

Cytotoxicity of Bovine Seminal Ribonuclease: Monomer versus Dimer[†]J. Eugene Lee[‡] and Ronald T. Raines^{*,‡,§}

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ABSTRACT: Bovine seminal ribonuclease (BS-RNase) is a homologue of bovine pancreatic ribonuclease (RNase A). Unlike RNase A, BS-RNase has notable toxicity for human tumor cells. Wild-type BS-RNase is a homodimer linked by two intermolecular disulfide bonds. This quaternary structure endows BS-RNase with resistance to inhibition by the cytosolic ribonuclease inhibitor protein (RI), which binds tightly to RNase A and monomeric BS-RNase. Here, we report on the creation and analysis of monomeric variants of BS-RNase that evade RI but retain full enzymatic activity. The cytotoxic activity of these monomeric variants exceeds that of the wild-type dimer by up to 30-fold, indicating that the dimeric structure of BS-RNase is not required for cytotoxicity. Dimers of these monomeric variants are more cytotoxic than wild-type BS-RNase, suggesting that the cytotoxicity of the wild-type enzyme is limited by RI inhibition following dissociation of the dimer in the reducing environment of the cytosol. Finally, the cytotoxic activity of these dimers is less than that of the constituent monomers, indicating that their quaternary structure is a liability. These data provide new insight into structure–function relationships of BS-RNase. Moreover, BS-RNase monomers described herein are more toxic to human tumor cells than is any known variant or homologue of RNase A including Onconase, an amphibian homologue in phase III clinical trials for the treatment of unresectable malignant mesothelioma.

Bovine pancreatic ribonuclease (RNase A;¹ EC 3.1.27.5) has likely been the most studied enzyme (1, 2). Although RNase A is still a popular model system in studies of protein chemistry and enzymology, recent research on RNase A has focused on its variants and homologues that have remarkable biological activities and potential medicinal applications (3–6). For example, several homologues of RNase A are endowed with natural cytotoxic activity. Bovine seminal ribonuclease (BS-RNase) has been shown to kill tumor cells in vitro and in vivo (7, 8). Onconase (ONC), a frog homologue of RNase A, is in phase III clinical trials for the treatment of unresectable malignant mesothelioma, an asbestos-related lung cancer (9, 10). The ability of these ribonucleases to enter cells and cleave cellular RNA leads to apoptosis (11, 12).

BS-RNase has 83% sequence identity with RNase A (Figure 1A) but possesses a distinct quaternary structure. Unlike RNase A (Figure 1B), BS-RNase exists as a homodimer cross-linked by disulfide bonds between Cys31 of one subunit and Cys32 of the other (Figure 1C). BS-RNase exists as a mixture of two distinct forms, M = M and M ×

M. In the M = M form, the two subunits are linked only by the two intersubunit disulfide bonds, whereas in the M × M form, the two subunits share additional noncovalent interactions established by the swapping of their N-terminal α -helices.² At equilibrium, the M × M form exists in a slight molar excess (18, 19).

Ribonuclease inhibitor (RI) is a 50 kDa protein present in the cytosol of mammalian cells (20–22). The binding between RI and RNase A is among the tightest of known protein–protein interactions, having an equilibrium dissociation constant (K_d) near 10^{-14} M (23–25). This high affinity has likely evolved to protect cells from rogue ribonucleases (26). Hence, for a ribonuclease to be cytotoxic, it must evade RI. Monomers of BS-RNase do not evade RI and are not cytotoxic (19, 27). In contrast, dimeric BS-RNase is resistant to RI (28) and is cytotoxic (8, 29, 30).

The two intersubunit disulfide bonds of BS-RNase are unlikely to be stable in the reducing environment of the cytosol. As a consequence, it is possible that internalized BS-RNase dissociates into monomers, which are subject to inhibition by RI. Indeed, the M = M form is known to dissociate more rapidly than does the M × M form (19, 31), which benefits from the noncovalent interactions derived from domain swapping. Moreover, the M × M form is more cytotoxic than is the M = M form, suggesting that RI does mediate the cytotoxicity of BS-RNase.

ONC provides the benchmark for the cytotoxic activity of ribonucleases. Its extraordinary cytotoxicity is believed to originate, in large part, from its effective RI evasion [estimated $K_d \geq 10^{-6}$ M (32)]. BS-RNase is less cytotoxic

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¹ Abbreviations: BS-RNase, bovine seminal ribonuclease; DTT, dithiothreitol; 6-FAM, 6-carboxyfluorescein; IPTG, isopropyl 1-thio- β -D-galactopyranoside; ONC, Onconase (a registered trademark of Alfacell, Inc.); PBS, phosphate-buffered saline; PDB, Protein Data Bank; RI, ribonuclease inhibitor protein; RNase A, bovine pancreatic ribonuclease; 6-TAMRA, 6-carboxytetramethylrhodamine.

