 Nucleic Acids Research, 1999, Vol. 27, No. 2 439–445

Alternative function of a protein kinase homology domain in 2′,5′-oligoadenylate dependent RNase L

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Received October 15, 1998; Revised and Accepted November 16, 1998

ABSTRACT

RNase L is the 2′,5′-oligoadenylate (2–5A)-dependent endoribonuclease that functions in interferon action and apoptosis. One of the intriguing, albeit unexplained, features of RNase L is its significant homology to protein kinases. Despite the homology, however, no protein kinase activity was detected during activation and RNA cleavage reactions with human RNase L. Similarly, the kinase plus ribonuclease domains of RNase L produced no detectable protein kinase activity in contrast to the phosphorylation obtained with homologous domains of the related kinase and endoribonuclease, yeast IRE1p. In addition, neither ATP nor pA(2′p5′A)$_3$ was hydrolyzed by RNase L. To further investigate the function of the kinase homology in RNase L, the conserved lysine at residue 392 in protein kinase-like domain II was replaced with an arginine residue. The resulting mutant, RNase L$_{K392R}$, showed >100-fold decreases in 2–5A-dependent ribonuclease activity without reducing 2–5A- or RNA-binding activities. The greatly reduced activity of RNase L$_{K392R}$ was correlated to a defect in the ability of RNase L to dimerize. These results demonstrate a critical role for lysine 392 in the activation and dimerization of RNase L, thus suggesting that these two activities are intimately linked.

INTRODUCTION

RNase L is a regulated endoribonuclease which functions in the antiviral and antiproliferative activities of IFNs and in multiple apoptotic pathways (1–6). In its native state, the N-terminal half of RNase L functions as a repressor of the ribonuclease domain in the C-terminal half (Fig. 1A) (7). Upon binding to 2–5A molecules, such as pA(2′p5′A)$_3$, the inactive monomeric form of RNase L is induced to dimerize into its catalytically active form (8–10). The N-terminal repressor half of RNase L contains nine ankyrin repeats, implicated in mediating protein–protein interactions, and a repeated P-loop motif, GKT, implicated in 2–5A binding (1,2,7). The 2–5A binding function of RNase L requires the ankyrin repeats including the conserved P-loop motif lysines 240 and 274 in the seventh and eighth ankyrin repeats (1,7). The repressor function can be mediated by just the last three ankyrin repeats (7). The ribonuclease domain present in the C-terminal half is homologous with the ribonuclease domains of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Homo sapiens* forms of the kinase/endoribonuclease, IRE1p, which functions in the unfolded protein response (11–14).

In addition to their homologous ribonuclease domains, IRE1p and RNase L share significant protein kinase homology. However, while IRE1p is an established protein kinase, the possible kinase function of RNase L is unknown (13,15,16). Protein kinase-like domains II, VI and VII, are conserved in both murine and human RNase L, but several highly conserved protein kinase residues are absent in RNase L (1,14,17). For example, the typical domain I sequence, GXGXXG, is not present in RNase L; in domain VII, kinase motif DFG appears as DFD in RNase L; in motif IX, an invariant aspartate is replaced with an arginine; and in motif XI, a conserved arginine is present in the human but not in the murine form of RNase L.

Protein kinase domain II contains a critical conserved lysine residue that functions in binding to the α and β phosphoryl groups of ATP (18,19). Substitution mutations of the conserved lysine in domain II with arginine typically results in partial or complete loss of kinase activity (19–21). Therefore, to investigate the significance and function of the protein kinase homology in RNase L, we replaced the conserved lysine in protein kinase domain II at residue 392 with an arginine. The resulting mutant RNase L$_{K392R}$ is defective for the ribonuclease and dimerization activities of RNase L, indicating a critical role of lysine 392. However, no protein kinase or ATP/2–5A hydrolysis activities were detected in reactions with wild type RNase L. These studies thus demonstrate a divergent, alternative function for a common protein kinase motif.

MATERIALS AND METHODS

Construction of RNase L$_{K392R}$

Site-directed mutagenesis involving overlapping polymerase chain reactions (PCR) was used to construct RNase L$_{K392R}$ (22). Sense and antisense primers corresponding to RNase L nt 1165–1186 (where 1 is the first coding nucleotide), with an A1175 to G1175 mutation in the coding strand and the complementary change in the non-coding strand, resulted in an arginine codon,

