Antitumor Action of Seminal Ribonuclease, Its Dimeric Structure, and Its Resistance to the Cytosolic Ribonuclease Inhibitor†

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ABSTRACT: Bovine seminal RNase (BS-RNase) is a homodimeric enzyme with a cytotoxic activity selective for tumor cells. In this study, the relationships of its cytotoxic activity to its dimeric structure and its resistance to the cytosolic RNase inhibitor (cRI) are investigated systematically by site-directed mutagenesis. The results show that (1) the dimericity of BS-RNase is essential for its full cytotoxic action; (2) the role of the dimeric structure in the antitumor activity is that of making the enzyme insensitive to the cytosolic RNase inhibitor; (3) a RNase may not be completely insensitive to cRI to exploit a full cytotoxic potential.

Bovine seminal RNase (BS-RNase)[1] is a 27-kDa protein purified from bull seminal vesicles and semen (see, for a review, ref *1*). Structurally, it is homodimeric enzyme with an amino acid sequence 83% identical to that of bovine pancreatic RNase (RNase A) (*2*), the most studied member of the superfamily of pancreatic-type RNases (*3*). BS-RNase is the only protein with a dimeric structure among all the pancreatic-type RNases. The main interactions that stabilize the dimeric structure of BS-RNase are (1) two disulfides between Cys-31 of each subunit chain and Cys-32 from the other; (2) the interchange or swap of the N-terminal helices between protomers. The latter feature characterizes about two-thirds of the molecules in solution that are in equilibrium with the molecules with no interchange.

BS-RNase is endowed with a potent cytotoxic activity specific for tumor cells (*4*). This has been demonstrated in several cell systems both in vitro and in animal models and has been attributed to its ability to permeate tumor cells and degrade ribosomal RNA, thus leading to cell death. In this regard, it has to be noted that the cytosol of mammalian cells contains the cytosolic RNase inhibitor (cRI), a 50-kDa protein that binds tightly to most pancreatic-type RNases and inhibits competitively their ribonucleolytic activity with K_i values in the fM range (*5*, *6*). It has been suggested that cRI protects cells from RNases that accidentally reach the cytosol (*5*, *6*). It has also been proposed that the cytotoxic action of BS-RNase may be based on its resistance to cRI (*7*−*9*). This proposal has been recently supported by the results of an experiment in which RNase A was rendered cytotoxic by decreasing its affinity for cRI (*10*). Kinetic data indicate that the dimeric structure of BS-RNase is incompatible with binding to cRI (*9*). This may explain how the seminal enzyme, upon cell internalization, can avoid inhibition by cRI and produce damage to cellular RNAs. However, the dimeric structure of BS-RNase has also been suggested to be important for its internalization by tumor cells (*11*). In this study, the relationships of the antitumor action of BS-RNase, its dimeric structure and resistance to cRI are investigated systematically. First, the stability of BS-RNase dimers was inquired. Then, molecular modeling and site-directed mutagenesis were used for preparing monomeric variants of the enzyme with various sensitivities to cRI. The results clarify important aspects of the mechanism of the antitumor action of BS-RNase and provide implications for the development of new antitumor RNases.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. Yeast RNA and cytidylyl-(3′-5′)-adenosine were from Sigma. cRI was from Promega. Human angiogenin was a gift of Dr. Robert Shapiro (Harvard University, Boston, MA). BS-RNase was purified as described (*12*). Noncovalent BS-RNase was prepared by biochemical methods (*13*). The G88R variant of RNase A was a generous gift of Dr. R. T. Raines (University of Wisconsin, Madison, WI). Cytotoxicity assays toward SVT2 and 3T3 fibroblasts (purchased from the American Type Culture Collection) were performed as previously described (*11*). Dissociation kinetics of ncBS-RNase were performed as described (*14*).

Molecular Modeling. The structural model for mKS/BS-RNase was prepared with the aid of SWISS-MODEL (*15*), a freely accessible server for automated comparative protein modeling (http://www.expasy.ch/swissmod/SWISS-MODEL.html).

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[1] Abbreviations: BS-RNase, bovine seminal RNase; cRI, cytosolic RNase inhibitor; RNase A, bovine pancreatic RNase; ncBS-RNase, noncovalent dimeric form of BS-RNase; mKS/BS-RNase, monomeric variant of BS-RNase with the mutations C31→K and C32→S; mKSRE/BS-RNase, monomeric variant of BS-RNase with the mutations C31→K, C32→S, G88→R, and S89→E; mKSREWW/BS-RNase, monomeric variant of BS-RNase with the mutations C31→K, C32→S, T87→W, G88→R, S89→E, and S90→W; RE/BS-RNase, dimeric variant of BS-RNase with the mutations G88→R and S89→E.

