THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 52, Issue of December 29, pp. 31097–31102, 1995

Printed in U.S.A.

Mechanism of Ribonuclease Cytotoxicity*

(Received for publication, August 3, 1995, and in revised form, September 27, 1995)

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Bovine seminal ribonuclease (BS-RNase), a dimeric homolog of bovine pancreatic ribonuclease A (RNase A), is toxic to mammalian cells. In contrast to dimeric BS-RNase, monomeric BS-RNase and RNase A are not cytotoxic and are bound tightly by cytosolic ribonuclease inhibitor. To elucidate the mechanism of ribonuclease cytotoxicity, we constructed a series of hybrid and semisynthetic enzymes and examined their properties. In five hybrid enzymes, divergent residues in BS-RNase were replaced with the analogous residues of RNase A so as to diminish an interaction with a putative cellular receptor. In a semisynthetic enzyme, the disulfide bonds that cross-link the monomeric subunits of dimeric BS-RNase were replaced with thioether bonds, which can withstand the reducing environment of the cytosol. Each hybrid and semisynthetic enzyme had ribonucleolytic and cytotoxic activities comparable with those of wild-type BS-RNase. These results suggest that dimeric BS-RNase (p*I* = 10.3) enters cells by adsorptive rather than receptor-mediated endocytosis and then evades cytosolic ribonuclease inhibitor so as to degrade cellular RNA. This mechanism accounts for the need for a cytosolic ribonuclease inhibitor and for the cytotoxicity of other homologs of RNase A.

Bovine seminal ribonuclease (EC 3.1.27.5, BS-RNase)[1] is a cytotoxic protein. In various assays, this cytotoxicity can manifest itself as an immunosuppressive, antitumor, embryotoxic, and aspermatogenic activity (Dostál and Motoušek, 1973; Souček *et al.*, 1986; Tamburrini *et al.*, 1990; Matoušek and D'Alessio, 1991; Laccetti *et al.*, 1992), each of which has potential therapeutic value (Youle *et al.*, 1993; Deonarain and Epenetos, 1994). Among these cytotoxic activities, the immunosuppressive activity is most likely to be physiologically significant, since this activity may be required to suppress the female immune response against components of bull seminal fluid (James and Hargreave, 1984).

The cytotoxicity of BS-RNase is related to its quaternary structure. BS-RNase is isolated as a dimer that is cross-linked by two disulfide bonds. RNase A and monomeric BS-RNase are not cytotoxic (Vescia *et al.*, 1980; Tamburrini *et al.*, 1990; Kim *et al.*, 1995b). In contrast, artificially dimerized RNase A is cytotoxic, but to a lesser extent than is BS-RNase (Bartholeyns and Baudhuin, 1976; Bartholeyns and Zenebergh, 1979; Vescia *et al.*, 1980; Di Donato *et al.*, 1994). At equilibrium, dimeric BS-RNase is a mixture of two distinct quaternary forms, M=M and MxM (Piccoli *et al.*, 1992). The conversion of M=M to MxM entails the exchange of *N*-terminal α-helices between subunits, as occurs during the artificial dimerization of RNase A. Recently, we and others demonstrated that the MxM form is responsible for the cytotoxicity of BS-RNase (Piccoli *et al.*, 1993; Cafaro *et al.*, 1995; Kim *et al.*, 1995b). We also demonstrated that the ribonucleolytic activity of BS-RNase is necessary for its cytotoxic activity (Kim *et al.*, 1995a).

The cytosol of cells contains a ribonuclease inhibitor (RI) (Roth, 1967; Blackburn and Moore, 1982; Lee and Vallee, 1993; Hofsteenge, 1994).[2] This protein binds tightly to RNase A and monomeric BS-RNase but not to dimeric BS-RNase (Murthy and Sirdeshmukh, 1992). The cytosol of cells is also a reducing environment, which is a hazard for disulfide bonds such as the two that cross-link the monomers of dimeric BS-RNase. If the disulfide bonds in dimeric BS-RNase are reduced, then the M=M form will be converted to monomers that are susceptible to inhibition by RI. In contrast, noncovalent interactions in the MxM form enable it to remain dimeric and retain its lethal enzymatic activity in the absence of its intersubunit disulfide bonds. These data lead us to propose that BS-RNase evolved its MxM form to evade RI and thereby retain its enzymatic activity *in vivo* (Kim, 1994; Kim *et al.*, 1995b). Here, we test this hypothesis with a semisynthetic dimer of BS-RNase that is cross-linked by thioether (C–S–C) rather than disulfide (S–S) bonds. Since thioether bonds are not cleaved by thiols, this semisynthetic enzyme will remain dimeric in the reducing environment of the cytosol.

