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Swapping the domains of exoribonucleases RNase II and RNase R: Conferring upon RNase II the ability to degrade ds RNA

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ABSTRACT

RNase II and RNase R are the two *E. coli* exoribonucleases that belong to the RNase II super family of enzymes. They degrade RNA hydrolytically in the 3′ to 5′ direction in a processive and sequence independent manner. However, while RNase R is capable of degrading structured RNAs, the RNase II activity is impaired by dsRNAs. The final end-product of these two enzymes is also different, being 4 nt for RNase II and 2 nt for RNase R. RNase II and RNase R share structural properties, including 60% of amino acid sequence similarity and have a similar modular domain organization: two N-terminal cold shock domains (CSD1 and CSD2), one central RNB catalytic domain, and one C-terminal S1 domain. We have constructed hybrid proteins by swapping the domains between RNase II and RNase R to determine which are the responsible for the differences observed between RNase R and RNase II. The results obtained show that the S1 and RNB domains from RNase R in an RNase II context allow the degradation of double-stranded substrates and the appearance of the 2 nt long end-product. Moreover, the degradation of structured RNAs becomes tail-independent when the RNB domain from RNase R is no longer associated with the RNA binding domains (CSD and S1) of the genuine protein. Finally, we show that the RNase R C-terminal Lysine-rich region is involved in the degradation of double-stranded substrates in an RNase II context, probably by unwinding the substrate before it enters into the catalytic cavity.

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Key words: ribonuclease; RNA; exosome; RNA degradation; protein domains; protein structure; RNA metabolism; protein modeling.

INTRODUCTION

RNase II and RNase R are the two *Escherichia coli* exoribonucleases that belong to the RNase II super family of enzymes. RNase II is the prototype of this family of exoribonucleases, and RNase II/R homologues are present in all domains of life.[1–5] The other member of the RNase II family, RNase R, has been shown to be required for virulence and is involved in mRNA degradation, and RNA and protein quality control.[5–10] In the nucleus and the cytoplasm of eukaryotic cells, the RNase II homologue—Rrp44/Dis3 is part of the exosome, an essential multiprotein complex of exoribonucleases, involved in processing, turnover, and quality control of different types of RNAs.[3] Most importantly, this enzyme was reported to be the only catalytically active nuclease in the yeast core exosome,[11] and studies have shown that this protein has a dual function since it comprises both an exo and an endoribonucleolytic activity.[5,12,13]

RNase II and RNase R share catalytic properties: they both processively degrade RNA hydrolytically in the 3′ to 5′ direction releasing 5′-nucleosite monophosphates. Both enzymes share structural properties, including 60% aminoacid sequence similarity, and 29% protein sequence identity.[14] Their activity is sequence independent but while RNase II is sensitive to secondary structures, RNase R is capable of degrading highly structured RNAs.[7,10,14,15] In fact, recent studies have shown that the RNB domain of RNase R is the one responsible for the degradation of double-stranded substrates.[16,17] It is known that RNase R needs a 3′- single-stranded overhang of at least five nucleotides in length to be able to attach to the substrate and proceed to the degradation of the structured RNA molecules.[18] However, it was recently shown that the CSDs and S1 domains are those responsible for the selective degradation of double-stranded substrates that contain a 3′ single-stranded overhang of five or more nucleotides.[16] Another difference between these two *E. coli* enzymes is that the final degradation product of RNase II is a 4 nucleotide fragment, whereas the end-product of RNase R is a 2 nucleotide fragment.[15,19,20] The same differences have been observed in *Salmonella*, which also has both RNase

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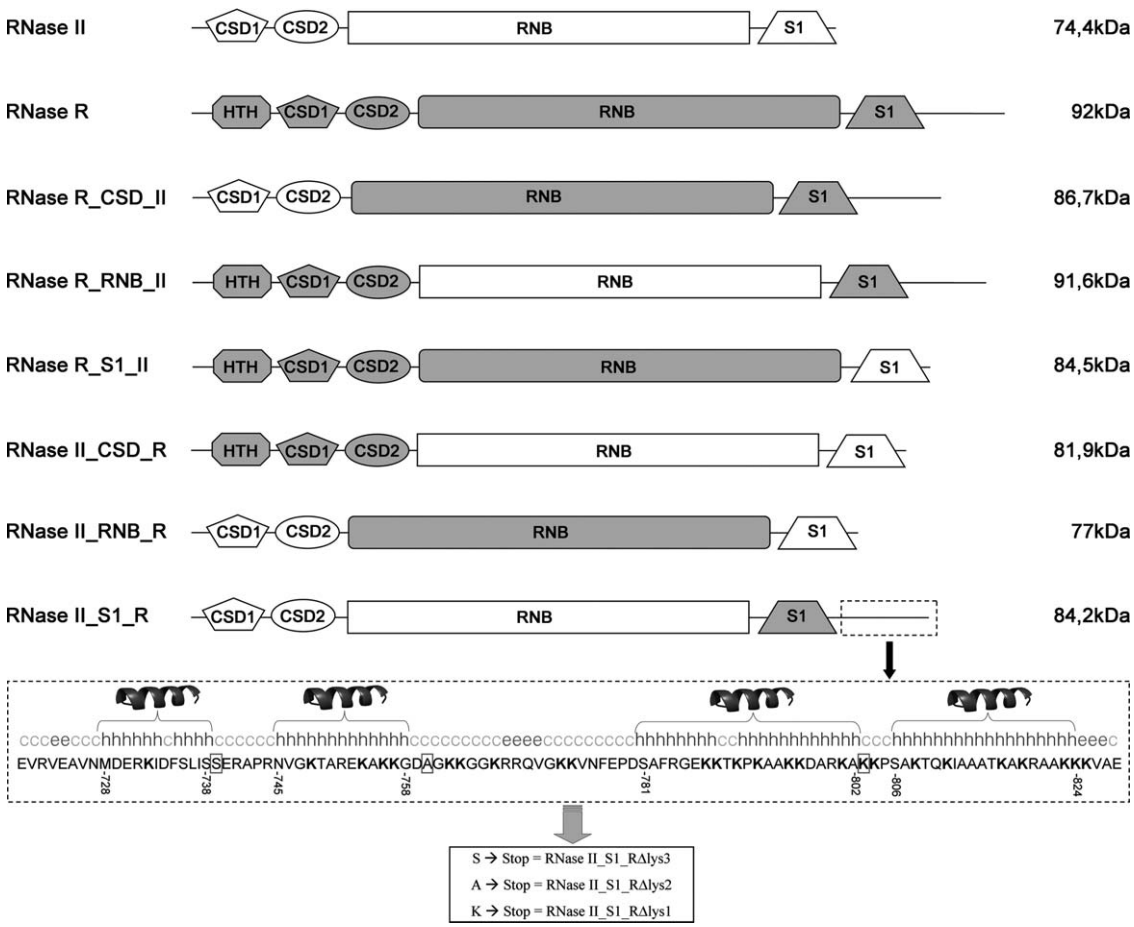

Figure 1

Linear representation of the domains of wild type RNase II, RNase R and its derivatives proteins. The lysine-rich tail of the S1 domain from RNase R is highlighted, and the protein structure is represented. The aminoacids that were substituted by stop codons to construct the proteins RNase II_S1_RΔLys1, RNase II_S1_RΔLys2, and RNase II_S1_RΔLys3 are boxed. On the top of the protein structure is the secondary structure prediction using the GOR 4 method of NPS@ as described in Experimental Procedures. h stands for alpha helix, e stands for extended strand and c stands for random coil.