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The model for the complex of mKS/BS-RNase and cRI was prepared with the program SWISS-PdbViewer (*16*).

Preparation of BS-RNase Mutants. Site-directed mutagenesis was performed by the overlap extension method of Ho et al. (*17*). Mutant DNAs were sequenced to establish the presence of the programmed mutations and rule out any spurious changes. Mutant proteins were expressed in *Escherichia coli*, recovered from inclusion bodies, and refolded as described for wild-type BS-RNase (*18*). Next, the Met-1 residue was removed by the use of *Aeromonas proteolytica* aminopeptidase (*19*). Finally, the proteins were purified by gel filtration as described for the isolation of recombinant human pancreatic RNase (*20*). Stock concentrations of the mutant proteins were determined by a colorimetric assay (BCA kit, Pierce), with reference to a calibration curve obtained with BS-RNase as standard. Final yields of homogeneous proteins were between 4 and 6 mg/L of bacterial culture.

Measurement of the Affinity of RNases for cRI. All assays were performed at 25 °C in 0.1 M 4-morpholinethanesulfonic acid, pH 6.0, containing 0.1 M NaCl and 10 μg/mL RNase-free bovine serum albumin. Inhibition of the monomeric variants of BS-RNase by cRI was assessed with a spectrophotometric method (*21*). Reaction mixtures contained 0.4 nM enzyme, 4−10 nM inhibitor, and the substrate cytidylyl-(3′-5′)-adenosine at a concentration (50 μM) well below K_m. The K_i values reported represent the -[I] intercepts of four-point plots of $1/v_0$ versus [I], where v_0 and [I] are the initial reaction velocity and the inhibitor concentration, respectively. The intercepts were obtained by linear regression. The rate constants for association and dissociation of the mKS/BS-RNase-RI complex were determined as described for the complex of cRI with human placental RNase (*22*).

RESULTS AND DISCUSSION

Contribution of the Intersubunit Disulfides to the Stabilization of the Dimeric Structure of BS-RNase. It has been previously reported that reduction of the two intersubunit disulfides of BS-RNase, followed by alkylation of the exposed sulfhydryls with iodoacetic acid, generates monomers and noncovalent dimers (*13*). The latter are stabilized by the exchange between subunits of their N-terminal helices and by a hydrophobic interaction that involves Leu-28 and Met-29 of the two subunits. In the present study, the stability of these noncovalent dimers (henceforth termed ncBS-RNase) was investigated by incubating them under pseudo-physiological conditions (*20*) and examining any dissociation into monomers by gel filtration. In a first assay performed after 24 h of incubation at 37 °C, ncBS-RNase was found to be completely dissociated into monomers. This result shows that ncBS-RNase is metastable, as previously described for the noncovalent dimers of RNase A obtained by lyophilization from concentrated solutions of acetic acid (*23*). Analyses of ncBS-RNase at shorter incubation times then revealed that dissociation of ncBS-RNase followed first-order kinetics with a $t_{1/2}$ of 3.3 h (data not shown). This value is 4.8−8.8-fold lower than those measured under the same conditions for the metastable dimers of RNase (*24*). This suggests that the noncovalent intersubunit interactions of BS-RNase are even less effective than those that characterize the artificial dimers of RNase A.

The contribution of the two disulfides to the stability of the dimeric structure of BS-RNase was further investigated by site-directed mutagenesis. Cys-31 and Cys-32 of seminal RNase were mutated to Lys and Ser, respectively, the residues present at corresponding positions in the polypeptide chain of RNase A. The mutant protein (henceforth named mKS/BS-RNase, where "m" denotes a monomeric variant of BS-RNase) was expressed in *E. coli*, recovered from inclusion bodies, and refolded. Then the amino-terminal Met-1 residue, that characterizes most of the heterologous proteins expressed in *E. coli*, was removed, and the processed protein was purified by gel filtration. This showed that mKS/BS-RNase has the molecular weight of a monomeric RNase, consistent with previous findings of S. A. Benner and colleagues (*25*). A sample of mKS/BS-RNase was then concentrated by ultrafiltration to a final concentration of 0.36 mM, and incubated in 50 mM Tris-Cl, pH 7.5, containing 130 mM NaCl, at 37 °C for 7 days. At the end of the incubation the stability of mKS/BS-RNase was analyzed by gel filtration to reveal the formation of any noncovalent dimeric species. mKS/BS-RNase was found to elute entirely as a monomer. On the basis of a detection limit of 9 μM for a dimeric RNase by gel filtration, a lower limit of dissociation constant for any dimeric species of mKS/BS-RNase in solution was found to be $K_d > 13$ mM.