² The M × M form of BS-RNase has served as an archetype for the evolution of oligomeric proteins via domain swapping (17).

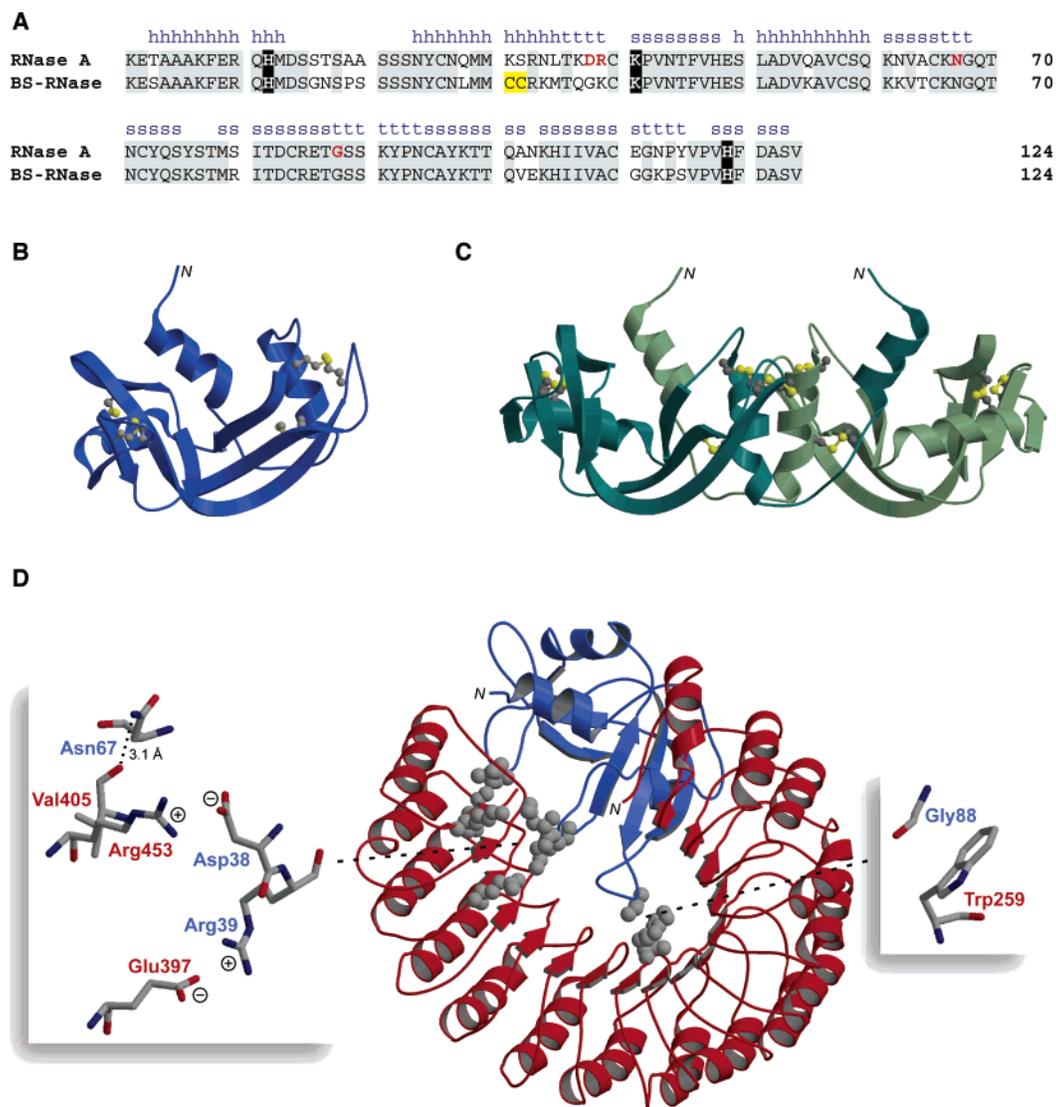


FIGURE 1: Amino acid sequences and three-dimensional structures of RNase A and BS-RNase. (A) Amino acid sequence alignment of RNase A and BS-RNase. The secondary structure of RNase A is identified with h (α -helix), s (β -strand), or t (turn). Residues conserved between the two ribonucleases are in gray boxes. Key active site residues are in black boxes. The two cysteine residues that participate in the intersubunit linkage of BS-RNase are in a yellow box. Residues near regions of high shape complementarity in the porcine RI•RNase A complex are in red. (B) Three-dimensional structure of RNase A [PDB entry 7RSA (13)]. (C) Three-dimensional structure of BS-RNase in its M \times M form [PDB entry 1BSR (14)]. (D) Three-dimensional structure of the porcine RI•RNase A complex [PDB entry 1DFJ (15)] highlighting regions disrupted herein. Images were created with the program MOLSCRIPT and rendered with the program RASTER3D (16).

than ONC, even though BS-RNase has greater ribonucleolytic activity than does ONC (33). Inhibition of BS-RNase by RI after reductive dissociation could be responsible for the lower cytotoxic activity of BS-RNase.

