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A Ribonuclease A Variant with Low Catalytic Activity but High Cytotoxicity*

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Onconase™, a homolog of ribonuclease A (RNase A) with low ribonucleolytic activity, is cytotoxic and has efficacy as a cancer chemotherapeutic. Here variants of RNase A were used to probe the interplay between ribonucleolytic activity and evasion of the cytosolic ribonuclease inhibitor protein (RI) in the cytotoxicity of ribonucleases. K41R/G88R RNase A is a less active catalyst than G88R RNase A but, surprisingly, is more cytotoxic. Like Onconase™, the K41R/G88R variant has a low affinity for RI, which apparently compensates for its low ribonucleolytic activity. In contrast, K41A/G88R RNase A, which has the same affinity for RI as does the K41R/G88R variant, is not cytotoxic. The nontoxic K41A/G88R variant is a much less active catalyst than is the toxic K41R/G88R variant. These data indicate that maintaining sufficient ribonucleolytic activity in the presence of RI is a requirement for a homolog or variant of RNase A to be cytotoxic. This principle can guide the design of new chemotherapeutics based on homologs and variants of RNase A.

Ribonuclease A (RNase A; EC 3.1.27.5 (1)) was perhaps the most studied enzyme of the twentieth century. Now homologs of RNase A are becoming important new chemotherapeutics (2–4). For example, low levels of bovine seminal ribonuclease (BS-RNase)[1] are cytotoxic (5, 6). More significantly, Onconase™ (ONC), which is isolated from the frog *Rana pipiens* (7), is currently in Phase III human clinical trials for the treatment of malignant mesothelioma. In addition, ONC inhibits HIV-1 replication in chronically infected human cells (8). Understanding the mechanism of ribonuclease cytotoxicity is vital for the further development of ribonucleases as chemotherapeutics.

Ribonuclease-mediated cytotoxicity is known to depend on several factors. Ribonucleases must enter the cell and reach the cytosol, where RNA degradation leads ultimately to cell death (9, 10). Indeed, injecting ribonucleases directly into *Xenopus* oocytes increases their cytotoxicity (11, 12). In the cytosol, ribonucleases encounter the ribonuclease inhibitor protein (RI). RI constitutes ≥0.01% of protein in the cytosol (13, 14) and inactivates ribonucleases by forming a tight complex that prevents RNA substrates from entering the active site (Fig. 1*A*) (15).

Ribonucleolytic activity is requisite for the cytotoxicity of BS-RNase and ONC (16, 17). Yet despite its relatively high ribonucleolytic activity, RNase A is not cytotoxic (9, 18). The cytotoxicity of BS-RNase and ONC has been attributed to the ability of BS-RNase A and ONC to evade RI (19, 20). RI is a potent inhibitor of RNase A with K_i near 10^{-14} M (21, 22). In contrast, ONC (estimated $K_i \geq 10^{-6}$ M (23)) and BS-RNase (24) escape inhibition by RI.

BS-RNase and ONC use different strategies to evade RI. BS-RNase forms a homodimer, which is stabilized by two intersubunit disulfide bridges. This dimeric form has a much lower affinity for RI than does the free monomer (24). ONC evades RI as a monomer. Only 3 of the 24 RNase A residues that contact RI are conserved in ONC (7, 25).

Cytotoxic variants of RNase A and its human homolog have been created with strategies inspired by BS-RNase and ONC. Dimers have diminished affinity for RI and are cytotoxic (26, 27). Also changes to contact residues by mutagenesis (18) or semisynthesis (28) have yielded monomeric variants with decreased affinity for RI and increased cytotoxicity.

Here, we probe for the first time the interplay between ribonucleolytic activity and RI affinity in the cytotoxicity of a ribonuclease. We find that cytotoxicity can be maintained in an RNase A variant with decreased catalytic activity if there is a concomitant decrease in the affinity for RI. This finding has important implications for understanding the biochemical basis for the cytotoxicity of ribonucleases.

