Increasing the potency of a cytotoxin with an arginine graft

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Variants and homologs of bovine pancreatic ribonuclease (RNase A) can exhibit cytotoxic activity. This toxicity relies on cellular internalization of the enzyme. Residues Glu49 and Asp53 form an anionic patch on the surface of RNase A. We find that replacing these two residues with arginine does not affect catalytic activity or affinity for the cytosolic ribonuclease inhibitor (RI) protein. This ‘arginine graft’ does, however, increase toxicity towards human cancer cells. Appending a nonaarginine domain to this cationic variant results in an additional increase in cytotoxicity, providing one of the most cytotoxic known variants of RNase A. These findings correlate the potency of a ribonuclease with its deliverance of ribonucleolytic activity to the cytosol, and indicate a rational means to enhance the efficacy of ribonucleases and other cytotoxic proteins.

Keywords: cancer/Coulombic interaction/protein transduction domain/ribonuclease/ribonuclease inhibitor

Introduction

Ribonucleases can be cytotoxic and thus have notable potential as chemotherapeutic agents (Youle and D’Alessio, 1997; Leland and Raines, 2001; Matoušek, 2001; Makarov and Ilinskaya, 2003; Benito et al., 2005; Arnold and Ulbrich-Hofmann, 2006). Bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) is the most-studied ribonuclease (Cuchillo and Hofmann, 2006). Although wild-type RNase A is not toxic to mammalian cells, properly engineered variants are cytotoxic (Rutkoski et al., 2005).

The cytotoxicity of a mammalian ribonuclease relies on several attributes (Bretsch et al., 2000; Dickson et al., 2003). First and foremost, ribonucleolytic activity is required for cytotoxicity (Ardelt et al., 1991; Kim et al., 1995). The cytotoxicity of a ribonuclease also correlates with its ability to evade the cytosolic ribonuclease inhibitor (RI) protein (Haigis et al., 2003; Dickson et al., 2005; Rutkoski et al., 2005).

Results

Instead of modifying the enzyme with a toxic agent, we created a ribonuclease variant that integrates a domain containing a cationic arginine patch. Here, we employ a new approach, ‘arginine grafting’ (Fuchs and Raines, 2007), to create a more cell-permeable variant of RNase A. The premise is that installing a patch of arginine residues improves affinity for the cell surface and hence cellular internalization, without affecting other properties of the enzyme. The resulting variant is a single, well-defined protein that can be produced by recombinant DNA technology alone. Previously, replacing five dispersed acidic residues with lysine was shown to endow a microbial ribonuclease with cytotoxic activity (Ilinskaya et al., 2002). By replacing only two proximal acidic residues with arginines, we create an arginine patch that increases the cytotoxicity of an RNase A variant. By also adding a protein transduction domain, we increase cytotoxicity even further.

Materials and methods

Cells and chemicals

Escherichia coli strains BL21(DE3) pLysS and BL21(DE3) were from Novagen (Madison, WI, USA). Human erythroleukemia cells (line K-562) were from the American Type Culture Collection (Manassas, VA, USA). [methyl-3H] Thymidine (6.7 Ci/mmol) was from NEN Life Science Products (Boston, MA, USA). All other chemicals and
reagents were of commercial reagent grade or better, and were used without further purification.

**Instruments**

The mass of each RNase A variant was ascertained by MALDI–TOF mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix in the campus Biophysics Instrumentation Facility. Fluorescence measurements were performed with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ, USA). Radioactivity was quantified with a Microbeta TriLux liquid scintillation counter (Perkin Elmer, Wellesley, MA, USA). Electrostatic potential maps were created with the program PyMol (DeLano Scientific, South San Francisco, CA, USA).

**Site-directed mutagenesis**

Oligonucleotides were obtained from Integrated DNA Technology (Coralville, IA, USA). cDNA encoding variants of RNase A were created in plasmid pBXR, which directs the production of RNase A in *E. coli* (delCardayre et al., 1995), by using the QuikChange mutagenesis kit from Stratagene (La Jolla, CA, USA). All variants of RNase A possessed either the native Lys1 residue (wild-type RNase A, G88R RNase A, E49/D53R RNase A and E49/D53R/G88R RNase A) or an N-terminal methionine residue (R9-tagged variants of RNase A), which has no effect on ribonucleaseolytic activity (Arnold et al., 2002). The C-terminal R9 tag was distanced from the remainder of a protein by a triglycine linker.

**Production and purification of protein variants**

Untagged variants of RNase A and Onconase [which is the most cytotoxic known homolog of RNase A (Matoušek et al., 2003b)] were produced in *E. coli* BL21(DE3) and purified as described previously (Leland et al., 1998). Variants of RNase A containing a C-terminal R9 tag were produced in *E. coli* BL21(DE3)pLysS cells and purified as described previously (Fuchs and Raines, 2005). The affinity of RNase A variants for human RI (hRI) was determined by using a competitive binding assay reported recently (Lavis et al., 2007). Unlike other assays (Abel et al., 2002), this assay exhibits negligible protein loss by nonspecific binding, allowing the determination of *Kd* values for the highly cationic R9-tagged variants of RNase A.