This hypothesis still leaves unresolved the mechanism by which BS-RNase enters mammalian cells. Homolog-scanning mutagenesis is a powerful method to distinguish the residues that give rise to particular properties in homologous proteins

* This work was supported by National Institutes of Health Grant GM-44783 (to R. T. R.) and Grant 506/93/1133 from the Grant Agency of the Czech Republic (to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BS-RNase, bovine seminal ribonuclease; DTT, reduced dithiothreitol; M, monomeric wild-type BS-RNase in which the sulfhydryl groups of Cys^{31} and Cys^{32} are carbamoylmethylated; M′, monomeric C31S BS-RNase in which the sulfhydryl group of Cys^{32} is carbamoylmethylated; M′–B, monomeric C31S BS-RNase in which the sulfhydryl group of Cys^{32} is alkylated with bimane; M′–B–M′, dimeric C31S BS-RNase in which the subunits are cross-linked by bimane; MxM, dimeric wild-type BS-RNase in which the NH_2-terminal tail of each subunit interacts with the COOH-terminal body of the other subunit; M=M, dimeric wild-type BS-RNase in which the NH_2-terminal tail of each subunit interacts with the COOH-terminal body of the same subunit; RNase A, bovine pancreatic ribonuclease A; RI, human placental ribonuclease inhibitor from human placenta; PAGE, polyacrylamide gel electrophoresis.

[2] Recently, the crystalline structures of RI and an RI·RNase A complex were determined by Kobe and Deisenhofer (1993, 1995).

(Cunningham *et al.*, 1989). We and others have applied this method to angiogenin, a homolog of RNase A that promotes blood vessel formation. The results from these studies suggest that angiogenin has a surface loop that directs receptor-mediated endocytosis (Harper and Vallee, 1989; Allemann *et al.*, 1991; Raines *et al.*, 1995). Here, we use homolog-scanning mutagenesis to search for residues in BS-RNase that could interact with a cellular receptor.

EXPERIMENTAL PROCEDURES

Materials

Dibromobimane and monobromobimane (as Thiolyte DB and MB, respectively) were from Calbiochem. Iodoacetamide, glutathione, and dithiothreitol were from Sigma. Human placental RI (as RNasin®) was from Promega (Madison, WI). All other materials were obtained as described (Kim *et al.*, 1995b).

Methods

General Procedures—Wild-type BS-RNase was purified from bull seminal plasma as described (Dostál and Motoušek, 1973; Kim and Raines, 1993). Wild-type and mutant BS-RNases were produced in *Escherichia coli* and purified as described (de Nigris *et al.*, 1993; Kim and Raines, 1993; Kim and Raines, 1994). The concentrations of all hybrid enzymes except for SRA-5 were determined by assuming that $\epsilon_{1\ cm}^{0.1\%} = 0.465$ at 278 nm (D'Alessio *et al.*, 1972). The concentration of SRA-5, which has an additional Tyr residue, was determined by assuming that $\epsilon_{1\ cm}^{0.1\%} = 0.567$, as calculated by the method of Gill and von Hippel (1989). The concentrations of the semisynthetic derivatives of C31S BS-RNases were determined with the protein assay kit from Bio-Rad, using the wild-type enzyme as a control. Manipulations of DNA were performed as described (Kim *et al.*, 1995b).

BS-RNase/RNase A Hybrids—Our strategy to search for evidence for a cellular receptor that recognizes BS-RNase but not RNase A was to determine the cytotoxicity of BS-RNase/RNase A hybrids in which divergent regions in BS-RNase were replaced with the analogous residues from RNase A. The amino acid sequences of BS-RNase and RNase A are shown in Fig. 1. The amino acid sequence of BS-RNase is 81% identical to that of RNase A, having only 23 substitutions in its 124 residues (Suzuki *et al.*, 1987). Seventeen of the 23 divergent residues in BS-RNase are located in three main regions: residues 16–20, 28–39, and 102–115. One is in position 3, and the remaining five are located in the region between residues 55 and 80.

Several of the substitutions in BS-RNase appear to enable the enzyme to acquire a dimeric structure. Residues 28–39 contain two substitutions that cross-link the monomeric subunits of the dimeric protein: Cys^{31} and Cys^{32}. The presence of Leu^{28} in BS-RNase, rather than a glutamine residue in RNase A, makes this region highly hydrophobic and thus facilitates the contact between the two subunits (Capasso *et al.*, 1983). Lys^{34} of BS-RNase is responsible for the high reactivity of Cys^{31} and Cys^{32} toward reducing agents (Parente *et al.*, 1985). Since the dimeric form of BS-RNase is responsible for its cytotoxic activities, we have left intact residues 28, 31, 32, and 34 of BS-RNase.

Most of the other divergent residues are located on the surface of the dimeric protein. Thus, these residues are capable of interacting with a putative cellular receptor. We divided these residues into five groups based on their location in the three-dimensional structure and then prepared five corresponding hybrids between BS-RNase and RNase A. These hybrid enzymes are designated SRA-1 to SRA-5, where SRA refers to seminal/ribonuclease A (Figs. 1 and 2).

