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Basis for regulated RNA cleavage by functional analysis of RNase L and Ire1p

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ABSTRACT

RNase L and Ire1p are members of a superfamily of regulated endoribonucleases that play essential roles in mediating diverse types of cellular stress responses. 2′-5′ oligoadenylates, produced in response to interferon treatment and viral double-stranded RNA, are necessary to activate RNase L. In contrast, unfolded proteins in the endoplasmic reticulum activate Ire1p, a transmembrane serine/threonine kinase and endoribonuclease. To probe their similarities and differences, molecular properties of wild-type and mutant forms of human RNase L and yeast Ire1p were compared. Surprisingly, RNase L and Ire1p showed mutually exclusive RNA substrate specificity and partially overlapping but not identical requirements for phylogenetically conserved amino acid residues in their nuclease domains. A functional model for RNase L was generated based on the comparative analysis with Ire1p that assigns novel roles for ankyrin repeats and kinase-like domains.

Keywords: interferon; ribonuclease; RNA stability; unfolded protein response

INTRODUCTION

RNA metabolism plays essential roles in several aspects of cell regulation, including RNA cleavage induced by certain stress responses. One such example is RNA degradation by the pppA2′p5′A2′p5′A (2-5A) system that occurs in interferon (IFN)-treated and virus-infected cells (Kerr & Brown, 1978; Zhou et al., 1993, 1997). Recently, an unprecedented type of mRNA splicing was discovered as a key regulatory step in the unfolded protein response (UPR; Cox & Walter, 1996; Sidrauski & Walter, 1997). Interestingly, RNase L and Ire1p, the enzymes that mediate RNA cleavage in the 2-5A system and in the UPR, respectively, have homologies in their kinase (or kinase-like) and ribonuclease domains (Fig. 1).

RNase L is the terminal enzyme in the 2-5A antiviral pathway. Interferon treatment of mammalian cells induces a family of 2-5A synthetases that share the ability to produce a series of 2′-to-5′-linked oligoadenylates known as 2-5A [$p_x(A2'p)_nA$; $x = 1–3$, $n > 2$] from ATP in response to viral or cellular double-stranded RNA (dsRNA) (Kerr & Brown, 1978). RNase L is converted from inactive monomers to active dimers after binding to 2-5A (Dong & Silverman, 1995; Cole et al., 1996). The N-terminal half of RNase L contains a series of nine ankyrin repeats encompassing the 2-5A binding domain whereas the C-terminal half is comprised of a kinase-like domain and a ribonuclease domain (Fig. 1A). The N-terminal region represses the ribonuclease domain, and consequently, RNase L has no ribonuclease activity in the absence of 2-5A. 2-5A is thought to cause a conformational change in the enzyme that releases and unmasks the ribonuclease and protein/protein interaction domains in RNase L (Dong & Silverman, 1997).

Homology between RNase L and a yeast protein, Ire1p, led to a prediction that yeast Ire1p was an endonuclease (Bork & Sanders, 1993). This prediction was later verified in studies with purified yeast Ire1p (Sidrauski & Walter, 1997). Ire1p, an integral membrane protein of the endoplasmic reticulum (ER), is an essential factor in mediating the UPR in yeast where it senses unfolded proteins in the lumen of the ER leading to the splicing of *HAC1* mRNA, coding for the UPR specific transcription factor (Cox et al., 1993; Mori et al., 1993; Sidrauski & Walter, 1997). Interestingly, yeast Ire1p has both serine/threonine kinase and endoribonuclease activities. Human Ire1α and Ire1β have also been shown to retain both kinase and nuclease activities (Niwa et al., 1999). Furthermore, conservation of

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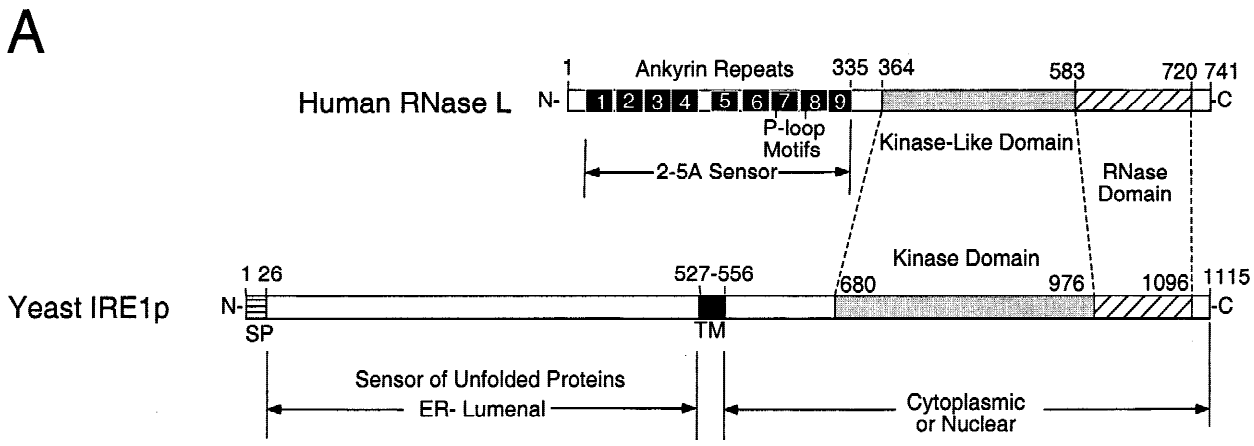


FIGURE 1. A: Comparison of the domains and motifs in human RNase L and yeast Ire1p. SP: signal peptide; TM: transmembrane domain. **B**: Amino acid sequences of the nuclease (or putative nuclease) domains of (from upper to lower sequence) human α (hIre1α) (Tirasophon et al., 1998), *Homo sapiens* Ire1β (M. Niwa and P. Walter), *Mus musculus* Ire1β (Wang et al., 1998), *Drosophila melanogaster* Ire1 (Adams et al., 2000), *Caenorhabditis elegans* (CE) Ire1 (*C. elegans* Sequencing Consortium, 1998), *Schizosaccharomyces pombe* (s.p.) Ire1p (GenBank accession number AL035248), *S. cerevisiae* (s.c.) Ire1p (Nikawa & Yamashita, 1992), *Arabidopsis thaliana* Ire1 (a.t.) (Lin et al., 1999), *H. sapiens* RNase L and *M. musculus* RNase L (Zhou et al., 1993, 2000) were aligned using the GeneWorks program. The nuclease consensus sequence is shown at the bottom. Closed circles: residues substituted with alanine in both RNase L and Ire1p; open circles: residues substituted with alanine in RNase L only. (*Figure continues on facing page.*)

sequence suggests that the nuclease activity of Ire1p is important for the UPR in a diverse group of eukaryotic organisms, including yeast, plants, nematodes, and mammals (Fig. 1B). The regulatory N-terminal half of Ire1p is present in the ER lumen, and the catalytic domains in the C-terminal half are in the cytoplasm or nucleus (Fig. 1A). Functional similarities between RNase L and Ire1p include sensor domains in the N-terminal regions, enzyme activation accompanied by oligomerization, and stimulation by adenosine nucleotides, such as ADP (Wreschner et al., 1982, Dong et al., 1994, Shamu & Walter, 1996; Sidrauski & Walter, 1997).