II and RNase R like proteins.[21] In *Streptococcus pneumoniae*, only one member of this family of enzymes is present. Its characterization showed that it behaves like RNase R, since it is able to degrade double-stranded substrates releasing a 2 nt fragment as its end-product.[21]

RNase II is a protein encoded by gene *rnb* with 72KDa. In *E. coli*, this protein is the major hydrolytic enzyme that is responsible for 90% of the exoribonucleolytic activity in crude extracts.[22] RNase II expression is differentially regulated at the transcriptional and post-transcriptional levels and the protein can be regulated by the environmental conditions.[23–26] The determination of the 3D structure of *E. coli* RNase II showed that RNase II consists of 4 domains: two N-terminal cold shock domains (CSD1 and CSD2), one central RNB catalytic domain, and one C-terminal S1 domain[27,28] (Fig. 1). The final degradation product of RNase II is 4 nt. The structure of the RNA bound complex showed that there is a tight packing of the five 3′-terminal nucleotides in the catalytic cavity, and the RNA "clamping" is mediated by the aromatic residues Tyr253 and Phe358.[27] Subsequently, it was demonstrated that Tyr253 is the residue responsible for setting the end-product of RNase II.[26,29] It was also demonstrated that Tyr-313 and Glu390 are important for the discrimination of cleavage of RNA versus DNA.[16,26,30] During the determination of key residues for catalysis, we have recently discovered that the substitution of the Glu-542 by alanine lead to a 110-fold increase in the exoribonucleolytic activity and 20-fold in RNA binding, turning RNase II into a "super-enzyme."[26,30,31]

RNase R is a 92KDa protein encoded by the *rnr* gene that is involved in the degradation of different types of RNAs such as rRNAs, small RNAs and mRNAs. It was shown that RNase R has *in vivo* affinity for polyadenylated RNA and can be a key enzyme involved in poly(A) metabolism.[32] It is a cold shock protein that is regulated

Table I
Plasmids Used in this Study

Plasmid	Relevant characteristic	Reference
pFCT6.1	gene *rnb* cloned into pET15b, Amp^R	Cairrão *et al.*,[23]
pABA-RNR	gene *rnr* cloned into pET15b, Amp^R	Amblar *et al.*,[19]
pFCT_SpeI514	pFCT6.1 with a SpeI restriction site in position 514 of *rnb* gene	This work
pFCT_SmaI1729	pFCT6.1 with a *Sma*I restriction site in position 1729 of *rnb* gene	This work
pFCT_SpeI514_SmaI1729	pFCT_SpeI514 with a *Sma*I restriction site in position 1729 of *rnb* gene	This work
pABA-RNR_SpeI547	pABA-RNR with a *Spe*I restriction site in position 547 of *rnr* gene	This work
pABA-RNR_SmaI1924	pABA-RNR with a SmaI restriction site in position 1924 of *rnr* gene	This work
pABA-RNR_SpeI547_SmaI1924	pABA-RNR_SpeI547 with a *Sma*I restriction site in position 1924 of *rnr* gene	This work
pFCT_CSD_R	Expresses RNase II with HTH, CSD1 and CSD2 domains from RNase R	This work
pFCT_RNB_R	Expresses RNase II with RNB domain from RNase R	This work
pFCT_S1_R	Expresses RNase II with S1 domain from RNase R	This work
pABA-RNR_CSD_II	Expresses RNase R with CSD1 and CSD2 domains from RNase II	This work
pABA-RNR_RNB_II	Expresses RNase R with RNB domain from RNase II	This work
pABA-RNR_S1_II	Expresses RNase R with S1 domain from RNase II	This work
pFCT_S1_R_ΔLys1	Expresses RNase II with S1 domain from RNase R without the last 26 aa	This work
pFCT_S1_R_ ΔLys2	Expresses RNase II with S1 domain from RNase R without the last 67 aa	This work
pFCT_S1_R_ ΔLys3	Expresses RNase II with S1 domain from RNase R without the last 87 aa	This work

at the transcriptional and post-transcriptional levels.[8,9] The activity of RNase R is modulated according the growth conditions of the cell[23] and its levels increase in stationary phase and under other stress conditions.[7,9,10] It has been shown that this protein is also involved in pathogenesis in different microorganisms.[6,33–35] The structural model of *E. coli* RNase R protein has been constructed based on the RNase II structure. It clearly indicates that these enzymes share a common three-dimensional arrangement, with all the critical residues for exoribonucleolytic activity located in equivalent spatial positions.[29] In fact, recent studies have shown that like in RNase II D209N mutant, Asp280 in RNase R is important for the activity of the enzyme but not for the RNA binding. Also it has been described that Tyr324 is also the conserved residue responsible for setting the final end product in RNase R, comparable with what is observed with RNase II.[16]

The aim of this work was to determine if the catalytical differences observed between RNase II and RNase R could be assigned directly to one of the domains. As such, we wanted to investigate which domain could be accounted for the setting of the different end-products (4 nt for RNase II and 2 nt for RNase R), and which domain could be responsible for the discrimination between single- and double-stranded RNA cleavage. For that, we constructed a set of six different hybrid proteins by swapping the cold shock domains, the catalytic domains, and the S1 domains between RNase II and RNase R (Fig. 1). The results presented here show that in fact the RNB domain from RNase R is the one responsible for the degradation of double-stranded substrates. However and more interestingly, our data shows that the C-terminal region from RNase R has a very important role in the degradation of double-stranded substrates. We show that this domain might contribute to the unwinding of the secondary structures, and this can explain why RNase R is capable of degrading RNA structured substrates.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 DNA ligase, Pfu DNA polymerase and T4 Polynucleotide Kinase were purchased from Fermentas. Unlabeled oligonucleotide primers were synthesized by STAB Vida, Portugal.

Strains

The *E. coli* strains used were DH5α (F′ fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17a)[36] for cloning experiments and BL21(DE3) (F^- r_B^- m_B^- gal ompT (int::P_{lacUV5} T7 gen1 imm21 nin5)[37] for protein expression.

Construction of hybrid proteins

The hybrid proteins were constructed by swapping the N-terminal region (corresponding to the Cold Shock Domains), the catalytic domain RNB or the C-terminal region (S1 domain) between $(His)_6$-RNase II and $(His)_6$-RNase R, thus obtaining the following six proteins: RNase II_CSD_R, RNase II_RNB_R, RNase II_S1_R, RNase R_CSD_II, RNase R_RNB_II, and RNase R_S1_II (Fig. 1).

For this purpose, the *Spe*I and *Sma*I restriction sites were introduced into the pFCT6.1 plasmid (Table I) at the 514 nt and 1729 nt positions respectively and in the pABA-RNR plasmid (Table I) at the 547 nt and 1924 nt positions respectively by overlapping PCR. The mutagenic primers used were pFCT¬_SpeI514_Fw, pFCT_SpeI514_Rev, pFCT_SmaI1729_Fw, pFCT_SmaI1729_Rev, RNR_SpeI547_Fw, RNR_SpeI547_Rev, RNR_SmaI1924_

Table II
Primers used in this Study

Primer	Sequence (5′ – 3′)[ab]	Purpose
pFCT_SpeI514_Fw	CACAATACATCACT**AG**TGGTGACG	Introduces SpeI restriction site into pFCT6.1 at the position 514
pFCT_SpeI514_Rev	CGTCACCA**CT**AGTGATGTATTGTG	Introduces SpeI restriction site into pFCT6.1 at the position 514
pFCT_SmaI1729_Fw	CCTGAAAGACAAA**C**CCGGGACCGACACCCG	Introduces SmaI restriction site into pFCT6.1 at the position 1729
pFCT_SmaI1729_Rev	CGGGTGTCGGTCCC**G**G**G**TTTGTCTTTCAGG	Introduces SmaI restriction site into pFCT6.1 at the position 1729
RNR_SpeI547_Fw	GTCGAAGTGCTGGGC**ACT**AG**T**ATGGGCACC	Introduces SpeI restriction site into pABA-RNR at the position 547
RNR_SpeI547_Rev	GGTGCCCATA**CT**AG**T**GCCCAGCACTTCGAC	Introduces SpeI restriction site into pABA-RNR at the position 547
RNR_SmaI1924_Fw	GTGTGACTTCATG**C**CCG**GG**CAGGTAGG	Introduces SmaI restriction site into pABA-RNR at the position 1924
RNR_SmaI1924_Rev	CCTACCTG**CC**CG**G**GCATGAAGTCACAC	Introduces SmaI restriction site into pABA-RNR at the position 1924
Δlys1_Fw	TTTAGCCTGATCTCC**TAA**GAACGCGCACCG	Introduces stop codon into pFCT_S1_R at the position 2172
Δlys1_Rev	CGGTGCGCGTTC**TTA**GGAGATCAGGCTAAA	Introduces stop codon into pFCT_S1_R at the position 2172
Δlys2_Fw	GAAAAAAGGCGAT**TA**AGGTAAAAAAGGCGG	Introduces stop codon into pFCT_S1_R at the position 2049
Δlys2_Rev	CCGCCTTTTTTACCT**TA**ATCGCCTTTTTTC	Introduces stop codon into pFCT_S1_R at the position 2049
Δlys3_Fw	GCGAGAAAAGCG**T**AAAAGCCATCGG	Introduces stop codon into pFCT_S1_R at the position 1989
Δlys3_Rev	CCGATGGCTTTT**A**CGCTTTTCTCGC	Introduces stop codon into pFCT_S1_R at the position 1989

Bases underlined indicate restriction sites.
Bases in bold indicate aminoacid changes.