Here, we probe the effect of quaternary structure on the cytotoxicity of BS-RNase. Specifically, we ask if monomers of BS-RNase could be endowed with potent cytotoxic activity. Guided by the structure of the RI•RNase A complex, we create monomeric variants of BS-RNase that have markedly decreased affinity for RI but retain full enzymatic activity. We then use these variants to demonstrate that the dimeric form of BS-RNase is not only unnecessary for cytotoxicity but can actually be *detrimental* to cytotoxic activity.

EXPERIMENTAL PROCEDURES

Materials. The fluorogenic ribonuclease substrate 6-carboxyfluorescein-dArUdAdA-6-carboxytetramethylrhod-

amine (6-FAM-dArUdAdA-6-TAMRA) was from Integrated DNA Technology (Coralville, IA). [*methyl*- ^3H]Thymidine was from PerkinElmer Life Sciences (Boston, MA). Phosphate-buffered saline (PBS) contained (in 1.00 L) KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g), and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.16 g) and had pH 7.4. All other chemicals and reagents were of commercial grade or better and were used without further purification.

K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA). Cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

Instrumentation. Protein molecular mass was determined by matrix-assisted laser desorption ionization—time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE-PRO biospectrometry workstation (Applied Biosystems, Foster City, CA) and a 3,5-dimethoxy-4-hydroxycinnamic acid

(sinapinic acid) matrix (Sigma Chemical Co., St. Louis, MO). Fluorescence measurements were performed with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Radioactivity was quantitated with a Microbeta TriLux liquid scintillation and luminescence counter (PerkinElmer, Wellesley, MA).

Production of Wild-Type BS-RNase and Its Variants. Wild-type BS-RNase was produced in *Escherichia coli* strain BL21(DE3) under the direction of pSR1, which is a pET-17b-based vector that contains a synthetic gene for BS-RNase, as described previously (34, 35). DNA encoding variants of BS-RNase was made from pSR1 with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Oxidative folding of wild-type BS-RNase and its dimeric variants was performed as described previously (36, 37). Briefly, protein produced as inclusion bodies was solubilized by adding 0.020 M Tris-HCl buffer (pH 8.0) containing guanidine hydrochloride (7.0 M) and EDTA (0.01 M). DTT was added to a final concentration of 0.1 M, and the resulting mixture was stirred for 2 h at room temperature. The solution was dialyzed overnight against 20 mM acetic acid, and the insoluble material was removed by centrifugation. The protein was folded oxidatively in the presence of glutathione (0.6 mM oxidized, 3.0 mM reduced) in 0.10 M Tris-HCl buffer (pH 8.4) containing L-arginine (0.5 M). After 18 h, dimerization was achieved by reduction of mixed disulfides between glutathione and Cys31 and Cys32 by the addition of a 10-fold molar excess of DTT, followed by air oxidation overnight. Oxidative folding of monomeric variants of BS-RNase was carried out in the same manner as for the dimeric enzyme but without the dimerization process.

Production of Human RI. Human RI was produced according to methods described previously with minor changes (35, 38). Briefly, DNA encoding human RI was cloned into a pET-22b(+) vector between the *NdeI* and *SalI* sites with a Met(-1) sequence. The resulting vector was used to transform cells of *E. coli* strain BL21(DE3). Transformed cells were grown to mid-log phase at 37 °C; expression of the hRI cDNA was induced by the addition of IPTG (to 0.5 mM), and the induced cells were grown overnight at 20 °C. Cells were then lysed with a French pressure cell, and insoluble material was removed by centrifugation. RI was purified using RNase A-affinity chromatography followed by anion-exchange chromatography. Purified RI was stored in PBS containing DTT (5 mM) to prevent oxidation. The molar concentration of active RI was determined by titration against a known concentration of RNase A (39), using 6-FAM-dArUdAdA-6-TAMRA as a substrate (vide infra).