There are also important functional differences between RNase L and Ire1p. In addition to their ribonuclease activities, Ire1 proteins transphosphorylate during the UPR (Shamu & Walter, 1996; Welihinda & Kaufman, 1996). In contrast, RNase L lacks several conserved protein kinase domain residues and to date has not been shown to have kinase activity (Dong & Silverman, 1999). Another major functional difference in the two enzymes is that Ire1p is a site-specific nuclease, whereas RNase L is relatively nonspecific (Floyd-Smith et al., 1981; Wreschner et al., 1981b; Carroll et al., 1996; Sidrauski & Walter, 1997). Thus, the difference in specificities between RNase L and yeast Ire1p present interesting questions, such as the molecular basis for cleavage site-selection. In the present study, we have probed the significance of similarities between human RNase L and yeast Ire1p through analysis of a series of substitution and deletion mutations. Results support an evolutionary and functional relationship between the RNase L/Ire1 family of regulated endoribonucleases while also highlighting important differences. Furthermore, several novel features of the RNase L activation mechanism mediated by ankyrin repeats and kinase-like motifs were uncovered.

RESULTS

Functional consequences of mutations in conserved amino acids in the RNase L/Ire1 superfamily

A conserved, nuclease signature sequence is apparent from a comparison between the carboxy terminal portions of RNase L and Ire1 from different organisms (Fig. 1B). To probe the functions of amino acid residues that are conserved in all members of the superfamily, alanine substitution mutations were made in human RNase L and yeast Ire1p. We chose to mutate residues that are both present in every member of the RNase L/Ire1 superfamily and based on the nature of functional groups presented by the amino acid side chains that could participate in the catalytic activity (Table 1). Histidines, for example, play critical roles in mediating RNA cleavage by different ribonucleases, including both the pancreatic ribonuclease and RNase T_1/T_2 superfamilies (reviewed in Cuchillo et al., 1997; Irie, 1997), and aspartate and arginine residues are involved in catalysis by pancreatic ribonuclease and RNase T_1, respectively. DNA encoding wild-type and mutant forms of human RNase L (H583A, P584A, W632A, D661A, R667A, and H672A) were expressed from the mammalian expression plasmid pcDNAneo3 by transient transfections of HeLa strain M cells that are deficient in endogenous RNase L. RNase L activity was deter-

B

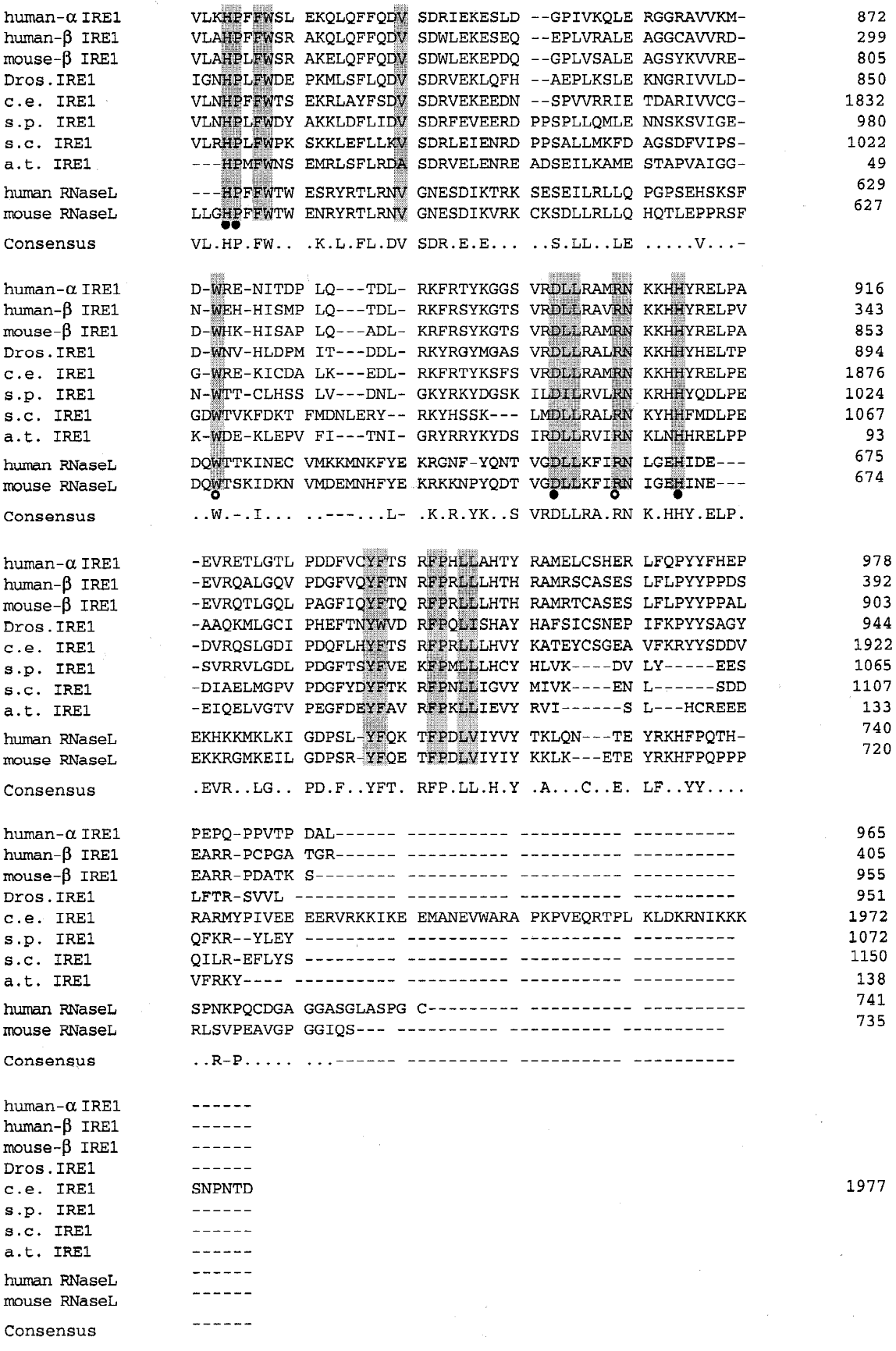


FIGURE 1. (*Legend on facing page.*)

TABLE 1. Effects of alanine substitution mutations on the ribonuclease activities of human RNase L and yeast IRE1p.

Enzyme	Homologous amino acid residue					
Human RNase L	H583 Active	P584 Active	W632 Inactive	D661 Inactive	R667 Inactive	H672 Inactive
Yeast IRE1p	H976 Inactive	P977 Inactive	W1025 N.D.	D1051 Inactive	R1056 N.D.	H1061 Slightly active

N.D.: not done; active: ribonuclease activity comparable to that observed with wild-type enzyme; slightly active: ribonuclease activity but at low levels compared to wild-type enzyme; inactive: no specific ribonuclease activity was observed.