These data indicate that mKS/BS-RNase is stably monomeric. Apparently, the noncovalent BS-RNase dimers, characterized by the exchange between subunits of their N-terminal helices, cannot be formed from monomers under physiological conditions. This provides further evidence that the noncovalent intersubunit interactions of BS-RNase play only a marginal role in stabilization of the dimeric structure, which is mainly maintained by the intersubunit disulfides.

These results confirm the proposal that in the evolution of a dimeric RNase such as BS-RNase, the primary mutation(s) (*26*) leading to dimerization involved very likely the location of Cys residues at appropriate positions for the formation of intersubunit disulfides (*27*).

Functional Characterization of Monomeric mKS/BS-RNase. As a first step in the functional characterization of mKS/BS-RNase, the mutant protein was tested for its ability to digest yeast RNA, a conventional RNase substrate (*28*). The enzyme was found to be somewhat more active than the wild-type dimeric protein (Table 1), showing that the two mutations, C31→K and C32→S, do not alter the architecture of the active site.

Next, the interaction of mKS/BS-RNase with cRI was investigated. One nanomolar mKS/BS-RNase was incubated with 1 M equiv of cRI and assayed for ribonucleolytic activity toward cytidylyl-(3′-5′)-adenosine, a very sensitive substrate of pancreatic-type RNases (*21*). The inhibitor was found to suppress the ribonucleolytic activity of mKS/BS-RNase to an undetectable level, indicating that its K_i for cRI is well below 1 nM. This tightness of binding precluded the use of standard kinetic procedures for K_i determination since such methods require the utilization of much lower inhibitor and enzyme concentrations, and this cannot be achieved in any of the available assay systems.

Binding of mKS/BS-RNase to cRI was hence investigated by an alternative approach based on the measurement of the rate constants for association and dissociation of the protein−inhibitor complex (*22*). The association rate constant k_{on} was

Table 1: Structural and functional properties of native and engineered RNases[a]

RNase	structure (M/D)[b]	RNase activity (units/mg)[c]	K_i for cRI (nM)	IC_{50} for SVT2 cells (μg/mL)
BS-RNase	D	33 ± 1	>2000[d]	30 ± 4
mKS/BS-RNase	M	52 ± 3	0.0093 ± 0.0032	80 ± 7
mKSRE/BS-RNase	M	34 ± 2	2.5 ± 0.5	20 ± 8
mKSREWW/BS-RNase	M	33 ± 3	3.1 ± 0.6	16 ± 2
RE/BS-RNase	D	40 ± 2	>2000[d]	32 ± 6
RNase A	M	90 ± 7	0.00004[e]	>250
G88R RNase A	M	N.M.[e]	0.41[e]	100 ± 11

[a] Values are the mean $\pm$ SEM. [b] M and D indicate a monomeric and dimeric structure, respectively, as evaluated by gel filtration. [c] RNase activity units were measured with yeast RNA as a substrate, according to Kunitz (*28*). [d] Value based on the finding that 0.2 μM cRI did not produce any detectable inhibition of the activity of 2 nM enzyme. [e] From ref *10*. [f] NM, not measured.

measured by examining the competition for binding to cRI between mKS/BS-RNase and human angiogenin, a RNase whose kinetics of binding to cRI are well characterized. It resulted to be 9×10^7 M^{-1} s^{-1}, a value somewhat lower than that reported for RNase A (*22*). The dissociation rate constant k_{off} was determined by first forming the mKS/BS-RNase-cRI complex, then adding a large excess of angiogenin to act as a scavenger for free cRI and, finally, by measuring the amount of free mKS/BS-RNase by enzymatic assays. It resulted to be 8.4×10^{-4} s^{-1}, a value that is about 60-fold higher than that measured for RNase A. Finally, from the values of k_{on} and k_{off}, a K_d of 9.3 pM was calculated. This value is 210-fold higher than that obtained for the complex of cRI with RNase A, but at least 200000-fold lower than that estimated for BS-RNase (Table 1).