Assays of Ribonucleolytic Activity. Ribonucleolytic activity was measured with a hypersensitive assay based on the cessation of fluorescence quenching (40). Briefly, the increase of fluorescence at 515 nm was measured upon adding enzyme to PBS containing 6-FAM-dArUdAdA-6-TAMRA (50 nM) at 23 ± 2 °C. Values for k_{cat}/K_M were calculated with the equation:

$$k_{\text{cat}}/K_M = \left(\frac{\Delta F/\Delta t}{F_{\text{max}} - F_0} \right) \frac{1}{[E]} \quad (1)$$

where $\Delta F/\Delta t$ is the initial slope of the reaction, F_0 is the initial fluorescence intensity, F_{max} is the fluorescence intensity

once the reaction is brought to completion, and $[E]$ is the concentration of the enzyme.

Assays of Binding to Human RI. The ability of monomeric variants of BS-RNase to bind to human RI was assessed by a competition assay reported previously with a minor modification (41). The assay was done in PBS containing DTT (5 mM) to prevent oxidation by air. First, the initial fluorescence (excitation, 491 nm; emission, 511 nm) of the fluorescein-labeled G88R RNase A (final concentration, 50 nM; K_d for the human RI•G88R RNase A complex, 0.54 nM) was measured. Next, human RI was added (to 50 nM), and the decrease of the fluorescence evoked by RI binding was measured after a 15 min equilibration. Finally, varying concentrations of a BS-RNase were added to compete with the fluorescein-labeled enzyme, and the increased fluorescence due to the dissociation of fluorescein-labeled enzyme from RI was measured after each addition and equilibration. The concentration of the remaining human RI-fluorescein-labeled G88R RNase A was calculated by comparing the fluorescence to the initial fluorescence. Values of K_d were obtained by a nonlinear least-squares analysis of the binding isotherm with the program DELTAGRAPH 5.5 (Red Rock Software, Salt Lake City, UT).

Assays of Cytotoxic Activity. The effect of wild-type BS-RNase, its variants, ONC, and RNase A on cell proliferation was determined by measuring the incorporation of [*methyl*-³H]thymidine into cellular DNA (26, 42, 43). K-562 cells were grown in RPMI 1640 medium (44) containing fetal bovine serum (10% v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cytotoxicity assays were performed using asynchronous log-phase cultures grown at 37 °C in a humidified incubator containing CO₂(g) (5% v/v). To assay toxicity, cells (95 µL of a solution of 5 × 10⁴ cells/mL) were incubated with a 5 µL solution of a ribonuclease or PBS in the 96-well plates. Cells were then grown for 44 h, and cell proliferation was monitored with a 4 h pulse of [*methyl*-³H]thymidine (0.25 µCi/well). Cells were harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Watertown, MA). Filters were washed with water and dried with methanol, and their ³H content was quantitated by liquid scintillation counting. Cytotoxicity data were analyzed with the programs SIGMAPLOT (SPSS Science, Chicago, IL) and DELTAGRAPH 5.5. Each data point represents the mean (±SE) of at least three experiments performed in triplicate. The IC₅₀ value of each variant was determined with the equation (45):

$$S = \frac{IC_{50}}{IC_{50} + [\text{ribonuclease}]} \times 100 \quad (2)$$

where S is the percent of total DNA synthesis during the 4 h pulse.

RESULTS

Rational Design of RI-Evading BS-RNase Variants. A recent analysis of the RI•RNase A complex revealed several regions of high shape complementarity between RI and RNase A (46), three of which were of interest to us here (Figure 1D). Gly88 and Asn67 of RNase A are proximal to Trp259 and Val405, respectively; Asp38 and Arg39 of RNase A form favorable Coulombic interactions with Arg453 and

Table 1: Properties of Wild-Type BS-RNase and Its Dimeric and Monomeric Variants

BS-RNase	$\Delta Z/\text{molecule}$	k_{cat}/K_M ($10^7 \text{ M}^{-1} \text{ s}^{-1}$) ^a	K_d (nM) ^b	IC_{50} (μM) ^c	m/z ^g	
					expected	observed
wild type	0	3.5 ± 0.3	$> 2 \times 10^3$ ^d	1.3 ± 0.1	27464	27472
G88R	+2	3.5 ± 0.1	ND ^e	0.28 ± 0.06	27662	27674
G38K/K39G/G88R	+2	3.4 ± 0.2	ND ^e	0.091 ± 0.02	27662	27671
G38R/K39D/G88R	0	4.1 ± 1.0	ND ^e	0.26 ± 0.04	27834	27850
C31A/C32A	0	1.9 ± 0.2	$(9.3 \pm 3.2) \times 10^{-3}$ ^f	>50	13660	13668
C31A/C32A/G88R	+1	2.3 ± 0.1	2.3 ± 0.6	0.11 ± 0.02	13769	13762
C31A/C32A/G38K/K39G/G88R	+1	1.7 ± 0.03	$(1.0 \pm 0.4) \times 10^2$	0.046 ± 0.009	13769	13763
C31A/C32A/G38R/K39D/G88R	0	1.4 ± 0.1	$(1.1 \pm 0.4) \times 10^2$	0.07 ± 0.01	13855	13864
C31A/C32A/G38K/K39G/N67R/G88R	+2	2.1 ± 0.2	$(9.3 \pm 1.1) \times 10^1$	0.048 ± 0.01	13811	13819