mined by transfecting cells with 2-5A and analyzing the integrity of the rRNA (Fig. 2A; Wreschner et al., 1981a). The cells that were transfected with the empty plasmid (plus and minus 2-5A) or with RNase L cDNA in the absence of 2-5A showed no cleavage of rRNA (Fig. 2A, lanes 1–3). In contrast, transfection with wild-type RNase L cDNA and 2-5A caused extensive breakdown of rRNA into discrete, characteristic cleavage products demonstrating 2-5A dependent activation of the RNase L (Fig. 2A, lane 4). Interestingly, two of the point mutants, H583A and P584, had undiminished 2-5A-dependent RNase activity despite the conserved presence of the histidine and proline in every known member of the RNase L/Ire1 superfamily (Fig. 2A, lanes 5 and 6). On the other hand, RNase L mutants W632A, D661A, R667A, and H672A, lacked ribonuclease activity (Fig. 2A, lane 7–10). Western blots probed with monoclonal antibody to human RNase L showed that all of these proteins were expressed to similar levels in the cells (Fig. 2B). Four of these mutant forms of RNase L (H583A, P584A, R667A, and H672A) were expressed in *Escherichia coli* and purified as GST fusion proteins (the other two mutants, W632A and D661A, could not be compared because these were expressed only at very low levels in *E. coli*). Assays were performed using a U_{25} substrate labeled at the 3′ terminus with [^{32}P]-pCp (see Materials and Methods). In the autoradiograms, both the intact RNA and a ladder of labeled RNA products are observed due to nonspecific degradation (Fig. 2C, lane 1). Mutant P584A was equally active to the wild-type enzyme [protein concentration required to cleave 50% of the RNA: (EC_{50}) < 0.01 μM], whereas H583A had slightly reduced activity (EC_{50} = 0.025 μM; Fig. 2C). These findings are in agreement with the intact cell results. In contrast, R667A and H672A lacked ribonuclease activity (Fig. 2C). In summary, H583 and P584 were not required for ribonuclease activity, whereas W632, D661, R667 and H672 were all essential (Table 1).

To determine the functions of homologous residues in yeast Ire1p, H976, P977, D1051, and H1061 were mutated to alanine (Fig. 1B and Table 1). RNA cleavage assays were performed with *HAC1*^{u} 508 RNA substrate containing both 5′ and 3′ splice sites (Fig. 3A). The wild-type Ire1p cleaved the substrate RNA at the two previously characterized sites (Fig. 3A, lanes 1–3). In contrast, H976A (Fig. 3A, lanes 4 and 5), P977A (Fig. 3A, lanes 6 and 7), and D1051A (Fig. 3A, lanes 8 and 9) were inactive. However, slight residual activity was observed with the H1061A mutant of Ire1p (Fig. 3A, lanes 10 and 11). Similar results were obtained with a mini stem-loop RNA substrate containing the *HAC1* 3′ splice site, that is, the mutant forms of Ire1p were inactive, except H1061A had a small amount of activity (Fig. 3B; Table 1). Therefore, although these amino acid residues are conserved between yeast Ire1p and human RNase L, mutations at two of these sites (H583 and P584 in RNase L and H976 and P977 in Ire1p) behaved very differently.

To compare the substrate specificity of the two nucleases, RNase L was incubated with *HAC1* RNA, and Ire1p was incubated with a poly(U) substrate of RNase L (Fig. 3C). RNase L was unable to cleave the *HAC1* mini stem-loop (Fig. 3C, lanes 4, 7, and 8), although it did efficiently cleave a polyuridylate substrate (Fig. 3C, lanes 9 and 10). Conversely, Ire1p did not cleave the polyuridylate under the condition where the *HAC1* mini substrate was efficiently cleaved (Fig. 3C, lanes 5 and 6). We also tested if the presence of Ire1p in the RNase L cleavage reactions would alter its specificity or level of activity. However, in a mixing experiment, RNase L only slightly inhibited the activity of Ire1p without altering specificity (Fig. 3C, lane 3). Thus, despite their amino acid similarities, both the mutant and substrate specificity experiments indicate functional differences between the two nucleases.

Mutations in the nuclease domain of RNase L do not affect 2-5A binding or dimerization

To determine if the lack of nuclease activity in RNase L mutants R667A and H672A was due to a defect in activator binding, covalent crosslinking to a ^{32}P-labeled and bromine-substituted 2-5A analog was performed under ultraviolet light (Nolan-Sorden et al., 1990). The result showed that R667A and H672A had 2-5A binding activities similar to that of wild-type RNase L and to the nuclease active mutants H583A and P584A (Fig. 4A).

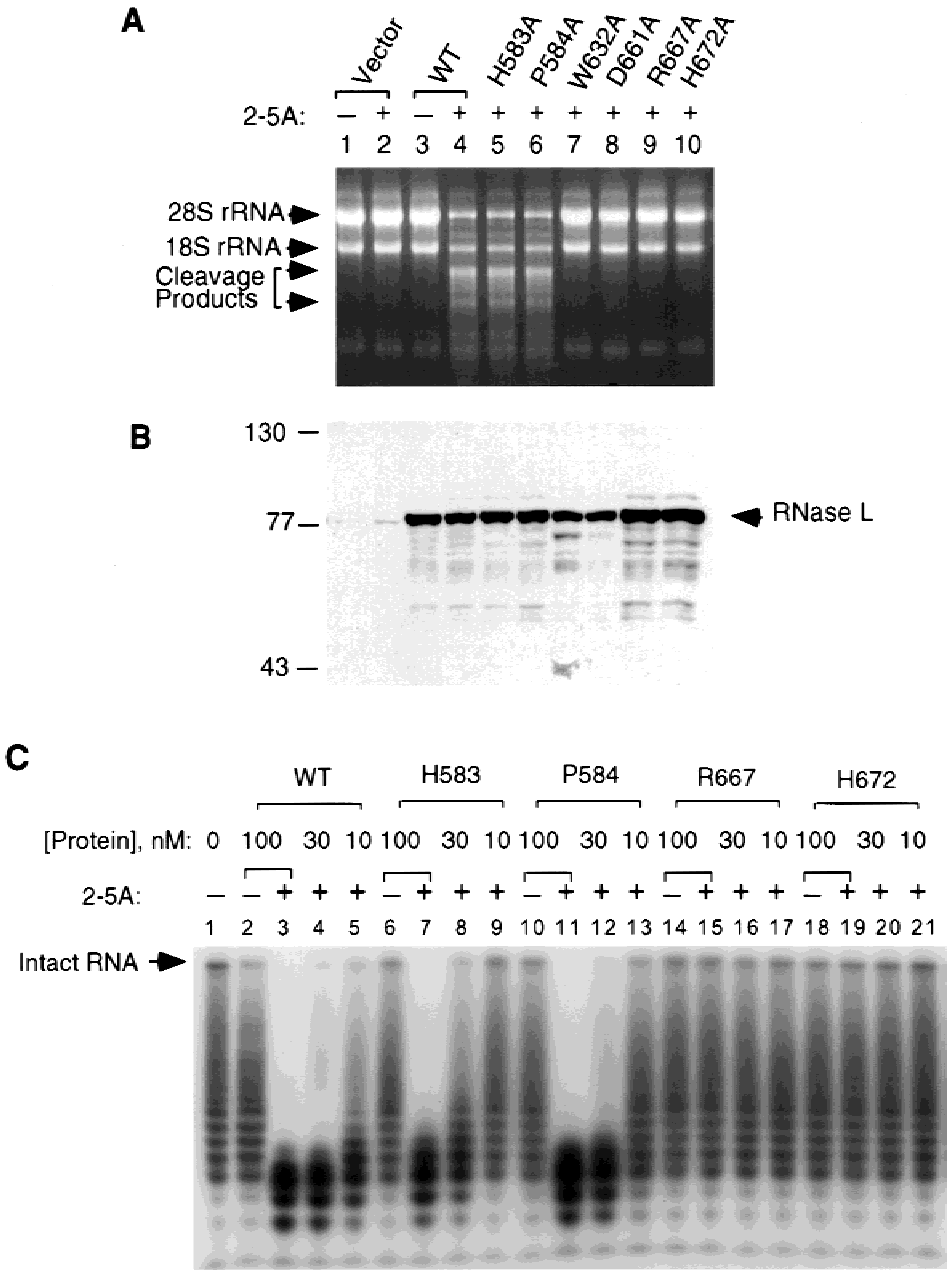
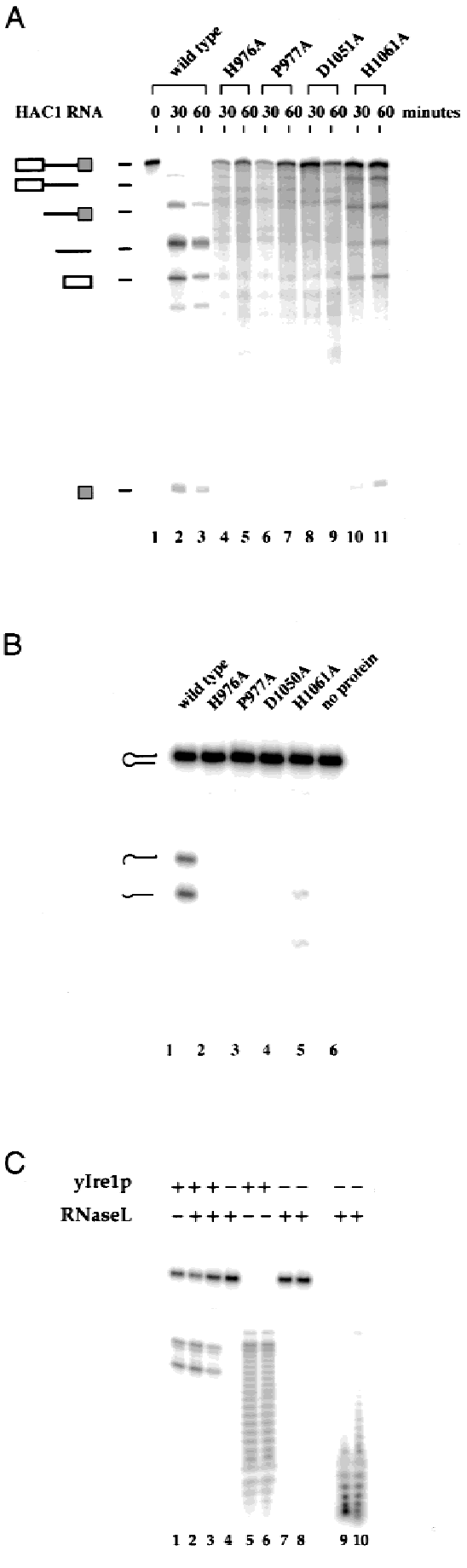


