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Primary Structure of a Ribonuclease from Porcine Liver, a New Member of the Ribonuclease Superfamily

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ABSTRACT: In most tissues, ribonucleases (RNases) are found in a latent form complexed with ribonuclease inhibitor (RI). To examine whether these so-called cytoplasmic RNases belong to the same superfamily as pancreatic RNases, we have purified from porcine liver two such RNases (PL_1 and PL_3) and examined their primary structures. It was found that RNase PL_1 belonged to the same family as human RNase U_s [Beintema et al. (1988) *Biochemistry 27*, 4530-4538] and bovine RNase K_2 [Irie et al. (1988) *J. Biochem. (Tokyo) 104*, 289-296]. RNase PL_3 was found to be a hitherto structurally uncharacterized type of RNase. Its polypeptide chain of 119 amino acid residues was N-terminally blocked with pyroglutamic acid, and its sequence differed at 63 positions with that of the pancreatic enzyme. All residues important for catalysis and substrate binding have been conserved. Comparison of the primary structure of RNase PL_3 with that of its bovine counterpart (RNase BL_4; M. Irie, personal communication) revealed an unusual conservation for this class of enzymes; the 2 enzymes were identical at 112 positions. Moreover, comparison of the amino acid compositions of these RNases with that of a human colon carcinoma-derived RNase, RNase HT-29 [Shapiro et al. (1986) *Biochemistry 25*, 7255-7264], suggested that these three proteins are orthologous gene products. The structural characteristics of RNases PL_1 and PL_3 were typical of secreted RNases, and this observation questions the proposed cytoplasmic origin of these RI-associated enzymes.

Ribonucleases (RNases)[1] that degrade RNA endonucleolytically via formation of nucleoside 2′,3′-cyclic phosphates can be found in almost any tissue and body fluid of mammals (Sierakowska & Shugar, 1977). The most widely studied members of this type of enzyme are the pancreatic RNases [Smyth et al., 1963; for a review, see Blackburn and Moore (1982) and Beintema (1987)]. In addition, the structures of four types of nonpancreatic RNases have been determined; in the bovine species, these correspond to seminal RNase (Suzuki et al., 1976), kidney RNase K_2 (Irie et al., 1988),

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[1] Abbreviations: BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-bromo-3-methylindolenine; RI, ribonuclease inhibitor; RNase, ribonuclease; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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brain RNase Brb (Watanabe et al., 1988), and angiogenin (Maes et al., 1988). Comparison of the amino acid sequences of these enzymes has shown that they form a superfamily of related enzymes (Beintema et al., 1988b). The structures of some of the homologous human enzymes have also been determined (Strydom et al., 1986; Beintema et al., 1984, 1988a). Correlation of this structural information with the results of immunological (Morita et al., 1987; Sorrentino et al., 1988) and molecular biological studies (Palmieri et al., 1985; Bond & Vallee, 1988) has led to an understanding of the tissue distribution of these enzymes.

Some questions remain, however, that need clarification at the structural level. One of these is whether the so-called intracellular or cytoplasmic RNases (Roth, 1956, 1958, 1962; Kraft & Shortman, 1970; Blackburn & Moore, 1982; Kumagai et al., 1983; Brockdorff & Knowler, 1987) belong to the above-mentioned superfamily or to a different one. In nearly all tissues, but not in extracellular fluids, RNases are found in a latent form complexed with a protein inhibitor (RI). RI has been found in every tissue examined, and it is widely distributed in the animal kingdom (Kraft & Shortman, 1970; Blackburn & Moore, 1982). The primary structures of RI from porcine liver (Hofsteenge et al., 1988) and human placenta (Lee et al., 1988; Schneider et al., 1988) have recently been determined. Partly due to the very low levels of RNases in most tissues, little is known about the enzymes that are the target of RI. RI has been found mainly in the cytosolic fraction of the cell, and it has been inferred from this that the associated RNases are located in the same fraction (Roth, 1956, 1967; Kraft & Shortman, 1970; Kumagai et al., 1983; Brockdorff & Knowler, 1987). Brockdorff and Knowler (1987) have purified a number of these RNases from rat liver and uterus, and characterized them enzymatically. However, the possibility that both the RNases and RI could have redistributed during tissue disruption compromises the conclusion that these RNases are normally present in the cytoplasm.

In this paper, we present a structural analysis of two RNases from porcine liver that can be found as a complex with RI. Comparison of the N-terminal sequence of one of these enzymes showed it to belong to the same family of RNases as human RNase U_s (Beintema et al., 1988a) and bovine RNase K_2 (Irie et al., 1988). The complete sequence of the second RNase was determined, and it was found that it belonged to a hitherto structurally unknown member of the RNase superfamily. Independently, Irie and co-workers have sequenced the same enzyme from bovine liver (M. Irie, personal communication), and the two sequences showed an unusual degree of identity (only 7 replacements out of 119 residues). The structural properties of these liver RNases are typical of secreted proteins.