Fw, and RNR_SmaI1924_Rev (Table II). To construct the hybrid proteins RNase II_RNB_R and RNase R_RNB_II, the resulting plasmids pFCT_SpeI514_SmaI1729 and pABA-RNR_SpeI547_SmaI1924 (Table II) were digested with *Spe*I and *Sma*I, obtaining degradation products of 6195 bps and 1216 bps for pFCT_SpeI514_SmaI1729 and 7005 bps and 1270 bps for pABA-RNR_SpeI547_SmaI1924. The restriction fragment with 1216 bps resultant of the restriction of pFCT_SpeI514_SmaI1729 was ligated to the restriction fragment of 7005 bps resultant of the digestion of pABA-RNR_SpeI547_SmaI1924, originating the hybrid protein RNase R_RNB_II. The restriction fragment with 6195 bps resultant of the restriction of pFCT_SpeI514_SmaI1729 was ligated to the restriction fragment of 1270 bps resultant of the digestion of pABA-RNR_SpeI547_SmaI1924, originating the hybrid protein RNase II_RNB_R.

To construct the hybrid proteins RNase II_CSD_R and RNase R_CSD_II, the plasmids with the insertion of *Spe*I restriction site only, pFCT_SpeI514 and pABA-RNR_SpeI547 (Table I) were digested with *Spe*I and *Xba*I (the restriction enzyme *Xba*I cleaves the pET15b plasmid upstream the insertion of the *rnb* or *rnr* genes), obtaining degradation products of 6860 bps and 551 bps for pFCT_SpeI514 and 7529 bps and 746 bps for pABA-RNR_SpeI547. The restriction fragment with 551 bps resultant of the restriction of pFCT_SpeI514 was ligated to the restriction fragment of 7529 bps resultant of the digestion of pABA-RNR_SpeI547, originating the hybrid protein RNase R_CSD_II. The restriction fragment with 6860 bps resultant of the restriction of pFCT_SpeI514 was ligated to the restriction fragment of 746 bps resultant of the digestion of pABA-RNR_SpeI547, originating the hybrid protein RNase II_CSD_R.

To construct the hybrid proteins RNase II_S1_R and RNase R_S1_II, the plasmids with the insertion of *Sma*I restriction site only, pFCT_SmaI1729 and pABA-RNR_SmaI1924 (Table I) were digested with *Sma*I and *Hind*III (the restriction enzyme *Hind*III cleaves the pET15b plasmid downstream the insertion of the *rnb* or *rnr* genes), obtaining degradation products of 7076 bps and 335 bps for pFCT_SmaI1729 and 7325 bps and 950 bps for pABA-RNR_SmaI1924. The restriction fragment with 335 bps resultant of the restriction of pFCT_SmaI1729 was ligated to the restriction fragment of 7325 bps resultant of the digestion of pABA-RNR_SmaI1924, originating the hybrid protein RNase R_S1_II. The restriction fragment with 7076 bps resultant of the restriction of pFCT_SmaI1729 was ligated to the restriction fragment of 950 bps resultant of the digestion of pABA-RNR_SmaI1924, originating the hybrid protein RNase II_S1_R.

The Δlys mutations in the RNase II_S1_R_Δlys1, RNase II_S1_R_Δlys2, and RNase II_S1_R_Δlys3 proteins were introduced into the pFCT_S1_R (Table I) by PCR overlapping. The primers used in the constructions were Δlys1_Fw, Δlys1_Rev, Δlys2_Fw, Δlys2_Rev, Δlys3_Fw, and Δlys3_Rev (Table II).

Overexpression and purification of wild type and hybrid proteins

The plasmid used for expression of wild-type *E. coli* histidine-tagged RNase II protein was pFCT6.1 plasmid (Table I). The plasmid used for expression of wild-type *E. coli* histidine-tagged RNase R protein was pABA-RNR (Table I).

All plasmids were transformed into BL21(DE3) *E. coli* strain (Novagen) to allow the expression of the recombinant proteins. Cells were grown at 30°C in 100 mL LB medium supplemented with 150 μg/mL ampicillin to an OD_{600} of 1.5. Then, they were transferred to 18°C for 30 min and then induced by addition of 0.5 m*M* IPTG; induction proceeded for 20 hours at 18°C. Cell cultures were pelleted by centrifugation at 6000 rpms for 15 min and stored at −80°C.

Purification of all proteins was performed by histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) and AKTA HLPC system (GE Healthcare) following the protocol previously described.[15,38] Protein concentration was determined by spectrophotometry using a Nanodrop device and measuring the OD at 280nm. Finally 50% (v/v) glycerol was added to the final fractions prior storage at −20°C. 0.5 μg of each purified protein was applied in an 8% SDS-PAGE and visualized by Coomassie blue staining (data not shown) to assess protein purity.

Activity assays

Exoribonucleolytic activity was assayed using three different RNA oligoribonucleotides as substrates.[15,38] The 30mer oligoribonucleotide (5′-CCCGACACCAACCACUAAAAAAAAAA AAAAA-3′), the 16mer oligoribonucleotide (5′-CCCGACACCAACCACU-3′) and the poly(A) chain of 35 nt were labelled at its 5′-end with [γ-^{32}ATP] and T4 polynucleotide kinase. The RNA oligomers were then purified using Microcon YM-3 Centrifugal Filter Devices (Millipore) to remove the nonincorporated nucleotide. The labelled 30mer and 16mer oligoribonucleotides were hybridized to the complementary 16mer oligodeoxyribonucleotide (5′AGT GGT TGG TGT CGG G 3′), thus obtaining the corresponding double stranded substrate 16–30ds and 16-16ds, respectively. The hybridization was performed in a 1:1 (mol:mol) ratio in the Tris component of the activity by 5 min of incubation at 68°C followed by 45 min at 37°C. The exoribonucleolytic reactions were carried out in a final volume of 10 μl containing 30 nm of substrate, 20 m*M* Tris-HCl pH 8, 100 m*M* KCl, 1 m*M* $MgCl_2$, and 1 m*M* DTT. The amount of each enzyme added to the reaction was adjusted to obtain linear conditions and is indicated in the respective figures. Reactions were started by the addition of the enzyme and incubated at 37°C. Samples were withdrawn at the time points indicated in the figures, and the reaction was stopped by adding formamide-containing dye supplemented with 10 m*M* EDTA. Reaction products were resolved in a 20% polyacrylamide/7 *M* urea and analyzed by autoradiography. The exoribonucleolytic activity of the enzymes was determined by measuring and quantifying the disappearance of the substrate in several distinct experiments in which the protein concentration was adjusted in order that, under those conditions, less than 25% of substrate was degraded. Each value obtained represents the mean of these independent assays.

Surface plasmon resonance analysis—BIACORE

Surface Plasmon Resonance analysis was developed for the study of the interaction between RNases and RNA molecules as previously described.[38] Biacore SA chips were obtained from Biacore Inc. (GE Healthcare). The Flow cells of the SA streptavidin sensor chip were coated with a low concentration of the following substrates. On flow cell 1, no substrate was added so this cell could be used as the control blank cell. On flow cell 2, a 5′ biotinylated 25-nucleotide RNA oligomer (5′-CCC GAC ACC AAC CAC UAA AAA AAA A-3′) was added to allow the study of the protein interaction with a single-stranded RNA molecule. On flow cell 3, a 5′ biotinylated 30-mer PolyA substrate. The target substrates were captured on flow cells 2 and 3 by manually injecting 20 μl of a 500 nm solution of the substrates in 1 *M* NaCl at a 10 μl/min flow rate, as described in previous reports.[31,38,39] The biosensor assay was run at 4°C in the buffer with 20 m*M* Tris-HCl pH8, 100 m*M* KCl, 1 m*M* DTT and 25 m*M* EDTA. The proteins were injected over flow cells 1, 2, and 3 for 2 min at concentrations of 10, 20, 30, 40, and 50 n*M* using a flow rate of 20μl/min. All experiments included triple injections of each protein concentration to determine the reproducibility of the signal and control injections to assess the stability of the RNA surface during the experiment. Bound protein was removed with a 60-s wash with 2 *M* NaCl, which did not damage the substrate surface. Data from flow cell 1 were used to correct for refractive index changes and nonspecific binding. Rate constants and equilibrium constants were calculated using the BIA EVALUATION 3.0 software package, according to the fitting model 1:1 Languimir Binding. Obtained and fitted data are represented in Supporting Information Figure 1.

Secondary structure prediction of the lysine-rich tail of RNase R, protein modeling and multiple sequence alignment

To predict the secondary structure of the Lysine-rich tail of RNase R, we used the NPS@ server (Network Protein Sequence Analysis)[40] with the GOR4 method[41] (http://npsa-pbil.ibcp.fr).

Structural model of the *E. coli* RNase R protein as well as RNase II_S1_R and RNase R_RNB_II constructions were performed by standard comparative modeling methods and the software DeepView,[42] using the crystal structures of wild-type RNase II and the RNase II D209N mutant complexed with a 13-nucleotide poly(A) RNA as templates (PDB codes: 2IX1 and 2IX0[27]). To optimize geometries, models were energy minimized using the GROMOS 43B1 force field implemented in DeepView,[42] using 500 steps of steepest descent minimization followed by 500 steps of conjugate-gradient minimization. Sequence identity between RNase II and modeled RNase R was 26%, with a Blast e-value of 3.3×10^{-43}. The quality of the model was checked using the analysis programs (Anolea, Gromos and Verify3D) provided by the SWISS-MODEL server (http://swissmodel.expasy.org/[43–45]).

