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Biochemistry of frog ribonucleases

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Abstract. Frogs have unique pyrimidine base-specific RNases, with structures similar to those of turtle, iguana and chicken RNases. Among the four frog RNases discussed here, three from *Rana pipiens*, *R. catesbeiana* and *R. japonica* oocyte cells show antitumour activity, and the latter two show lectin activity towards sialic acid-rich glycoproteins. In this review, (i) we compare their unique primary structures with respect to the locations of their disulphide bridges, three-dimensional structure, base specificity and heat stability as compared with RNase A, and (ii) we summarize current knowledge about the mode of action of lectin and the antitumour activities of the three frog RNases.

Key words. Frog RNase; lectin activity; antitumour activity; pyrimidine base-specific RNase; amphibian RNase.

Introduction

Most of pyrimidine base-specific RNases as typified by bovine pancreatic RNase A have four intramolecular disulphide bridges, and their locations are very well conserved. Since the discovery of turtle pancreatic RNase, which lacks one disulphide bridge [1], the primary structures of several RNases lacking one disulphide bridge of RNase A Cys65–Cys72, have been elucidated. They are angiogenin [2], chicken bone marrow RNase [3], an RNase derived from v-mys-transformed myeloma monocytic cells [4], chicken liver RNase [5], iguana pancreatic RNase [6] and four proteins (RNases) derived from frog liver and oocytes [7–10]. Among them, frog proteins conserved the three disulphide bridges among the four of RNase A, and have another novel one near their C termini. These structurally exceptional RNases lacking a disulphide linkage (Cys65–Cys72) might be temporarily designated as angiogenin/turtle/chicken/frog type RNases. These structurally unique RNases also showed highly characteristic enzymological, physical and physiological properties (angiogenesis, lectin activity and antitumour activity). In this review, we will summarize the unique nature of frog-derived RNases with respect to structure, enzymatic nature and physiological function in comparison with turtle and chicken RNases and angiogenin.

The biochemistry of this group of RNases was historically studied employing three different approaches. Beintema et al. elucidated the amino acid sequence of turtle pancreatic RNase in 1985 [1], and, subsequently, that of the iguana [6], from the viewpoint of comparative and evolutionary biochemistry. Levy et al. described the presence of cytosine base-specific RNase from chicken liver [11]. Miura et al. further purified this RNase [12], and later Hayano et al. elucidated the

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amino acid sequence of this RNase (RNase CL2) and showed it to be a pyrimidine-specific and cytosine-preferential RNase [5]. As for the frog RNases, Nagano et al. purified bullfrog (*Rana catesbeiana*) liver RNase and showed it to be a pyrimidine base-specific RNase [13, 14]. Roth [15] and Nagano et al. [13, 14] also detected the presence of an RNase inhibitor in frog tissues. The bullfrog RNase inhibitor does not inhibit mammalian RNases and vice versa, as frog RNases are insensitive to the mammalian RNase inhibitor [15]. Malica Blaszkiwicz also purified an RNase from *R. esculenta* [16]. Nitta et al. further purified the RNase from *R. catesbeiana* liver and elucidated its primary structure (RC liv.) [9]. Riordan et al. isolated a factor that has angiogenicity from human carcinoma cells and determined its amino acid sequence, which showed it to belong to the RNase A family [2]. The development of research on angiogenin was reviewed by Riordan [2].

On the other hand, Sakakibara et al. found proteins derived from bullfrog, *Rana catesbeiana* (cSBL), and Japanese frog (*R. japonica*) (jSBL) eggs that showed agglutination of tumour cells as well as lectin activity towards cells with sialic acid residues [17–19]. The primary structures of cSBL and jSBL were determined by Titani et al. [7] and Kamiya et al. [8], and their RNase activities were demonstrated by these investigators [20].

Quite independently, Shogen and his associates showed that a protein from *R. pipiens* oocyte cells (onconase; P30) had antitumour activity and was toxic to cells. They also determined its amino acid sequence [10]. The structure indicated that this protein is also a member of the RNase A superfamily [10].

Primary structure of frog RNases

The primary structure of a frog RNase, cSBL, was first recognized by Titani et al. [7] as that of a protein which is homologous with RNase A, and which confers lectin activity to it. Subsequently, the primary structures of three frog RNases, liver RNase from *R. catesbeiana* [9] (Rc liv.), a lectin from *R. japonica* (jSBL) [8] and onconase from *R. pipiens* [10] were elucidated. The structures of these proteins indicated that they are homologous proteins belonging to the RNase A superfamily of enzymes, based on their high homology with RNase A and having most of the amino acid residues involved in the catalysis of RNase A as well as some base recognition functions, particularly components of the B_1 base recognition site of RNase A (fig. 1). Frog RNases have six half-cystine residues (thus three disulphide bridges) at the same locations as in RNase A, but they lack one disulphide bridge connecting the 65th and 72nd positions (RNase A numbering). They have a fourth disulphide bridge (Cys107–Cys126), and one of the half-Cys residues (Cys126) is located near the C-terminus of each enzyme. The locations of the three conserved disulphide bridges are the same as those of chicken, turtle and iguana RNases. However, chicken, turtle and iguana RNases do not appear to have the novel disulphide bridge observed in frog RNases. As shown in figure 1, catalytic components of the P_1 site (His12, His119 and Lys41, RNase A numbering), are all conserved, while some amino acid residues which are thought to augment activity, though their roles have yet to be clarified, such as Asp121 and Gln11 are replaced.