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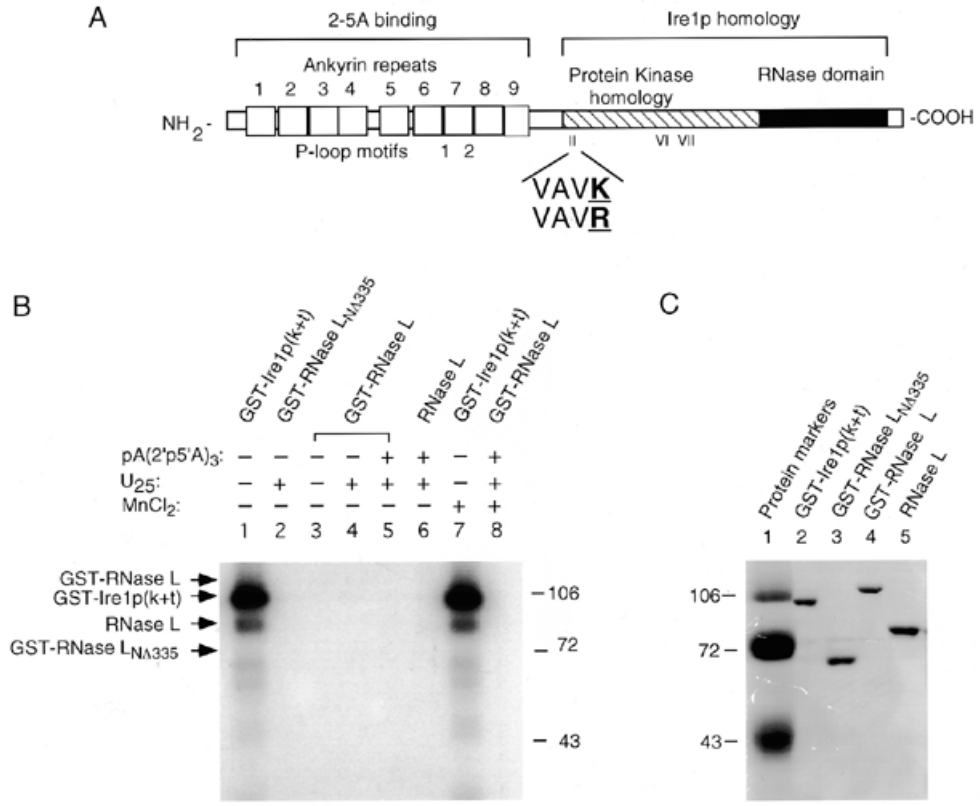

Figure 1. RNase L activation does not require kinase activity. (**A**) Diagram of RNase L showing regions of homology with protein kinases and IRE1p and showing the sequence that includes the K to R mutation. (**B**) GST–Ire1p(k+t), GST–RNase $L_{N\Delta335}$, GST–RNase L and RNase L were incubated with [γ-^{32}P]ATP with (+) or without (–) pA(2′p5′A)$_3$, U_{25} or $MnCl_2$. An autoradiogram of the dried SDS–polyacrylamide gel is shown. The positions of the proteins (arrows) and molecular weight markers (in kDa) are indicated. (**C**) Coomassie blue staining of the proteins is shown.

AGG, in place of a lysine codon, AAG, at codon 392. Two DNA fragments, A and B, with overlapping ends were produced in the first round of PCR. Fragment A was made with the 3′-complementary primer: 5′-CAC AGA ACG TCC TCA CAG CTA C-3′ and a 5′ primer with the RNase L sequence from nt 661 to 678. Fragment B was with the 5′-complementary primer: 5′-GTA GCT GTG AGG ACG TTC TGT G-3′ and a 3′ primer with the RNase L sequence from nt 1694 to 1671. These fragments were isolated from agarose gels and used as both templates and primers in the second round of PCR with the same outside primers used in the first round PCR. The overlapping ends annealed allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The extended DNA product was further amplified by PCR using the outside primers. The final PCR product with the mutated sequence was subcloned to vector containing wild type RNase L cDNA, pGEX-4T-3/pZC5, with *Nco*I and *Dra*III restriction enzymes.

As a control, RNase L_{K392R} was reverse mutated to wild type RNase L using a similar procedure with a pair of complementary primers containing the wild type sequence, A1175, instead of G1175. The final PCR product was cloned into the vector containing the RNase L_{K392R} mutation. The wild type enzyme generated by this method is referred to as RNase L_{R392K}. The sequences of the mutant and reconstructed wild type RNase L cDNAs were confirmed by DNA sequencing.

Expression and purification of RNase L and RNase L_{K392R}

Briefly, cDNAs for wild type RNase L, RNase L_{K392R} or RNase L_{R392K} in vector pGEX-4T-3 were transformed into *Escherichia coli* strain DH5α. The glutathione *S*-transferase (GST) fusion proteins were expressed and purified by binding to glutathione–Sepharose 4B (Pharmacia, Piscataway, NJ), washing, and eluting in 20 mM glutathione, 50 mM Tris–HCl, pH 8.0 as described (7). Expression and purity of the protein preparations were determined by SDS–PAGE with Coomassie blue staining and by western blots probed with monoclonal antibody to RNase L (8).