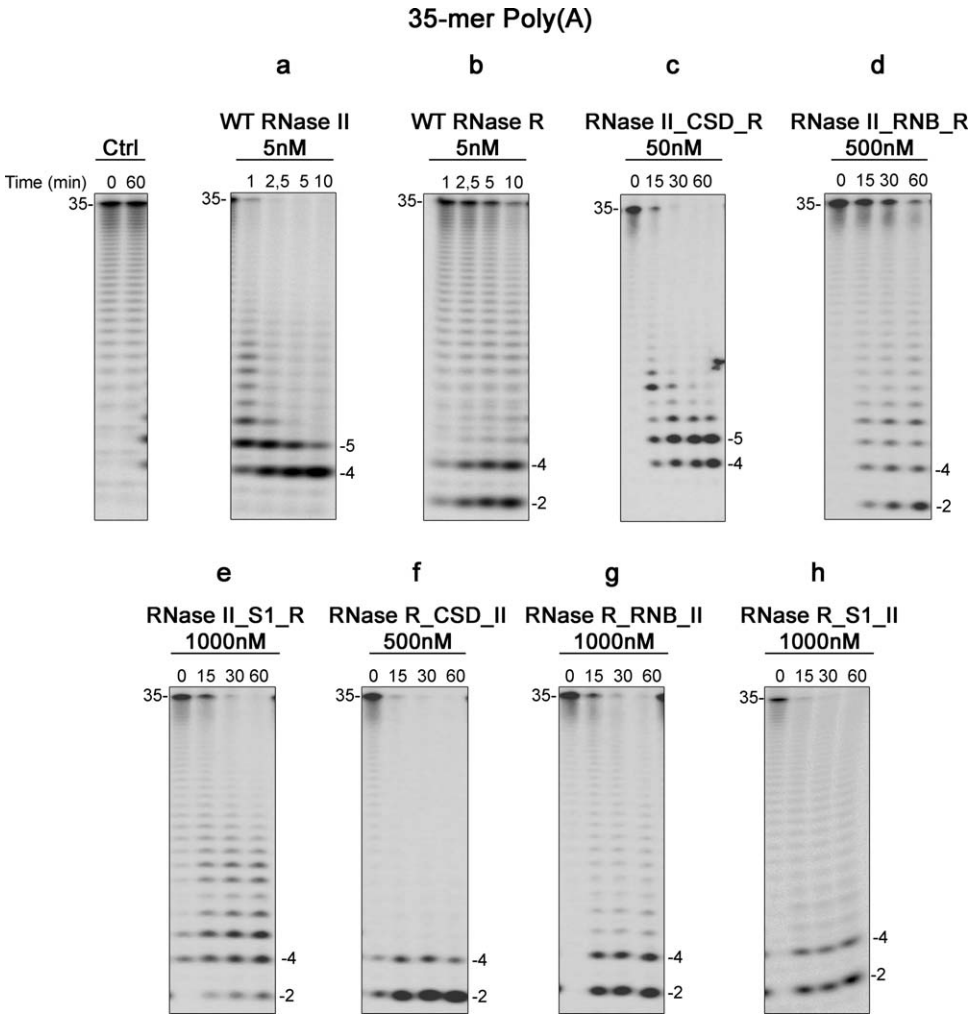

Figure 2

Exoribonuclease activity with a 35ss Poly(A) substrate: comparison of wild-type with hybrid enzymes. Activity assays were performed as described under Materials and Methods using a poly(A) chain of 35 nt. The proteins used and their respective concentrations are shown. The wild-type enzymes were used as control. Samples were taken during the reaction at the time points indicated, and reaction products were analyzed in a 20% polyacrylamide/7 *M* urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

Structures were manipulated using the Swiss-PDB viewer and were rendered using Pymol.[46]

Homologous sequences belonging to the to RNase II family of proteins in protein databases were obtained using Blast,[47] and they were aligned using ClustalW[48] and T-COFFEE[49] algorithms.

RESULTS

Characterizing the exoribonucleolytic activity of the hybrid proteins using poly(A) substrate

RNase II and RNase R are members of the same family of exoribonucleases and therefore share catalytic and structural properties. However, they behave differently regarding the final end-product released: while RNase II releases a 4 nt fragment as its end-product, RNase R releases a mixture of 2 nt and 4 nt fragments, but 2 nt is the predominant product [Fig. 2(a, b)].[15,19,20] The mechanism of RNase II has been elucidated and the size of the final product released depends on the aromatic residues Tyr-253 and Phe-358. that "clamp" the RNA.[27,29] In RNase R, Phe-429 is located in the position immediately downstream of the equivalent residue of Phe-358 in RNase II.[29] This residue may allow a partial "clamp" of the RNA and a 4 nt end-product is released. However, other oligoribonucleotides may still bind to the catalytic cavity and are able to be degraded up to the final 2 nt fragment.[16] Since these two enzymes

Table III
Exoribonucleolytic Activity of Wild Type and Hybrid Proteins

Protein	Protein Activity (pmol subst/nmol prot/min)
wt RNase II	299.4 ± 36.0
RNase II_CSD_R	0.5 ± 0.05
RNase II_RNB_R	0.05 ± 0.002
RNase II_S1_R	0.04 ± 0.001
RNase II _S1_R_ΔLys1	0.01 ± 0.001
RNase II _S1_R_ΔLys2	0.01 ± 0.001
RNase II _S1_R_ΔLys3	0.01 ± 0.001
RNase II D209N	<0.0001[29]
wt RNase R	130.8 ± 6.3
RNase R_CSD_II	0.04 ± 0.001
RNase R_RNB_II	0.02 ± 0.001
RNase R_S1_II	0.08 ± 0.002
RNase R D280N	<0.0001[16]

Exoribonucleolytic activity was assayed using a 35 nt poly(A) chain as substrate. Activity assays were performed in triplicate as described in Experimental Procedures.

also share a common 3D arrangement and have the same domain organization,[29] we were interested in studying which domain(s) would be responsible for these differences. For this purpose, we exchanged the domains between RNase II and RNase R and constructed six hybrid proteins. In the designation chosen, the first word represents the protein that "received" the other domain as explained in Figure 1. The constructed proteins are: RNase II_CSD_R, which consists of RNase II with the two cold shock domains and the helix turn helix region from RNase R; RNase II_RNB_R, which consists of RNase II with the RNB domain of RNase R; RNase II_S1_R that is RNase II with the S1 and basic region from RNase R; RNase R_CSD_II, in which the CSDs from RNase II substitute those of RNase R; RNase R_RNB_II, which consists of RNase R with the RNB from RNase II; and finally, RNase R_S1_II, in which the S1 domain from RNase II substitutes the S1 domain from RNase R (Fig. 1).

We started to analyze the protein activity by using a single-stranded poly(A) substrate. This substrate was chosen to determine the activity of the proteins, since it has been shown that RNase II family members reflect a marked preference for poly(A) substrates.[16,30] To determine the activity of the hybrid proteins, we tested several different protein concentrations, and performed the calculations in triplicate using the values where less than 25% of the substrate was degraded. The results obtained are presented in Table III and the units of activity refer to the pmol of substrate which is degraded by 1 n*M* of protein in 1 min. When compared with their wild type counterparts, we can see that the six engineered proteins have a reduced activity (Table III). The decrease in the activity of the hybrid proteins ranges from 600-fold (RNase II_CSD_R protein) to 7000-fold (RNase II_S1_R protein). However, when compared with the inactive mutants RNase II D209N and RNase R D280N (Table III), we can safely say that, although bearing a low activity, these proteins are still able to degrade RNA substrates. Since we are working with engineered proteins that do not exist in nature, we also need to consider the possibility that only a fraction of the hybrid proteins adopt a catalytically active conformation. In this case, the determination of the activity could be underestimated. Another objective of this work was to identify the end-product of each of these engineered proteins. To do so, we tested different conditions to ensure that all the enzymes had reached their end-product. As a consequence, we had to use higher protein concentrations for the hybrid proteins than those used for both RNase II and RNase R. Figures 2–5 show the representative assays from the several ones performed that we believe better illustrate the results obtained.

In RNase II_CSD_R, when we changed the CSDs of RNase II by the ones from RNase R we observed that the protein behaved like RNase II [Fig. 2(c)]. However, when we changed the RNB or S1 domains of RNase II by the equivalents of RNase R (as is the case in the RNase II_RNB_R and RNase II_S1_R proteins), we were able to see that the final product changed to 2 nt [Fig. 2(d, e)]. For RNase II_S1_R protein, it was possible to observe that the majority of the product released was a 4 nt fragment and only a small portion of substrate was able to be degraded until the 2 nt of length, contrary to what happened for RNase R [Fig. 2(e)]. Thus, we can conclude that the S1 and RNB domains from RNase R are each sufficient to allow RNase II to behave like RNase R, and generate the 2 nt end-product.