Among the B_1 site components – Thr45, Phe120 and Ser123 – the first two are conserved in frog RNases. In the P_2 site, Lys7 is partly conserved. In the B_2 site, only His119 and Glu111 are conserved. The effects of some of the replacements on base specificity are discussed below. The disulphide bridge located near the C-terminus with the active site His119 (RNase A numbering) is probably responsible for stability and increased enzymatic activity at higher temperatures such as 60 °C (see below).

The most characteristic feature of the four frog RNases is a γ-pyroglutamyl residue at the N-terminus. Several highly characteristic biological properties, including enzyme activity, appear to be related to this γ-pyroglutamyl residue. Frog lectins and onconase are insensitive to the placental RNase inhibitor, and show cytotoxicity towards several cell lines. They also have lower enzymatic activities than RNase A. However, a recombinant onconase with an N-terminal Met instead of the γ-pyroglutamy residue showed decreased cytotoxicity and lower enzymic activity. In contrast, a recombinant onconase without this Met and with a cyclized γ-glutamyl residue again showed cytotoxicity and enzymatic activity [21]. Thus, the γ-pyroglutamyl residue appears to be important for these biological roles.

Cloning of cDNA of frog RNases

Complementary DNA (cDNA) encoding complementary sialic acid-binding lectin (cSBL) was cloned from a cDNA library prepared from bullfrog liver. The cDNA consisted of 853 base pairs containing 224 nucleotides of a 5′-flanking region, followed by a leader sequence for the signal peptide (22 amino acid residues) and a 333-nucleotide region coding mature cSBL, a 3′-flanking region and a poly(A) tail [22]. The amino acid sequence deduced from the cDNA was completely in agreement with that of protein sequencing [7]. Because the amino terminal of the cSBL protein was a γ-pyroglutamyl residue, processing of mature cSBL involves

cleavage of the signal peptide and further cyclization of newly formed Gln [22] (K. Nitta et al. unpublished observations). Expression of onconase in inclusion body of *Escherichia. coli* was performed with synthesized cDNA assembled by 12 oligonucleotides using *E. coli* biase [23].

Three-dimensional structures of onconase and cSBL

X-ray crystallographic data of onconase were presented by Mosimann et al. [24]. The three-dimensional structure of this protein, consisting of two sets of triple-stranded antiparallel β sheets and three α helices, is very similar to that of RNase A. The location of active-site amino acids, such as His12, His119, Lys41 and Thr45, are well conserved in onconase. Chen et al. [25] determined the secondary structure of cSBL by means of H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy and assigned the signal for each proton precisely. The results were in good agreement with the results of Mosimann et al. [24]. The position of the receptor-binding site (sialic acid-binding site) of cSBL remained uncertain, because the structures of homologous proteins, like human angiogenin and onconase, were determined to be non-liganded forms. Nonaka et al. [26] crystallized cSBL in the presence of 4 mM sialic acid at pH 7.6. X-ray diffraction data were collected up to 1.62 Å resolution [R − merge(I) = 5.23%, completeness = 89.4%, redundancy = 2.67]. The crystal structure of cSBL was solved by molecular displacement techniques using the structure of the onconase as the search model [24]. In the current refined model, it was found that two sialic acid molecules bind with cSBL (fig. 2). One sialic acid binds at a site very near to the active site, mainly by hydrogen bonds (site I), and the other near a loop consisting of amino acid residues 64 to 78 (RNase A numbering) via hydrophobic interactions (site II). The binding of the latter sialic acid was very weak as judged from the