Materials and Methods

Enzyme Purification. Porcine liver RNases were purified by a modification of the method published by Brockdorff and Knowler, which was a modification of a method developed by H. G. Schleich and P. Blackburn [see personal communication in Brockdorff and Knowler (1987)]. The extraction of 1 kg of liver and the purification of the RNase–RI complexes by chromatography on DEAE-Sepharose as well as the dissociation of these complexes and the ion-exchange chromatography on SP-Sephadex were performed essentially as described by Brockdorff and Knowler (1987). The following minor modifications were made: 120 mM NaCl was substituted for sucrose in the extraction buffer, and a linear gradient of 20–700 mM NaCl was used to develop the DEAE column. The final purification of the porcine liver RNases was achieved

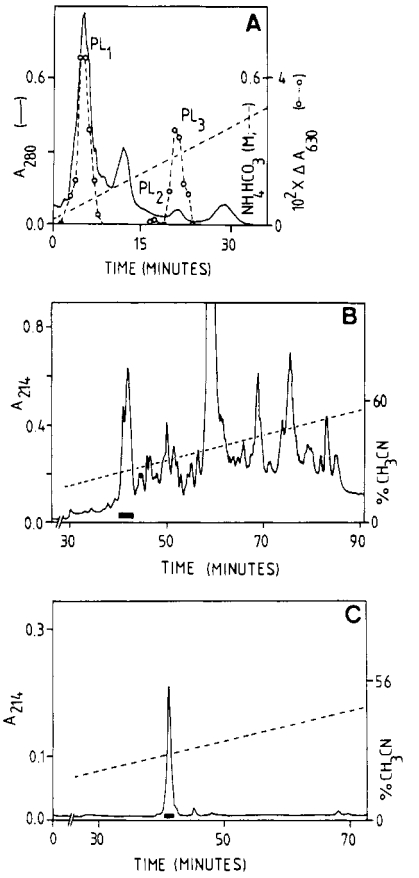

FIGURE 1: Purification of RNases PL_1 and PL_3. RNase inhibitor associated RNase activity was purified as described by Brockdorff and Knowler (1987) (see Materials and Methods). (A) Chromatography of the fraction from SP-Sephadex on a Mono S FPLC column. The column was equilibrated with 20 mM NH_4HCO_3, pH 8.5, and eluted with a gradient of 20–600 mM NH_4HCO_3 over 45 min at a flow rate of 1 mL/min: (—) A_{280}; (O---O) RNase activity. (B) Purification of RNase PL_1 by reversed-phase HPLC on a C_4 column. The column was equilibrated in 0.1 TFA and eluted with a linear gradient of 10.5–56% CH_3CN in the same solvent, at a flow rate of 1 mL/min. Fractions containing RNase activity are indicated by a bar. (C) RNase PL_3 was purified by reversed-phase HPLC as described for RNase PL_1.

by cation-exchange chromatography using a Mono S FPLC column, and reversed-phase HPLC chromatography on a C_4 column.

The eluate of the SP-Sephadex column was dialyzed against 20 mM NH_4HCO_3, pH 8.5, and loaded onto a Mono S FPLC column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. The elution was performed with a gradient of 20–600 mM NH_4HCO_3 over 45 min at a flow rate of 1 mL/min (Figure 1A). The three peaks containing RNase activity were pooled, lyophilized, and dissolved in a minimal volume of 0.1% TFA. RNases PL_1 and PL_3 were further purified by reversed-phase chromatography on a C_4 column (Vydac, Hisperia, CA, or Brownlee, Santa Clara, CA) using a linear gradient of 10.5–56% CH_3CN in 0.1% TFA over 90 min at a flow rate of 1 mL/min (Figure 1B,C). Throughout the purification procedure, the method of Shapira (1962) was used for the determination of RNase activity at pH 7.5, using pancreatic RNase A as a standard. Protein concentrations were determined by using the method of Bradford (1976). Electrophoresis on polyacrylamide–SDS gels followed by staining with Coomassie brilliant blue (Laemmli, 1970) was used to assess the purity of the preparations.

Fragmentation of RNase PL_3 and Purification of the Peptides. Removal of the N-terminal pyroglutaminyl residue

Table I: Purification of RNase PL_1 and RNase PL_3[a]

	total protein (mg)	act. (μg equiv of RNase A)	yield (%)
DEAE-Sepharose			
−pHMB treatment		0	
+pHMB treatment	6560	143	100
SP-Sephadex	4.2	114	80
FPLC Mono S			
RNase PL_1	6.0	50	35
RNase PL_3	ND[b]	38	27
reversed-phase HPLC			
RNase PL_1	ND	34	24
RNase PL_3	0.031[c]	36	25

[a] Purification from 1 kg of porcine liver. Homogenization of the tissue and purification of the RNase–RI complexes were performed essentially as described by Brockdorff and Kowler (1987) (see Materials and Methods). The RI–RNase complexes obtained from the DEAE-Sepharose column were dissociated by the use of *p*-(hydroxymercury)benzoate (pHMB), and the amount of activity obtained in this way has been set at 100%. Protein concentrations were determined by using the method of Bradford (1976) and RNase activity by the method of Shapira (1962). [b] Not determined due to insufficient quantities. [c] Determined by amino acid analysis.

from native RNase PL_3 with pyroglutamate aminopeptidase (Sigma, St. Louis, MO) was performed as described by Podell and Abraham (1978), and the RNase was repurified by reversed-phase HPLC as described above.