When we changed the RNase R domains by the ones in RNase II (RNase R_CSD_II, RNase R_RNB_II and RNase R_S1_II proteins), we observed that the final end-product released was always a 2 nt [Fig. 2(f–h)]. These results indicate that the RNB domain of RNase R is responsible for setting the final end-product. However, when the RNB domain of RNase II was inserted into RNase R (RNase R_RNB_II), the final product was not altered as expected, which suggested to us that the RNA binding domains of RNase R are also involved, probably by inducing a different conformation of the protein.

The hybrid proteins prefer poly(A) substrates

To see if the binding ability was affected in the hybrid proteins, we determined the dissociation constants by SPR using two different single-stranded substrates, a 25-nt RNA oligomer and a 30 nt poly(A) oligomer and compared them with the wild type enzymes (Table IV). In all the cases, and for all the proteins, the affinity constants determined using the 30 nt poly(A) substrate were always lower when compared with those detected using the other ssRNA substrate. This was expected, and indicates that, like in the cases of wild-type RNase II and

Table IV
RNA Binding Affinity of the Hybrid Proteins

Protein	K_D (nM) 25-mer	K_D (nM) PolyA
wt RNase II	6.5 ± 0.4	1.3 ± 0.4
RNase II_CSD_R	16.0 ± 0.4	4.3 ± 0.3
RNase II_RNB_R	7.0 ± 0.9	2.0 ± 0.3
RNase II_S1_R	3.2 ± 0.5	1.3 ± 0.1
wt RNase R	3.2 ± 0.4	1.2 ± 0.1
RNase R_CSD_II	8.6 ± 0.7	5.1 ± 0.1
RNase R_RNB_II	5.4 ± 0.6	4.4 ± 0.1
RNase R_S1_II	10.1 ± 1.4	3.2 ± 0.2

The dissociation constants (K_D) were determined by Surface Plasmon resonance using BIACORE 2000 with a 25 nt RNA oligomer (5′-Biotin-CCC GAC ACC AAC CAC UAA AAA AAA A-3′) and a 30 nt poly(A) RNA oligomer.

RNase R, the hybrid proteins constructed reflect their preference for poly(A) type substrates. However, in the case of RNase R_RNB_II and RNase R_S1_R that preference is not so marked (Table IV).

The results obtained also showed that, for almost all proteins, the affinity is slightly reduced for both substrates when compared with the wild type enzymes (Table IV). These differences are not significant, however, they can help us explain the reduction in the activity of the hybrid proteins, since the affinity of a protein is not always correlated with its activity. For example, the RNase II_D209N and RNase R_D280N mutants were inactive but the RNA affinity was not altered.[16,29,50]

Degradation of double-stranded substrates by hybrid proteins

To study which domains could be responsible for the differences regarding the ability to cleave double-stranded substrates, we tested the hybrid proteins against a 16–30ds substrate, that consists of a 30-mer ribonucleotide hybridized with a complementary 16-mer oligonucleotide, as described in Experimental Procedures. To detect the ribonucleolytic activity with this substrate, we tested several different concentrations of the various proteins to find the optimal conditions. In our assays, the hybrid proteins were active with double stranded substrates when using as little as 10 nm of protein. However, in such conditions it was difficult to determine which was the real final product released and if, in fact, the protein was degrading the substrate. For this reason, in Figure 3 the protein concentrations used in the assays are extremely high.

The results showed that when RNase II has the N-terminal region of RNase R (RNase II_CSD_R) the hybrid protein behaved like RNase II since it was not able to degrade double-stranded substrates. However, the protein was capable to degrade a few more nucleotides than RNase II, releasing a 20 nt fragment instead of the usual 23 nt fragment [Fig. 3(a, c)]. RNase II_RNB_R protein was able to cleave the 16–30ds substrate just like RNase R [Fig. 3(d)]. It was previously described that RNase R needs a 3′-single-stranded overhang of at least 7 nucleotides of length to attach to the substrate and proceed with the degradation[18] and it is not able to cleave a substrate in the absence of a 3′-end tail [Fig. 4(a)]. However, the hybrid protein RNase II_RNB_R did not present this requirement since it was able to degrade double-stranded substrates in the absence of a 3′-overhang [Fig. 4(c)], even though only a small percentage of the 16–16ds substrate was degraded. This fact confirms that, as previously demonstrated,[16] the RNB domain from RNase R is the one responsible for the degradation of double-stranded substrates. The substitution of only one RNase R binding domain (CSD or S1 domains) eliminates the requirement of a 3′ single-stranded overhang for the degradation of dsRNA substrates. Surprisingly, the RNase II_S1_R protein was also able to cleave double-stranded substrates releasing a fragment with 2 nt of length [Fig. 3(e)]. Nevertheless, for this protein the requirement for a 3′-overhang was essential since it was not able to cleave the perfect double-stranded substrate [Fig. 4(d)]. This suggests that the catalytic cavity of the protein is only accessible to single-stranded substrates, similarly to that which occurs with RNase II.[27] This implies that the RNA is entering into the catalytic cavity in a single-stranded form, which means that the substrate is being unwound before its entry. Thus, the domain responsible for this action must be the S1 domain from RNase R.

RNase R_CSD_II, RNase R_RNB_II and RNase R_S1_II proteins were able to cleave the double-stranded substrates similar to RNase R [Fig. 3(f–h)]. When the RNB domain of RNase R was substituted by the one of RNase II (RNase R_RNB_II), the protein was still able to cleave the double-stranded substrate [Fig. 3(g)]. In this case, the requirement for a 3′-overhang was also verified, since the protein was not able to degrade the 16–16ds substrate [Fig. 4(f)]. This suggests that the RNB domain from RNase R is not the only one responsible for the degradation of structured RNA.

In our work, we showed that the simple substitution of the RNB domain in RNase II for that of RNase R lead to the construction of a protein that was active against double-stranded substrates. The RNB domain does not seem to be acting alone in this process, because when switching the S1 domain from RNase II for the S1 domain from RNase R, the respective protein is also able to cleave double-stranded substrates (Fig. 3). However, it seems that the RNB and S1 domains may act differently against structured RNAs when they are in an RNase II context. While RNase R S1 domain requires a 3′single-stranded overhang to bind to the substrate and degrade it, the RNB domain from RNase R is able to bind and degrade structured RNAs in the absence of a tail, as previously demonstrated.[16] The results obtained led us to