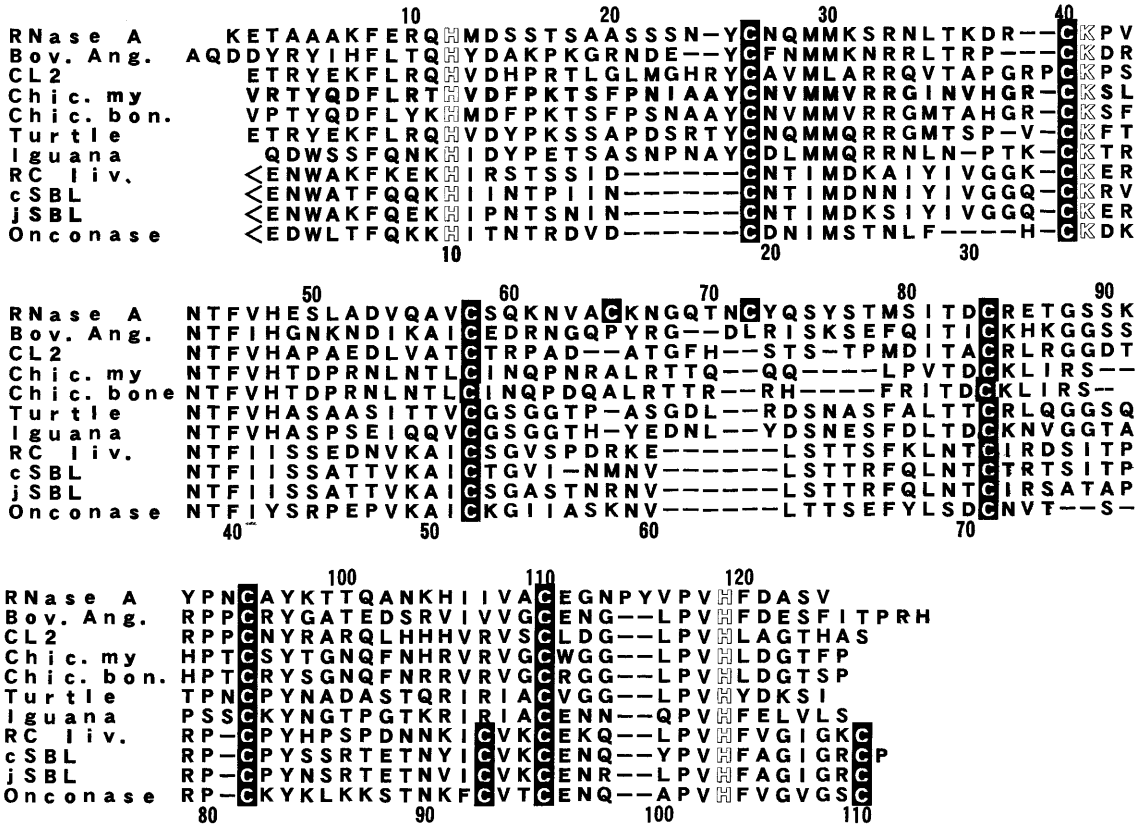

Figure 1. Amino acid sequences of four frog RNases, turtle, iguana and chicken RNases, and angiogenin. RNase A, bovine pancreatic RNase A; Bov. Ang., bovine angiogenin; CL2, chicken liver RNase: Chic. my, chicken RNase derived from v-*mys*-transformed myelomamonocytic cells; Chic. Bon., chicken bone marrow RNase; Turtle, turtle RNase; Iguana, iguana RNase; RC liv., RNase from bullfrog liver; cSBL, sialic acid-binding lectin from bullfrog oocytes; jSBL, sialic acid-binding lectin from Japanese frog; onconase, RNase from leopard frog oocytes. Amino acid residues essential for catalysis are denoted by white letters, and half-cystine residues by white letters with shading. Figures at the top and bottom of the matrix are the RNase A and cSBL numberings, respectively.

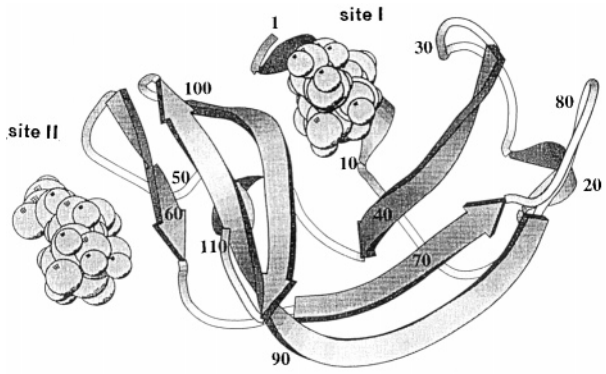

Figure 2. Ribbon diagram of the molecular conformation of cSBL protein-sialic acid complex. The sialic acid was denoted by shaded spheres.

temperature factor (88.6 Å^2) as compared with the former (41.0 Å^2). The weakly binding sialic acid was easily washed out by soaking in buffer without sialic acid. Comparisons of cSBL with onconase and RNase A are shown in figure 3. The main chain of cSBL is highly superimposable on that of onconase (fig. 3), and the main differences between cSBL and RNase A are observed at the N-terminal and four loops from residues 21–24, 31–39, 57–70, and 88–95. The third loop position comprises the sialic acid binding site II. Main differences between cSBL and onconase are the presence of two loops in cSBL from the 29–34 and 75–81 (cSBL numbering). A comparison between the conformations of the main chain and side chains of the active site of RNase A and cSBL is shown in figure 3c, showing the B_1 site (major binding site of RNase) constituents to be well conserved in their locations except for a slight skewing of the His119 (RNase A numbering) side chain due to binding with sialic acid. With regards to the B_2 site, Asn71 of RNase A was replaced by Lys95 in cSBL.

In onconase and egg lectins the γ-pyroglutamyl residue folds back against the N-terminal α helix and forms hydrogen bonds with Lys11 and Val118 (RNase A numbering) at the C-terminal, as well as with the phosphate group of the substrate [24, 26]. However, it is very interesting that we have no data excluding the possiblity of the binding of sialic acid with onconase except for some substitution of amino acid residues at positions 4, 6 and 10 (RNase A numbering). Thus, for receptor binding with cSBL, cooperation between these two binding sites or other forces may be necessary.