FIGURE 2. Effects of substitution mutations in the nuclease domain of human RNase L. **A**: Cleavage of rRNA in HeLa M cells after transfection with RNase L cDNA and 2-5A. HeLa cells were transfected with pcDNA3 (vector) or with vector containing wild-type and mutant RNase L cDNA (as indicated). Total RNA was isolated and separated on formaldehyde/1% agarose gel, stained with ethidium bromide, and photographed under UV light. Intact 18S and 28S rRNA and cleavage products are indicated with arrows. **B**: The western blot of the cell extracts in **A** probed with a monoclonal antibody to human RNase L. **C**: RNA cleavage activities of purified GST-RNase L wild-type and mutant RNase L at different concentrations (as indicated) performed by preincubation of the proteins with (+) or without (−) 0.1 μM 2-5A on ice for 30 min followed by incubation with U_{25}-[^{32}P]-pCp at 30 °C for 30 min. The RNA cleavage products were separated in 20% acrylamide sequencing gels. An autoradiogram of a gel is shown.

To establish whether the substitutions of these residues affected dimerization, GST fused to wild-type and to mutant RNase L were incubated with wild-type RNase L (not a fusion protein) in the presence or absence of 2-5A. After immobilization on glutathione-Sepharose, an 83-kDa band distinguished the native, wild-type RNase L from the 110-kDa GST-RNase L as detected by a monoclonal antibody to RNase L (Fig. 4B). The wild-type and mutant forms of RNase L (H583A, P584A, R667A, and H672A) all associated with untagged RNase L in the reactions with 2-5A, but not in the reactions without 2-5A. Therefore, the loss of RNase activities in R667A and H672A are not the result of deficiencies in either enzyme dimerization or 2-5A binding activity.



Nuclease domain mutants of RNase L do not inhibit wild-type RNase L

Because the mutant RNase L can form complexes with wild-type RNase L, we tested if such heterodimeric complexes are active for nuclease activity (Fig. 5). Wild-type RNase L (10 nM) was mixed with different amounts of either R667A or H672A (10–500 nM) in the presence of 1 or 10 nM 2-5A on ice for 30 min followed by incubation with RNA at 30 °C for 30 min. Surprisingly, in the presence of 10 nM 2-5A, the ribonuclease activity of RNase L was not inhibited by a 50-fold molar excess of RNase L mutants R667A or H672A (Fig. 5, lanes 13–20). However, in the presence of a 10-fold lower amount of 2-5A (1 nM) and excess mutant RNase L (500 nM), there was a slight inhibition of RNA breakdown, presumably due to sequestration of a limiting amount of 2-5A (Fig. 5, lanes 7 and 11). Therefore, the mutant RNase L in heterodimeric complexes with the wild-type protein did not display a dominant negative effect on the nuclease. These results also suggest that a single active nuclease domain in a dimer is all that is required for RNA cleavage activity.

Functions mediated by the protein kinase-like domains in RNase L

To investigate the function of the protein kinase-like domains in RNase L, deletion analysis was performed using purified, GST fusion proteins. Because the ankyrin repeat/2-5A binding domain was removed in these truncated proteins, it was not necessary to add 2-5A to the cleavage reactions (Dong & Silverman, 1997). Deletion of 510 amino acid residues from the N-terminus

FIGURE 3. Cleavage reaction of *HAC1* RNA by mutant forms of Ire1p and comparison of substrate specificities to RNase L. **A**: Cleavage reactions were carried out with radiolabeled *HAC1*^{u} 508 RNA substrate containing both 5′ and 3′ splice sites (Sidrauski & Walter, 1997). After cleavage reactions at 30 °C for 30 min, RNA was separated on 5% urea denaturing polyacrylamide gel. Different cleavage products were indicated by the icons (upper-to-lower): precursor, 5′exon + intron, 3′exon + intron, intron, 5′exon, and 3′exon. **B**: Radiolabeled mini stem-loop substrate RNA containing the *HAC1* 3′ splice site was incubated with 1 μg of either wild-type or mutant Ire1p (as indicated) in cleavage buffer (see Materials and Methods) for 2 h at 30 °C. The cleavage products were separated on 12% urea denaturing polyacrylamide gel and were visualized by autoradiography. **C:** The mini stem-loop structure and the [^{32}P]-U_{25}-pCp substrates were incubated with either Ire1p or RNase L (NΔ385). The 3′ mini stem loop substrate RNA and the [^{32}P]-U_{25}-pCp RNase L substrate were incubated in the presence of both Ire1p (LKT) (0.4 μg) and RNaseL (50 ng for lane 2, 150 ng for lane 3) or with either 0.4 μg of yIre1p or 150 ng of RNase L alone as indicated. The cleavage reactions were carried out in the Ire1p cleavage buffer (lanes 1, 2, 3, 6, 7, and 9) or in the RNase L cleavage buffer (lanes 4, 5, 8, and 10) (see Materials and Methods). The nature and origin of the nonspecific cleavage products seen in the reactions with yIre1p is presently not clear. It is possible that the Ire1p preparation contained a minor contaminant of a nonspecific nuclease, or, alternatively, that the mutant Ire1p can cleave RNA with a relaxed substrate specificity.