Assays for protein kinase activity

GST–Ire1p(k+t) which contains kinase and C-terminal nuclease domains (a kind gift from M. Niwa and P. Walter, UCSF) (12), GST–RNase $L_{N\Delta335}$ (7), RNase L (non-fusion protein from baculovirus) (23) and GST–RNase L (7) (0.5 μM each) were incubated with 5 μCi of [γ-^{32}P]ATP in 20 μl of kinase buffer (12) or kinase buffer plus 2 mM $MnCl_2$ in the presence or absence of 0.1 μM pA(2′p5′A)$_3$ or 80 nM U_{25} at 30°C for 30 min. Proteins were separated on SDS–polyacrylamide gel and autoradiograghy was done. Proteins (5 μg) were run on separate SDS–polyacrylamide gels and stained with Coomassie blue.

Incubations with $^{32}pA(2'p5'A)_2$

$^{32}pA(2'p5'A)_2$ was prepared by incubating 1 mM $A(2'p5'A)_2$ (Sigma Co., St Louis, MO), 125 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, DuPont NEN, Boston, MA) and 3 μl of T4 polynucleotide kinase in 50 μl reaction buffer (Gibco BRL, Gaithersburg, MD) at 37°C for 90 min. The $^{32}pA(2'p5'A)_2$ was purified by HPLC as described (24). The purified $^{32}pA(2'p5'A)_2$ (3000 Ci/mmol) was observed to bind to RNase L in a radiobinding filter assay (25). To determine if 2–5A was hydrolyzed during RNA cleavage reactions, GST–RNase L (2 μg) or BSA (2 μg) was incubated with 0.6 nM $^{32}pA(2'p5'A)_2$ (0.2 μCi) with or without U_{25} (80 nM) in 100 μl buffer A (20 mM Tris–HCl, pH 7.5, 10 mM magnesium acetate, 8 mM 2-mercaptoethanol, 90 mM KCl, 0.1 mM ATP, 10 μg/ml leupeptin) at 30°C for 15 min. The reactions were boiled for 3 min, centrifuged at 16 700 *g* for 5 min, and 50 μl was injected into an HPLC Aquasil C18 column (Keystone Scientific Inc., Bellefonte, PA). The material was eluted in 50 mM ammonium phosphate, pH 6.0, with a 20–50% gradient of 1:1 methanol:H_20 in 25 ml at 1 ml per min. The radioactivity in the fractions was determined by liquid scintillation counting.

To determine if the 5′-phosphoryl group of 2–5A can be transferred to GST–RNase L, GST–RNase L_{K392R} or RNase L (not a fusion protein) 1 μg each was incubated with 0.6 nM purified $^{32}pA(2'p5'A)_2$ (0.4 μCi) with and without ATP (10 nM) in 20 μl of buffer A on ice for 15 min or at 30°C for 15 min. The proteins were separated by SDS–PAGE and the dried gels were placed with X-film. To determine if 2–5A transferred phosphoryl groups to RNA cleavage products, 80 nM unlabeled U_{25} was incubated with 0.6 nM $^{32}pA(2'p5'A)_2$ (0.4 μCi) and GST–RNase L (0.1 μg) in 20 μl of buffer A at 30°C for 0, 2, 5 and 15 min. The RNA was separated in 20% polyacrylamide–8% urea gels and exposed to X-ray film for autoradiography.

Assays for ATP hydrolysis

[α-^{32}P]ATP (0.1 μCi) was incubated in the absence or presence of 0.2, 0.1 and 0.05 μM GST–RNase L. Assays containing 10 nM $pA(2'p5'A)_2$ and 80 nM U_{25} in 10 μl of buffer A were at 30°C for 30 min. One microliter of each reaction and 0.5 μl of 20 mM unlabeled ATP, ADP and AMP as markers were spotted to TLC plates of polyethylenimine cellulose on polyester (Sigma). Plates were rinsed in distilled water, dried and developed in 2 M Tris–HCl, pH 8.7, for 90 min. The markers of ATP, ADP and AMP were visualized under UV light and the [α-^{32}P]ATP was visualized in autoradiograms.