Base specificity of frog RNase in comparison to RNase A

The RNases discussed above are essentially pyrimidine base-specific RNases. However, detailed studies of substrate specificities have been performed on three frog RNases (RC liv., cSBL and jSBL) [27] and onconase [28], as well as on chicken RNase CL2 [5], turtle RNase [29] and iguana RNase [6]. Kinetic studies on the base specificities of three frog RNases (RC Liv., cSBL and jSBL) with eight dinucleoside phosphates showed that frog RNases are uracil-preferential RNases, since the rates of hydrolysis of the dinucleosdie phosphate UpX consistently exceeded that of CpX (X = A, G, U and C). Similar experiments on onconase demonstrated a similar uracil base preference [28]. Experiments on iguana RNase with UpA, CpA, uridylic acid (cUMP) and cytidylic acid (cCMP) indicated that iguana RNase is also a uracil-preferential enzyme [6]. Separate experiments performed for turtle and chicken RNases indicated that both are essentially cytosine base-preferential, like RNase A (C preference) [5, 29].

As regards the B_1 base recognition site of frog RNases, Thr45 is well conserved, and Phe120 is also conserved or replaced by Tyr (turtle) or Leu (chicken). The latter plays a role functionally similar to that of an aromatic ring in many RNases. However, Ser123 in RNase A, which is thought to be very important for pyrimidine base specificity via hydrogen bonding with a pyrimidine base, either directly or via a water molecule, is replaced by Val and Ile in frog and iguana RNases. Although we have no definite explanation on the uracil preference of frog RNases, such substitutions by hydrophobic side chains may perturb the hydrogen-bonding network for base recognition.

As regards the B_2 site (a base recognition site adjacent to the 3′ side of the B_1 site), in contrast to the case of RNase A (A preference) in kinetic studies Okabe et al. [27] showed this site to be guanine base-preferential in frog RNases, because the k_{cat}/K_m values of U(C)pG were remarkably higher than those of other base combinations, that is U(C)pX (X = U, C, A). Such results were also shown by Liao [30], employing hydrolysis of yeast 5S RNA and in other experiments on cSBL/egg RNase. The major sites of 5S RNA cleavage were UpG and CpG. An experiment with phosphorylated dinucleoside phosphate cleavage indicated that the presence of 5′-phosphates such as in pUpG or pCpG enhances the susceptibility of phosphodiester linkage in UpG and CpG approximately 10-fold. In RNase A, the 5′-side phosphate of pXpY is thought to bind with Lys66 [31]. In cSBL and onconase, we could not find any amino acid residue which occupied the position of Lys66 in RNase A, because the loop including Lys66 was absent in these frog RNases. In cSBL, Arg109 (cSBL numbering) is most closely located to the P_o position. However, even for this amino acid residue direct interaction with the 5′-phosphate of pUpX appears to be too difficult. X-ray crystallographic analysis of the cSBL-pUpX analogue complex might thus be necessary to identify the amino acid residue corresponding to Lys66 in cSBL.