These data show that, in contrast with native, dimeric BS-RNase, mKS/BS-RNase binds to cRI tightly, but much less than other ribonucleases, such as RNase A and angiogenin. The different affinity values found for the complexes of cRI with mKS/BS-RNase or RNase A may in part be attributed to the absence in mKS/BS-RNase of acidic residues such as Asp-38 and Glu-111. These residues, present in RNase A, and replaced by glycine residues in the seminal protein, have been found to make several favorable contacts with the inhibitor in the crystal structure of the RNase A−cRI complex (*29*).

Finally, mKS/BS-RNase was tested for antitumor activity in vitro. The monomeric protein was found to inhibit the proliferation of malignant STV2 fibroblasts in a dose-dependent manner with an IC_{50} value of 80 μg/mL (Figure 1). It should be added that mKS/BS-RNase did not affect the growth of normal 3T3 fibroblasts (data not shown). These results show that mKS/BS-RNase is endowed with the same selective cytotoxicity for malignant cells that characterizes wild-type dimeric BS-RNase, but is much less potent as a cytotoxic agent (Table 1).

The albeit low cytotoxic activity of mKS/BS-RNase may be explained by comparing its picomolar K_d with the much lower, femtomolar K_d values that characterize the complexes of cRI with RNase A or other monomeric RNases. A RNase whose K_d value is in the pM range, such as mKS/BS-RNase, would not be completely inhibited and could display a moderate cytotoxicity. This interpretation is supported by an estimate of the number of RNase molecules that could evade cRI once internalized by the tumor cell, as detailed below.

Let us assume that all the RNase molecules that are not positively localized in specific cell compartments could in principle enter the cytosolic compartment. It has been

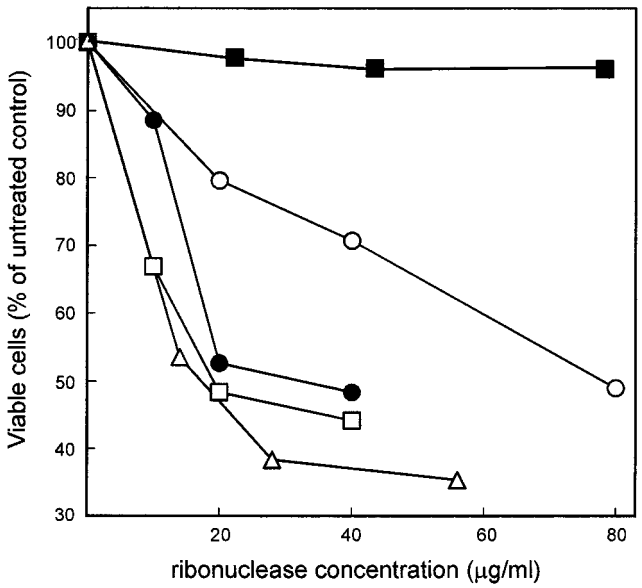

FIGURE 1: Effects of BS-RNase (closed circles) and its monomeric variants, mKS/BS-RNase (open circles), mKSRE/BS-RNase (open squares), and mKSREWW/BS-RNase (triangles), on the proliferation of malignant SVT2 fibroblasts. The cytotoxic activity of RNase A is also shown (closed squares). Cells were grown in the presence of each RNase for 48 h and counted. Each value is the mean of three determinations and is expressed as percentage of the control, in which cells were grown in the absence of RNases. The average standard error of the means was 9%.

reported (*30*) that tumor cells incubated with 50 μg/mL RNase A for 24 h internalize an amount of enzyme that represents about 0.04% of the total cellular proteins and that about two-thirds of the internalized RNase molecules are associated with the nuclear and microsomal intracellular compartments. As 1 mg of cellular proteins corresponds to about 10^7 cells, it can be estimated that about 1×10^{-18} mol of RNase, equivalent to about 6×10^5 molecules, can reach the cytosol of each cell. On the basis of the concentration of intracellular cRI of about 1 μM (*10*), and on the 40 fM K_d value calculated for RNase A, we can estimate that 99.999996% of the cytosolic RNase A molecules would be complexed to cRI. The remaining amount, equivalent to 0.034 molecule/cell, can reasonably be considered as unable to produce any significant damage to intracellular RNA. On the other hand, the 210-fold higher K_d of mKS/BS-RNase (compared to RNase A) would produce a proportionally higher amount of free cytosolic RNase molecules. This amount, of about 10 enzymatically active molecules/cell, may well produce some damage to cellular RNA, thus accounting for the modest cytotoxic activity of mKS/BS-RNase.