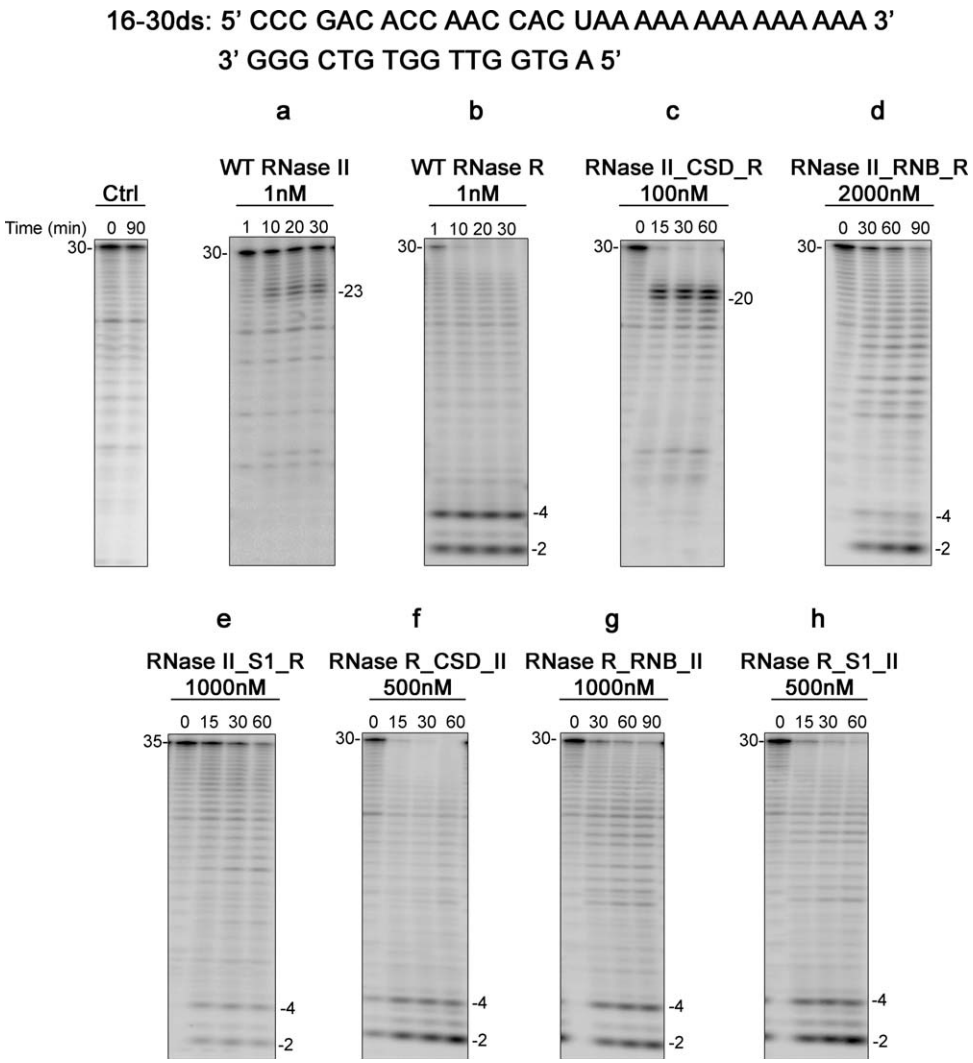

Figure 3

Exoribonuclease activity with 16–30ds substrate: comparison of wild-type with hybrid enzymes. Activity assays were performed as described under Material and Methods using a 30-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16–30ds. The proteins used and their respective concentrations are shown. The wild-type enzymes were used as control. Samples were taken during the reaction at the time points indicated, and reaction products were analyzed in a 20% polyacrylamide/7 *M* urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

further analyze the role of the C-terminal region of RNase R in the mechanism of degradation of double-stranded substrates.

Unravelling the role of the lysine-rich tail of RNase R

Since the S1 domain from RNase R is allowing the cleavage of double-stranded substrates, we wanted to discriminate which region could be conferring that property. By comparing the C-terminal region of both RNase II and RNase R proteins, it is possible to observe that, apart from the S1 domain, RNase R has an extra lysine-rich tail (Fig. 1). To understand the role of this region in RNA degradation, we predicted its secondary structure in the Network Protein Sequence Analysis as described in Experimental Procedures. The results predicted showed that this region is formed by four alpha-helices: the first (from the C-terminal end) comprises the last 22 aa of the protein, the second is formed by the aa 781 to 802, the third comprises aa 745 to 758 and the forth, which is smaller when compared with the others, is constituted by aa 728 to 738 (note that this numbering refers to RNase R and not to the hybrid protein, as indicated in the figure) (Fig. 1). To investigate the contribution of the three major alpha-helices in the degradation of double-stranded substrates, we

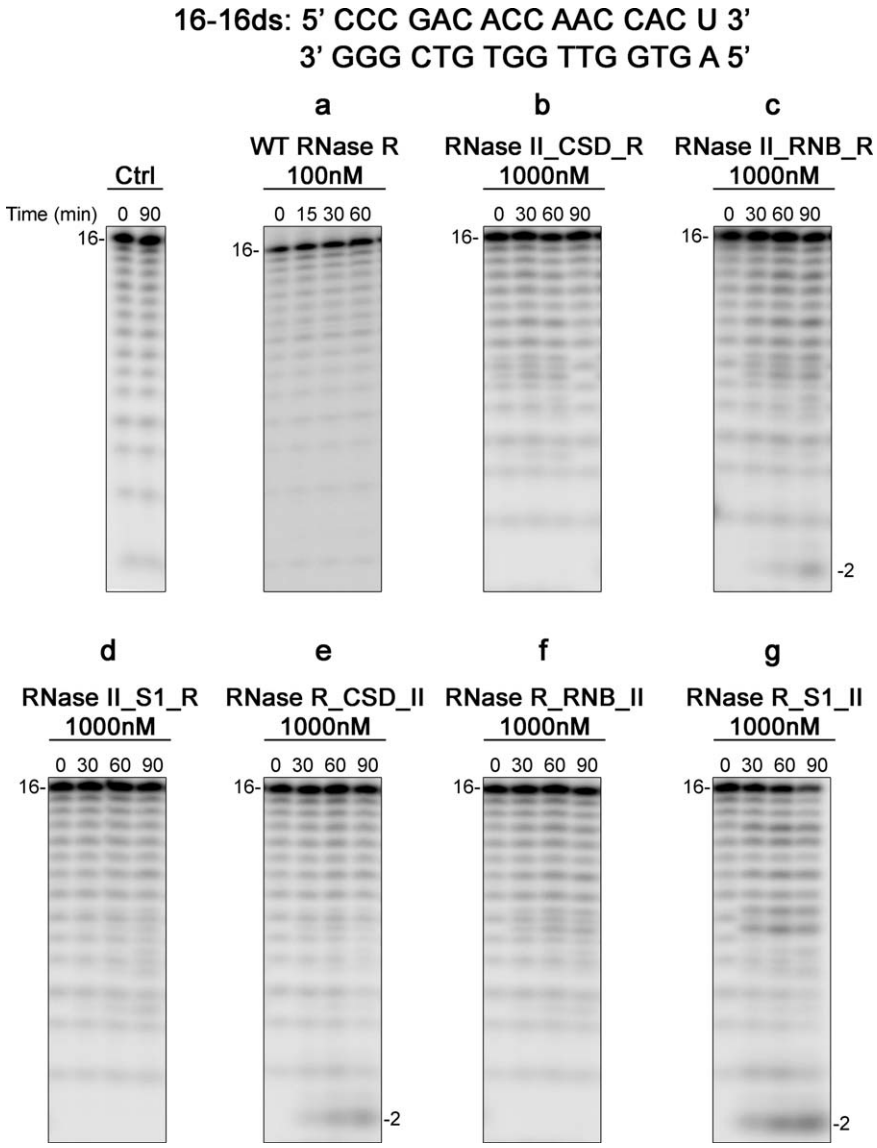

Figure 4

Exoribonuclease activity with 16–30ds substrate: comparison of wild-type with hybrid enzymes. Activity assays were performed as described under Material and Methods using a 16-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16–16ds. The proteins used and their respective concentrations are shown. The wild-type enzymes were used as control. Samples were taken during the reaction at the time points indicated, and reaction products were analyzed in a 20% polyacrylamide/7 *M* urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

introduced three stop codons into the hybrid protein RNase II_S1_R as indicated in Figure 1, thus obtaining the three proteins, RNase II_S1_RΔlys1, RNase II_S1_RΔlys2 and RNase II_S1_RΔlys3, which lack one, two or three alpha-helices, respectively. The determination of the role of the C-terminal region of the S1 domain from RNase R in the degradation of double-stranded substrates was performed in an RNase II context, using the hybrid protein RNase II_S1_R. The mutations were introduced in this protein and not in RNase R wt because the RNB domain of RNase R by itself is able to degrade double-stranded substrates[16] and this characteristic could bias the results obtained. The proteins were then analyzed regarding their activity against single- and double-stranded substrates and RNA affinity.

We also determined the exoribonucleolytic activity of these three proteins, and we could observe that the activity is 4-fold reduced when we compare with RNase II_S1_R (Table III). This indicates that the Lysine-rich tail is also contributing for the activity of RNase II_S1_R