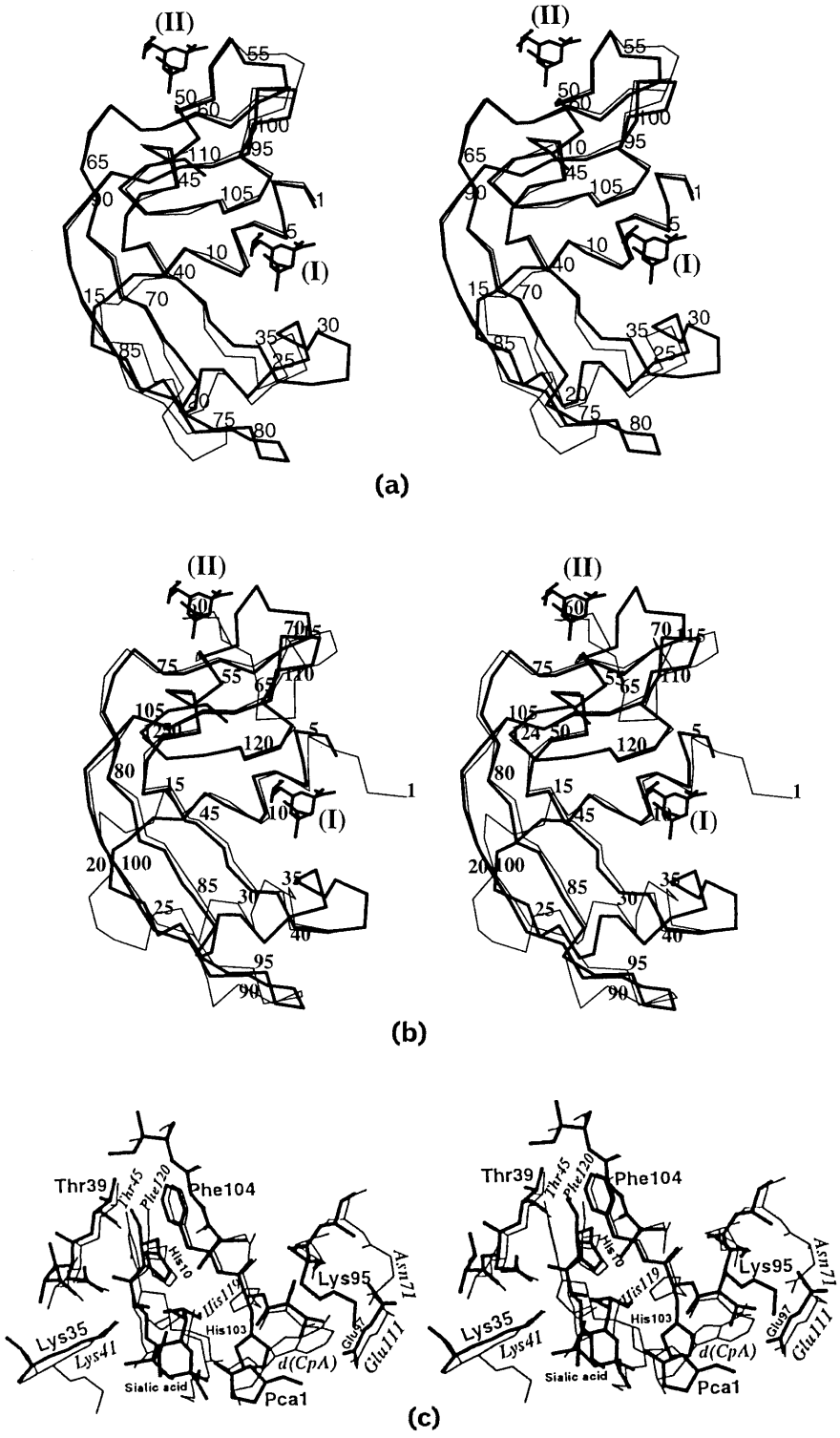

Figure 3. (*a*) Superposition of the cSBL-sialic acid complex (thick lines) and onconase (PDB-ID 10NC) (thin lines). Numbers represent the residue number of cSBL. (*b*) Superposition of the cSBL-sialic acid complex (thick lines) with RNase A (PDB-ID IRPG) (thin lines). Numbers represent the residue number of RNase A. (*c*) Superposition of the active site of the cSBL-sialic acid complex (thick lines) over that of the RNase-deoxycytidyl-3′,5′-deoxyadenosine (d(CpA)) complex (PDB-ID: 1RPG) (thin lines). Bold letters indicate amino acid residues and sialic acid of cSBL-sialic acid complex, and italics indicate the amino acid residue and d(CpA) of RNase-d(CpA) complex. (I) and (II) are sialic acid-binding sites.

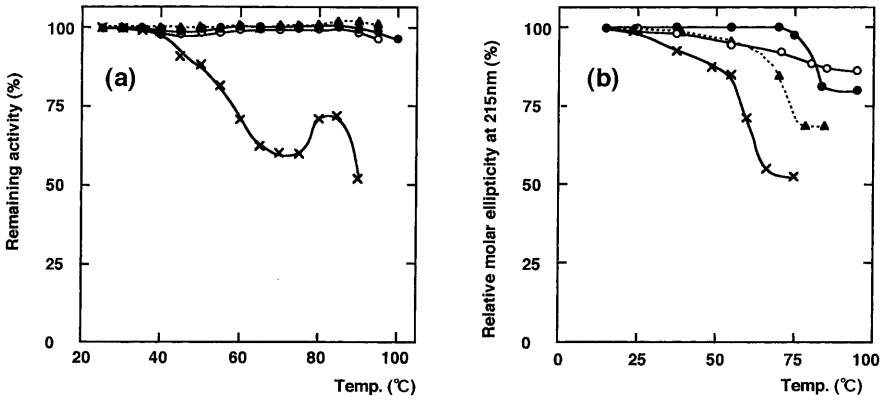

Figure 4. Heat stability of three frog RNases. (*a*) Samples of each protein were incubated in sodium acetate buffer (pH 6.0) for 5 min at various temperatures. The enzymatic activity of each sample was measured at pH 7.5 and 37 °C, and expressed as a percentage (25 °C = 100%). (*b*) Temperature dependence of CD spectra of three frog RNases at pH 7.5. Relative molar ellipticity expressed as a percentage (14 °C = 100%). ●, RC liv.; ○, cSBL; ▲, jSBL; ×, RNase A.

The mode of interaction at the B_2 site of frog RNases is not clear at present. Since His119 and Glu111 are well conserved in frog RNases, large differences in this site of RNase A and other frog RNases may be attributable to the shortening of a loop around Asn71. Shortening seems to be responsible for replacement of the role of Asn71 in the B_2 site of RNase by Lys95 (in frog RNases; residue 109 RNase A numbering) and Thr95 (in onconase) (fig. 3c). In terms of B_2 site preferences in chicken RNase CL2 [5] and turtle RNase [29], guanine is preferred rather than adenine, as is the case in RNase A. Glu111 is substituted for Leu (chicken) and Val (turtle) in these RNases. Therefore, shortening of the loop around Asn71 might at least in part be responsible for the guanine preference of frog RNases. The stacking of purine bases with His119 and hydrogen bonding with Glu111 might be very important factors determining the B_2 site preference. But these interactions might be perturbed by the shortening of this loop around Asn71. Further research is needed to resolve this problem.