EXPERIMENTAL PROCEDURES

Cells and Chemicals—Escherichia coli strain BL21(DE3) and the pET22b(+) expression vector were from Novagen (Madison, WI). K-562 cells, which are from a continuous human erythroleukemia line, were from the American Type Culture Collection (Manassas, VA). Enzymes for DNA manipulation were from Promega (Madison, WI) or New England Biolabs (Beverly, MA). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Nucleic Acids—[*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from NEN Life Science Products. Poly(cytidylic acid) (poly(C)) was from Midland Certified Reagent (Midland, TX). The fluorogenic substrate, 6-FAM~dArUdAdA~6-TAMRA (where 6-FAM is a 5′ 6-carboxyfluorescein group and 6-TAMRA is a 3′ 6-carboxytetramethylrhodamine group), was supplied by Integrated DNA Technologies (Coralville, IA). DNA oligonucleotides for sequencing and site-directed mutagenesis were from Integrated DNA Technologies. Other reagents for DNA sequencing were from PE Biosystems.

Instruments—Absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA) equipped with a Cary temperature controller. Fluorescence measurements were performed with a QuantaMaster 1 photon-counting fluorometer (Photon Technology International, South Brunswick, NJ). DNA was sequenced using an Applied Biosystems Automated DNA Sequencer at the University of Wisconsin-Madison Biotechnology Center.

Rationale for Varying Residue 41—The side chain of Lys-41 in RNase A donates a hydrogen bond to a nonbridging phosphoryl oxygen in the transition state during RNA cleavage (Fig. 1*B*) (29). Replacing Lys-41 with an arginine or alanine residue decreases ribonucleolytic activity by 10^2- and 10^4-fold, respectively. The side chain of Lys-41 also interacts

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[1] The abbreviations used are: BS-RNase, bovine seminal RNase; ONC, Onconase™; RI, ribonuclease inhibitor protein; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

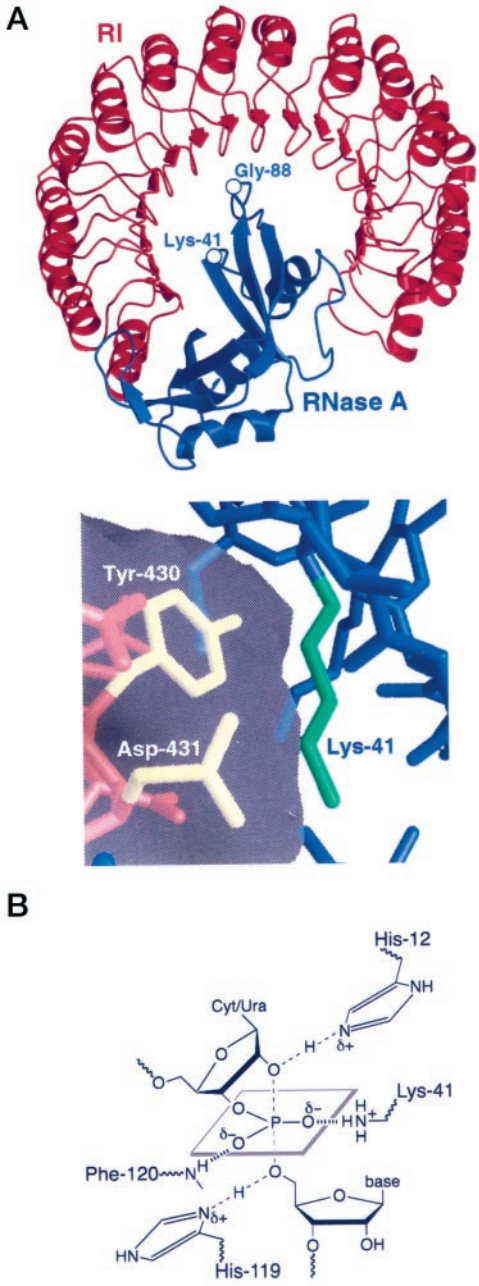

FIG. 1. **Interactions of Lys-41 of ribonuclease A.** *A*, three-dimensional structure of the crystalline RI·RNase A complex, overall (*top*) and near Lys-41 (*bottom*) (25). RI is in *red*; RNase A is in *blue*. *B*, putative structure of the transition state during catalysis of RNA cleavage by RNase A (1).