Ribonuclease assays

A chemically-synthesized oligouridylic acid, U_{25} (Midland Certified Reagent Co., Midland, TX) was labeled at its 3′-terminus with 5′-^{32}P-pCp (3000 Ci/mmol) (Du Pont NEN) with T4 RNA ligase (Gibco BRL). The U_{25}-[^{32}P]pCp, 80–160 nM, was incubated with different amount of purified GST fusion proteins of RNase L, RNase L_{K392R} or RNase L_{R392K} in the presence and absence of 100 nM of $pA(2'p5'A)_3$ in 20 μl of buffer A at 30°C for 30 min. Reaction mixtures were heated to 100°C for 5 min in loading buffer and RNA and RNA degradation products were separated in 20% polyacrylamide–8% urea sequencing gels (7). For the kinetics analysis, purified GST–RNase L and GST–RNase L_{K392R} were preincubated with and without 20 μM of $pA(2'p5'A)_3$ on ice for 30 min (26). Reaction mixtures containing 0, 10, 30 and 100 nM GST–RNase L or GST–RNase L_{K392R} were further incubated with 80 nM U_{25}-[^{32}P]pCp in the presence or absence of 5 μM $pA(2'p5'A)_3$ in buffer A for 0, 3, 10 and 30 min at 30°C. Portions of each reaction sample were analyzed in sequencing gels to measure the extent of RNA degradation and by western blots to monitor GST–RNase L 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, CA) and the computer program, NIH Image 1.6. The western blots were probed with monoclonal antibody to RNase L (8).

2–5A binding activity assays

A ^{32}P-labeled and bromine-substituted 2–5A analog, $p(A2'p)_2(br8A2'p)_2A3'[^{32}P]pCp$, (the unlabeled 2–5A derivative was a kind gift of P. F. Torrence, NIH) was crosslinked to GST–RNase L and GST–RNase L_{K392R} (10 μg each) under UV light (24). The RNase L fusion proteins were incubated with the probe (0.3 μCi; 3000 Ci/mmol) in 500 μl of buffer A on ice. After 5, 15 and 45 min of incubation on ice, 50 μl aliquots were placed under 308 nm light on ice for 5, 15 or 45 min. Protein separation was by electrophoresis on SDS–8% polyacrylamide gels followed by autoradiography of the dried gels.

RNA binding assay

Poly(U)–Sepharose 4B, 10 μl of a 60% v/v suspension containing 0.5 mg poly(U)/ml with ~100 uridyl residues/chain (Pharmacia Biotech, Piscataway, NJ), or 10 μl of a 20% v/v glutathione–Sepharose 4B suspension was added to 50 μg of cell extracts containing GST fusion proteins in PBS-C (PBS with 10% glycerol, 0.1 mM ATP, 5 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 1 μg/ml leupeptin). Reactions were incubated at room temperature for 20 min with shaking and gentle vortexing every 5 min. The bound proteins were washed three times with 0.3 ml PBS-C, eluted in SDS–gel sample buffer with boiling for 5 min, separated by electrophoresis on SDS–8% polyacrylamide gels, transferred to nitrocellulose membrane, and probed with monoclonal antibody to human RNase L (8).

RNase L–RNase L interaction assays

Cell extracts (100 μg of protein) containing GST–RNase L, GST–RNase L_{K392R} and GST–RNaseL_{R392K} were incubated with 60 μg of extract containing recombinant, non-fusion human recombinant RNase L produced in insect cells (23) in the presence and absence of $pA(2'p5'A)_3$ (4 μM) in buffer A on ice for 1 h. Subsequently, bovine serum album (250 μg) and 5 μl of 20% v/v glutathione–Sepharose 4B were 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. 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 (7,8).

RESULTS

Lack of kinase and ATP- or 2–5A-hydrolysis activities during reactions with RNase L

To determine whether activation of RNase L involves a protein kinase activity, a series of experiments was performed with [γ-^{32}P]ATP (Fig. 1 and data not shown). For comparison, GST linked to the kinase plus ribonuclease domains of yeast IRE1p was included in the analysis (Fig. 1B, lanes 1 and 7). Autophosphorylation of IRE1p(k+t) was demonstrated as described previously (12). In contrast, no kinase activity was observed with a homologous region of human RNase L in GST–RNase $L_{N\Delta 335}$ (lane 2) (7). Furthermore, GST–RNase L, and RNase L which was not a fusion protein also lacked kinase activity in these assays (lanes 3–6 and 8). There was no kinase activity from RNase L even in the presence of 2–5A and an RNA substrate. Addition of manganese, which can stimulate some protein kinases, also had no effect (lanes 7 and 8). RNase L activity was demonstrated in separate reactions with radiolabeled RNA, thus demonstrating the ribonuclease activity does not involve or require kinase activity (data not shown). Staining of aliquots of the proteins used for this experiment showed a high degree of purification (Fig. 1C).