^a Values of k_{cat}/K_M (\pm SE) are for catalysis of 6-FAM-dArUdAdA-6-TAMRA cleavage at 23 ± 2 °C in PBS (pH 7.4). ^b Values of K_d (\pm SE) are for the complex with human RI at 23 ± 2 °C. ^c Values of IC_{50} (\pm SE) are for incorporation of [*methyl*-³H]thymidine into the DNA of K-562 cells exposed to a BS-RNase and were calculated with eq 2. The IC_{50} value for ONC is 0.2 ± 0.1 μM . ^d From ref 49. ^e ND, not determined. ^f For C31K/C32S BS-RNase (49). ^g Values of m/z were determined by MALDI-TOF mass spectrometry.

Glu397 of RI. Without any detailed structural information of the complex with monomeric BS-RNase, we reasoned that RNase A can serve as a good model for monomeric BS-RNase because of its high sequence identity (83%) and similar three-dimensional structure (47).

Our design strategy was as follows. First, we prepared C31A/C32A/G88R BS-RNase. Replacing the two cysteine residues with alanine excises two sulfur atoms and thereby prevents dimer formation. As in G88R RNase A (35), the bulky arginine residue at position 88 should create a steric clash with RI residues (Figure 1D). Substitutions in this region are known to diminish the affinity of RNase A (35, 46, 48), BS-RNase (49), and RNase 1 [which is a human homologue (50)] for RI. Next, we attempted to disrupt a favorable Coulombic interaction between RI and C31A/C32A/G88R BS-RNase by swapping residues 38 and 39. In RNase A, swapping Asp38 and Arg39 converts two attractive interactions into two repulsive ones (46). The corresponding residues in BS-RNase are Gly38 and Lys39. Accordingly, two distinct sets of substitutions were introduced in this region. We employed the strategy validated with D38R/R39D RNase A (46), incorporating arginine and aspartate residues at the corresponding positions in BS-RNase to create C31A/C32A/G38R/K39D/G88R BS-RNase. In addition, we swapped the endogenous BS-RNase residues, Gly38 and Lys39, to create C31A/C32A/G38K/K39G/G88R BS-RNase. Then, we replaced Asn67 with an arginine residue in the C31A/C32A/G38K/K39G/G88R variant. This substitution is known to generate an additional steric clash in the RNase A•RI (46). Finally, we generated dimers of these variants by reinstalling Cys31 and Cys32. It is noteworthy that none of the altered residues are known to contribute to the catalysis of RNA cleavage by BS-RNase.

Protein Production. Wild-type BS-RNase and its variants were produced in *E. coli* to yield ≥ 20 mg of purified enzyme/L of culture. Purified enzymes appeared as a single band after electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate (data not shown), and each had the expected mass to within 0.065% according to MALDI-TOF mass spectrometry (Table 1). Wild-type BS-RNase and its dimeric variants ran in the gel as a dimer in the absence of a reducing agent and as a monomer if boiled in reducing sample buffer (data not shown). This result indicates that the quaternary structure of the dimers is maintained by disulfide bonds.

Human RI was produced in *E. coli* to yield 5 mg of purified protein/L of culture.

Ribonucleolytic Activity. Each monomeric variant of BS-RNase had ribonucleolytic activity within 30% of that of C31A/C32A BS-RNase (Table 1). Each dimeric variant had ribonucleolytic activity within 20% of that of wild-type BS-RNase. Thus, none of the substitutions had an adverse affect on the ability of BS-RNase to catalyze RNA cleavage. The dimeric enzymes had approximately 2-fold greater ribonucleolytic activity on a molar basis than did the monomeric enzymes, reflecting their two independent active sites (14, 51).

Affinity for Human RI. The C31A/C32A/G88R substitution decreased the affinity of monomeric BS-RNase for RI by 250-fold. The C31A/C32A/G38K/K39G/G88R, C31A/C32A/G38R/K39D/G88R, and C31A/C32A/G38K/K39G/N67R/G88R substitutions each decreased the affinity for RI by 10^4 -fold. Our competition assay does not provide an accurate measure of the affinity of the dimeric BS-RNases (too low) and C31A/C32A BS-RNase (too high) for RI (41). The dimeric BS-RNases are likely to form a complex with $K_d > 2$ μM^3 (41), as reported previously for wild-type BS-RNase (49). Similarly, C31A/C32A BS-RNase is likely to form a complex with K_d near 9.3 ± 3.2 pM, as reported for C31K/C32S BS-RNase (49).