extensively with RI residues Tyr-430 and Asp-431 in the RI·RNase A complex (25). Angiogenin is a ribonuclease with 35% sequence identity to RNase A that binds RI with high affinity ($K_i = 7 \times 10^{-16}$ M (30–32)). Lys-40 in angiogenin is homologous to Lys-41 in RNase A. Replacing Lys-40 with an arginine residue decreases the affinity of angiogenin for RI by 10^2-fold (33). Thus, Lys-41 of RNase A likely plays an important role in both the manifestation of ribonucleolytic activity and its inhibition by RI. To probe the interplay between activity and its inhibition, we chose to create variants of RNase A that have a lysine, arginine, or alanine residue at position 41. These substitutions were all made in G88R RNase A, which is cytotoxic (18).

Production of Ribonucleases—Wild-type RNase A, its G88R, G88R/K41R, and G88R/K41A variants, and ONC were produced and purified by methods analogous to those described (18). Protein concentrations were determined by UV spectroscopy using $\epsilon_{277.5} = 0.72$ ml mg^{-1} cm^{-1} for RNase A (34) and its variants and $\epsilon_{280} = 0.87$ ml mg^{-1} cm^{-1} for ONC (18).

Assays of Conformational Stability—Data from cytotoxicity assays performed at 37 °C (see below) can be interpreted properly only with knowledge of the conformation of the protein at that temperature. The conformational stability of each RNase A variant was measured by UV spectroscopy as described (18). Data were fitted to a two-state model for denaturation, and this fit was used to determine the value of T_m, which is the temperature at the midpoint of the thermal transition.

Assays of Ribonucleolytic Activity—The embedded-ribonucleotide substrate 6-FAM~dArUdAdA~6-TAMRA exhibits a 200-fold increase in fluorescence upon cleavage by ribonucleases and is ideal for the rapid and accurate determination of k_{cat}/K_m and K_i values for RNase A and its homologs and variants (35). Values of k_{cat}/K_m for the RNase A variants were determined by measuring the increase in the fluorescence of 6-FAM~dArUdAdA~6-TAMRA (excitation, 490 nm; emission, 515 nm) over time as described (35). Data were fitted to the equation: $k_{cat}/K_m = (\Delta I/\Delta t)\{(I_f - I_o)[E]\}$, where $\Delta I/\Delta t$ is the measured activity, I_o is the initial fluorescence intensity before addition of enzyme, I_f is the fluorescence intensity upon depletion of the substrate following addition of wild-type RNase A, and [E] is the ribonuclease concentration (35).

Preparation of Ribonuclease Inhibitor—Knowing the solution concentrations of active RI is necessary for the calculation of K_i values. The buffer of commercial RI contains the viscogen glycerol, which may lessen the accuracy of volumes delivered by pipette. The viscous buffer was exchanged for 20 mM HEPES-HCl buffer (pH 7.6) containing KCl (50 mM) and DTT (8 mM) by using a Microcon concentrator (10,000 M_r cut-off; Amicon). The glycerol-free solution of RI was diluted 10:1 with 20 mM HEPES-HCl buffer (pH 7.6), and aliquots were stored at −78 °C.

The concentration of active RI was determined by titration with RNase A. Specifically, the ribonucleolytic activity was measured in the presence of aliquots of RI. A solution of RNase A (0.42 nM) and RI in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 mM) and DTT (5 mM) was incubated at 25 °C for 5 min. Poly(C) was added (to 0.10 μM), and the increase in absorbance at 250 nm was measured over time. The concentration of active RI was calculated by assuming that the number of moles of RI equals one-half the number of moles of RNase A at 50% activity.