In addition, there was no detectable hydrolysis of [α-^{32}P]ATP to ADP or AMP by GST–RNase L incubated in the presence or absence pA(2′p5′A)$_3$ and U_{25} as determined by thin layer chromatography (Materials and Methods; data not shown). To rule out the possibility that phosphorylation was not necessary because of prior *in vivo* kinase activity, GST–RNase L was immobilized and treated with bacterial alkaline phosphatase. However, the phosphatase-treated RNase L showed undiminished 2–5A-dependent ribonuclease activity (data not shown). In addition, no phosphorylation was detected in reactions with [γ-^{32}P]ATP containing both RNase L and RNase L_{K392R} (data not shown).

The possible hydrolysis of 2–5A by RNase L was investigated. A radiolabeled 2–5A, ^{32}pA(2′p5′A)$_2$, was analyzed by HPLC before and after incubation with RNase L or with bovine serum albumin as a control (Materials and Methods). The HPLC profile of ^{32}pA(2′p5′A)$_2$ incubated with either of the two proteins indicated that there was no hydrolysis or modification of the oligoadenylate (Materials and Methods, and data not shown). In addition, neither RNase L nor the cleavage products of U_{25} was labeled during the incubation with the 2–5A probe. These experiments indicate that RNase L activation and ribonuclease activity does not involve or require kinase activity.

Lysine 392 in protein kinase-like domain II is essential for RNase L activity

To determine the function of protein kinase-like domain II in RNase L, the conserved lysine residue at position 392 was replaced with an arginine. GST–RNase L and GST–RNase L_{K392R} were compared for their abilities to degrade U_{25}-[^{32}P]pCp (Fig. 2). The RNase L fusion proteins were pre-incubated in the presence or absence of a relatively high concentration of pA(2′p5′A)$_3$ (20 μM) on ice for 30 min to allow the activator to bind to the protein (26). After 3 min at 30°C in the presence of pA(2′p5′A)$_3$ and 10 nM of GST–RNase L there was no intact RNA detected, only cleavage products ≤6 nt in length (Fig. 2A, lane 3, and B). In the absence of pA(2′p5′A)$_3$, even 100 nM of GST–RNase L failed to degrade the RNA after

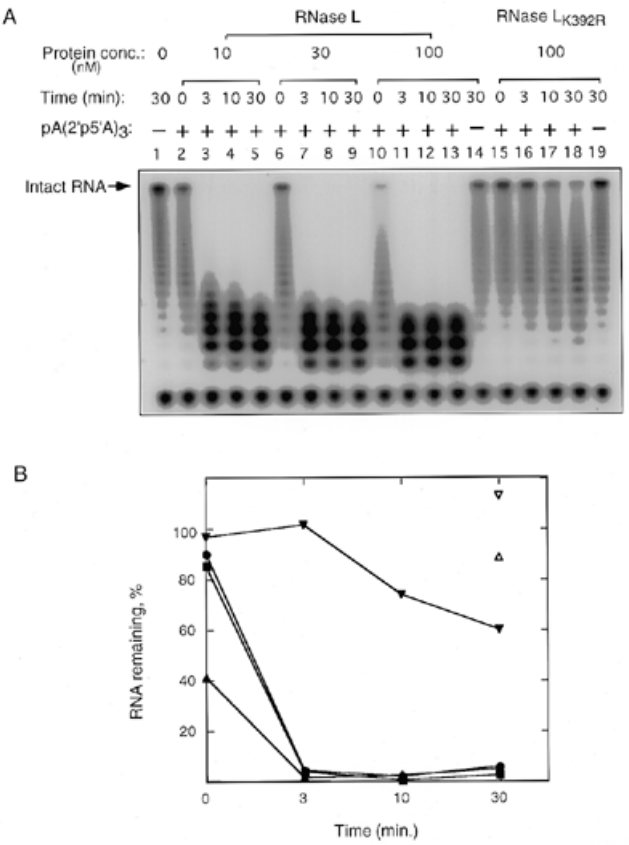

Figure 2. Kinetics of RNA cleavage activities of GST–RNase L and GST–RNase L_{K392R}. (**A**) RNase L and RNase L_{K392R} pre-incubated in the absence (–) or presence (+) of pA(2′p5′A)$_3$ were incubated with U_{25}-[^{32}P]pCp for 0, 3, 10 and 30 min as indicated. An autoradiogram is shown. (**B**) The intact RNA, U_{25}-[^{32}P]pCp, remaining after the incubations in the presence of pA(2′p5′A)$_3$ with 10 nM (●), 30nM (■) and 100 nM (▲) of GST–RNase L and 100 nM (▼) GST–RNase L_{K392R} or in the absence of pA(2′p5′A)$_3$ with 100 nM (Δ) of GST–RNase L or 100 nM (∇) of GST–RNase L_{K392R} was measured from the autoradiograms and expressed as a percentage of the input amount of intact RNA.