Semisynthetic Enzymes—Our strategy to reveal the importance of evading RI was to determine the cytotoxicity of a semisynthetic BS-RNase that would remain dimeric (and hence resistant to RI) in a reducing environment. Since the sulfhydryl group of a cysteine residue has unique reactivity and alkylation of a sulfhydryl group produces a thioether bond (C–S–C) that does not suffer reduction, we reasoned that a bifunctional alkylating reagent could provide the necessary cross-link. Still, we had to create a cross-link between, rather than within, subunits.

Previously, we had prepared C31S BS-RNase (Kim *et al.*, 1995b). Monomers of this mutant enzyme have only one free sulfhydryl group, that in Cys^{32}. To reduce the sulfhydryl group in Cys^{32}, this protein was treated for 20 min with a 10-fold molar excess of DTT in 10 mM Tris-HCl buffer, pH 8.5. The reduced monomer was then reacted with a 10-fold molar excess of dibromobimane, a fluorescent bifunctional alkylating reagent (Kosower and Kosower, 1987). The resulting nonreducible dimer (M′–B–M′; Structure 1) of C31S BS-RNase was purified as we described previously (Kim and Raines, 1995). The reduced monomer was also treated with a 10-fold molar excess of monobromobimane and iodoacetamide under similar conditions to yield M′–B and M′, respectively. The reactions were quenched by the addition of DTT to a final concentration of 20 mM. The three semisynthetic enzymes were then dialyzed exhaustively against 10 mM sodium phosphate, pH 7.0, containing NaCl (0.10 M).

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                10          20          30          40
BS-RNase  KESAA AKFER QHMDS GNSPS SSSNY CNLMM CCRKM TQGKC
RNase A   ..T.. ..... ..... ST.AA ..... ..Q.. KS.NL .KDR.
SRA                       11 11                2  222

                50          60          70          80
BS-RNase  KPVNT FVHES LADVK AVCSQ KKVTC KNGQT NCYQS KSTMR
RNase A   ..... ..... ....Q ..... .N.A. ..... ..... Y...S
SRA                           3 3                   4

                90          100         110         120
BS-RNase  ITDCR ETGSS KYPNC AYKTT QVEKH IIVAC GGKPS VPVHF DASV
RNase A   ..... ..... ..... ..... .AN.. ..... E.N.Y ..... ....
SRA                           44          5 5 5
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FIG. 1. **Sequence alignment of BS-RNase and RNase A.** The residues that were replaced in BS-RNase to create the hybrid enzymes SRA-1 to SRA-5 are indicated.

Quaternary Structure—We assessed the ability of the two quaternary forms of wild-type BS-RNase, M=M and MxM, to withstand a reductive environment. The M=M and MxM dimers were prepared by selective reduction and gel filtration chromatography as described (Kim *et al.*, 1995b). Each form (final concentration, 1.0 mg/ml; 74 μM monomer) was incubated for 30 min at room temperature in 100 mM sodium phosphate buffer, pH 7.2, containing NaCl (0.10 M) and DTT (0, 74, 370, or 740 μM). The reaction was quenched by adding iodoacetamide to a final concentration of 25 mM. The resulting proteins were analyzed by SDS-PAGE under nonreducing conditions.

Similarly, each form (final concentration, 1.0 mg/ml; 74 μM monomer) was incubated at room temperature in 100 mM sodium phosphate buffer, pH 7.2, containing NaCl (0.10 M), reduced glutathione (9.0 mM), and oxidized glutathione (0.15 mM). These conditions mimic the glutathione concentrations in the cytosol of mammalian cells (Hwang *et al.*, 1992). At various times, aliquots of each reaction were quenched by adding iodoacetamide to a final concentration of 25 mM and analyzed by SDS-PAGE under nonreducing conditions. After 24 h of incubation, the remaining material was quenched and analyzed by gel filtration chromatography.

Ribonucleolytic Activity and Its Inhibition—Ribonucleolytic activity was assayed by the method of Kunitz (1946) using yeast RNA (80 μg/ml) as substrate. Inhibition of the ribonucleolytic activity by RI was assessed by a modification of the method of Shortman (1961), who defined one unit of RI as the amount of inhibitor required to decrease by 50% the activity of 5 ng of RNase A. A solution of M′, M′–B, or M′–B–M′ with ribonucleolytic activity equivalent to that of 20 ng of RNase A was incubated for 5 min at room temperature with RI (5–1000 units) in 50 mM imidazole-HCl buffer, pH 7.0, containing NaCl (0.10 M) and DTT (2 mM). The solution was then assessed for remaining ribonucleolytic activity. The low concentration of DTT in this assay was necessary to maintain the activity of RI and had no effect on the ribonucleolytic activity of M′, M′-B, or M′-B-M′ (data not shown).