Assays of Inhibition by Ribonuclease Inhibitor—Values of K_i were determined by measuring the effect of RI on ribonucleolytic activity. Reaction mixtures (2.0 ml) consisted of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), DTT (5 mM), enzyme (3.4 pM or 6.8 pM G88R RNase A; 60 pM K41R/G88R RNase A; 0.66 nM or 1.1 nM K41A/G88R RNase A), 6-FAM~dArUdAdA-6~TAMRA (0.60 μM), and RI (24 pM–3.0 nM for G88R RNase A; 0.24 nM–30 nM for K41R/G88R RNase A and K41A/G88R RNase A). Eight additions of RI were performed, doubling the concentration of RI with each addition and allowing at least 5 min between additions. The apparent K_i values were determined by nonlinear least squares regression analysis of data fitted to the equation: $\Delta I/\Delta t = (\Delta I/\Delta t)_o\{K_i/(K_i + [I])\}$, where $(\Delta I/\Delta t)_o$ is the activity before inhibitor addition, $\Delta I/\Delta t$ is the activity after inhibitor addition, and [I] is the concentration of RI, which was corrected for changes in volume (35).

Assays of Cytotoxicity—The cytotoxicity of a ribonuclease was assessed by measuring its effect on the incorporation of [*methyl*-^{3}H]thymidine into DNA newly synthesized by K-562 cells. A ribonuclease in phosphate-buffered saline (KCl (0.20 g/liter), KH_2PO_4 (0.20 g/liter), NaCl (8.0 g/liter), $Na_2HPO_4{\cdot}7H_2O$ (2.16 g/liter)) was added to cells growing at 37 °C, and cytotoxicity was evaluated after 44 h as described (18). Results were expressed as the percentage of *methyl*-^{3}H incorporated into the DNA of cells treated with phosphate-buffered saline alone. Data represent the average of three repetitions of each individual assay.

RESULTS

Conformational Stability—K41R/G88R RNase A and K41A/G88R RNase A were found to have T_m values of (63 ± 2) °C and (64 ± 2) °C, respectively. The T_m values for wild-type RNase A (63 °C), G88R RNase A (60 °C), and ONC (90 °C) were reported previously (18). All of these ribonucleases are >99% folded at 37 °C.

Ribonucleolytic Activity—The value of $k_{cat}/K_m = 3.6 \times 10^7$ M^{-1} s^{-1} for the cleavage of 6-FAM~dArUdAdA~6-TAMRA by wild-type RNase A was reported previously (35). Here the value of k_{cat}/K_m for G88R RNase A was found to be 1.4×10^7 M^{-1} s^{-1}, which is 2-fold lower than that of the wild-type enzyme. Previous measurements of catalytic activity with poly(C) as substrate gave a similar ratio, with the k_{cat}/K_m values of RNase A

TABLE I

Values of k_{cat}/K_m, K_i, $(k_{cat}/K_m)_{cytosol}$, and IC_{50} for ribonucleases

Values of k_{cat}/K_m were determined for catalysis of 6-FAM~dArUdAdA~6-TAMRA cleavage at pH 6.0 and 25 °C. Values of K_i were determined for inhibition of catalysis by ribonuclease inhibitor. The parameter $(k_{cat}/K_m)_{cytosol}$ is defined in Equation 1. Values of IC_{50} were determined for the toxicity of K-562 cells (Fig. 2).

Ribonuclease	k_{cat}/K_m	K_i	$(k_{cat}/K_m)_{cytosol}$	IC_{50}
	10^7 M^{-1} s^{-1}	nM	10^3 M^{-1} s^{-1}	μM
RNase A	3.6 ± 0.4[a]	0.000044[b]	0.002	
G88R RNase A	1.4 ± 0.1	0.18 ± 0.04	3	6
K41R/G88R RNase A	0.05 ± 0.01	3.0 ± 0.4	1	2
K41A/G88R RNase A	0.00065 ± 0.00004	3.2 ± 0.5	0.02	
ONC	0.000077 ± 0.000009	≥1 × 10^3[c]	≥ 0.4	0.3

[a] Ref. 35.
[b] Ref. 21.
[c] Ref. 23.

