediate antitumor activity against a broader profile of malignancies. The compromised induction of apoptosis or senescence observed in RNase-L-deficient cells in culture is predicted to confer a survival advantage on tumor cells with inactivating mutations in RNase-L. Consistent with this idea, stable knockdown of RNase-L increased tumor number and size as compared to control cells in nude mouse xenografts of human cervical cancer cells and ectopic expression of RNase-L reduced tumorigenesis in a murine xenograft model (BAH unpublished and (132)). RNase-L can also influence the response of cancer cells to chemotherapeutic agents, as stable knockdown of RNase-L in prostate cancer cells conferred resistance to apoptosis induced by a combination of camptothecin and TRAIL (133). However, a role for RNase-L in response to chemotherapeutic agents *in vivo*, either in mouse models or as a correlation with treatment outcome in human patients, has not been reported. On a molecular level, microarray analyses of RNase-L-regulated mRNAs identified transcripts encoding proliferation-associated proteins that may contribute to its tumor suppressor function. For example RNase-L activation increased transcription of macrophage inhibitory cytokine-1 that was reported to mediate apoptosis in prostate cancer (24) and downregulated mRNAs encoding ribosomal proteins that are associated with increased protein biosynthesis observed in rapidly proliferating cancer cells (25). Two recently identified targets of RNase-L regulation that may play important roles in its antitumor activity are the RNA binding proteins HuR and TTP (Sanduja *et al.* and Srikantan and Gorospe this series). These proteins bind AU-rich elements in the 3'UTR of labile mRNAs including those encoding oncogenes, cytokines and growth factors. HuR stabilizes its targets resulting in increased expression and is elevated in human cancers (134). In contrast, TTP binds AREs in a subset of mRNAs that is partially overlapping with HuR targets and functions to recruit decay enzymes and promote mRNA degradation. TTP expression is low in cancer cells and is implicated in tumor suppressor activities as ectopic TTP expression in cervical cancer cells reduces proliferation and enhances sensitivity to chemotherapeutic agents (135). The mechanisms by which RNase-L impacts HuR, TTP and the regulation of ARE mRNAs appear to involve complex feedback and feed-forward loops as RNase-L was reported to regulate HuR and is itself regulated by HuR (22, 26). In addition, our recent studies indicate that RNase-L functions in concert with TTP to cleave TTP mRNA and a subset of TTP targets (BAH unpublished). The antitumor activity of RNase-L thus appears to involve significant reprogramming of the gene expression profile with distinct mRNA subsets targeted in cancers of different origin or stage of progression. The contribution of specific RNase-L substrates to its antiproliferative activities has not been determined, therefore upstream steps in pathway activation may represent optimal therapeutic targets. Towards this goal, studies to determine the mechanism of RNase-L-substrate recognition represent an important area of research.

While most studies describe a tumor suppressor role for RNase-L, it was reported to be upregulated in premalignant familial adenomatous polyposis polyps and adenocarcinomas as compared to prostate epithelium (136) and RNase-L knockdown reduced tumor formation in human leukemia xenografts (BAH unpublished). These observations suggested that RNase-L may play an oncogenic role in certain contexts. Indeed, opposing activities in tumor suppression and oncogenesis are observed for innate immune effectors in which a regulated inflammatory response mediates tumor suppression by recruiting and activating innate immune cells to kill tumor cells. In contrast, a dysregulated inflammatory response can result in tissue damage, chronic proliferative repair and increased tumor invasion to promote tumorigenesis (137). Consistent with an inflammatory mechanism in RNase-L-associated prostate cancer, a mutation downstream of *RNASEL* was correlated with an increase in both prostate cancer risk and levels of inflammatory biomarkers (126). The potential for pathologic effects in conditions of dysregulated RNase-L activity suggests that pharmacologic RNase-L inhibitors may be effective as anti-inflammatory agents in certain contexts.

RNase-L functions in antiproliferative, antiviral and immunomodulatory activities that may differentially impact tumor incidence and progression in distinct settings. As RNase-L knockout mice do not spontaneously develop tumors, studies to analyze the consequences of RNase-L inactivation in defined mouse models of tumorigenesis will help to determine which activities mediate key antitumor functions. This information will also provide insights into the specific types of human malignancies in which RNase-L may play an important role. In this regard, studies of RNase-L function in human cancers have been limited to mutational analyses of *RNASEL* sequence, as expression of its mRNA and protein are typically low and provide no information on activity. Reagents to directly measure RNase-L activity in clinical samples (e.g. via detection of enzyme activity or loss of endogenous substrate) will provide an accurate assessment of its association with human cancers and treatment outcome and may reveal a broader role than has been previously estimated.