Cytotoxic Activity—The ability of ribonucleases to kill human lymphocytes (stimulated by mixed lymphocyte culture), human tumor cells (line K-562), bovine embryos, and spermatogenic mouse cells was assayed as described (Kim *et al.*, 1995a, 1995b).

RESULTS

BS-RNase/RNase A Hybrids—All hybrid enzymes had ribonucleolytic activity identical (±10%) to that of wild-type BS-RNase.

Semisynthetic BS-RNases—Monomeric C31S BS-RNase was reacted with dibromobimane to generate a dimer (M′–B–M′) that is cross-linked by thioether bonds. This dimer was highly fluorescent (Kim and Raines, 1995) and remained as a dimer in the presence of reducing agents (data not shown). Monomeric C31S BS-RNase was also alkylated with monobromobimane and iodoacetamide to generate monomers M′–B and M′, respectively. The two chemically modified monomers had a ribonucleolytic activity identical (±10%) to that of intact monomeric

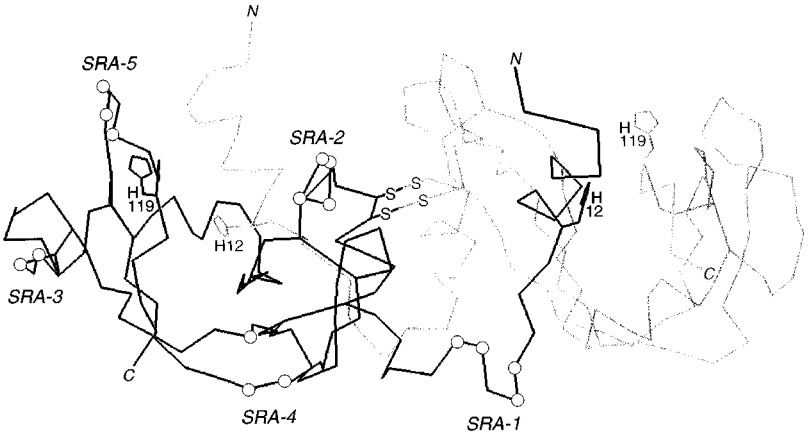
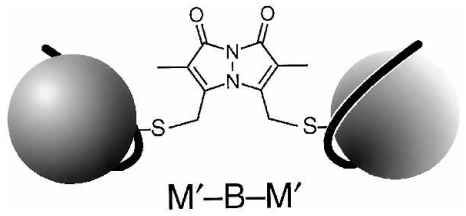


FIG. 2. **Structure of the crystalline MxM form of wild-type BS-RNase (Mazzarella *et al.*, 1987).** The residues that were replaced in BS-RNase to create the hybrid enzymes SRA-1 to SRA-5 are indicated in one subunit.



STRUCTURE 1.

C31S BS-RNase; M′–B–M′ had 5-fold less ribonucleolytic activity. Similarly, wild-type BS-RNase dimers have less ribonucleolytic activity than does the wild-type monomer (Piccoli *et al.*, 1988).

Ribonuclease Inhibition—Semisynthetic BS-RNases (M′, M′–B, and M′–B–M′) were incubated with various amounts of RI and then assayed for remaining ribonucleolytic activity. The monomers, M′ and M′–B, were effectively inhibited by RI. Apparently, the chemical modification of the sulfhydryl group of Cys^{32} with either iodoacetamide or monobromobimane did not severely impair the interaction of RI with the enzyme. In contrast the dimer, M′–B–M′, was highly resistant to inhibition by RI (Table I). At least 50-fold more RI was required to inhibit equivalent ribonucleolytic activity in M′-B-M′ than in M′ or M′–B.

Quaternary Structure of BS-RNase—The integrity of the quaternary structure of BS-RNase in a reducing environment was assessed by exposing the enzyme to reducing agents and analyzing the results by SDS-PAGE. After incubation for 30 min in the presence of a 10-fold molar excess of DTT, >90% of the M=M and MxM forms of wild-type BS-RNase were converted to monomers (Fig. 3*A*). This result suggests that the reduction potential of the intrasubunit disulfide bonds in the M=M and MxM forms is greater than that of DTT.

Next, the two forms were incubated with a mixture of reduced and oxidized glutathione that mimicked the reducing environment of the mammalian cytosol (Hwang *et al.*, 1992). Aliquots were removed at various time points and analyzed by SDS-PAGE under nonreducing conditions (Fig. 3*B*). Up to 70% of M=M gradually converted to a monomer. No further increase in the fraction of monomer was observed after 24 h. In contrast, >90% of MxM remained as a dimer after 24 h of incubation. This result suggests that the intrasubunit disulfide bonds of MxM, but not those of M=M, can survive in the cytosol. After 24 h of incubation, the protein samples were analyzed by gel filtration chromatography. By that time, approximately 70% of the M=M form had been converted to a monomer, while the MxM form remained dimeric (data not shown). This result is consistent with the results of SDS-PAGE.