Reduction and carboxymethylation of the protein prior to tryptic cleavage were performed under the conditions of Crestfield et al. (1963) in a total volume of 50 μL. The reaction mixture was diluted to 1 mL with H_2O before digestion with trypsin as previously described (Hofsteenge et al., 1988). Reaction with 4-vinylpyridine after reduction with β-mercaptoethanol and performic acid oxidation were performed by published methods (Hermodson et al., 1973; Hirs, 1967), and excess reagents were removed by dialysis and lyophilization, respectively. The procedures used for the cleavages with CNBr and BNPS-skatole have been described previously (Hofsteenge et al., 1988). Cleavage with the endoproteinase Asp-N (4% w/w) (Boehringer, Mannheim, FRG) was done in 100 mM sodium phosphate buffer, pH 8, for 21 h at 37 °C (Drapeau, 1978). For each of the digests, approximately 30 μg of RNase PL_3 was used, except for the cleavage with BNPS-skatole where 15 μg was used.

The procedures for purification of peptides by reversed-phase HPLC using C_4 or C_{18} columns have been described in detail previously (Hofsteenge et al., 1988).

Amino Acid Analysis and Sequence Determination. Peptides and proteins were hydrolyzed in the vapor of 6 N HCl for 18 h at 110 °C, and the amino acid composition was determined with (dimethylamino)azobenzenesulfonyl chloride (Knecht & Chang, 1986). Amino acid sequencing using an Applied Biosystems Model 470A sequencer was performed as described previously (Hofsteenge, 1988).

Results

Purification and Characterization of Porcine Liver RNases. The scheme used for the purification of ribonucleases from rat liver (Brockdorff & Knowler, 1987) could also be applied to pig liver. However, it was observed that the use of heparin–Sepharose resulted in RNases that no longer bound to cation exchangers. This could have been due to leakage of heparin from the Sepharose column. Therefore, a modified purification scheme was used in which chromatography on heparin–Sepharose was replaced by ion-exchange chromatography using a Mono S FPLC column (see Materials and Methods). This step resulted in the fractionation of the RNase activity into two major peaks (Figure 1A: RNases PL_1 and PL_3), comparable to those observed on heparin–Sepharose in the case of rat liver (Brockdorff & Knowler, 1987). The final purification of RNase PL_1 and RNase PL_3 was achieved by using chromatography on a C_4 reversed-phase column. RNase PL_1 separated into an asymmetric peak with RNase activity (Figure 1B), whereas RNase PL_3 gave a single active peak (Figure 1C). The purification of these RNases has been summarized in Table I. RNase PL_2 was obtained in a low and variable yield, and no attempt was made to purify this protein further.

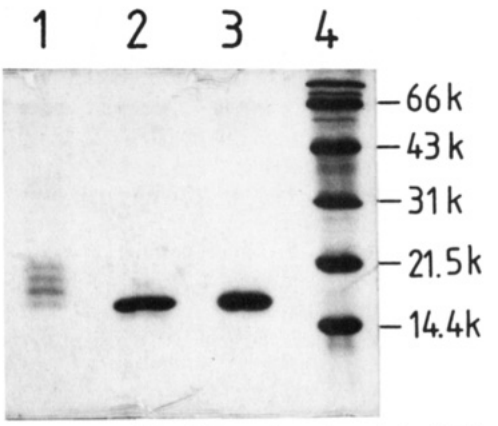

FIGURE 2: Polyacrylamide gel electrophoretic analysis of RNases PL_1 and PL_3. Lane 1, RNase PL_1; lane 2, RNase PL_3; lane 3, pancreatic RNase A; lane 4, standard proteins.

Table II: Determination of the N-Terminal Amino Acid Sequence of RNase PL_1

cycle	amino acid	pmol	cycle	amino acid	pmol
1	Ile	152	18	Pro	70
2	Pro	182	19	Ser	ND
3	Lys	148	20	Pro	46
4	Asn	26[c]	21	Leu	20
5	Leu	90	22	Gln	27
6	Thr	ND[a]	23	X[b]	
7	Arg	49	24	Asn	23
8	Ala	114	25	Lys	16
9	Gln	83	26	Ala	16
10	Trp	50	27	Met	5
11	Phe	71	28	Asn	23
12	Thr	ND	29	Gly	9
13	Ile	47	30	Val	6
14	Gln	60	31	Asn	6
15	His	23	32	X	
16	Ile	37	34	Tyr	ND
17	Gln	49	35	Thr	ND

[a] ND, not determined. [b] Unmodified RNase PL_1 was sequenced without modification of the half-cystines. [c] The relatively low yield of Asn-4 could be due to partial glycosylation, since it occurs in the recognition sequence Asn-Leu-Thr.