4.3. Immunomodulatory activities

4.3.1. Antibacterial and proinflammatory functions

In addition to a direct effect on a virus-infected cell or a tumor cell, a role for RNase-L in modulating the immune response has emerged as an important mechanism by which it mediates biologic activities. RNase-L-deficient mice have increased numbers of thymocytes due to reduced apoptosis but no overt phenotype has been associated with this condition and all other immune cell subsets appear normal in the absence of pathogens (7). However, rejection of class II major histocompatibility complex-disparate skin allografts was delayed in RNase-L-/- mice, whereas contact hypersensitivity was unaffected. These results suggested a role for RNase-L in CD4+ but not CD8+ -mediated proinflammatory responses (138). Established immune mediators (e.g. chemokines, cytokines, ISGs) were identified as targets of RNase-L regulation in microarray studies, providing further support for an immunomodulatory function (9, 24, 25). The finding that RNase-L produced small RNAs that could serve as RLH activators to amplify IFN-beta induction provided the first mechanistic insights into its regulation of innate immune mediators (35). A subsequent study revealed a novel role for RNase-L in antibacterial immunity that involved multiple components of the immune response. Specifically, RNase-L-/- mice exhibited increased susceptibility to *Escherichia coli* and *Bacillus anthracis* infections which corresponded with a compromised induction of proinflammatory cytokines, altered recruitment of immune cells and disrupted clearance of internalized bacteria in macrophages (9). The mRNA encoding cathepsin-E, an endolysosomal protease that functions in pathogen targeting to the lysosome, was identified as a RNase-L substrate in macrophages. This regulation of cathepsin-E may mediate, in part, the effect of RNase-L on bacterial clearance. In a separate report linking the 2-5A system to the proinflammatory response to bacteria and virus-derived stimuli, the OAS2 protein was found to interact with the nucleotide-binding oligomerization domain-2 protein (NOD2) (139, 140). NOD2 is a member of the NLR family of proteins that coordinate the sensing of endogenous and exogenous danger-associated stimuli with signal transduction pathways to induce transcription of proinflammatory mediators. OAS2 forms a complex with NOD2 and other signaling components at the mitochondrial membrane that, when activated by dsRNA, produces 2-5A to activate RNase-L; in turn, RNase-L generates RNA activators of RIGI to stimulate signaling and induction of proinflammatory cytokines (141). In addition to their transcriptional induction in response to innate immune stimuli, the expression of proinflammatory cytokines including TNF-alpha is regulated by the TTP-dependent control of their mRNA stability (142)(Sanduja *et al.*, this issue). We recently determined that RNase-L regulates TTP and is part of a network that regulates ARE-mRNAs including those encoding proinflammatory cytokines. This observation suggested that RNase-L regulates cytokine mRNAs through TTP, however the mechanistic details remain to be determined. Interestingly, the regulation of proinflammatory cytokines by RNase-L may provide an explanation for the reduced antigenicity of a replicase-based DNA vaccine in RNase-L-/- mice (143). Specifically, dsRNA produced by the vaccine is thought to induce 2-5A production and RNase-L activation that in turn enhances an immune response to the DNA vaccine via increased cytokine production and the induction of apoptosis in WT mice. A compromised cytokine induction and apoptotic response in RNase-L-/- mice may contribute to the diminished antigenicity observed.

4.3.2. Association with Chronic Fatigue Syndrome

Chronic fatigue syndrome (CFS) is a debilitating, long term disorder with no known etiology. It is diagnosed by the occurrence of defined symptoms including extreme fatigue, short-term memory loss, musculoskeletal pain, impaired sleep, tender lymph nodes, low-grade fever, headache, and postexertional malaise, and the exclusion of medical factors that may cause overlapping symptoms such as depression, severe obesity, hepatitis B or C, or hypothyroidism (144). CFS currently affects 17 million

people worldwide, predominantly people aged 18-50 with a higher incidence in women. Patients with CFS often display signs of chronic immune activation, such as elevated $CD8^+$ T cell counts and diminished natural killer (NK) cell function. These indicators and altered cytokine expression led to the hypothesis that chronic infection with a viral agent may be associated with this disorder (144).