Cytotoxic Activity—The abilities of wild-type BS-RNase from seminal plasma and *E. coli*, BS-RNase/RNase A hybrids (SRA-1 to SRA-5), and monomeric RNase A and BS-RNase to kill human lymphocytes, human tumor cells, bovine embryos, and mouse spermatogenic cells were compared. As shown in Fig. 4*A*, the dimeric enzymes had a strong and equivalent ability to kill mixed lymphocyte culture-stimulated human lymphocytes. The two monomeric ribonucleases (M and RNase A) had modest immunosuppressive activity, but only when present at high concentration (100 μg/ml). As shown in Fig. 4*B*, wild-type BS-RNase was toxic to human leukemic cell line

TABLE I
Inhibition of ribonucleolytic activity by RI

Units of RI required to decrease by 50% the ribonucleolytic activity of semisynthetic BS-RNases are shown.

Semisynthetic BS-RNase	I_{50}
	units
M′	12
M′–B	14
M′–B–M′	760

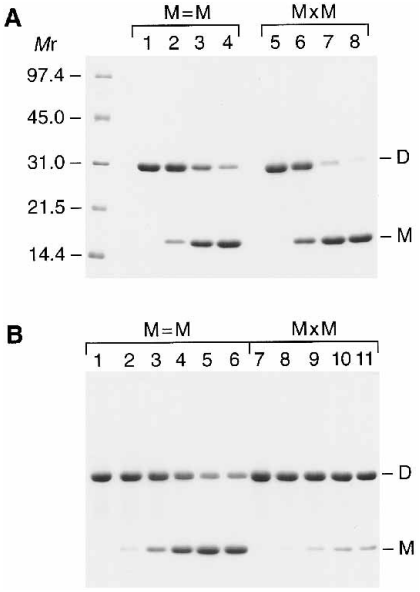


FIG. 3. **Effects of reducing agents on MxM and M=M forms of wild-type BS-RNase.** *A*, each dimeric form was treated with various amounts of DTT and analyzed by SDS-PAGE under nonreducing conditions. *Lanes 1* and *5*, 0-fold molar excess of DTT; *lanes 2* and *6*, 1-fold; *lanes 3* and *7*, 5-fold; *lanes 4* and *8*, 10-fold. *B*, each form was treated with (*lanes 2–6* and *8–11*) or without (*lanes 1* and *7*) glutathione. At various time points, aliquots were removed and analyzed by SDS-PAGE under nonreducing conditions. *Lane 2*, 10 min; *lanes 3* and *8*, 1 h; *lanes 4* and *9*, 5 h; *lanes 5* and *10*, 24 h; *lanes 6* and *11*, 48 h.