30 min of incubation (Fig. 2A, lane 14, and B). In contrast, 30 min incubations with 100 nM of GST–RNase L_{K392R} in the presence of pA(2′p5′A)$_3$ caused only a 40% decline in levels of intact RNA (Fig. 2A, lane 18, and B). Therefore, RNase L_{K392R} retains <1% of the activity of the wild type enzyme (the activity of 10 nM of wild type RNase L for 3 min exceeded that of 100 nM of RNase L_{K392R} for 30 min).

To verify that the Lys to Arg mutation was responsible for the defect, the single nucleotide change in RNase L_{K392R} cDNA was confirmed by DNA sequencing. In addition, a wild type enzyme was reconstructed from RNase L_{K392R} by site-directed mutagenesis to an enzyme with the original wild type sequence, RNase L_{R392K}. The reconstructed wild type enzyme had comparable activity with original wild type RNase L as determined in assays in which RNA degradation was determined as a function of protein concentration (Fig. 3). In this experiment, no ribonuclease activity was detected using RNase L_{K392R}, perhaps due to the fact that pA(2′p5′A)$_3$ was not pre-incubated with the enzyme but added only at the beginning of the RNA cleavage reactions.

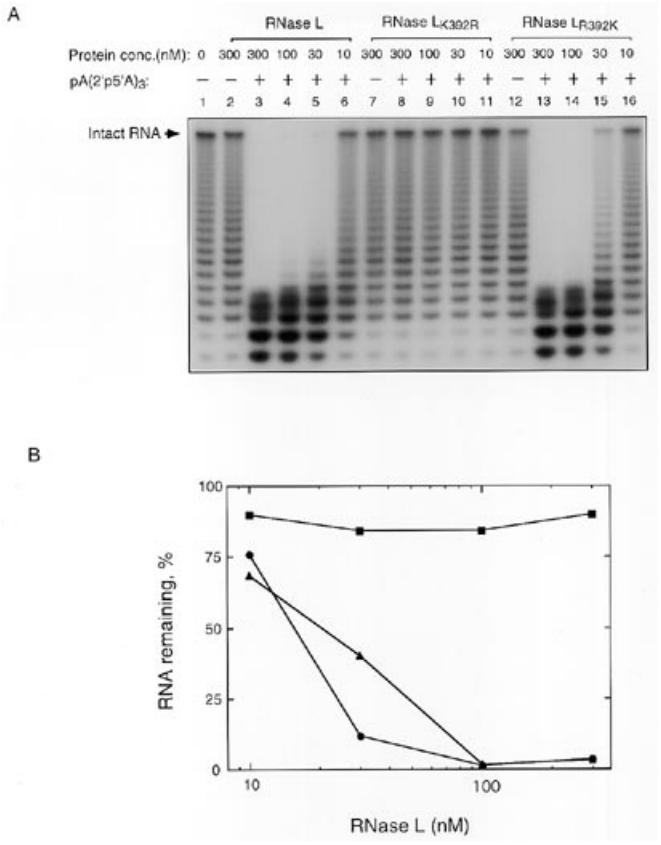

Figure 3. RNA cleavage activity of RNase L_{R392K}. (**A**) The RNA cleavage activities of GST–RNase L, GST–RNase L_{K392R} and GST–RNase L_{R392K} at different concentrations were compared in the absence (–) or presence (+) of 100 nM pA(2′p5′A)$_3$. The intact RNA, U_{25}-[^{32}P]pCp, is indicated with an arrow. An autoradiogram is shown. (**B**) The intact RNA, U_{25}-[^{32}P]pCp, remaining after incubations with GST–RNase L_{K392R} (■), GST–RNase L (●) and GST–RNase L_{R392K} (▲) was measured from the autoradiograms and expressed as a percentage of the input amount of intact RNA.

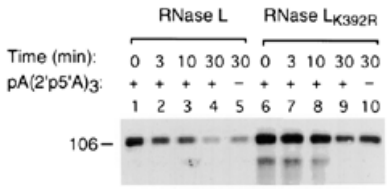

Figure 4. Stability of GST–RNase L_{K392R} and GST–RNase L during RNA cleavage reactions detected in a western blot probed with monoclonal antibody to RNase L. The position of a 106 kDa protein marker is shown to the left. The upper bands near the position of the marker protein are intact GST–RNase L (lanes 1–5) and GST–RNase L_{K392R} (lanes 6–10). The analysis was done with aliquots of the assay shown in Figure 2, lanes 10–19.