(5.7×10^6 M^{-1} s^{-1}) and G88R RNase A (2.9×10^6 M^{-1} s^{-1}) differing by 2-fold (18).

Substitutions at position 41 in RNase A are known to reduce catalytic activity (29, 36, 37). The cleavage of poly(C) by the K41R and K41A variants was reported previously to be 10^2- and 10^4-fold slower, respectively, than by wild-type RNase A (29, 37). Here the activities of K41R/G88R RNase A and K41A/G88R RNase A were measured using the 6-FAM~dArUdAdA~6-TAMRA substrate. The k_{cat}/K_m value of K41R/G88R RNase A is 5×10^5 M^{-1} s^{-1}, which is 70-fold lower than that of the wild-type enzyme. The k_{cat}/K_m value of K41A/G88R RNase A is 6.5×10^3 M^{-1} s^{-1}, which is 5,500-fold less than that of RNase A.

Ribonuclease Inhibitor Binding—The complex of RI with wild-type RNase A is extremely tight ($K_i \approx 10^{-14}$ M (21, 22)). The G88R RNase A variant has a weaker affinity for RI. The K_i for G88R RNase A measured with the fluorogenic substrate was 0.18 nM, which is similar to that determined previously with poly(C) as substrate (18). The double variants K41R/G88R and K41A/G88R RNase A have even lower affinities for RI with K_i values of 3.0 and 3.2 nM, respectively (Table I).

Cytotoxicity—The toxicity of the RNase A variants to K-562 cells was compared with wild-type RNase A as a negative control and ONC as a positive control. G88R RNase A has an IC_{50} value of 6 μM, which is comparable to that reported previously (18). Wild-type RNase A is not toxic to K-562 cells, even at 50 μM. Like the wild-type enzyme, K41A/G88R RNase A is not cytotoxic. Surprisingly, K41R/G88R RNase A, which has 28-fold less ribonucleolytic activity than G88R RNase A, has an IC_{50} of 2 μM, which is 3-fold lower than that of G88R RNase A (Fig. 2). Both of the single variants, K41R RNase A and K41A RNase A, were not cytotoxic (data not shown). Lys-31 in ONC is homologous to Lys-41 in RNase A. K31R ONC has an IC_{50} value of 0.5 μM, which is somewhat higher than the IC_{50} of ONC (0.3 μM) (data not shown).

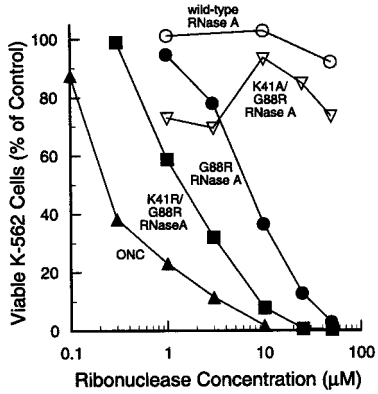

FIG. 2. **Cytotoxicity of ribonucleases.** The proliferation in culture of K-562 cells, which are from a human erythroleukemia line, was evaluated by measuring the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA following a 44-h incubation with the ribonucleases. Values are the mean from three cultures and are expressed as a percentage of the control, which is the mean from cultures lacking exogenous ribonuclease.

DISCUSSION

The ability to evade RI is a necessary attribute for the cytotoxicity of ribonucleases (3). ONC evades RI exceptionally well and is highly cytotoxic, despite its low ribonucleolytic activity relative to RNase A. Wild-type RNase A is 10^4-fold more active as a catalyst of RNA cleavage than is ONC (Table I). Yet, RNase A is not cytotoxic, even at concentrations that are 10^2-fold higher than the IC_{50} of ONC (Fig. 2). The critical biochemical distinction is likely to be the differential affinity for RI. Wild-type RNase A forms an extremely tight complex with RI ($K_i \approx 10^{-14}$ M (21, 22)) in comparison to ONC ($K_i \geq 10^{-6}$ M (23)).