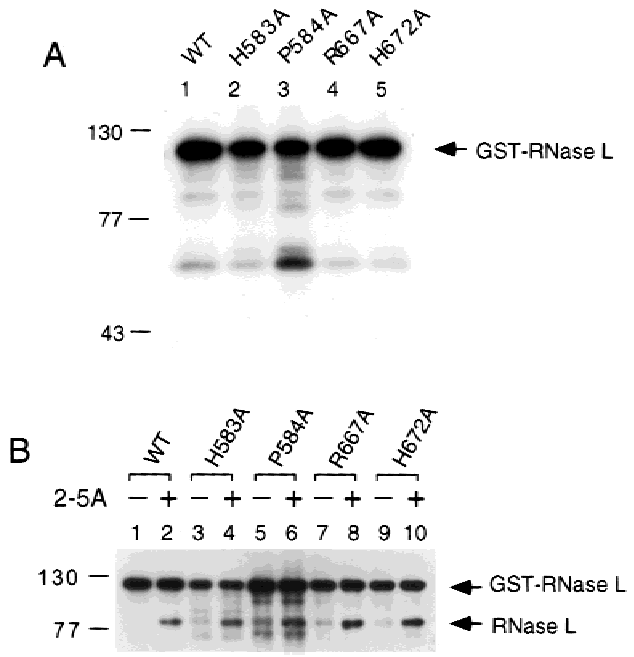


FIGURE 4. 2-5A binding and dimer formation activity of wild-type and mutant RNase L. **A**: The 2-5A binding activities of 2 μg each of WT (wild type RNase L), and mutants H583A, P584A, R667A, and H672A. A ^{32}P-labeled 2-5A analog was covalently crosslinked to the proteins under UV light. An autoradiogram of the gel with the reactions is shown. **B**: Wild-type and mutant RNase L GST fusion proteins were incubated with native RNase L (not a fusion protein) in the presence (+) or absence (−) of 2-5A. The immobilized RNase L and RNase L GST fusion proteins on the beads were detected by western blot probed with a monoclonal antibody to human RNase L. Native (untagged) RNase L and GST-RNase L fusion proteins are indicated (arrows).

(mutant NΔ510) produced a polypeptide lacking ribonuclease activity (Fig. 6). Mutant NΔ435 showed slight ribonuclease activity ($EC_{50} < 1$ μM). However, mutants NΔ392, NΔ385, and NΔ335 showed relatively higher levels of ribonuclease activity ($EC_{50} = 0.2$, 0.1, and 0.2 μM, respectively; Fig. 6). These results show that the kinase-like motifs that are on the C-terminal side of residue 392 are needed for nuclease activity.

Previously we observed that a K392R mutation in the kinase-like domain of complete RNase L (in motif II that mediates ATP binding in protein kinases) failed to dimerize with wild-type RNase L and was catalytically inactive (Dong & Silverman, 1999). The possible contribution of the conserved K392 to the catalytic activity of RNase L was further investigated in N-terminal truncated forms of RNase L. Surprisingly, the K392R mutation when combined with an NΔ385 truncation retained ribonuclease activity, ($EC_{50} = 0.2$ μM; Fig. 6B). In addition, NΔ392, which lacks protein kinase-like motif II including K392, also retains ribonuclease activity (Fig. 6B,C). Therefore, kinase-like motif II is required for ribonuclease activity in the full-length RNase L, but not in N-terminal truncated forms of the enzyme.

DISCUSSION

Comparative analysis of the nuclease domains within the RNase L/Ire1 superfamily

Mammals evolved at least three genes with ribonuclease activities, all related to the yeast Ire1p gene, that are involved in cellular responses to challenges caused by the presence of unfolded protein in the ER (Ire1α, Ire1β) or to viral infections (RNase L). Accordingly, the nuclease domains in the RNase L/Ire1 superfamily members each contain a highly conserved signature sequence (Fig. 1B). Surprisingly, two residues that are essential for the activity of yeast Ire1p (H976 and P977) are not required in human RNase L (H583 and P584; Table 1). The absence of an effect of substituting alanine for the proline residue at position 583 in RNase L was also surprising because prolines can have strong influences on the folding of polypeptide chains. Because the homologous histidine and proline are present in every member of the superfamily, it is likely that these residues serve some function that was dispensable for the activities of RNase L measured in this study. Possible alternative functions include involvement in mediating protein/protein or protein/substrate interactions, intracellular localization or enzyme stability. Another four RNase L residues were found to be essential: W632, R667, D661, and H672. Amino acids homologous to the last two of these residues are also important for activity in Ire1p (D1051 and H1061), with D1051 inactivating the enzyme and H1061A exhibiting some residual activity (Fig. 3 and Table 1). What could account for these differences in the catalytic domain of the two nucleases? Previous studies indicate that although Ire1p leaves 2′,3′-cyclic phosphoryl groups at the cleavages site, RNase L leaves 3′-phosphoryl groups (Wreschner et al., 1981b; Carroll et al., 1996; Gonzalez et al., 1999). Therefore, it is plausible that such differences in the cleavage mechanisms pose different requirements on the structure of the active sites of the two nucleases and hence may account for the different effects of the mutations observed here. Alternately, perhaps the nuclease domain begins on the C-terminal side of residue P584 in RNase L.

Although our mutagenesis results presented here indicate that residues between position 392 and 720 are important for nuclease activity in human RNase L, we consider it likely that the actual nuclease domain is much smaller and maps to the C-terminal region that follows the kinase-like domain and is unique to all members of the RNase L/Ire1 superfamily. Using a sequence line-up with known kinases as a guide to define a phylogenetically conserved domain boundary, the nuclease domains would map between residues 583 and 720 for RNase L and between residues 976 and 1096 for Ire1p (Fig. 1A). Interestingly, truncation of the N-terminal sequences to residue 392 remained active,