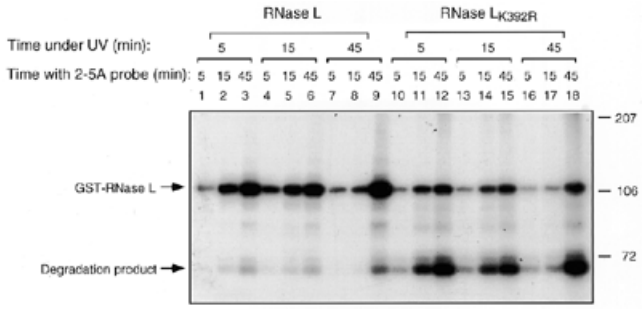

Figure 5. 2–5A binding activities of GST–RNase L_{K392R} and GST–RNase L incubated with p(A2′p)$_2$(br^{8}A2′p)$_2$A3′[^{32}P]pCp for 5, 15 and 45 min (labeled in figure as 'Time with 2–5A probe') followed by exposure to UV light for 5, 15 or 45 min (labeled as 'Time under UV'). The protein markers are indicated to the right.

To rule out the possibility that the substitution of lysine 392 might cause the protein to be less stable, protein levels were monitored by western blots probed with monoclonal antibody to human RNase L (Fig. 4). However, in reactions containing 100 nM of protein, GST–RNase L appeared slightly less stable than GST–RNase L_{K392R}. There was only minimal breakdown of GST–RNase L_{K392R} after 3 min of incubation, a time at which the RNA was almost completely degraded by GST–RNase L (Fig. 2, lane 11). Therefore, the lack of ribonuclease activity of GST–RNase L_{K392R} is not due to its enhanced breakdown. The western blot shown was performed on aliquots of the same RNA decay assay as in Figure 2.

2–5A- and RNA-binding activities of RNase L and RNase L_{K392R}

The cause of the defect in the ribonuclease function of RNase L_{K392R} was investigated. The 2–5A binding activity of GST–RNase L_{K392R} was determined by crosslinking to a ^{32}P-labeled and bromine substituted 2–5A analog under UV light (24). These assays indicated that GST–RNase L_{K392R} was able to bind with the 2–5A probe (Fig. 5). However, the mutation created a UV sensitive site in the protein resulting in labeling of both intact GST–RNase L_{K392R} and a breakdown polypeptide of ~60 kDa (Fig. 5, arrows). However, when the relative signals in the two bands were added together, it was apparent that the 2–5A binding activity of GST–RNase L_{K392R} was not reduced compared with that of GST–RNase L (data not shown).

To determine if RNase L_{K392R} retained the ability to bind RNA, the wild type and mutant proteins were immobilized on poly(U)–Sepharose and, for comparison, on glutathione–Sepharose (Fig. 6). RNA binding activities were observed with both proteins (Fig. 6, lanes 2 and 4). These results showed that the mutation of lysine 392 does not significantly impair RNA binding activity.

Mutation of lysine 392 resulted in a defect in the ability of RNase L_{K392R} to associate with RNase L in the presence of 2–5A

The activation of RNase L by 2–5A is accompanied by the induction of homodimers of the enzyme (7–10). To determine if the substitution of lysine 392 with arginine affected the ability to dimerize, GST–RNase L fusion proteins were incubated with wild type RNase L (not a fusion protein), in the presence or absence of pA(2′p5′A)$_3$ (Fig. 7). After immobilization on glutathione–Sepharose, the bound RNase L (at 83 kDa) was observed and could be distinguished from GST–RNase L (at 109 kDa) in western blots probed with monoclonal antibody to human RNase L. A 2–5A dependent binding of RNase L to wild

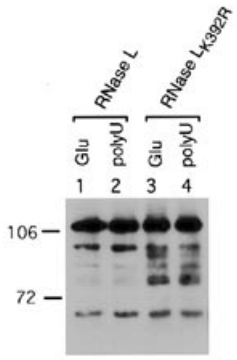

Figure 6. RNA binding activity of GST–RNase L_{K392R} and GST–RNase L. Proteins were bound to poly(U)–Separose (polyU) or glutathione–Sepharose (Glu) and measured by probing western blots with a monoclonal antibody to human RNase L. The positions of the molecular weight markers (in kDa) are indicated.