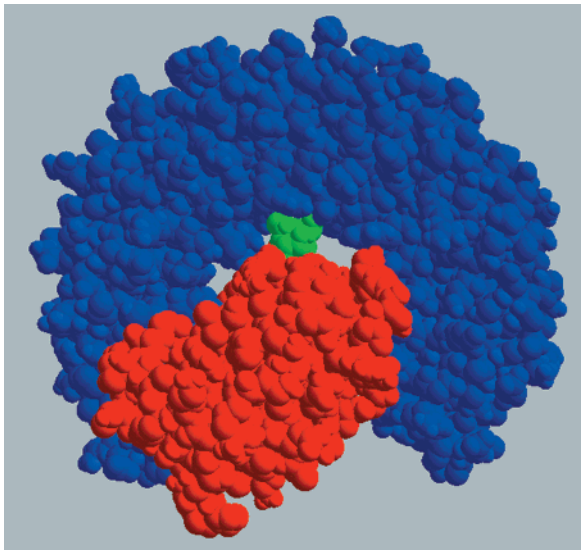

FIGURE 2: Modeled complex of cRI (blue) and a monomeric variant of BS-RNase (mKS/BS-RNase). The RNase is colored in red, with a close contact between the inhibitor and the RNase (loop 87−90) in green. Each atom is represented by its van der Waals surface. The model was prepared with the programs SWISS-MODEL and SwissPdbViewer (*16*).

Design of Monomeric Variants of BS-RNase That Are Resistant to cRI. The relationship between cRI-evasiveness and cytotoxicity of BS-RNase was further investigated by constructing variants of monomeric mKS/BS-RNase with a lower affinity for the inhibitor. These molecules were designed by using an approach previously employed for the construction of RNase A variants partially resistant to cRI (*10*). First, a model for mKS/BS-RNase was obtained by comparative protein modeling based on the atomic coordinates of the crystal structure of RNase A (PDB code 7RSA). The choice was based on the availability of the structure of the RNase A-cRI complex (*29*), and on the high sequence identity between mKS/BS-RNase and the pancreatic enzyme. The root-mean-square deviation between the two proteins for the position of homologous α-carbons is of about 0.2 Å. Next, a model for the complex of mKS/BS-RNase and cRI was obtained by superimposing the model of mKS/BS-RNase to the crystal structure of RNase A in its complex with cRI. The monomeric seminal enzyme could be accommodated in the horseshoe cavity of cRI very similarly to RNase A (Figure 2). In the resulting model of the complex, the enzyme loop 87−90 is packed against the middle part of the inhibitor and, as in RNase A, it contains only residues with short side chains (Thr-87, Gly-88, Ser-89, and Ser-90). To design variants of mKS/BS-RNase in which the shape complementarity between cRI and the monomeric enzyme was disrupted, bulky residues were substituted for those originally present in the loop 87−90. In a first mutant, mKSRE/BS-RNase, Gly-88 and Ser-89 were mutated to Arg and Glu, respectively. A second mutant, mKSREWW/BS-RNase, had the same two mutations plus two tryptophan residues replacing Thr-87 and Ser-90 of the wild-type protein.

Preparation and Characterization of mKSRE/BS-RNase and mKSREWW/BS-RNase. The two monomeric variants of BS-RNase, mKSRE/BS-RNase and mKSREWW/BS-RNase, were expressed in *E. coli* and purified as monomeric proteins with full enzymatic activity (Table 1). Their interaction with cRI was then investigated. The ribonucleolytic activity of mKSRE/BS-RNase was found to be inhibited by cRI much less than that of KS/BS-RNase. The K_i of mKSRE/BS-RNase for cRI resulted to be 2.5 nM, a value 270-fold higher than that of KS/BS-RNase (Table 1). This result shows that the two mutations G88→R and S89→E are indeed able to weaken dramatically the interaction of the RNase molecule with cRI. When mKSREWW/BS-RNase was tested for inhibition by cRI, its K_i resulted to be 3.1 nM, a value only slightly higher than that of mKSRE/BS-RNase. This result shows that the two additional mutations that characterize mKSREWW/BS-RNase, T87→W and S90→W, are not able to enhance the evasiveness to cRI. Likely, in the cRI-mKSRE/BS-RNase complex the RNase loop 87−90 already adopts a conformation that makes the interaction with the inhibitor weaker, so that the insertion in such conformation of the two tryptophan residues can be accommodated without further disturbing the formation of the complex.