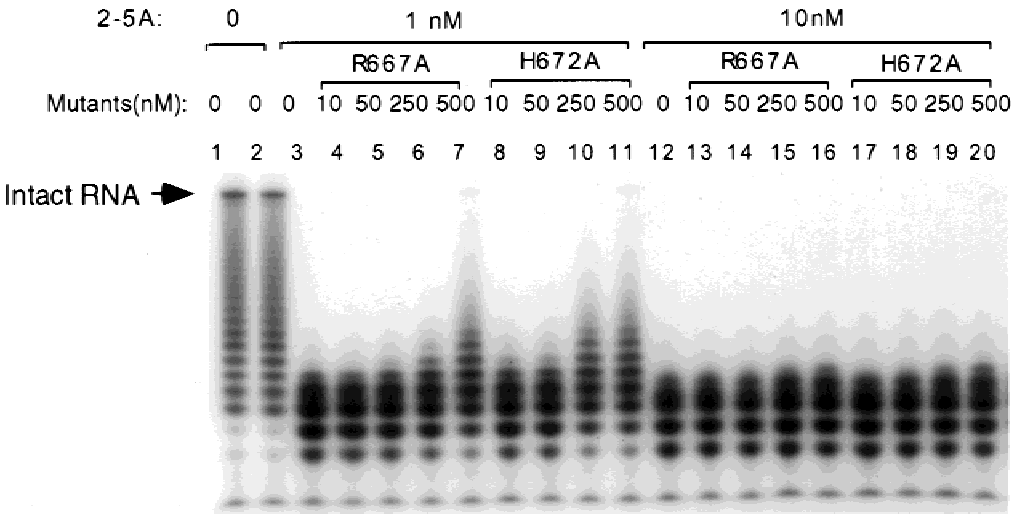


FIGURE 5. Effects of inactive RNase L mutants R667A and H672A on the RNA cleavage activity of wild-type RNase L. Wild-type RNase L (10 nM) was mixed with different amounts of mutant RNase L (as indicated) and then with either 1 nM or 10 nM 2-5A on ice for 30 min before incubation with 80 nM [^{32}P]-U_{25}-pCp at 30 °C for 30 min. RNA products were separated in sequencing gels. An autoradiograph of a gel is shown.

truncation to residue 435 reduced activity, and truncation to residue 510 resulted in a loss of activity (Fig. 6). This requirement for amino acid residues N-terminal to the proposed nuclease domain suggests that portions of the kinase-like domain beyond residue 392 play a role in maintaining an active conformation for the catalytic domain, possibly by functioning as a structural scaffold for the nuclease domain. Alternatively, the partial deletions create an unfolded domain that is deleterious for nuclease function perhaps by causing nonspecific aggregation.

The substrate specificities of Ire1p and RNase L are mutually exclusive

Despite homology at the amino acid levels in the nuclease domains of RNase L and Ire1p, both enzymes have different substrate specificities (Fig. 3C). RNase L cleaves single-stranded RNA with a preference for UU and UA dinucleotides, but also at other sequences at lower frequencies (Floyd-Smith et al., 1981; Wreschner et al., 1981b; Carroll et al., 1996). Although RNase L is only moderately specific for the sequences it cleaves in naked single-stranded RNA, rRNA in intact ribosomes is precisely cleaved by RNase L at a limited number of sites (Fig. 2A; Wreschner et al., 1981a; Silverman et al., 1983; Iordanov et al., 2000). Therefore, within the context of a highly structured ribonucleoprotein complex, RNase L can cleave RNA with a high degree of specificity. The 28-nt *HAC1* mini stem-loop containing the 3′ splice site was not cleaved by RNase L, perhaps because the loop sequence (5′-CCGAAGC-3′) lacks UU or UA (Fig. 3C). In sharp contrast, Ire1p is a highly specific endoribonuclease that cleaves *HAC1*^{u} mRNA at two precise sites, allowing splicing of the mature mRNA by tRNA ligase (Sidrauski et al., 1996). As expected from the previous characterization of its substrate specificity, polyuridylate, a substrate for RNase L, was not cleaved by Ire1p (Fig. 3C). The marked differences in the substrate specificities are consistent with the view that the two enzymes evolved for very different biological functions. In response to unfolded proteins in the ER, Ire1p acts as a *HAC1*^{u} mRNA splicing factor, allowing for the synthesis of Hac1p protein, a leucine zipper transcription activator for UPR target genes. In contrast, RNase L, which is activated in interferon-treated and virus-infected cells, limits viral infections by destruction of the viral and cellular RNA, thus, preventing viral protein synthesis. The RNase-L-mediated degradation of cellular RNA also induces apoptosis, thus allowing elimination of virus-infected cells (Rusch et al., 2000).

A model for the activation of RNase L by 2′,5′-oligoadenylates

Together with data from previous studies, the results presented here allowed us to develop a functional model for the activation of RNase L by 2-5A (Fig. 7). In the absence of 2-5A, RNase L is a monomer that lacks ribonuclease activity (Dong & Silverman, 1995). The 2-5A binding domain maps to a series of ankyrin repeats in the N-terminal portion of RNase L (Hassel et al., 1993; Zhou et al., 1993; Dong & Silverman, 1997). Ankyrin repeats 7 and 8 contain a repeated P loop motif (GKT) shown to be critical for 2-5A binding by the observation that mutations of both conserved lysines to asparagines resulted in loss of 2-5A binding (Zhou