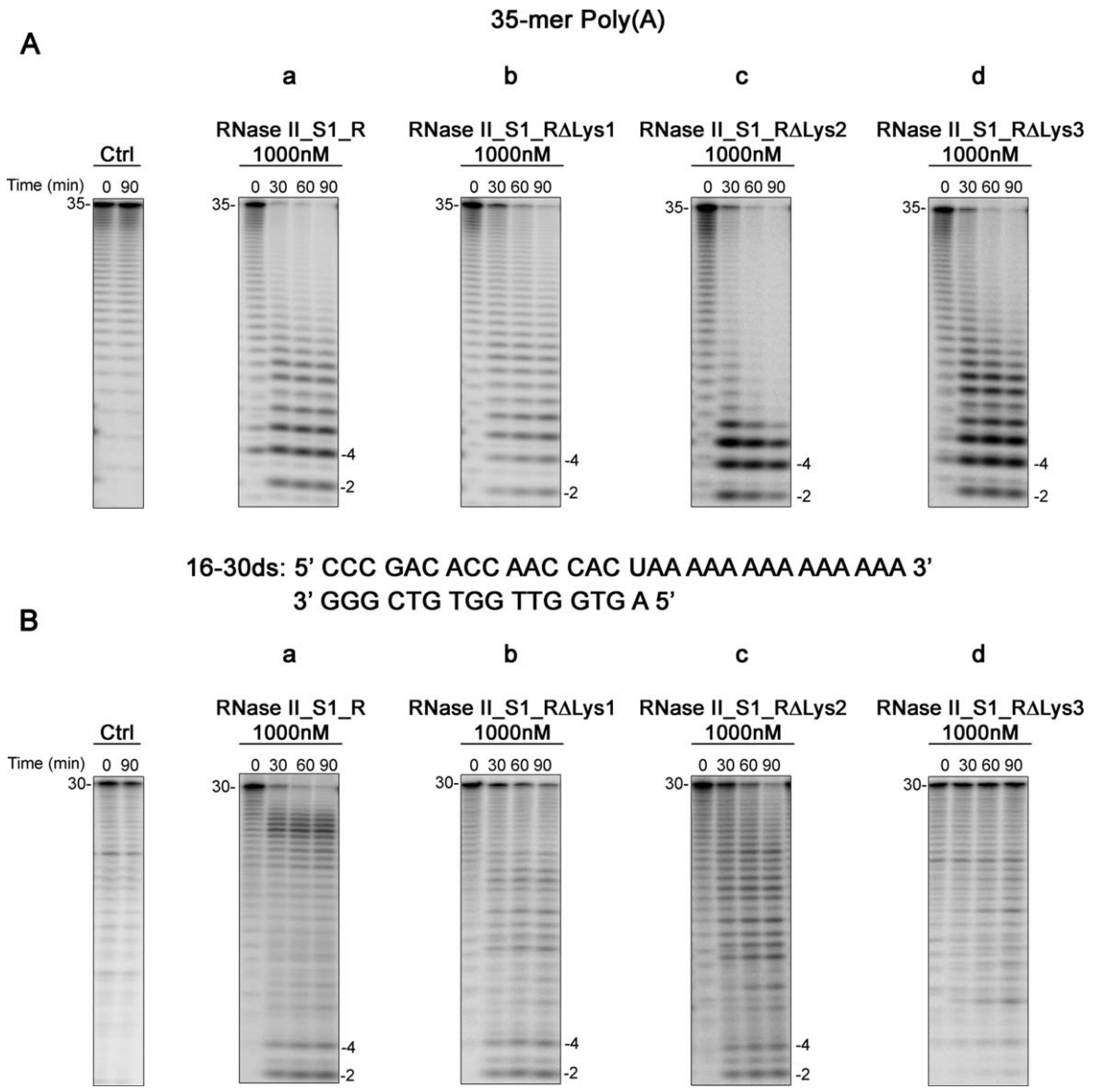

Figure 5

Exoribonuclease activity of RNase II_S1_R and ΔLys mutants. Activity assays were performed as described under Materials and Methods using a poly(A) chain of 35 nt (A), and a 30-mer oligoribonucleotide hybridized to the complementary 16mer oligodeoxyribonucleotide, thus obtaining the corresponding double stranded substrate 16–30ds (B). The proteins used and their respective concentrations are shown. The wild-type enzyme was used as control. Samples were taken during the reaction at the time points indicated, and reaction products were analyzed in a 20% polyacrylamide/7 *M* urea gel. Control reactions with no enzyme added (*Ctrl*) were incubated at the maximum reaction time for each protein. Length of substrates and degradation products are indicated in the figure.

protein. When the substrate used was the single-stranded one, the results obtained were similar to those obtained for the RNase II_S1_R hybrid protein, since all the three proteins tested released a 2 nt fragment as end-product [Fig. 5(A)]. When we tested the double stranded-substrate 16–30ds, we could observe some differences between these proteins. As already mentioned, the RNase II_S1_R is able to cleave double-stranded substrates. The same behaviour is observed when the first and second alpha helices are absent (in RNase II_S1_R_Δlys1 and RNase II_S1_R_Δlys2 proteins) [Fig. 5B(b, c)]. However, when we removed all three helices, the protein RNase II_S1_R_Δlys3 was not able to degrade the substrate tested [Fig. 5B(d)]. These results indicate that the Lysine-rich region can be involved in the degradation of double-stranded substrates in RNase R. We also measured the RNA affinity of these proteins with the two different substrates tested previously and compared the data with the values obtained with the RNase II_S1_R protein (Table V). For both substrates used it was possible to see that the K_D value raised slightly when the helices were removed. In the case of the 25ss substrate, the hybrid

Table V
RNA Binding Affinity of the Δlys Hybrid Proteins

Protein	K_D (nM) 25-mer	K_D (nM) PolyA
RNase II_S1_R	3.2 ± 0.5	1.3 ± 0.1
RNase II _S1_RΔLys1	4.2 ± 0.4	1.4 ± 0.3
RNase II _S1_RΔLys2	5.5 ± 0.9	1.4 ± 0.1
RNase II _S1_RΔLys3	6.9 ± 0.2	2.4 ± 0.3

The dissociation constants (K_D) were determined by Surface Plasmon resonance using BIACORE 2000 with a 25 nt RNA oligomer (5′-Biotin-CCC GAC ACC AAC CAC UAA AAA AAA A-3′) and a 30 nt poly(A) RNA oligomer.

protein II_S1_R presented a K_D value that was equivalent to the one presented by RNase R, which was 3.2 nm for both proteins (Table V). When the three helices were absent, the K_D value presented by the II_S1_R_Δlys3 protein became closer to the one presented by RNase II wt (6.9 ± 0.2 and 6.5 ± 0.4 nm, respectively). The results obtained indicate that the lysine rich tail from RNase R is also responsible for a higher affinity for some RNA substrates.

DISCUSSION

To understand which domains are responsible for the differences observed between RNase II and RNase R regarding the RNA degradation, we switched domains between them and analyzed the activity of the six hybrid proteins against single- and double-stranded substrates.

When we used a single-stranded substrate, we observed that the presence of the RNB domain from RNase R in RNase II changed the final product from 4 to a mixture of 4 and 2 nt [Fig. 2(d)]. This result clearly indicates that it is in the RNB domain of both proteins that resides the difference regarding the releasing of the final product after cleavage. However, when the RNB domain from RNase II was inserted into RNase R, the end-product was not altered to 4 nt as expected by the previous result [Fig. 2(g)]. One possible explanation for this could be the fact that the differences in both RNB domains can not be related with the amino acid sequence but with the conformation that the protein acquires when it is folded. In the RNase R_RNB_II protein, the presence of the CSD and S1 domains of RNase R can lead to a subtle conformational rearrangement of the catalytic cavity in the RNB domains of RNase II. To elucidate this question, we modelled both proteins and compared them with RNase II structure[27] and RNase R model[29] [Fig. 6(a)]. In fact, by comparing the four models, it is possible to see that all proteins share an almost identical structure. If we analyze in more detail the catalytic cavity of all proteins, no dramatic changes are observed (data not shown). So, it appears that the differences observed in the activity of these enzymes are not due to changes in the overall protein structure. A suitable explanation could be the different nature of the residues located in S1 domain in close contact to RNA [Fig. 6(b–d), purple residues]. In RNase II, the residues in S1 domain which are in close contact with the RNA molecule are Ser572, Gly574, Phe588, Pro590, Pro592, Phe593, Arg632, Thr635, and Ser637 as indicated in Figure 5B. In the protein RNase II_S1_R, the residues from the S1 domain from RNase R that contact the RNA molecule are different (Thr655, Phe657, Leu671, His673, Ser675, Ser676, Asn714, Glu717, Lys719), that implies differences in the properties of the relationship between RNA and S1 [Fig. 6(c)]. When compared with other proteins of the family, the nature of RNA-contacting residues is, as expected, more conserved among members of the same sub-family (RNase II sub-family versus RNase R sub-family) and, to some extent, different between these sub-families [Fig. 6(d)]. Moreover, when comparing the interactions between the RNB and the S1 domains of RNase II [Fig. 6(b)] and the RNB of RNase II with the S1 from RNase R [Fig. 6(c)], it is possible to observe differences regarding the residues involved in the contact of the S1 domain with the RNB. While in S1 domain from RNase II the residues in contact with the RNB domain are Arg577, Arg579, Asn583, Glu606, Lys619, Val620, and Thr621, in the RNase II_S1_R protein we can see that Phe660, Arg662,Leu666, Ile668, Gln689, Arg701, Leu702, and Gly703 are the ones involved in this interaction [Fig. 6(b–d)]. The residues responsible for the domain-domain interactions in the S1 domain are conserved between sub-families but not conserved in RNase II and RNase R [Fig. 6(d)]. The different nature of the residues in S1 domain that contact to the same residues in RNB domain (as this domain is conserved both in RNase II and RNase II_S1_R proteins) could be responsible for changes in the relative position of S1 domain from RNase in the overall structure of the RNase II_S1_R protein [Fig. 6(c)]. It could not be discarded that the alterations in the interactions of the RNB and S1 domains in this hybrid protein may induce a subtle conformational change in some residues near the catalytic cavity. These modifications may result in a higher affinity for RNA in the catalytic cavity, and a fraction of the 4 nt fragments can still bind to the protein and be cleaved until they reach the 2 nt of length.