The relatively high cRI-evasiveness of mKSRE/BS-RNase and mKSREWW/BS-RNase represented a valuable tool for probing the relationship between the ability of BS-RNase to resist inhibition by cRI and its performance as a cytotoxic agent. The two monomeric engineered enzymes, when tested for cytotoxic activity toward SVT2 fibroblasts, resulted to be 4−5-fold more effective than mKS/BS-RNase and even more active than wild-type BS-RNase (Figure 1 and Table 1). This result shows a clear correlation between cRI-evasiveness and cytotoxic activity: the two proteins, mKSRE/BS-RNase and mKSREWW/BS-RNase are both more evasive to cRI than mKS/BS-RNase, and more effective cytotoxic agents. Furthermore, mKSRE/BS-RNase and mKSREWW/BS-RNase are still much more sensitive to cRI than BS-RNase and as active cytotoxic agents as the wild-type protein (Table 1). This result has two important implications: (1) the dimeric structure of BS-RNase appears to be essential for its antitumor activity because it makes the protein insensitive to the inhibitor: its significance clearly depends on cRI resistance; (2) an RNase does not need to be fully cRI-evasive to exploit in full its cytotoxic potential. In fact, we found that a monomeric variant of RNase A, engineered into a cRI-evasive RNase by substituting Arg for Glu at position 88 (*10*), is less cytotoxic than the monomeric BS-RNase variants described here, although its K_i value for cRI is about 10-fold lower (see Table 1). It should be noted that the IC_{50} value obtained for this RNase A variant by testing its cytotoxicity on SVT2 malignant fibroblasts is virtually identical to that previously obtained with K-562 erythroleukemic cells (*10*).

Preparation and Characterization of RE/BS-RNase. The results reported above indicate that mKSRE/BS-RNase and mKSREWW/BS-RNase are more active cytotoxic agents than mKS/BS-RNase since they are more cRI-evasive. However, it could not be ruled out that the amino acid substitutions that make mKSRE/BS-RNase and mKSREWW/BS-RNase less sensitive to cRI may have had other effects on the RNase molecule and on its cytotoxic action. These mutations might have enhanced unexpectedly some other properties of mKS/BS-RNase, such as its stability, or its ability to enter tumor cells. These possible effects were investigated by preparing a dimeric variant of BS-RNase provided with the same substitutions that characterize the monomeric variant mKSRE/BS-RNase, i.e., G88→R and S89→E. If these mutations enhanced some properties of BS-

RNase other than the cRI-evasiveness, then the new variant of BS-RNase should be more effective as a cytotoxic agent than the wild-type protein. The dimeric variant RE/BS-RNase was prepared and characterized (Table 1). No significant differences were found between wild-type BS-RNase and the mutated enzyme. In particular, the cytotoxic activity of RE/BS-RNase was found to be equivalent to that of native BS-RNase. This result is in line with the conclusion that the substitutions G88→R and S89→E increase the cytotoxic activity of mKS/BS-RNase only by making it less sensitive to cRI.

Concluding Remarks. Four variants of BS-RNase endowed with levels of enzymatic activity similar to that of the wild-type protein, but showing different sensitivities to cRI, and various degrees of cytotoxicity, were used to probe the relationship between the dimeric structure of BS-RNase and its antitumor activity. The data show that dimericity is an essential prerequisite for the cytotoxic activity of BS-RNase solely because it provides the enzyme with the ability to avoid the inhibitory action of cRI. As the cytotoxic action is exerted in the cytosol (*11*), the data reported here also suggest that the ability of BS-RNase to reach the cytosol of tumor cells does not appear to depend on its dimeric structure. Further, the data show that RNases do not need to be fully cRI-evasive in order to exhibit an antitumor activity similar to that of BS-RNase. A value of K_i for cRI of about 10 pM, such as that calculated for mKS/BS-RNase, is sufficient to confer to a RNase a significant degree of cytotoxicity. Values of K_i in the low nanomolar range, such as those calculated for mKSRE/BS-RNase and mKSREWW/BS-RNase, enable a RNase to exert a cytotoxic action equivalent to that of naturally cRI-evasive BS-RNase, whose K_i is $> 2\ \mu$M. In conclusion, while a threshold effect is apparent, in that a significant degree of insensitivity to cRI must be reached by a RNase to acquire a cytotoxic action, a rooftop effect also applies, as above a significant level of resistance to cRI no clear relationship is evident between the cytotoxic action of a RNase and its cRI-evasiveness. This may have important implications for the development of new therapeutics based on engineered RNases.

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