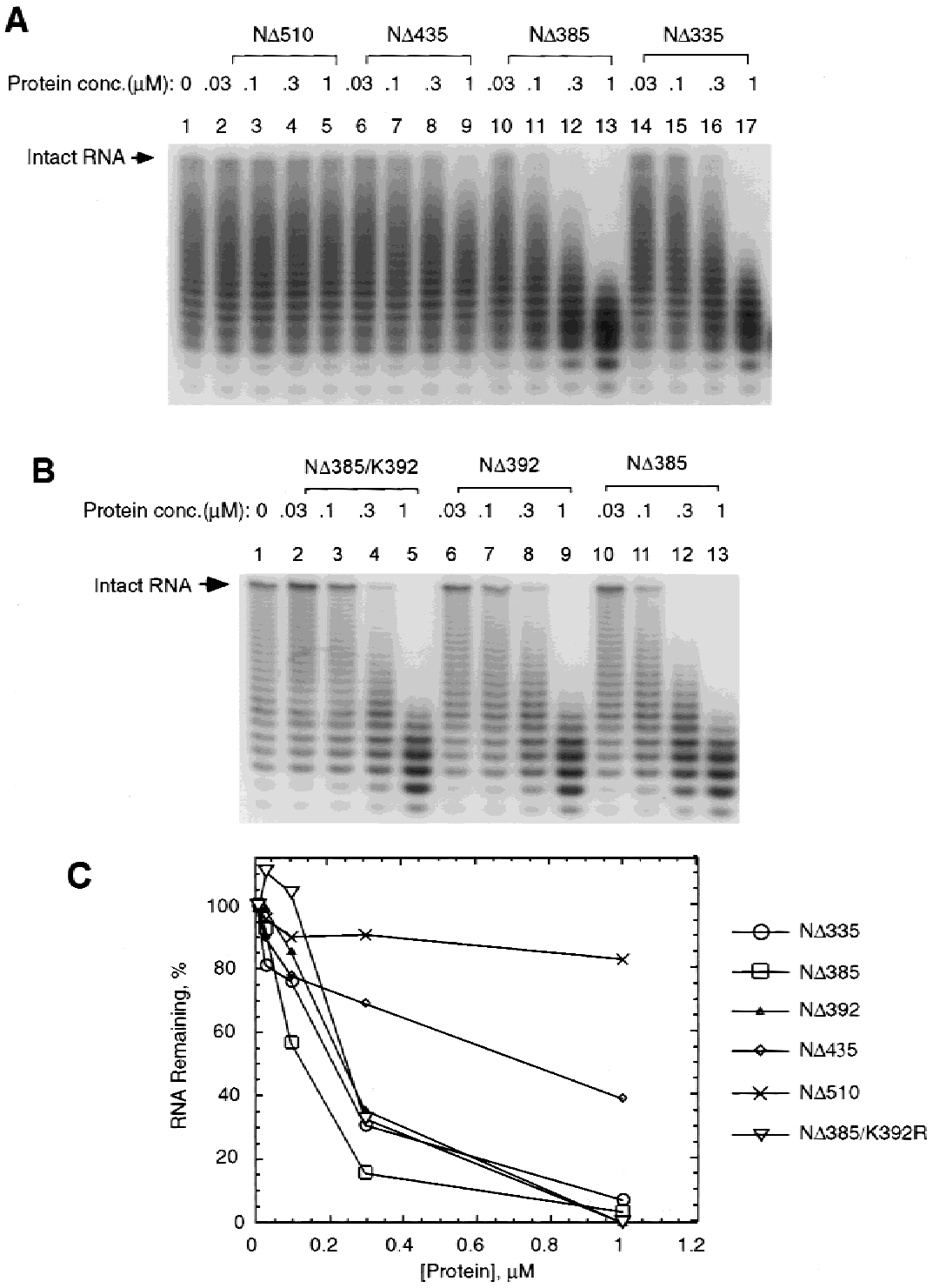


FIGURE 6. Ribonuclease assays of (**A**) truncated and (**B**) truncated and lys-to-arg mutants of RNase L. Different amounts (as indicated) of purified mutant RNase L GST fusion proteins were incubated with 80 nM [^{32}P]-U_{25}-pCp at 30 °C for 30 min. RNA cleavage products were separated in 20% sequencing gel. Autoradiograms of gels are shown. **C**: Quantitation by densitometry of intact RNA remaining after incubations expressed as percentage of the input level of RNA.

et al., 1993). Internal deletions in human RNase L of ankyrin repeats, 3–4 or 7–9, also resulted in a loss of 2-5A binding activity while maintaining repression of the ribonuclease domain (B. Dong and R.H. Silverman, unpubl.). In addition, N-terminal truncation mutants of RNase L showed that ankyrin repeats 7–9 by themselves can mediate repression (Dong & Silverman, 1997; Diaz-Guerra et al., 1999). As expected from the

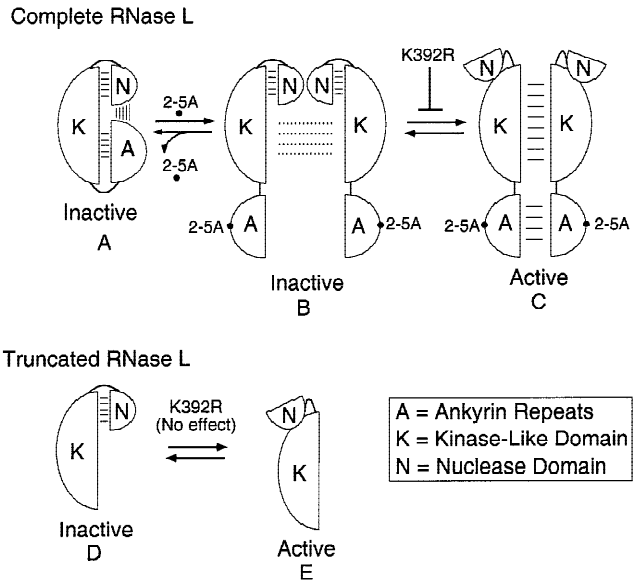


FIGURE 7. A functional model for activation of RNase L.

N-terminal location of the 2-5A binding domain, mutations in the C-terminal nuclease domain of RNase L did not affect 2-5A binding activity (Fig. 4A). Ankyrin repeats typically function in mediating protein/protein interactions (Sedgwick & Smerdon, 1999), whereas in RNase L they also mediate 2-5A binding. Deletion of the ankyrin repeats produces a constitutively active nuclease (Dong & Silverman, 1997), indicating that these motifs act by suppressing the nuclease function, which we propose in Figure 7A to result from binding to the kinase-like domain. Binding of 2-5A releases the repression caused by the ankyrin repeats, while at the same time allowing the enzyme to reconfigure for dimerization via an interaction of two kinase-like modules and ankyrin motifs (Fig. 7B).

This proposed function for the protein kinase-like domain in RNase L is indicated from the analysis of deletion mutants and the K392R mutant (Fig. 6). Previously, a lysine-to-arginine mutant of complete RNase L (at residue 392 in protein kinase-like motif II; VAV$\underline{K}$ $\rightarrow$ VAV$\underline{R}$) failed to dimerize with wild-type RNase L, and it lacked catalytic activity (Dong & Silverman, 1999). This was in sharp contrast to the identical mutation in N-terminal truncated RNase L (NΔ385/K392A), which retained ribonuclease activity (Fig. 6B,C). Similarly, RNase L mutant NΔ392, which lacks protein kinase-like domain II, remained active. Apparently, lysine 392 does not contribute directly to the catalytic activity of RNase L, but rather is required for dimer formation, a prerequisite for nuclease activity in the complete enzyme but not in the truncated enzyme. In addition, the kinase-like domain suppresses nuclease activity because deletion of the ankyrin repeats, leaving only the kinase-like and nuclease domains, results in a constitutively active nuclease with several-fold lower activity than the complete, 2-5A-activated enzyme (Figs. 2 and 6; Dong & Silverman, 1999). We propose that the inactive and active forms of the truncated protein are in equilibrium owing to the absence of the inhibitory ankyrin repeats (Fig. 7D,E). Therefore, the K393R mutation in the truncated protein, NΔ385/K392R, does not further diminish activity because the lysine is required for dimerization that does not occur when the ankyrin repeats are absent, as shown previously by Dong and Silverman (1997). Our findings suggest that the dimeric form of RNase L (Fig. 7C) is in a stabilized, active conformation, whereas the putative intermediate (Fig. 7B) is unstable and lacks activity.

Because dimers are stable, wild-type RNase L is able to form an active heterodimer complex with inactive, nuclease domain mutants of RNase L, R667A and H672A (Figs. 4B and 5). Therefore, dimers containing only a single active nuclease domain are able to cleave RNA. However, an isolated nuclease domain in truncation mutant NΔ510 is inactive, suggesting that the kinase-like motifs that are C-terminal of residue 392 also play a scaffolding role requiring maintaining the nuclease domain in an active conformation (Fig. 7C,E). Interestingly, a mutant form of the mouse RNase L, ZB1, is an effective dominant negative inhibitor of wild-type mouse RNase L (Hassel et al., 1993). RNase L mutant ZB1 is truncated at residue 651 and contains an additional six amino acids (KPLSG) at the C-terminus not present in the wild-type enzyme. Perhaps the inhibitory activity of RNase L ZB1 is the result of suppression of the nuclease domain by the heptameric peptide sequence or to differences in the mouse and human RNase L dimeric structures.

In comparison, both C-terminal truncated and kinase mutants of yeast Ire1p function as dominant negative inhibitors (Shamu & Walter, 1996). Regulation of Ire1p by unfolded proteins in the ER involves oligomerization in the plane of the membrane inducing trans-phosphorylation (Shamu & Walter, 1996; Welihinda & Kaufman, 1996). Therefore, the dominant negative effect can be explained by the requirement for kinase activity to trans-phosphorylate, which in turn may be a prerequisite for oligomerization in Ire1p.