Heat stability of frog RNases in comparison with RNase A

In the course of purifying pancreatic RNase A, we often use heating aimed at denaturing contaminating proteins, because RNase A is a very stable enzyme. Heat stability of frog RNases (RC Liv., cSBL and jSBL) was tested by heating for 5 min at pH 6.0 and at various temperatures. The remaining activity was then measured at pH 7.5 and 37 °C. The results are shown in figure 4 [27]. The activity of RNase A fell sharply above 50 °C, increased slightly from 75 to 80 °C, then decreased again. On the other hand, the three frog RNases were very stable up to 85 °C. To eliminate factors influencing reversible renaturation in the above experiment, circular dichroism (CD) spectra of three frog RNases were measured at pH 7.5 and various temperatures. $[\Theta]_{215\ nm}$ value-temperature profiles are shown in figure 4b. The $[\Theta]_{215\ nm}$ values of RNase A decreased gradually from 30 to 55 °C, then fell sharply above 50 °C. For jSBL, $[\Theta]_{215\ nm}$ decreases gradually from 25 to 60 °C, then more sharply above 60 °C. RC liv. was very stable up to 75 °C, then $[\Theta]_{215\ nm}$ decreased sharply. For cSBL, $[\Theta]_{215\ nm}$ diminished gradually from 20 to 90 °C without a sharp decrease. The final $[\Theta]_{215\ nm}$ value was more than 80% of the initial value. These results indicate that RC liv. and cSBL retain their native conformation up to 70 °C. It appears that frog RNases are more heat-stable than RNase A [27]. Similarly, the three frog RNases are more resistant to urea denaturation than RNase A. cSBL, RC liv., jSBL and RNase A retained their native conformations at 5, 3 and 1.5 M urea, respectively. Optimal temperatures for the activity measured were about 70 °C for both cSBL and RC liv. proteins, and those of jSBL and RNase A were slightly lower at 66 and 50 °C, respectively. The higher temperature optimum of frog RNases may due to the disulphide bridges near the C-terminus, because retaining the location of His119 and other residues in proximity to the active site is important for activity [27].

Physiological properties/enzymatic activity

Among the RNases belonging to angiogenin/turtle/chicken/frog-type RNases enzyme, angiogenin, onconase and frog egg RNases showed very unique physiological properties besides RNA degradation, such

as angiogenesis by angiogenin, antitumour activity by onconase and SBL, and lectin activity by SBL. Several observations provide evidence which shows that the enzymatic activity of individual enzyme is indispensable for its physiological activity.

Both bromoacetate and diethylpyrocarbonate, followed by hydroxylamine, eliminated >95% of the RNase activity of angiogenin [32, 33], and mutations of active-site residues Lys 40, His13 and His114 of angiogenin virtually abolished the enzymatic activity [34, 35], as occurs in other members of the RNase A superfamily. These changes also abolish the angiogenic activity of these mutants. Modification of ε-amino groups of lysyl residues of cSBL by acetylation, maleylation and carbamylation appear to destroy not only the tumour cell agglutination activity but also RNase activity [34, 35]. The ε-amino group of Lys, especially that of Lys35 of cSBL, may play a very important role in lectin activity as well as RNase activity [20]. Similarly, alkylated onconase retains only 2% of its enzymatic activity. Onconase inhibits cell proliferation and causes cell death [36, 37], but alkylated onconase was at least 100-fold less potent in inhibiting protein synthesis in 9L glioma cells. Thus, the toxicity of onconase is also dependent on RNase activity [38].

Lectin activity of frog RNase

The sialic acid-binding lectin, cSBL and jSBL, preferentially agglutinate a large variety of human and animal tumour cells, but not normal red blood cells, lymphocytes or fibroblasts [17, 18, 39]. This phenomenon correlated with a higher binding activity of SBLs to tumour cells. Tumour cell agglutination induced by SBLs was strongly inhibited by ganglioside, bovine submaxillary mucin, heparin and spermidine; to a lesser degree by fetuin and keratan sulphate; and not at all by less sialylated glycoproteins, such as transferrin [17–19]. Inhibition by sialomucin or fetuin was markedly reduced by desialylation by sialidase from *Athrobacter ureafaciens* [17, 40].

Treatment of tumour cells with sialidase greatly reduced cSBL-dependent agglutination, and the sialidase-dependent reduction of tumour cell agglutination was inhibited by the sialidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid [17]. Thus, cSBL-dependent tumour cell agglutination is due to a high density of sialic acid at the cell surface. The receptor glycoprotein that interacts with cCBL has been demonstrated in the Triton X-100-insoluble matrix-SDS extract fraction of a variety of tumour cells by SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blotting with cSBL and anti-cSBL antibodies [17]. Some cell lines, such as MKN-28, MKN-45 and Sarcoma 180, showed high molecular weight (180 kD) and low molecular weight (22 kD) receptor components reacting with cSBL. A 22-kD receptor component was presumed to be caveolin, the major structural component of caveolae [41].