Analysis of RNase PL_1 by electrophoresis on an SDS–polyacrylamide gel showed a number of closely spaced bands (Figure 2, lane 1). However, N-terminal sequence analysis yielded only one sequence (Table II). The heterogeneity observed on SDS–polyacrylamide gels could be due to the presence of different amounts of carbohydrate or to differences in the amino acid sequences in the C-terminal part of the molecules. Asn-4 occurs in a carbohydrate attachment sequence (Asn-X-Thr/Ser), and the low yield of this residue during Edman degradation of RNase PL_1 could be due to partial glycosylation occurring at this position (Table II). Gel electrophoretic analysis of RNase PL_3 showed a single band with a mobility slightly lower than that of bovine pancreatic RNase A (Figure 2, lane 2). RNase PL_3, both unmodified and also reduced and carboxymethylated, was not susceptible

Table III: Amino Acid Composition of the Peptides Used To Prove the Amino Acid Sequence of RNase PL_3[a]

peptide: position:	CB1 1–4	T2 4–7	T3 8–10	T4 11–32	D4 25–38	D5 39–48	T6 42–54	T7 55–65	T8 66–82
Asx	0.9 (1)			5.1 (5)	1.3 (1)	0.8 (1)	3.2 (3)	1.0 (1)	2.5 (3)
Glx	1.0 (1)	1.1 (1)		2.1 (2)	1.2 (1)	0.8 (1)	1.0 (1)	0.9 (1)	2.0 (2)
Ser		0.1			0.7 (1)	0.2		2.7 (3)	0.2
Thr				1.5 (1)	1.0 (1)	0.9 (1)	1.1 (1)	1.1 (1)	1.3 (1)
Gly				2.4 (2)			0.2		2.9 (2)
Ala				1.9 (2)		0.6			
Arg	1.1 (1)	1.1 (1)	nd (1)	1.5 (2)	2.1 (2)	1.4 (1)	1.1 (1)		1.4 (1)
Pro				1.1 (1)					
Val				1.0 (1)					2.1 (3)[b]
Met	0.8[b] (1)	0.6 (1)		1.1 (1)	3.3 (3)	0.3			(1)
Ile				0.9		0.8 (1)	2.2 (3)[c]	1.8 (2)	
Leu			nd (1)	1.1 (1)	1.1 (1)				
Phe			nd (1)	0.9		1.5 (2)	1.8 (2)		
Cys				0.6 (1)	1.3 (1)	0.8 (1)		0.9 (2)	1.5 (2)
Lys					0.9 (1)	0.7 (1)		1.0 (1)	1.0 (1)
His				1.2 (1)	0.8 (1)	0.6 (1)	0.8 (1)		0.9 (1)
Tyr		0.8 (1)		0.6 (1)	1.0 (1)				
Trp						nd (1)			

peptide: position:	D10 80–91	T9 83–95	D11 92–106	D12 107–117	T10A 102–119	CB2 98–119
Asx	1.6 (2)	1.0 (1)		1.0 (1)	2.2 (2)	1.9 (2)
Glx	0.7 (1)	1.3 (1)		2.1 (2)	2.2 (2)	1.8 (2)
Ser	1.8 (2)	1.5 (2)	0.9 (1)			1.1 (1)
Thr	1.0 (1)	1.1 (1)	1.1 (1)			1.0 (1)
Gly	1.2 (1)	1.2 (1)		0.9 (1)	1.9 (1)	1.0 (1)
Ala	1.0 (1)	1.1 (1)	2.8 (3)	0.4	1.2 (1)	2.1 (2)
Arg	2.0 (2)	2.9 (3)	4.0 (4)		0.9 (1)	2.1 (2)
Pro	1.1 (1)	1.1 (1)		2.0 (2)	1.9 (2)	2.1 (2)
Val			1.4 (2)	1.9 (2)	3.1 (3)	2.8 (4)[c]
Met			0.7 (1)			
Ile			1.2 (1)		1.0 (1)	0.8 (1)
Leu					0.3	0.3
Phe				1.2 (1)	1.1 (1)	1.0 (1)
Cys	0.8 (1)	0.3 (1)	1.3 (1)	1.0 (1)	0.6 (1)	n.d (1)
Lys					1.1 (1)	0.9 (1)
His				0.8 (1)	0.9 (1)	0.7 (1)
Tyr		0.6 (1)	1.2 (1)			
Trp						

[a] Peptides were hydrolyzed for 24 h at 110 °C and analyzed as described under Materials and Methods. [b] Determined as homoserine + homoserine lactone. [c] These peptides contained pairs of hydrophobic amino acids.

to Edman degradation, suggesting the presence of a blocked N-terminal residue. The only blocked amino acid residue found in RNases has been pyroglutamic acid (Beintema et al., 1988b). Treatment of RNase PL_3 with pyroglutaminase yielded a protein that showed a single amino acid sequence, permitting the determination of the primary structure up to and including residue 24 (Figure 3).

Determination of the Primary Structure of RNase PL_3. The detailed proof of the primary structure of RNase PL_3 is shown in Figure 3. Tryptic digestion of the reduced and carboxymethylated protein and fractionation of the peptides by reversed-phase HPLC (see Materials and Methods) yielded a nearly complete set of peptides. The only peptide that was not recovered was the blocked polar tripeptide <Glu-Asp-Arg. Sequence analysis of the peptides showed that a number of them contained uncleaved lysyl and arginyl bonds (T8 and T9), indicating that the digestion had not proceeded to completion. In addition, peptides that contained one or more methionyl residues separated into several peaks due to oxidation of these residues. This was most likely caused by the presence of iodoacetic acid during the tryptic digestion. The majority of overlapping peptides was obtained from two digests by using the endoproteinase from *Pseudomonas fragi* that cleaves N terminally of aspartic and cysteic acid residues (Drapeau, 1978). The peptides D4 and D5, obtained from the performic acid oxidized protein, overlapped peptides T4, T5, and T6, whereas the digest of the protein modified with vinylpyridine yielded peptide D* which overlapped peptides T8(A), T9, and T10. The remainder of the peptides from these digests confirmed the amino acid sequence of the tryptic peptides.