When we determined the K_D values of the hybrid proteins we observed that with the poly(A) substrate the affinity was reduced when compared with the 25-mer. This indicates that, like for wild-type RNase II and RNase R, the hybrid proteins also reflect their marked preference for poly(A) type substrates.

Regarding the degradation of the double-stranded substrate, five of the six hybrid proteins tested were able to overcome the secondary structures and degrade the substrates totally. It was previously described that the RNB domain from RNase R is able, by itself, to degrade double-stranded substrates.[16,17] So, it was not a surprise to see that when we switched the RNB domain from RNase

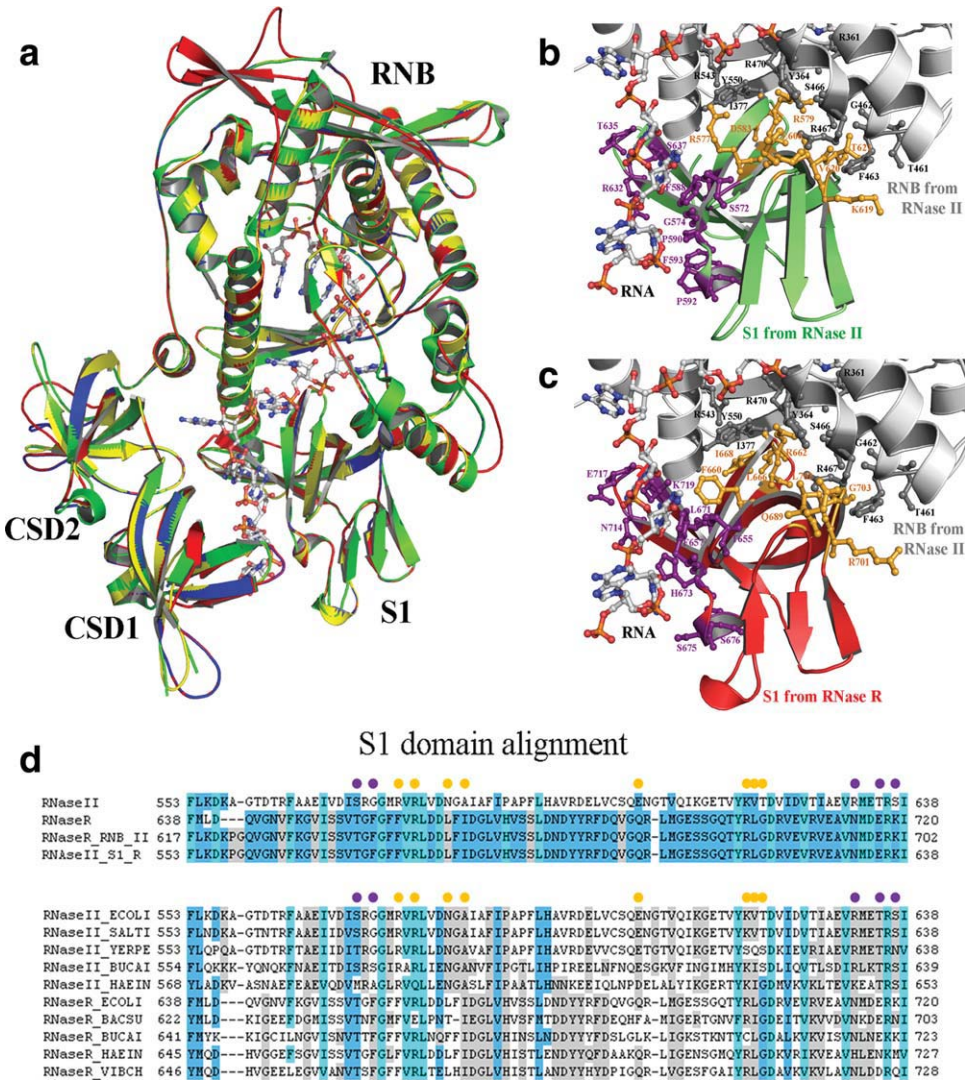

Figure 6

Modelling the hybrid proteins RNase II_S1_R and RNase R_RNB_II. **(a)** Representation of RNase II structure (green) and the predictive 3D models of the *E. coli* RNase R (red), RNase II_S1_R (yellow) and RNase R_RNB_II (blue) proteins **(b)** Residues of S1 domain from RNase II (green cartoon, residues in purple) in close contact to RNA: Ser572, Gly574, Phe588, Pro590, Pro592, Phe593, Arg632, Thr635 and Ser637; Residues of S1 domain from RNase II (residues in orange—Arg577, Arg579, Asn583, Glul606, Lys619, Val620 and Thr621) in close contact to RNB domain from RNase II (residues in dark grey—Arg361, Tyr364, Ile377, Thr461, Gly462, Phe463, Ser466, Arg467, Arg470, Arg543, and Tyr550). **(c)** Residues of S1 domain from RNase R (red cartoon, residues in purple) in close contact to RNA: Thr655, Phe657, Leu671, His673, Ser675, Ser676, Asn714, Glu717 and Lys719); Residues of S1 domain from RNase R (residues in orange—Phe660, Arg662, Leu666, Ile668, Gln689, Arg701, Leu702 and Gly703) in close contact to RNB domain from RNase II (residues in dark grey—Arg361, Tyr364, Ile377, Thr461, Gly462, Phe463, Ser466, Arg467, Arg470, Arg543, and Tyr550). **(d)** Top: structure alignment of the S1 domain of RNase II, RNase R and the constructed polypeptides RNase R_RNB_II and RNaseII_S1_R. Bottom: alignment of the S1 domain of RNase II and RNase R from different bacteria (*Escherichia coli* –ECOLI-, *Salmonella typhimurium* –SALTI-, *Yersinia pestis* –YERPE-, *Buchnera aphidicola* -BUCAI-, *Haemophilus influenzae* –HAEIN-, *Bacillus subtilis* –BACSU- *and Vibrio cholerae* –VIBCH-). Sequences from *E. coli* are included in both alignments for comparison purposes. Multiple alignments are coloured according to conservation. Position of some important residues is highlighted: purple dots—residues in S1 domain contacting RNA molecule; orange dots—residues in S1 domain contacting RNB domain.

II for the one from RNase R, the resultant protein was able to cleave double-stranded substrates. The same was valid for the hybrid proteins where the RNB of RNase R is present (RNase R_CSD_II and RNase R_S1_II). We also observed that the requirement of a single-stranded 3′ overhang to degrade dsRNA is a property resulting from the association of the RNB domain with the CSD and S1 RNA binding domains of RNase R.

The most intriguing result was obtained with the RNase II_S1_R and RNase R_RNB_II proteins. In both cases, the RNB from RNase R is not present and the proteins are still able to overcome secondary structures (Fig.

3). The common element between these proteins is the S1 domain from RNase R, thus this domain has to be the one responsible for the behaviour observed for these proteins. Moreover, the degradation of structured RNA molecules by the S1 domain implies the existence of a 3′-single-stranded overhang for cleavage to occur. In fact, in the C-terminal region of RNase R there is a lysine-rich region positioned after the S1 domain, and this feature is absent in S1 from RNase II (Fig. 1). Recently it was shown that the Lysine-rich region of RNase R is important for recruitment of stalled ribosomes and for the selection of defective transcripts to be degraded.[51] If we analyze other proteins from the RNase II-family of enzymes, we can see that this Lysine-rich tail is only present in RNase R-like proteins, which led us to hypothesize that it could be also involved in the degradation of structured RNAs. The results obtained confirm that, in an RNase II context, the lysine-rich region is important for the degradation of double-stranded substrates. Our results are in agreement with recent findings that show that RNase R could have some helicase activity which is conferred by the binding domains.[52] Our experiments indicate that the helicase activity could be of the responsibility of the S1 domain of RNase R, namely of the lysine-rich tail in the C-terminus of the protein, which can be responsible for the unwinding of the substrate. Moreover, we showed that this activity is intrinsic to RNase R, since this protein was able to degrade double-stranded substrates in the absence of ATP, in contrast to other helicases.

With this work we intended to unravel the different modes of action between the two major *E. coli* exoribonucleases, RNase II and RNase R, namely we aimed to explain their different behaviours. With the lack of the 3D structure of RNase R, only biochemical and modelling studies can help disclose the differences between these two homologue enzymes, belonging to the same RNase II family of proteins. In this report we show that both S1 and RNB domains from RNase R, in separate, allow the appearance of the characteristic 2 nt end-product and the degradation of double-stranded substrates. Finally, we demonstrated that the degradation of structured RNAs is tail-independent when the catalytic domain from RNase R is no longer associated with the RNA binding domains from RNase R.

As such, the results obtained in this report can be extrapolated for the comprehension of the mode of action of other members of the RNase II family. Moreover, this work represents a major breakthrough in the distinction between these two so close but yet so different exoribonucleases.

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