In light of a potential link between CFS and a viral agent, researchers began to examine host antiviral proteins that may be disrupted in CFS and contribute to its pathogenesis. Early studies found that the OAS/RNase-L pathway was dysregulated in peripheral blood mononuclear cells (PBMC) isolated from CFS patients. They detected upregulated active OAS, elevated levels of RNase-L, and downregulated RLI, which collectively suggested a significant increase in RNase-L activity (52, 145). The amount of OAS/RNase-L/RLI deregulation observed was found to negatively correlate with NK cell function and the dysregulation of apoptosis (146, 147). Furthermore, a low molecular weight variant of RNase-L was discovered in CFS patients but not healthy controls. This protein was identified as a 37 kDa fragment of RNase-L (denoted here as RNL-37) (148). This 37 kDa protein contains the N-terminal ankyrin repeat domains, which are responsible for binding to 2-5A, and its ratio to full length RNase-L correlated with the level of patient disability (53). RNL-37 is generated by the cleavage of RNase-L by human leukocyte elastase, calpain, or other proteases found in PBMCs from CFS patients (149, 150). One hypothesis suggests that infection may activate monocytes, neutrophils, and T cells to produce elastase and cleave RNase-L. This proteolysis results in the production of two fragments, RNL-37 and a 30 kDa protein that encodes the C-terminal nuclease domain of RNase-L. Based on the current model of RNase-L activation (figure 2B), RNL-37 may bind and inhibit the nuclease activity of the 30 kDa fragment. Once 2-5A binds RNL-37, it disassociates from the nuclease domain, allowing RNA hydrolysis at a rate three times that of the wild type enzyme (151). In addition to this increased rate of RNA decay, this truncated endonuclease may not be regulated in the same way as the full length protein, possibly exhibiting altered target specificity that may contribute to CFS pathology. Related to the cleavage of RNase-L is the report of Fremont *et al* demonstrating that binding of 2-5A trimer or tetramer protects RNase-L from proteolysis whereas RNase-L bound to 2-5A dimer is susceptible to cleavage (152). 2-5A dimer is preferentially made by the p100 isoform of OAS (153); furthermore, the distribution of 2-5A species produced may also be influenced by the characteristics of the activating dsRNA (154, 155). Thus information on the relevant OAS isoforms and their activators in CFS may shed light on the mechanisms controlling the production of RNL-37 and identify novel therapeutic targets.

While one line of investigation focused on host immune defects as a risk factor for CFS, others looked for the presence of specific pathogens in CFS patients. These studies suggested links to human herpes virus-6, Epstein-Barr virus and *Mycoplasma spp*. but none suggested a causal role (51, 52, 156, 157). Following on the finding that the dysregulation of RNase-L in prostate cancer is associated with XMRV, Lombardi *et al.* looked for the presence of this virus in CFS patients that also exhibited altered RNase-L activity. The initial study reported a striking positive correlation between XMRV and CFS; specifically 67% of CFS patients were positive for virus as compared to 3.7% of healthy controls (158). XMRV was detected by multiple techniques including nested PCR, immunoblotting, transmission of virus from PBMCs to uninfected LNCaP cells, transmission of virus from patient plasma, and the detection of an antibody response to XMRV's envelope protein (158). Since the publication of these original findings, several papers have been published challenging this association, and some reports have supported it (159-162). Several factors may play a role in these seemingly disparate results. They include possible contamination from strains of highly similar murine leukemia viruses, geographical differences in strain and incidence, and standardized methods of detection. At this time, the relationship between XMRV and CFS, as well as a role for RNase-L in this disease, remains unresolved and requires further investigation. Particularly, has this association identified an etiologic agent for CFS or is it simply an indicator of RNase-L dysfunction?

5. CONCLUSIONS AND THERAPEUTIC IMPLICATIONS

RNase-L functions in diverse physiologic processes that are essential for the health and viability of the host including the control of cell proliferation and defense against pathogens. These activities are disrupted in pathologic conditions suggesting that RNase-L represents a viable target for therapeutic intervention. In this regard studies into the mechanisms of RNase-L action will provide tools to assess its activity as a prognostic or diagnostic indicator of therapeutic response or disease progression respectively. For example, as a mediator of the cellular response to cancer chemotherapeutic agents the expression and activity of RNase-L may be a good predictor of treatment outcome. The requirement of RNase-L for natural small molecule activators (i.e. 2-5A) makes it an attractive target for pharmacologic manipulation. In fact, cell-permeable RNase-L activators have been developed and exhibit broad spectrum antiviral activity (163). The targeting of a host immune component, like RNase-L, as therapeutic approach is particularly advantageous in the context of emerging pathogens or biowarfare in which the etiologic agent is not immediately known. Direct RNase-L activators may also show efficacy against bacterial infections or cancer; indeed, RNase-L activation in combination with CPT or TRAIL resulted in increased killing of cultured prostate cancer cells (133). Finally, an important role for RNase-L in the regulation of multiple components of the immune response has emerged from recent studies (9, 28, 35). In the role of a proinflammatory mediator, strategies to inhibit RNase-L may be appropriate in pathologic conditions associated with chronic inflammation. Studies to elucidate the mechanisms by which RNase-L impacts different aspects of an immune response may reveal novel therapeutic opportunities.

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