In order to obtain an overlap for peptides T6, T7, and T8, the native protein was cleaved with BNPS-skatole. Edman degradation of the unfractionated digest yielded a single sequence corresponding to residues 52–80, which provided the overlap of these peptides.

To confirm the N- and C-termini of the polypeptide chain, protein modified with vinylpyridine was digested with CNBr and fractionated on a C_4 reversed-phase column. The amino acid composition of peptide CB1 (Table III) was in agreement with the presence of a pyroglutaminyl residue at the N-terminus of the molecule. Peptide CB2 terminated with the same sequence (-Asp-Lys) as did peptide T10, thus establishing the C-terminus of the molecule. The absence of homoserine in the amino acid composition of this peptide further confirmed it to be the C-terminal peptide (Table III). Amino acid analysis of peptides that prove the sequence presented in Figure 3 is given in Table III. Generally, a good agreement between the calculated and observed values was found.

The amino acid composition of RNase PL_3 calculated from the sequence was in good agreement with the one determined experimentally (Table IV), confirming the correctness of the sequence. Moreover, no obvious weaknesses occur in the presented structure (Figure 3), and each amino acid residue was determined at least twice.

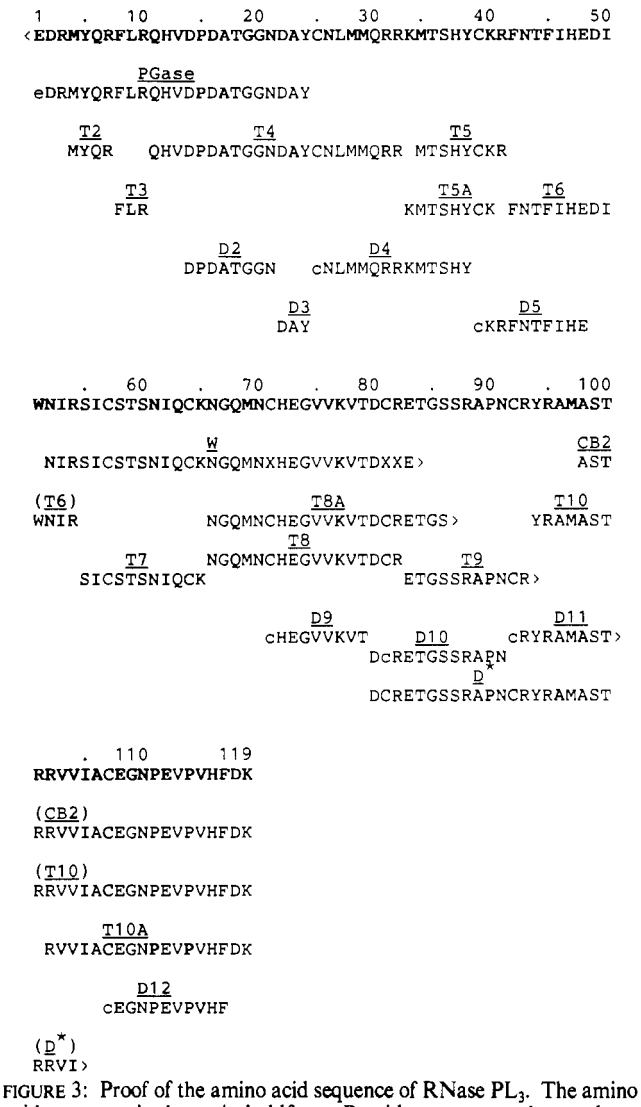

FIGURE 3: Proof of the amino acid sequence of RNase PL_3. The amino acid sequence is shown in boldface. Peptide names are shown above the sequences and are underlined. They are numbered according to their occurrence in the polypeptide chain. The following codes have been used: PGase, pyroglutamate aminopeptidase; T, trypsin; D, endoproteinase from *P. fragi*; CB, cyanogen bromide; W, BNPS-skatole. (>) indicates that the peptide has not been sequenced up to its C-terminus. Amino acid residues that have been inferred from the amino acid composition only are shown in lower case letters. "<E" indicates the N-terminal pyroglutaminyl residue.

Discussion

In homogenates of a variety of tissues, a significant fraction of the RI is in a complex with RNase(s) (Roth, 1956, 1958, 1962; Kraft & Shortman, 1970). Generally, these enzyme–inhibitor complexes behave heterogeneously during anion-exchange chromatography, and it has been shown that this is due to the presence of different RNases, rather than heterogeneity of the inhibitor (Blackburn & Moore, 1982; Brockdorff & Knowler, 1986, 1987). It has been assumed that, like RI, the RNases of these complexes are of cytoplasmic origin. Brockdorff and Knowler (1987) have found that in rat liver, the majority of RNase activity associated with RI consists of two distinct RNases. Using a very similar procedure, we have purified two RNases (PL_1 and PL_3) from porcine liver.