Amino acid sequences of SBLs are homologous to those of onconase, RC liv., human angiogenin and RNase A as described above [2, 7–10]. SBLs showed not only lectin activity but also strong RNase activity with pyrimidine base specificity [8, 20, 27]. However, RNase A and RC liv. showed no lectin activity [20, 27]. 2′,(3′)-CMP and 2′,(3′)-UMP, very effective competitive inhibitors of RNase activity, were also effective inhibitors of cSBL-induced tumour cell agglutination [27]. These findings indicated that the site of agglutination of cSBL was, though not identical, related to the active site of frog RNase. RNase A and angiogenin are strongly inhibited by an RNase inhibitor (RNasin) from human placenta, whereas the RNase activity and tumour cell agglutination activity of cSBL are not affected by this inhibitor as already mentioned [20].

Antitumour activity of frog RNases

Among the four frog RNases, three (cSBL, jSBL and onconase) show antitumour activity. Antitumour activities common to these RNases include first binding with the tumour cell surface, internalization and cytotoxicity, probably due to RNA cleavage. However, the mechanism of cytotoxicity of cSBL and jSBL differs from that of onconase, because onconase cytotoxicity was not affected by sialic acid or other small sugar compounds (Y. N. Wu et al., unpublished data, cited in Youle and D'Alessio [42]), whereas those of SBLs were greatly reduced by sialylated glycoprotein such as sialomucin [43].

Antitumour activities of onconase, an egg protein of the leopard frog (*R. pipiens*) have been studied since the discovery by Darzynkiewicz et al. [37] that onconase inhibits cell division at the G1 phase at concentrations as low as 1 μg/ml. The results were briefly summarized in the review by Youle and D'Alessio [42].

Wu et al. [38] showed that onconase inhibited protein synthesis of 9L glioma cells with a 50% inhibitory concentration (IC_{50}) of about 10^{-7} M [38]. ^{125}I-labelled onconase binds to 9L glioma cell in a biphasic way with two different K_ds, 6×10^{-8} and 2.5×10^{-7} M. The low K_d value was very similar to the IC_{50} for onconase, and Wu et al. showed that onconase inhibition of protein synthesis in 9L glioma cells coincided with the degradation of 28S and 18S ribosomal RNA (rRNA). As suggested in early work of Roth [15], the cellular RNase inhibitor of the glioma cell did not work on onconase, and thus did not inhibit internalized onconase.

The most interesting property of this enzyme, in terms of its therapeutic potential, was that the cytotoxicity of onconase was affected synergistically with that of other reagents, such as tamoxifen, trifluoroperazine, lovastatin and retinoic acid [44, 45].

Both SBLs exhibited significant inhibition of solid tumour growth and ascites accumulation after inoculation of Ehrlich cells and sarcoma 180 cells. Thus, administration of SBLs prolonged the life spans of tumour-bearing mice [40]. SBLs inhibited protein synthesis in mouse leukaemia P388 and L1210 cells in vitro [40]. RNase A did not show these toxicities either in vitro and in vivo. Sialidase treatment eliminated the cytostatic effects of SBL. In addition to this phenomenon, P388 cells in the presence of benzyl-α-*N*-acetylgalactosamine (GalNAc) became resistant to cSBL. The results suggested that internalization of SBL is mediated by O-linked carbohydrate chain(s) of the sialoglycoprotein receptors, and that SBLs once internalized inhibit protein synthesis [43]. In support of this idea, ammonium chloride treatment of tumour cells suppressed the cytostatic effect of SBLs. However, this treatment had no effect on onconase [42]. Thus, internalization of SBLs via high-density sialoglycoprotein at the tumour cell surface may result in inhibition of tumour cell growth.

In order to investigate possible mechanisms underlying cytostatic or tumoricidal activity of cSBL, the cSBL-resistant P388 cell variant RC-150 was established [43]. On electron microscopic observation, the surfaces of P388 cells revealed that 90% of the cell population was microvilli-enriched and that 8% consisted of microplicae-dominant cells. In contrast to this finding, RC-150 cells were devoid of microvilli and microplicae. This difference in the surface structures of the two cell lines suggested that the RC-150 cell membrane is more stable than that of P388 cells. The doubling time, tumorigenicity, and lethality of RC-150 cells were similar to those of P388 cells.

cSBL agglutinated both P388 and RC-150 cells in the logarithmic growth phase rather than at confluence [43]. No difference was found between the sialidase-labile sialic acid levels in RC-150 and P388 cells. Although cSBL had no effect on RC-150 cell growth even at a concentration of 100 mM, the 50% inhibitory concentration for P388 cells was approximately 3.1–6.2 mM. Both cells were incubated with dansylcadaverine (DC)-labelled cSBL at 0 °C for 30 min and then washed and harvested for fluorescence analysis. In both cells, fluorescence was observed at the cell surface. After incubation with DC-labelled cSBL at 37 °C for 3 h, fluorescence was incorporated into P388 but not RC-150 cells. This indicated that RC-150 cells may have a defective internalization mechanism [43]. The lack of a cSBL effect on RC-150 cell growth could be due either to imperfection of cSBL endocytosis or to alterations in its mode of binding to the plasma membrane.