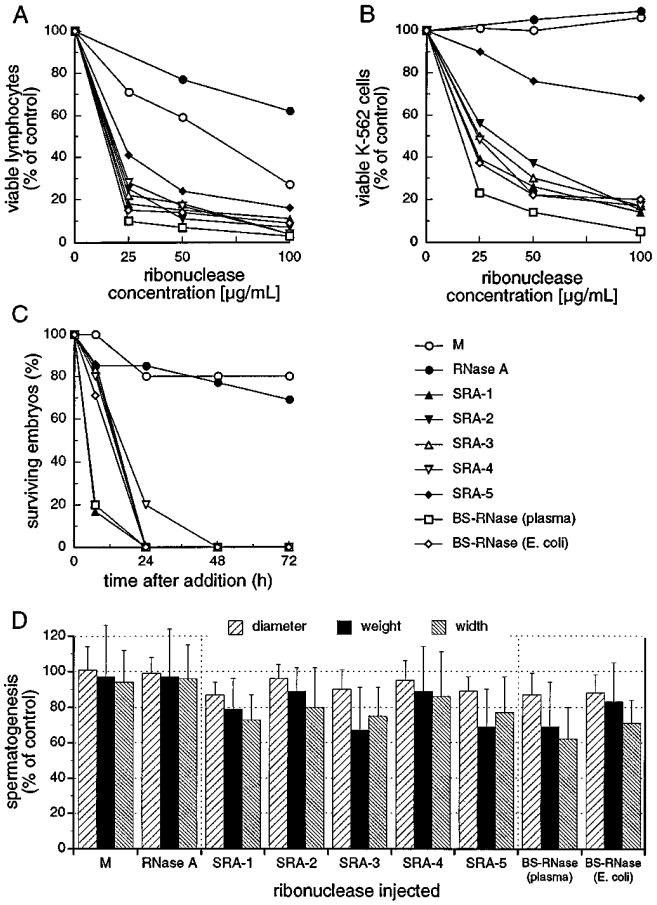


FIG. 4. **Effect of various forms of BS-RNase on the proliferation in culture of human lymphocytes (*A*) and of human tumor cell line K-562 (*B*), on the viability of bovine embryos (*C*), and on mouse spermatogenesis (*D*).** Proliferation was evaluated by the incorporation of [6-^{3}H]thymidine into cellular DNA. Proliferation data (*A* and *B*) are the mean from three cultures and were recorded 3 days after the addition of ribonuclease to the culture. Spermatogenesis data (*D*) are the mean from five injected testes and were recorded 10 days after injection. Controls were medium (*A* and *B*) or testes (*D*) containing no added ribonuclease.

K-562. All of the hybrids displayed a similar antitumor activity except for SRA-5, which had a more modest effect. The two monomers had no effect on the growth of the tumor cells. As shown in Fig. 4*C*, wild-type BS-RNase and each hybrid enzyme caused ≥80% mortality of bovine embryos 24 h after injection and 100% mortality 48 h after injection. Monomeric BS-RNase and RNase A had only minimal embryotoxic activity. As shown in Fig. 4*D*, the dimeric enzymes had a deleterious effect on the diameter of seminiferous tubules, the weight of testes, and the width of spermatogenic layers of injected mice. The monomeric enzymes displayed no aspermatogenic activity.

We also compared the abilities of semisynthetic derivatives of C31S BS-RNase and wild-type BS-RNase to inhibit the growth of human lymphocytes and human tumor cells. Previously, we had shown that dimeric C31S BS-RNase dimer has only a modest effect on the growth of these cells (Kim *et al.*, 1995b). In contrast, the data in Fig. 5 show that M′–B–M′ has an immunosuppressive and antitumor activity comparable with that of wild-type BS-RNase. The two monomers, M′ and M′–B, showed negligible cytotoxic activity.

In summary, the various cytotoxicity assays yielded the same result; all dimers were as active as wild-type BS-RNase, and all monomers were relatively inactive.

DISCUSSION

Ribonucleases have biological activities that appear to extend far beyond their role as catalysts of RNA degradation (D'Alessio *et al.*, 1991; D'Alessio, 1993). For example, onconase from the frog *Rana pipiens* is a potent antitumor agent now in phase III human clinical trials against pancreatic and liver cancer (Ardelt *et al.*, 1991; Mikulski *et al.*, 1995). Angiogenin promotes blood vessel formation (Fett *et al.*, 1985; Raines *et al.*, 1995). S-RNases from plants are responsible for self-incompatibility (McClure *et al.*, 1989; Taylor *et al.*, 1993). The mechanism by which ribonucleases effect these and other seemingly diverse biological actions has been a mystery.

Foremost, ribonucleases are enzymes. We believe that the apparently unusual biological activities of ribonucleases are manifestations of cytotoxicity that results from the catalytic degradation of cellular RNA. Here, we have elucidated the molecular mechanism by which a particular ribonuclease, BS-RNase, effects its cytotoxicity.

In contrast to other ribonucleases, BS-RNase is isolated as a dimer. Monomeric BS-RNase formed by the selective reduction of the intersubunit disulfide bonds displayed negligible cytotoxicity in various assays (Figs. 4 and 5), confirming previous reports (Vescia *et al.*, 1980; Tamburrini *et al.*, 1990; Kim *et al.*, 1995b). Since the dimeric structure seems to be necessary for the cytotoxicity of BS-RNase, it is possible that only dimers of BS-RNase are recognized by an as yet unidentified cellular receptor. Artificial dimers of RNase A have only modest cytotoxic activity (Vescia *et al.*, 1980). To discern whether the differing cytotoxicity of dimers of BS-RNase and RNase A originates from differences in their amino acid sequences, we used homolog-scanning mutagenesis.

Homolog-scanning mutagenesis is a useful method to reveal the structural determinants that cause functional variation among homologous proteins (Cunningham *et al.*, 1989). In this method, the primary sequences of two homologs are compared, and a series of hybrids between the two homologs are constructed by substituting residues from one homolog into the other. The properties of the hybrid can reveal which amino acid residues are critical for function. For example, a hybrid enzyme in which a divergent surface loop of RNase A was replaced with the analogous residues of angiogenin endowed RNase A with angiogenic activity (Raines *et al.*, 1995). Likewise, the reciprocal hybrid in which the loop of angiogenin was replaced with residues of RNase A eliminated the angiogenic activity of angiogenin (Harper and Vallee, 1989). These results suggest that the loop of angiogenin interacts with a specific cellular receptor.