In Figure 4A, the N-terminal sequence of RNase PL_1 is compared with that of bovine RNase K_2 (Irie et al., 1988) and human RNase U_s (Beintema et al., 1988a). The high degree of identity between these three enzymes and the fact that the same additions and deletions must be made to obtain an optimal alignment with pancreatic RNase A show that they belong to the same family of RNases. Although this family has been termed "non-secretory" (Sierakowski & Shugar, 1977; Beintema et al., 1988a; Irie et al., 1988), immunological studies have shown that they occur in body fluids, as well as in many tissues (Morita et al., 1987). The conclusion that RNase PL_1 is the porcine counterpart of human RNase U_s is supported by the presence in human liver of an RNase that has an N-terminal sequence identical with RNase U_s (Sorrentino et al., 1988). Interestingly, this latter enzyme seems to be identical with a neutrophil-derived neurotoxin (Gleich et al., 1986; Beintema et al., 1988a). Previously, it has been pointed out that this family of RNases has structural characteristics typical of secreted or lysosomal enzymes (Beintema et al., 1988a,b; Sorrentino et al., 1988; Irie et al., 1988), i.e., (i) the positions of the half-cystinyl residues have been exactly conserved compared with bovine pancreatic RNase A, suggesting the formation of the same set of disulfide bridges; (ii) the occurrence of N-glycosidically linked carbohydrate. Both disulfide bridge formation and carbohydrate attachment take place on the luminal side of the endoplasmic reticulum (Freedman, 1984, 1989; Kornfeld & Kornfeld, 1985). These considerations suggest that RNase PL_1, although found in association with RI in liver extracts, is a secreted or lysosomal molecule, and unlikely to be located in the cytoplasm. The finding of a small amount of RNase K_2 and RNase U_s in serum by immunological methods would be in agreement with this interpretation (Morita et al., 1987; Sorrentino et al., 1988).

In Figure 4B, the sequence of RNase PL_3 has been compared with that of porcine and bovine pancreatic RNase (Jackson & Hirs, 1970; Phelan & Hirs, 1970; Smyth et al, 1963) and bovine liver RNase (RNase BL_4; M. Irie, personal communication). A difference matrix summarizing this comparison is shown in Figure 5. The amino acid sequences of the bovine and the porcine liver RNase were found to be very similar, differing in only 7 out of 119 positions. In comparison, 26 substitutions were found between the pancreatic enzymes from these two species. The seven substitutions occur in loops connecting elements of secondary structure, and/or can be considered to be of a conservative nature. Since the function of the liver RNases is unknown, it is difficult to

Table IV: Amino Acid Composition of Pig and Bovine Liver RNase and of the Human Colon Carcinoma (HT-29) Derived RNase

	RNase PL_3[a]	RNase BL_4[b]	RNase HT-29[c]
Asx	14.9 (16)	16	14.0
Glx	10.9 (11)	13	11.2
Ser	8.3 (7)	8	6.7
Thr	7.1 (7)	7	7.8
Gly	6.4 (6)	6	9.0
Ala	6.6 (6)	4	4.6
Arg	12.7 (13)	14	13.0
Pro	4.3 (4)	4	4.7
Val[d]	7.4 (8)	8	6.6
Met	6.2 (6)	5	4.3
Ile[d]	5.7 (6)	5	6.2
Leu[d]	2.1 (2)	3	3.3
Phe	4.1 (4)	4	3.9
Cys	5.8 (8)	8	8.0
Lys	4.7 (5)	5	5.2
His	4.6 (5)	5	5.5
Tyr	3.2 (3)	3	3.9
Trp	ND (1)	1	1.1
total	119	119	119

[a] Average values obtained from four different preparations. [b] Calculated from the sequence shown in Figure 4; personal communication from Dr. M. Irie. [c] Calculated from Shapiro and Vallee (1986) assuming a length of 119 amino acids for the polypeptide chain. [d] The values for these residues were obtained after 72-h hydrolysis.