Furthermore, it is possible (i) that there is a defect in sialiylation of a specific glycoprotein that mediates endocytosis in RC-150 cells and (ii) that the sialylation of other glycoproteins is unaffected, resulting in residual lectin binding but no endocytosis [43].

The RC-150 cell line is an internalization-defective mutant possessing a qualitatively different receptor. Thus, the antitumour effect of cSBL involves two processes, binding (agglutination) and internalization of cSBL.

Wu et al. reported that onconase toxicity results from onconase binding to cell surface receptors, followed by internalization into the cytosol where it degrades rRNA, thereby inhibiting protein synthesis and causing cell death [38]. Although the mechanism of the toxic action of onconase is similar to that of cSBL, the onconase receptor is unknown. Because onconase toxicity was not inhibited by sialic acid or other simple sugars, and onconase kills not only untreated tumour cells but also the sialidase-treated cells [42], sialoglycoproteins at the tumour cell surface may be excluded from the onconase receptor candidate molecules.

We have set forth two working hypotheses regarding the mechanism of the antitumour activity of cSBL. Both hypotheses are schematically shown in figure 5. The first can be stated as follows. cSBL, once internalized, functions directly in RNA degradation. The RNA degradation affects cell proliferation, leading to cell death via inhibition of protein synthesis. cSBL-treated P388 cells showed extensive RNA degradation over the course of 1 h. In contrast, cSBL-treated RC-150 cells

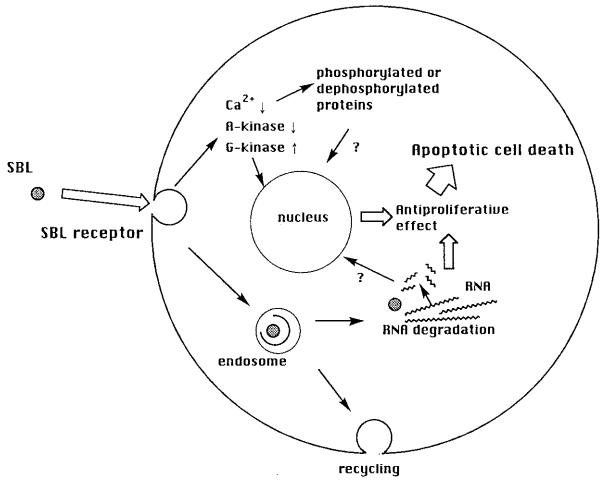

Figure 5. Proposed scheme for cytostatic or tumoricidal effect of SBL. Upper route: Change in signal transduction triggered by SBL binding to its receptor through protein kinase cascade. Lower route: Direct action of internalized SBL on RNA leading to inhibition of protein synthesis and induction of cell death.

showed no RNA degradation even over the course of 24 h [46]. Total RNA extracted from either P388 or RC-150 cells showed equal degradation when treated directly with cSBL. These results suggest that the cytostatic or tumoricidal activity of cSBL is due to its bifunctional nature, that is (i) binding to the cell surface glycoprotein receptor followed by (ii) internalization and RNA degrading.

The second hypothesis can be stated as follows. Binding of cSBL to the SBL receptor of tumour cells causes tumour cell agglutination, leading to alteration of the normal signalling pathway, consequently changing interacellular protein kinase cascade reactions, and thereby inhibiting cell growth. To determine whether cSBL affects growth stimulatory or inhibitory signals, we compared the intracellular Ca^{2+} concentration with protein kinase A, G and C activities. Treatment of P388 cells with cSBL leads to a decrease in the intracellular Ca^{2+} concentration and in protein kinase A activity, but increases protein kinase G activity [46]. Treatment of RC-150 cells with cSBL had no effect on any of these parameters. The decrease in intracellular Ca^{2+} in cSBL-treated P388 cells might be essential not only for protein kinase A inactivation but also protein kinase G activation and cell growth inhibitory signalling. Presumably, increases in guanylic acid (cGMP) enhanced Ca^{2+} pumping and influenced the protein kinase cascade reaction [47, 48].

Apoptosis is usually characterized by an early collapse of the nucleus, with extreme condensation of chromatin and loss of the nucleolus [49]. Apoptotic cells have an increased rate of RNA turnover, and it has been suggested that RNA degradation may play a role in the progression to cell death [50, 51]. When P388 cells were treated with cSBL for 24 h, not only RNA degradation but also DNA fragmentation were observed (K. Nitta, unpublished observations). The internalization of cSBL correlated with an increase in the rate of DNA fragmentation and the induction of apoptotic cell death. While P388 cells were tumour necrosis factor (TNF) receptor- and Fas-negative, treatment with cSBL increased expression of the TNF receptor and Fas antigen on the cells. Therefore, it is possible that SBL-induced cell death is involved in TNF receptor-mediated apoptosis.

cSBL is selectively internalized into tumour cells having the SBL receptor via receptor-mediated endocytosis and subsequently induces apoptotic cell death. In addition, the introduction of exogenous nucleases into tumour cells [52, 53], or activation of an endogenous enzyme in the cells, subsequently induces apoptotic cell death. The clarification of these mechanisms may allow the application of frog RNases to cancer therapy.

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