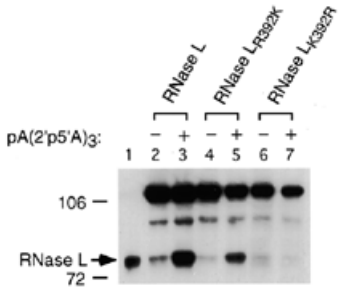

Figure 7. RNase L_{K392R} is defective for dimerization. GST–RNase L, GST–RNase L_{R392K} and GST–RNase L_{K392R} were incubated with native human RNase L in the presence and absence of pA(2′p5′A)$_3$. Analysis of the glutathione–Sepharose bound proteins was by SDS–PAGE and western blot analysis probed with monoclonal antibody to RNase L. The positions of native RNase L (arrow) and the molecular weight marker proteins are indicated.

type GST–RNase L and the reconstructed wild type GST–RNase L_{R392K} was observed (Fig. 7, compare lanes 2 and 3, and 4 and 5). In contrast, GST–RNase L_{K392R} failed to bind to RNase L in the presence or absence of pA(2′p5′A)$_3$ (Fig. 7, lanes 6 and 7). These same results were reproduced in seven separate experiments. Therefore, the defect in the ability of GST–RNase L_{R392K} to bind with RNase L correlated with its lack of ribonuclease activity.

DISCUSSION

We have determined a critical and unexpected role for protein kinase-like domain II in the dimerization and activation of RNase L. The significance of the resemblance of RNase L to protein kinases has remained unknown since the time it was initially recognized (1). The domain II lysine is conserved in protein kinases where it functions in binding α and β phosphoryl groups in MgATP (reviewed in 19). Mutations of the lysine in domain II to arginine typically leads to partial or complete losses of kinase activity (16,19–21). Apparently, RNase L evolved, at least in part, from a protein kinase. In this regard, the homology in the protein kinase and ribonuclease domains of RNase L with the yeast, *C.elegans* and human forms of the kinase/endoribonuclease, IRE1p, suggests an evolutionary relationship between these proteins (12,13). Activation of both IRE1p and RNase L are believed to be accompanied by oligomerization, although IRE1p is activated in response to unfolded proteins in the endoplasmic reticulum and is accompanied by transphosphorylation and site-specific cleavage of Hac1 mRNA. In contrast, RNase L activation by 2–5A was not accompanied by autophosphorylation (Fig. 1). However, while we have thus far been unable to detect any kinase activity from RNase L, it is difficult to completely rule out that the enzyme could have kinase activity *in vivo.* Nevertheless, the absence of several highly conserved residues of protein kinases in RNase L makes such a possibility seem unlikely at present (17). The protein kinase homology region is not involved in 2–5A binding because an isolated N-terminal half of RNase L, lacking the entire kinase-like region, binds 2–5A nearly as well as the complete protein (1,7). In addition, RNase L_{K392R} is capable of binding 2–5A, RNA and ATP (Figs 5 and 6, and data not shown).

The domain II lysine in IRE1p is essential for protein kinase activity and endonuclease activities as determined by mutations to arginine or alanine (13,16,21). The lysine to alanine mutant of human IRE1p is catalytically inactive and functions as a dominant negative mutant. A mutation to arginine of the domain II lysine in *S.cerevisiae* IRE1p reduced the unfolded protein response to ~25% of the wild type level (15,16). It was suggested that the lysine to arginine mutation in yeast IRE1p probably decreased its kinase activity to very low, undetectable levels (16). The yeast IRE1p amino acid sequence between domains VII and VIII, which contains the serine phosphorylation sites, is not conserved in RNase L. Therefore, while the domain II is essential for both IRE1p and RNase L activities, the particular role of the lysine in catalysis appears to have diverged. The conserved lysine is necessary for dimerization of RNase L while in IRE1p it functions in the kinase reactions.

Mutation of lysine 392 with arginine resulted in >100-fold decrease in RNase activity and a defect in the ability of RNase L to oligomerize in response to 2–5A binding (Figs 2, 3 and 7). The loss of activity was not due to trivial explanations such as an increased rate of protein degradation (Fig. 4). Neither the activator, pA(2′p5′A)$_3$, nor ATP was hydrolyzed during the RNA degradation reaction catalyzed by RNase L. The role of 2–5A is presumably to induce a conformational change in RNase L that unmasks the protein–protein interaction and ribonuclease domains. Our findings show a critical role for lysine 392 in the dimerization and activation of RNase L, thus providing support for a model in which these activities are linked. The domain II lysine in RNase L is involved in generating the catalytic form of the enzyme, but its role clearly differs from that of protein kinases. Nevertheless, the similarities between RNase L and the IRE1p proteins suggest that they are members of the same family of enzymes. These proteins apparently diverged to acquire separate enzymatic and biological functions. Comparative studies should provide further insight into the mechanisms and functions of these regulated endoribonucleases.

ACKNOWLEDGEMENTS

We thank M. Niwa and P. Walter (UCSF) for the gift of GST–Ire1p(k+t), P. F. Torrence (NIH) for the bromine derivative of 2–5A and A. Zhou (Cleveland) for preparing the 2–5A probe. This investigation was supported by a grant from the United States Public Health Service, Department of Health and Human Services, National Cancer Institute (CA 44059).

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