Cytotoxic Activity. Each monomeric variant of BS-RNase was toxic to cultured K-562 cells (Figure 2). C31A/C32A/G88R BS-RNase had an IC_{50} value of 0.11 ± 0.02 μM (Table 1). C31A/C32A/G38R/K39D/G88R BS-RNase had an IC_{50} value of 0.07 ± 0.01 μM . The C31A/C32A/G38K/K39G/G88R and C31A/C32A/G38K/K39G/N67R/G88R variants were more cytotoxic, having IC_{50} values of 0.046 ± 0.009 and 0.048 ± 0.01 μM , respectively. In contrast, C31A/C32A BS-RNase had an IC_{50} value of > 50 μM . ONC had an IC_{50} value of 0.2 ± 0.1 μM (Figure 2), which is similar to values reported previously (26, 42, 43).

All three dimeric BS-RNase variants were more cytotoxic than wild-type BS-RNase, which had an IC_{50} value of 1.3 ± 0.1 μM . G88R BS-RNase and G38R/K39D/G88R BS-RNase had 4-fold greater cytotoxic activity than did the wild-

³ Values of K_i in ref 49 were determined by assaying the inhibition of ribonucleolytic activity by RI. Such values are equivalent to values of K_d for the RI•RNase A complex determined by the competition assay used herein.

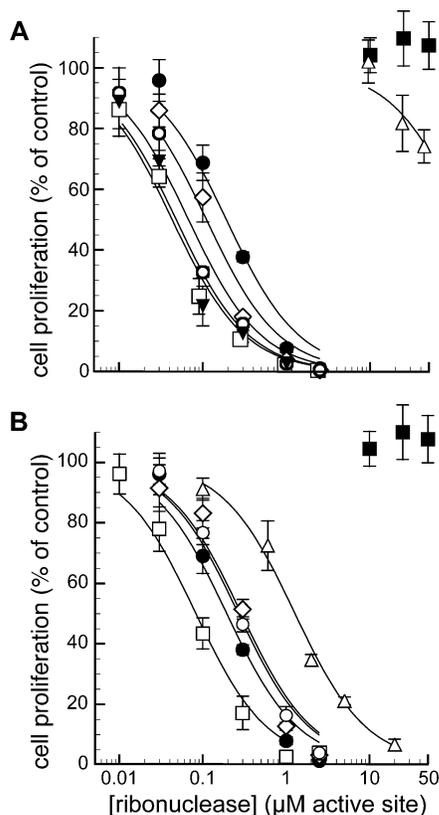


FIGURE 2: Effect of BS-RNase, its variants, RNase A, and ONC on the proliferation of cultured K-562 cells. Cell proliferation was determined by incorporation of [*methyl*-³H]thymidine into cellular DNA after a 44 h incubation with a ribonuclease. Each value is the mean (\pm SE) of three or more independent experiments with triplicate samples and is expressed as a percentage of the PBS control. Enzyme concentration is expressed in terms of micromolar active site to account for the two active sites in dimeric BS-RNases. (A) Monomeric BS-RNases: (Δ) C31A/C32A BS-RNase; (\diamond) C31A/C32A/G88R BS-RNase; (\square) C31A/C32A/G38K/K39G/G88R BS-RNase; (\circ) C31A/C32A/G38R/K39D/G88R BS-RNase; (\blacktriangledown) C31A/C32A/G38K/K39G/N67R/G88R BS-RNase; (\blacksquare) RNase A; (\bullet) ONC. (B) Dimeric BS-RNases: (Δ) wild-type BS-RNase; (\diamond) G88R BS-RNase; (\square) G38K/K39G/G88R BS-RNase; (\circ) G38R/K39D/G88R BS-RNase; (\blacksquare) RNase A; (\bullet) ONC.

type enzyme, and G38K/K39G/G88R BS-RNase had 15-fold greater cytotoxic activity.

DISCUSSION

The widely accepted mechanism for BS-RNase-mediated cytotoxicity requires that the enzyme exhibit ribonucleolytic activity in the cytosol. In support of this mechanism, catalytically inactive BS-RNase is not cytotoxic (29). In addition, the dimeric structure of the enzyme enables evasion of cytosolic RI (19, 30, 31). Monomers of wild-type BS-RNase derived from reduction of the two intersubunit disulfide bonds are inhibited by RI (27, 28) and are not cytotoxic (19). Thus, we reasoned that monomers of BS-RNase that resist RI as well as dimers composed of such monomers could provide new insight into the structure and function of BS-RNase.