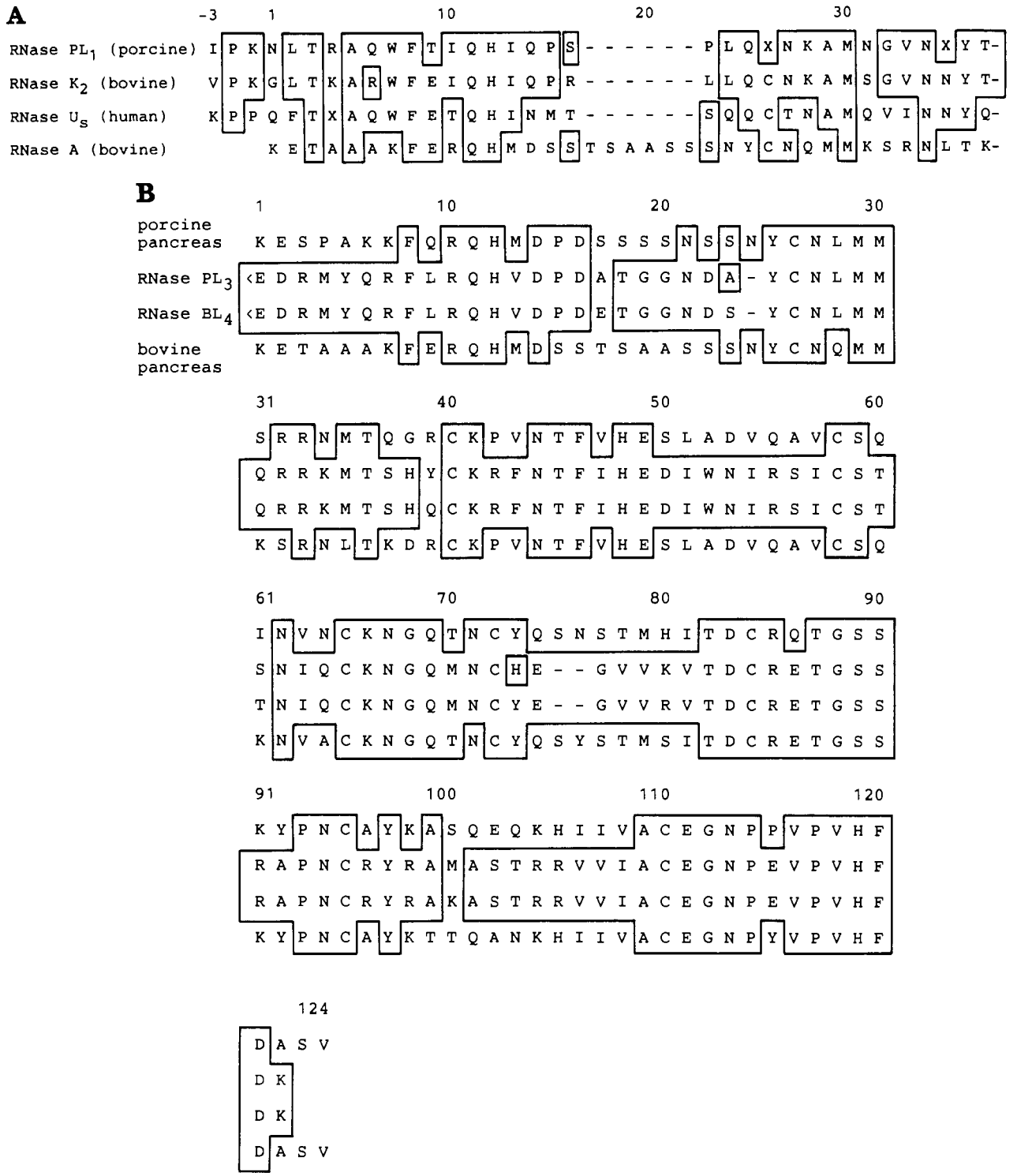

FIGURE 4: Sequence comparison of RNase PL_1 and RNase PL_3. (A) The N-terminal amino acid sequence of RNase PL_1 has been compared with those of bovine RNase K_2, human RNase U_S, and bovine pancreatic RNase A. The numbering scheme used is that of pancreatic RNase. (B) The complete amino acid sequence of RNase PL_3 is compared with that of porcine pancreatic RNase, bovine liver RNase BL_4 (M. Irie, personal communication), and bovine pancreatic RNase A. The numbering scheme used is that of pancreatic RNase.

	RNase PL_3	Porcine pancreas	RNase BL_4	Bovine pancreas
RNase PL_3	-	63	7	70
Porcine pancreas	-	-	61	26
RNase BL_4	-	-	-	67

FIGURE 5: Difference matrix of the sequences shown in Figure 4B.

explain this high degree of sequence conservation. Shapiro et al. (1986) have purified a pancreatic RNase-like activity which is secreted by human colon carcinoma cells (HT-29). The amino acid composition of this enzyme is strikingly similar to that of RNase BL_4 and RNase PL_3 (Table IV). In addition, RNase HT-29 has been found to be N-terminally blocked. Although its C-terminal residue has been reported to be glycine (Shapiro et al., 1986), these three RNases may well be orthologous gene products.

Optimal alignment of the pancreatic and liver enzymes required the introduction of small deletions, at positions 24 and 75–76. Moreover, the liver enzymes are two residues

shorter at the C-terminus. All eight half-cystines in RNase PL_3 and RNase BL_4 occur at exactly the same position as in pancreatic RNase, probably forming the same set of disulfide bridges (Spackman et al., 1960; Phela & Hirs, 1970). As with RNase PL_1, this suggests that RNase PL_3 is a secreted rather than a cytoplasmatic enzyme. This hypothesis is supported by the immunological and chromatographic evidence that RNase BL_4, the bovine counterpart to RNase PL_3 (Figure 4B), occurs both in liver and in serum (Morita et al., 1987).