Encouraged by this previous success, we used homolog-scanning mutagenesis to identify any amino acid residues that are essential for the cytotoxicity of BS-RNase. We prepared and characterized five hybrid enzymes in which segments of RNase A replaced divergent segments of BS-RNase (Figs. 1 and 2). Each hybrid enzyme retained full ribonucleolytic activity (data not shown) and cytotoxicity (Fig. 4). We therefore conclude that mammalian cells do not have a receptor specific for BS-RNase.

BS-RNase may permeate cells by adsorptive, rather than receptor-mediated, endocytosis. The glycosaminoglycans that cover the surface of mammalian cells are polyanions. BS-RNase has pI = 10.3 (D'Alessio *et al.*, 1972; Kim *et al.*, 1995b). RNase A (pI = 9.3 (Ui, 1971)) is less cationic than BS-RNase, and a dimer of RNase A is less cytotoxic than BS-RNase. Further, dimeric BS-RNase binds to and destabilizes membrane bilayers *in vitro* (Mancheno *et al.*, 1994). Recently, Youle, D'Alessio, and co-workers (Wu *et al.*, 1995) illuminated the precise route that ribonucleases take to reach the cytosol. The ability of cationic ribonucleases to invade cells by adsorptive

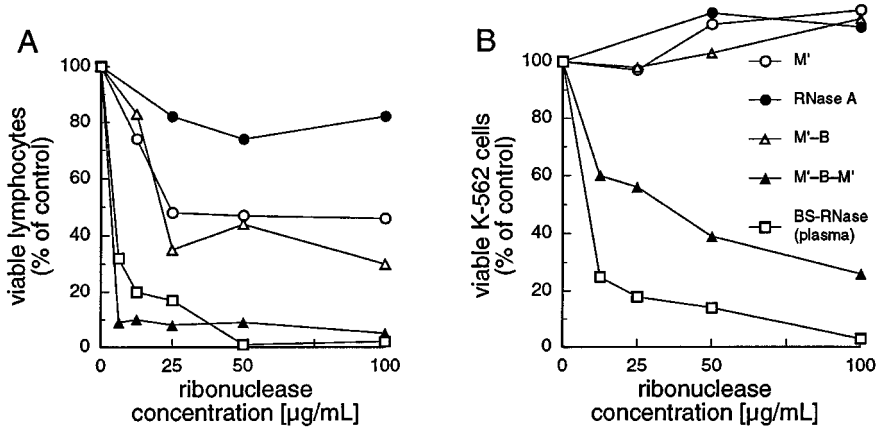


FIG. 5. **Effect of various forms of BS-RNase on the proliferation in culture of human lymphocytes and of human tumor cell line K-562.** Proliferation was evaluated by the incorporation of [6-^{3}H]thymidine into cellular DNA. Values are the mean from three cultures and are reported as a percentage of the control, which was the mean value from medium containing no exogenous ribonuclease. Data were recorded 3 days after the addition of ribonuclease to the culture.

endocytosis presages the co-evolution of a cytosolic ribonuclease inhibitor (Beintema *et al.*, 1988).

Once inside the cytosol, ribonucleases are cytotoxic because they degrade cellular RNA (Kim *et al.*, 1995a; Wu *et al.*, 1995). In BS-RNase, it is the MxM form that is responsible for this lethal enzymatic activity. In a reducing environment similar to that of the cytosol, the two intersubunit disulfide bonds of M=M were reduced, while those of MxM remained intact (Fig. 3*B*). These results indicate that the two dimeric forms of BS-RNase meet with different fates in the cytosol; M=M dissociates into monomers, while MxM remains as a dimer. These dissimilar fates arise because reducing the intersubunit disulfide bonds of MxM results in a dimer that is maintained by noncovalent interactions. Held in proximity, the sulfhydryl groups in the two subunits can apparently reform disulfide bonds in the presence of a weak reducing agent such as reduced glutathione ($E'_0 = -0.252$ V (Lees and Whitesides, 1993)), but not in the presence of a strong reducing agent such as DTT ($E'_0 = -0.327$ V). In contrast, reducing the intersubunit disulfide bonds of M=M results in monomers, making the reduction essentially irreversible in a reducing environment.[3] A nonreducible dimer of BS-RNase is cytotoxic. We had proposed that the MxM form of BS-RNase is cytotoxic because it can remain as a dimer, and therefore resistant to RI, in the cytosol (Kim *et al.*, 1995b). We based this proposal in part on the behavior of a mutant of BS-RNase in which Cys^{31} is changed to a serine residue. In C31S BS-RNase, the population of the MxM form at equilibrium is reduced markedly from that in wild-type BS-RNase, and so is the cytotoxicity (Kim *et al.*, 1995b). Still, this proposal does not preclude other properties of the MxM form from giving rise to the cytotoxicity of BS-RNase. Here, we prepared a nonreducible covalent dimer (M′–B–M′) of C31S BS-RNase by using a thiol-specific cross-linking reagent, dibromobimane (Kim and Raines, 1995). The nonreducible dimer of C31S BS-RNase is unlikely to be in the MxM form to any significant extent because the MxM form of C31S BS-RNase itself is relatively unstable and because its two subunits are now separated by a bimane group. Yet, this semisynthetic enzyme has cytotoxic activities comparable with those of wild-type BS-RNase (Fig. 5). Thus, we have enhanced dramatically the cytotoxicity of C31S BS-RNase simply by changing the atoms that cross-link the two subunits. This result demonstrates that the domain swapping of the MxM form is not *per se* responsible for the cytotoxicity of BS-RNase. Significantly, we also found that M′–B–M′ is resistant to inhibition by RI (Table I). The crystalline structure of an RI·RNase A complex (Kobe and Deisenhofer, 1995) suggests that BS-RNase dimers such as MxM and M′–B–M′ cannot bind to RI because the dimer interface is buried in the complex. We propose that the cytotoxicity of M′–B–M′, like that of MxM, arises from its ability to remain dimeric and therefore resistant to RI in the cytosol.