RI Affinity of Monomeric BS-RNase Variants. In previous work, we designed semisynthetic monomers of BS-RNase that evade RI (52). Specifically, we modified the sulfhydryl group of Cys31 in C32S BS-RNase or Cys32 in C31S BS-RNase with a carboxymethyl group. The resulting semisyn-

thetic enzymes were shown to evade RI in a qualitative assay and to be toxic for spermatogenic layers in mice. The semisynthetic enzymes were not, however, toxic to K-562 or other mammalian cells. Here, we have extended this approach.

In RNase A, the G88R substitution decreases the affinity for RI by nearly 10^4 -fold (35). In BS-RNase, the same substitution decreases the affinity for RI by 250-fold (Table 1). Thus, changing Gly88 in an RNase A homologue provides a reliable means to diminish affinity for RI without decreasing ribonucleolytic activity.

Perturbing the presumed Coulombic interactions between RI and residues 38 and 39 of BS-RNase further enhances RI evasion. Both C31A/C32A/G38K/K39G/G88R BS-RNase and C31A/C32A/G38R/K39D/G88R BS-RNase have 50-fold less affinity for RI than does C31A/C32A/G88R BS-RNase (Table 1). Swapping residues 38 and 39 of RNase A had a similar effect on the affinity of that enzyme for RI (46). Thus, substitutions to residues 38 and 39 in an RNase A homologue can be used to diminish affinity for RI without decreasing ribonucleolytic activity. In accord with our experimental results, a recent computational analysis by Camacho and co-workers designated Arg39 of RNase A as a secondary "anchor residue" in the RI•RNase A complex (53).

Asn67 of RNase A is in close contact with Val405 of RI in the RI•RNase A complex (Figure 1D) and was identified by Camacho and co-workers as the primary anchor residue in the RI•RNase A complex (53). Replacing Asn67 with an arginine was found to decrease the affinity of RNase A for RI (46). We tried to enhance the ability of C31A/C32A/G38K/K39G/G88R BS-RNase to evade RI by installing an arginine residue at position 67. Surprisingly, this substitution had a negligible effect on the affinity for RI.

Cytotoxicity of Monomeric BS-RNase Variants. Monomeric BS-RNase variants that evade RI are cytotoxic (Figure 2A, Table 1). C31A/C32A/G88R BS-RNase has $>10^3$ -fold greater cytotoxic activity than does C31A/C32A BS-RNase. The addition of a cationic residue (arginine) could enhance the affinity of the enzyme for the anionic surface of a mammalian cell (54, 55) and, thus, be partly responsible for the enhanced cytotoxicity of G88R BS-RNase. The even greater cytotoxic activity of C31A/C32A/G38R/K39D/G88R BS-RNase, which has the same molecular charge as C31A/C32A BS-RNase, demonstrates that evasion of RI does indeed play a key role in the process. Likewise, C31A/C32A/G38K/K39G/G88R BS-RNase is a more potent cytotoxin than is C31A/C32A/G88R BS-RNase, even though the two enzymes have the same molecular charge. Thus, the substitution of three residues converts a nontoxic enzyme into a cytotoxin that is not only potent but more potent than ONC (Figure 2A), which provides the benchmark for the cytotoxic activity of ribonucleases (33). Indeed, C31A/C32A/G38K/K39G/G88R BS-RNase is the most cytotoxic variant or homologue of RNase A reported to date.

Cytotoxicity of Dimeric BS-RNase Variants. The cytotoxic activity of wild-type (that is, dimeric) BS-RNase is greater if the enzyme is composed of monomers that evade RI (Figure 2B, Table 1). Interestingly, the same trends in cytotoxic activity observed with monomeric BS-RNases are also evident with their dimeric counterparts. For variants of the same molecular charge, constituent monomers with a higher K_d value always give rise to a dimer with greater cytotoxic activity. For example, G38R/K39D/G88R BS-