RNase PL_3 and porcine pancreatic RNase differ at 63 positions (counting deletions as 1 difference). When the bovine enzymes are included, it becomes obvious that a large number of these substitutions occurs in six clusters (residues 1–7, 17–20, 37–39, 50–57, 74–81, and 100–109). A number of the substitutions are worth noting. (i) Porcine pancreatic RNase is a heavily glycosylated molecule, containing carbohydrate at Asn-21, Asn-34, and Asn-76. RNase PL_3 is free of carbohydrate due to mutation or deletion of the aspariginyl residues (positions 34 and 76) or mutation of the hydroxyl amino acid in the sequence Asn-X-Ser/Thr (position 23). (ii) The region 75–78 forms a bend between the β-strands 69–76 and 79–87 in bovine pancreatic RNase (Richards & Wyckoff, 1972). Ser-75 had been found to be invariant in all members of the superfamily (Beintema et al., 1988b). Its hydroxyl side chain is hydrogen bonded to CO-75 and NH-106 and contributes to maintenance of the three-dimensional structure of the enzyme. However, RNase PL_3 and RNase BL_4 contain a deletion in this region and thus are missing this residue that was thought to be essential. (iii) The role of Lys-66 in binding P_0, the 5′-phosphate on the ribose 5′ to the cleaved bond, has been pointed out previously (Iwahashi et al., 1981). In members of the superfamily lacking Lys-66, the presence of a positively charged residue at position 122 is thought to compensate for such a mutation [e.g., turtle pancreatic RNase and human RNase U_s (Beintema, 1987)]. The absence of both positively charged residues in human angiogenin has been suggested to account for its low ribonucleolytic activity (Beintema et al., 1988b). Interestingly, RNase PL_3 and RNase BL_4 contain lysyl residues at both positions. RNase K_2 was also found to contain two positively charged residues. (iv) From a comparison of the binding of RNases from different species to human placental RI, it was suggested that Tyr-92 was essential (Blackburn & Gavilanes, 1980). However, both RNase PL_3 and RNase BL_4 which bind RI have substituted Ala for Tyr-92.

Morita et al. (1987) have characterized seven RNases in the bovine species by chromatographic and immunological techniques. The sequences of both RNase PL_3 and RNase BL_4 differ at 60–80 positions with those of the other 5 structurally defined (bovine) RNases: pancreatic RNase (Smyth et al., 1963), seminal RNase (Suzuki et al., 1976), kidney RNase K_2 (Irie et al., 1988), brain RNase Brb (Watanabe et al., 1988), and angiogenin (Maes et al., 1988), showing that these liver enzymes form the sixth structurally defined member of the RNase superfamily. All structures can be considered to be typical of secreted proteins, a proposition that is confirmed in the case of pancreatic and brain RNase, and (human) angiogenin by the finding of a signal sequence in the mRNA (Carsana et al., 1988; Confalone et al., 1989; Kurachi et al., 1985). At present, it is unclear whether the superfamily contains still more members, and the physiological role of the majority of the enzymes remains to be established. An intriguiging hypothesis that suggests that these RNases play a role in the metabolism of short-lived extracellular RNA-containing messengers (Benner, 1988) has been put forward, but no direct experimental evidence bearing on this question is available.

The function of the cytoplasmic RI has to be established as well. This inhibitor binds very tightly to a number of RNases (Shapiro et al., 1986; Lee et al., 1989a,b; J. Hofsteenge and S. R. Stone, unpublished observations) and could be considered to act as a safeguard against RNases that are destined for secretion but inadvertedly appear in the cytoplasm. RNases PL_1 and PL_3 could be two such enzymes. Alternatively, RI could be involved in the regulation of RNA metabolism by binding to cytoplasmic RNases. At present, nothing is known about the identity of such RNAses.

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Registry No. Ribonuclease, 9001-99-4; ribonuclease PL_3 (pig liver reduced), 123205-67-4.

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NMR Studies of Arc Repressor Mutants: Proton Assignments, Secondary Structure, and Long-Range Contacts for the Thermostable Proline-8 → Leucine Variant of Arc†

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ABSTRACT: Arc repressor is a 53-residue sequence-specific DNA binding protein. We report the assignment of the proton NMR spectrum and the secondary structure for the thermostable PL8 variant of Arc. This mutant, which differs from wild type by a Pro-8 → Leu substitution, was chosen for study because its enhanced stability allows spectra to be acquired at elevated temperatures where spectral resolution is higher. The first five residues of the protein play important roles in DNA binding but appear to be disordered in solution. Residues 6–14 form the remaining part of the N-terminal DNA binding region of the protein and assume an antiparallel β-conformation. This indicates that Arc is a member of a new class of DNA binding proteins. The observed interresidue nuclear Overhauser effects are consistent with a β-strand, γ-turn, β-strand structure for the residue 6–14 region, although other structures are also consistent with the data. The remaining portion of the protein is predominantly α-helical. Residues 16–26 and 35–50 form amphipathic α-helices which may pack together in a four-helix bundle in the protein dimer.

Arc is a small DNA binding protein responsible in part for the regulation of lysogeny in the *Salmonella* phage P22 (Susskind, 1980). The 53 amino acid protein (Chart I) binds to its operator as a tetramer (Brown, unpublished results) but is dimeric in solution (Vershon et al., 1985). Studies of mutant proteins show that Arc uses residues at its N-terminal end to mediate most of its interactions with operator DNA (Vershon et al., 1986; Bowie & Sauer, 1989). Moreover, a chimeric protein containing the N-terminal nine amino acids of Arc

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