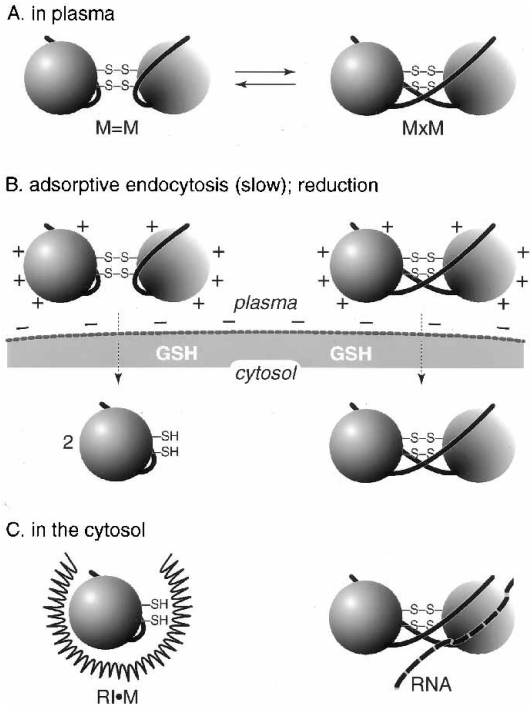


FIG. 6. **Mechanism for the cytotoxicity of bovine seminal ribonuclease.** *A*, dimeric BS-RNase is a mixture of two quaternary forms, M=M and MxM. The conversion of M=M to MxM entails the exchange of *N*-terminal α-helices between subunits. *B*, in a slow step, cationic BS-RNase adsorbs to anionic cells and enters by endocytosis. Reduced glutathione (*GSH*) in the cytosol cleaves the intersubunit disulfide bonds of the M=M but not the MxM form. *C*, the MxM form is resistant to endogenous cytosolic RI and is able to exert its lethal enzymatic activity on cellular RNA.

A molecular mechanism for the cytotoxicity of BS-RNase that is consistent with all known data is shown in Fig. 6. Several pieces of evidence suggest that adsorptive endocytosis is the rate-limiting step in this mechanism. First, ribonucleases injected into cells are $>10^3$-fold more toxic than ribonucleases that enter cells of their own accord (Saxena *et al.*, 1991). Second, onconase has 10^3-fold less ribonucleolytic activity than RNase A (Ardelt *et al.*, 1991), which is approximately as active as BS-RNase. Still, onconase is 30-fold more cytotoxic than BS-RNase (Mosimann *et al.*, 1994), perhaps because onconase is smaller than BS-RNase and hence more endocytotic. Finally,

[3] The function of other proteins, most notably certain eukaryotic transcription factors, is also regulated by oxidation and reduction of a specific disulfide bond (Abate *et al.*, 1990; Hayashi *et al.*, 1993).

we have recently shown that angiogenin is toxic to lymphocytes (Matoušek *et al*., 1995). Unlike BS-RNase and onconase (Murthy and Sirdeshmukh, 1992; Wu *et al*., 1993), angiogenin is bound tightly by RI (Lee and Vallee, 1993). Also in contrast to BS-RNase and onconase, angiogenin appears to enter cells by receptor-mediated endocytosis (Harper and Vallee, 1989; Raines *et al*., 1995). An influx of angiogenin may be able to overwhelm RI, which constitutes only 0.01% of the protein in the cytosol (Blackburn and Moore, 1982). Similarly, exposure to high concentrations of RNase A and monomeric BS-RNase is somewhat toxic to lymphocytes (Figs. 4*A* and 5*A*). Thus, the mechanism in Fig. 6 suggests that the cytotoxicity of ribonucleases can be enhanced by increasing the rate at which uninhibited ribonucleolytic activity enters the cytosol. We are using this strategy to create new cytotoxins of potential therapeutic value.

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