In the RI·RNase A complex, the side chain of Lys-41 lies in a cavern that ends at the side chain of Asp-431 (Fig. 1*A*). RI has a nearly 20-fold higher K_i for both K41R/G88R RNase A and K41A/G88R RNase A than for G88R RNase A (Table I). The larger side chain at position 41 in the K41R/G88R variant may weaken the interaction with RI by steric repulsion or by a less optimal Coulombic interaction with Asp-431. Replacing Lys-41 with the smaller, uncharged alanine residue in K41A/G88R RNase A shows a similar decrease in RI affinity as K41R/G88R RNase A. Removing the Asp-431(RI)-Lys-41(RNase A) Coulombic interaction could contribute to this decrease in affinity.

The lower ribonucleolytic activity of K41R/G88R RNase A does not cripple this enzyme as a cytotoxin. Despite having a 30-fold lower k_{cat}/K_m value than G88R RNase A, K41R/G88R RNase A is 3-fold more cytotoxic (Table I). The likely explanation is the 20-fold better ability of the K41R/G88R variant to evade RI. In contrast, K41A/G88R RNase A is not cytotoxic, despite its ability to evade RI to the same extent as does the K41R/G88R variant. The basis for the dramatic difference in the cytotoxicity of K41A/G88R RNase A and K41R/G88R RNase A is likely to reside in the 80-fold lower ribonucleolytic activity of the K41A/G88R variant.

Ribonucleolytic activity would be destructive in the cytosol, where RNA is prevalent. Yet RI is also present in the cytosol, and only an uninhibited ribonuclease is able to catalyze RNA cleavage. The cytotoxic ribonucleases herein display decreases in k_{cat}/K_m values in the order: G88R RNase A > K41R/G88R RNase A > ONC, but concomitant increases in K_i values in the reverse order: ONC > K41R/G88R RNase A > G88R RNase A (Table I). Interestingly, the decreases in k_{cat}/K_m values are similar in magnitude to the increases in K_i values. This finding suggests that these two parameters, k_{cat}/K_m and K_i, are related to IC_{50} in a simple manner.

To relate ribonucleolytic activity and its inhibition by RI to cytotoxicity, we define $(k_{cat}/K_m)_{cytosol}$ as a measure of the ribonucleolytic activity that is manifested in the presence of cytosolic RI,

$$(k_{cat}/K_m)_{cyto} = \frac{k_{cat}}{K_m(1 + ([RI]/K_i))} \quad \text{(Eq. 1)}$$

To use Equation 1, we use the values of k_{cat}/K_m and K_i in Table I and assume that [RI] = 1 μM (3, 13, 14). Then, the values of $(k_{cat}/K_m)_{cytosol}$ for ONC, K41R/G88R RNase A, and G88R RNase A are $>4 \times 10^2$ M^{-1} s^{-1}, 3×10^3 M^{-1} s^{-1}, and 1×10^3 M^{-1} s^{-1}, respectively (Table I). The similarity of the $(k_{cat}/K_m)_{cytosol}$ values for the G88R and K41R/G88R variants is in gratifying agreement with the similarity of their IC_{50} values (Table I). This agreement suggests that the $(k_{cat}/K_m)_{cytosol}$ parameter has predictive value for variants of RNase A. The values of $(k_{cat}/K_m)_{cytosol}$ for wild-type RNase A and the K41A/G889 variant, which are not cytotoxic, are 2 M^{-1} s^{-1} and 2×10^1 M^{-1} s^{-1}, respectively. A threshold of $(k_{cat}/K_m)_{cytosol} > 2 \times 10^1$ M^{-1} s^{-1} may be necessary for an RNase A variant to be cytotoxic.

Conclusions—A homolog or variant of RNase A can be made cytotoxic by increasing its ribonucleolytic activity in the presence of RI. Lesser intrinsic catalytic activity can be overcome by greater RI evasion. New chemotherapeutics may arise from homologs or variants of RNase A with low catalytic activity (such as ONC) if the ribonuclease also has a low affinity for RI.

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