The comparison of human RNase L and yeast Ire1p is leading to an appreciation of how these intriguing enzymes have retained sequence homology and striking similarities in their modes of action while also diverging in their functions during evolution. The origins of the complete 2-5A system, in particular how the requirement for 2′,5′-oligoadenylates emerged, remains a mystery. Cloning and analysis of precursor genes to the higher vertebrate forms of 2-5A synthetases and RNase L may provide important clues (Wiens et al., 1999). Evidence strongly suggests that at least the nuclease portion of RNase L evolved from the RNA splicing kinase/endoribonuclease, Ire1p. The regulatory part of RNase L seems to have derived from a separate,

ankyrin-like protein, which evolved to respond to 2-5A (Zhou et al., 2000), whereas this function for Ire1 is provided by the ER-lumenal unfolded protein-sensing domain. Yet the activation mechanism may be very similar: (1) the most N-terminal domains prevent dimerization until activated, (2) they then allow the kinase-domains to dimerize (which for Ire1 may requires autophosphorylation, and for RNase L is constitutive as the kinase-like domain is inactive), and (3) dimerization relieves inhibition of the nuclease domain. Thus in RNase L, both the kinase-like domain and the ankyrin repeat domain function as inhibitors, allowing RNase L to be a regulated nuclease.

MATERIALS AND METHODS

Plasmids, mutagenesis, and DNA cloning

The alanine substitution mutants of human RNase L were constructed by performing an overlapping PCR method (Ho et al., 1989) and subcloning. Amino acid residues of human RNase L that were individually replaced with alanines were H583, P584, W632, D661, R667, H672, except for K392, which was replaced with an arginine. For each mutant form of RNase L, a pair of complementary primers were designed and synthesized. The PCR products with the mutated sequences were subcloned into pGEX-4T-3 vector containing wild-type RNase L cDNA using *Dra*III and *Xho*I. The N-terminal deletion mutants of RNase L, NΔ385, NΔ392, NΔ435 and NΔ510, were constructed with PCR by creating a *Bam*HI restriction site upstream of the sequences for each mutant (Dong & Silverman, 1997). The PCR products were subcloned into the vector containing wild-type RNase L into *Bam*HI and *Xho*I sites. NΔ385/K392R was produced with the same PCR primers used to create NΔ385, but with the RNase L K392R mutant as template, instead of the wild-type sequence (Dong & Silverman, 1999). All mutants were confirmed by sequencing.

The cytoplasmic portion of *Saccharomyces cerevisiae* Ire1p containing its linker, kinase, and C-terminal tail domains, Ire1(LKT), was cloned into pGEX-2T (Pharmacia, Uppsala, Sweden) as described in Shamu and Walter (1996). Mutations were introduced using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California). The presence of each mutation was confirmed by sequencing.

Mutant and wild-type forms of RNase L and Ire1p were expressed as GST fusion proteins in *E. coli* strain DH5α and purified with Glutathione-Sepharose 4B (Pharmacia) as previously described (Dong & Silverman, 1997). The wild-type and mutant RNase L cDNAs were subcloned from pGEX-4T-3 vector into pcDNA3 vector (Invitrogen) for transfections of HeLa M cells.

RNase L in vitro assays

Assays for RNase L activity used an oligouridylic acid, U_{25} (Midland Certified Reagent Co.), labeled at its 3′ terminus with [5′-^{32}P]-pCp (3,000 Ci/mmol; Du Pont/NEN) with T4 RNA ligase (Gibco/BRL) as described (Dong & Silverman, 1999). Briefly, different amount of purified GST fusion proteins of RNase L or mutant RNase L were incubated in the presence and absence of 100 nM of 2-5A on ice for 30 min. Reaction mixtures were further incubated with 80 nM U_{25}-[^{32}P]pCp (unpurified) for 30 min at 30 °C. RNA was analyzed in sequencing gels to measure the extent of RNA degradation. The amounts of intact U_{25}-[^{32}P]pCp remaining after the incubations was determined from autoradiograms of the dried gels with a Sierra Scientific high resolution CCD camera (Sunnyvale, California) and the computer program, NIH Image 1.6.

2-5A binding activity assays

A ^{32}P-labeled and bromine-substituted 2-5A analog, p(A2′p)$_2$(br^{8}A2′p)$_2$A3′[^{32}P]pCp, was crosslinked to GST-RNase L and mutant RNase L (2 μg each) under ultraviolet light (Nolan-Sorden et al., 1990). The RNase L fusion proteins were incubated with the probe (0.3 μCi; 3,000 Ci/mmol) on ice for 60 min and then under 308 nm light on ice for 60 min. Protein separation was by electrophoresis on SDS/8% polyacrylamide gels followed by autoradiography of the dried gels.

RNase L/RNase L interaction assays

Cell extracts (100 μg of protein) containing GST-RNase L or GST-mutant RNase L were incubated with 60 μg of extract containing human recombinant RNase L (untagged) produced in insect cells (Dong et al., 1994) in the presence and absence of 2-5A (0.4 μM) on ice for 1 h. Subsequently, bovine serum album (250 μg) and 5 μL of 20% (v/v) glutathione-sepharose 4B was added and the mixtures were incubated with shaking at room temperature for 20 min, with gentle vortexing every 5 min, followed by washing three times with 0.3 mL of PBS-C (Dong & Silverman, 1997). The bound proteins were eluted in SDS/gel sample buffer with boiling for 5 min, separated by electrophoresis in SDS/8% polyacrylamide gels, transferred to nitrocellulose membrane and probed with monoclonal antibody to human RNase L (Dong & Silverman, 1995).

rRNA cleavage assay in HeLa M cells

HeLa M cells grown in DMEM/10% FBS were plated on 100 mm dishes 24 h before DNA transfection of cells (at about 70% confluency) using lipofectamine PLUS (Gibco/BRL). Four micrograms of DNA were incubated with 20 μL of Plus reagent in 0.6 mL of serum-free DMEM at room temperature for 20 min. The DNA and Plus complexes were mixed with 30 μL of lipofectamine diluted in 0.6 mL of serum-free DMEM and incubated at room temperature for another 20 min. The cells were washed with PBS, 4 mL of serum-free DMEM was added, then the DNA complexes were added to the cells. After 3 h incubation at 37 °C, the medium with DNA was replaced with fresh DMEM with 10% FBS and the cells were incubated for another 14 h. The cells were then transfected with 1 μM 2-5A [consisting primarily of trimer, ppp(A2′p)$_2$A, and tetramer, ppp(A2′p)$_3$A] by the same procedure for a total 6 h incubation. The RNA of the cells was isolated with 3 mL of Trizol (BRL) followed by chloroform extraction and isopropanol precipitation. Fifteen micrograms

of RNA from each sample were separated on formaldehyde/1% agarose gel. RNA was visualized under UV light after staining with ethidium bromide.

In vitro Ire1p nuclease reactions

Expression and purification of wild-type and various mutant forms of yeast Ire1(LKT) was performed as described previously (Sidrauski & Walter, 1997). The nuclease assays were carried out using in vitro-transcribed [^{32}P]-labeled 508-nt *HAC1* RNA [*HAC1*^{u} 508] (Sidrauski & Walter, 1997) and 3′ mini stem-loop substrates (Gonzalez et al., 1999). One microgram of purified Ire1(LKT) was incubated with 1 fmol of in vitro-transcribed RNA substrates in cleavage buffer [20 mM HEPES, pH 7.6, 50 mM KOAc, 1 mM $MgCl_2$, 1 mM DTT, 2 mM ADP, 40 U RNasin (Promega, Madison, Wisconsin)] at 30 °C for 2 h (unless otherwise indicated). The reaction was terminated with 10 vol of stop buffer followed by extraction with phenol-chloroform, and ethanol precipitation. The cleavage reactions were then electrophoresed through either 5% or 12% denaturing polyacrylamide gels and visualized by autoradiography.

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