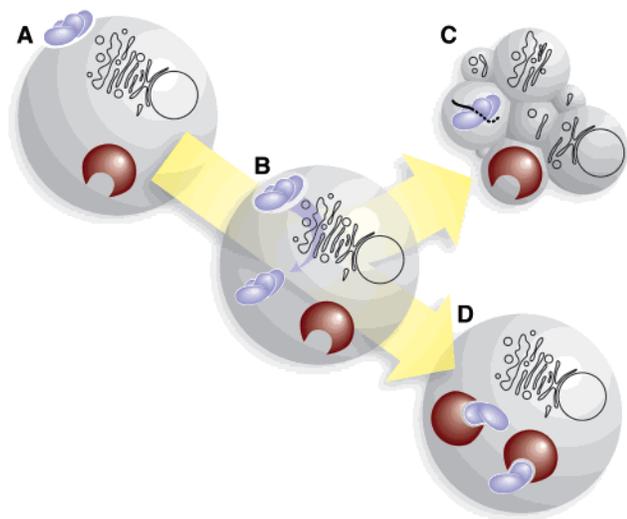


FIGURE 3: Putative mechanism for the cytotoxic activity of wild-type BS-RNase (12, 56). (A) BS-RNase binds to the cell surface and is internalized by dynamin-independent endocytosis. (B) BS-RNase reaches the cytosol by translocation from an endosome or the Golgi apparatus. (C) Intact BS-RNase degrades cellular RNA, leading to apoptosis. (D) Dissociated BS-RNase is inhibited by cytosolic RI (this work).

Table 2: Molecular Charges of RNase A and Its Monomeric Homologues

ribonuclease	Arg	Lys	Asp	Glu	Z	Z/residue (%)
RNase A (cow)	4	10	5	5	+4	+3.2
RNase 1 (human)	10	8	6	6	+6	+4.7
Onconase	3	12	6	3	+5 ^a	+4.8
BS-RNase, monomer	4	14	4	5	+9	+7.3

^a The N-terminus is uncharged because residue 1 is a pyroglutamate.

RNase is more cytotoxic than is the wild-type enzyme, and G38K/K39G/G88R BS-RNase is more cytotoxic than is the G88R variant. In other words, the cytotoxic activity of a BS-RNase variant correlates with the ability of its constituent monomers to evade RI. BS-RNase is known to dissociate into monomers in a buffer that mimics the reducing environment of the cytosol (30). Our data indicate that a significant fraction of BS-RNase dissociates in the cytosol and enable us to elaborate the mechanism of BS-RNase cytotoxicity (Figure 3) (56).

Recently, D'Alessio and co-workers reported that G88R/S89E BS-RNase is not significantly more cytotoxic than wild-type BS-RNase, even though this variant can evade RI after monomerization [$K_d = 2.5 \pm 0.5$ nM (49)]. The apparent discrepancy with our work can be explained by the greater cytotoxic activity of G88R BS-RNase arising largely from its acquisition of a positive charge, which could enhance cellular uptake of the enzyme (vide supra). The S89E substitution in G88R/S89E BS-RNase negates the benefit of the additional positive charge. We conclude that the evasion of RI accrued from the G88R substitution alone is not sufficient to endow dimeric BS-RNase with enhanced cytotoxicity.

Molecular Charge and Cytotoxicity. Some ribonucleases are highly cationic proteins (Table 2). This cationic character is thought to facilitate cellular uptake of the enzyme. Moreover, increasing this cationic character by chemical modification (54), site-directed mutagenesis (55), or addition of an Arg₃ tag (57) enhances its cytotoxicity. G38K/K39G/

G88R BS-RNase and C31A/C32A/G38K/K39G/G88R BS-RNase are both more cationic and more cytotoxic than G38R/K39D/G88R BS-RNase and C31A/C32A/G38R/K39D/G88R BS-RNase, respectively, but do not differ significantly in molecular size, ribonucleolytic activity, or affinity for RI (Figure 2, Table 1). It is noteworthy that, in creating a monomeric BS-RNase variant, D'Alessio and co-workers increased the cationicity of the enzyme by replacing Cys31 with a lysine residue (49). The resulting variant, C31K/C32S BS-RNase, exhibited significant cytotoxic activity ($IC_{50} = 80 \pm 7$ μ g/mL or 5.9 ± 0.5 μ M) even though it had high affinity for RI ($K_d = 9.3 \pm 3.2$ pM).

Monomer versus Dimer. Monomeric BS-RNase variants that evade RI display greater cytotoxic activity than do their dimeric BS-RNase counterparts (Figure 2, Table 1). This finding was unexpected, as the ability of a dimeric BS-RNase to evade RI should always exceed that of its constituent monomers. Other factors that contribute to the cytotoxicity of a ribonuclease, such as efficient internalization or translocation (Figure 3), must be responsible for this discrepancy. Regardless, our data demonstrate that BS-RNase monomers that evade RI can be supremely potent cytotoxins.

Conclusions. We have investigated the significance of the dimeric structure of BS-RNase in its cytotoxicity. In so doing, we created monomeric BS-RNases with greater cytotoxic activity than ONC, an amphibian ribonuclease in phase III trials as a cancer chemotherapeutic, or any dimeric BS-RNase. Thus, the dimeric structure of BS-RNase provides a means to evade RI but is not necessary for cytotoxicity and can actually decrease cytotoxic activity. Finally, our data indicate that the BS-RNase dimer is severed in the cytosol, where RI limits its cytotoxicity.

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