**Assay of cytotoxicity**

The effect of ribonucleases on the proliferation of K-562 cells was determined by measuring the incorporation of [methyl-3H]thymidine into cellular DNA as described previously (Leland et al., 1998; Rutkoski et al., 2005).

**Results and discussion**

**Design of cationic RNase A variants**

The ability of polycations to effect the cellular internalization of a macromolecule has been known for over 40 years (Rysor and Hancock, 1965; Fuchs and Raines, 2006). Recently, we used an arginine ‘graft’ to endow the green fluorescent protein from the jellyfish *Aequorea victoria* with cell permeability (Fuchs and Raines, 2007). We have now applied the arginine grafting strategy to RNase A, a mammalian protein that can be cytotoxic.

We used the cytotoxic activity of an RNase A variant as a measure of its cellular internalization. This measure, though indirect, requires the internalized ribonuclease to retain its catalytic activity (and, thus, its three-dimensional structure) to elicit cytotoxicity (Ardelt et al., 1991; Kim et al., 1995). Accordingly, most of our variants contained the G88R substitution, which makes RNase A cytotoxic by raising the *Kd* value for the hRI-RNase A complex (Leland et al., 1998).

We sought to increase the cationicity of RNase A without disrupting catalytic activity. In an electrostatic potential map of RNase A, we noted the presence of two acidic residues on its molecular surface (Fig. 1A and B). These residues, Glu49 and Asp53, are in close proximity to one another but remote from the active site. We hypothesized that replacing these acidic residues with basic ones would create a cationic patch without compromising catalytic activity. We chose to replace Glu49 and Asp53 with arginine, which is the most effective residue for facilitating cellular internalization (Mitchell et al., 2000; Suzuki et al., 2002). Recently, we showed that appending an R9 tag to the C-terminus of RNase A increased its internalization (Fuchs and Raines, 2005). Hence, we also determined if an arginine graft and an R9 tag have an additive effect on cytotoxic activity.

**Biochemical properties of RNase A variants**

The E49R/D53R-containing variants of RNase A exhibited wild-type catalytic activity (Table I), as expected. The conformational stability of the E49R/D53R and E49R/D53R/G88R variants (*Tm* = 54°C) was, however, significantly lower than that of either wild-type RNase A (*Tm* = 64°C) or the G88R variant (*Tm* = 60°C). Appending an R9 tag to the E49R/D53R or E49R/D53R/G88R variant decreased stability...
even further. Still, all variants were >99% folded at physiological temperature.

The ability to evade the RI protein within mammalian cells is a strong determinant of ribonuclease cytotoxicity (Leland et al., 1998; Rutkoski et al., 2005; Johnson et al., 2007). Glu49 and Asp53 are remote from the molecular interface in the porcine RI RNase A complex (Fig. 1C). Nevertheless, we measured the affinity of both E49R/D53R/G88R RNase A and E49R/D53R/G88R RNase A–R9 for RI (Table I), finding these affinities ($K_d = 2.6$ nM and $K_d = 3.0$ nM, respectively) to be comparable to that of G88R RNase A ($K_d = 2.8$ nM).

### Cytotoxicity of RNase A variants

The toxicity of a ribonuclease increases as its cellular internalization becomes more efficient (Fuchs and Raines, 2005). We monitored the internalization of the RNase A variants by measuring their toxicity to K-562 cells (Fig. 2; Table I). Wild-type RNase A is not toxic to mammalian cells due to

### Table I. Biochemical parameters of RNase A and its variants

<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>$pI^a$</th>
<th>$T_m$ (°C)$^b$</th>
<th>$k_{cat}/K_M$ (10$^6$ M$^{-1}$s$^{-1}$)$^c$</th>
<th>IC$_{50}$ (μM)$^d$</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase A</td>
<td>8.6</td>
<td>64°</td>
<td>2.34 ± 0.09</td>
<td>&gt;25</td>
<td>44 × 10$^{-6}$</td>
</tr>
<tr>
<td>G88R RNase A</td>
<td>8.8</td>
<td>60°</td>
<td>2.9 ± 0.1</td>
<td>6.2 ± 0.5</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>E49R/D53R RNase A</td>
<td>9.2</td>
<td>54°</td>
<td>2.21 ± 0.02</td>
<td>&gt;25</td>
<td>&lt;2.8$^g$</td>
</tr>
<tr>
<td>E49R/D53R/G88R RNase A</td>
<td>9.3</td>
<td>54°</td>
<td>2.5 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>RNase A–R9</td>
<td>9.6</td>
<td>56°</td>
<td>1.0 ± 0.2$^e$</td>
<td>&gt;25$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>G88R RNase A–R9</td>
<td>9.8</td>
<td>54°</td>
<td>9.6 ± 0.2$^e$</td>
<td>1.9 ± 0.1$^b$</td>
<td>ND</td>
</tr>
<tr>
<td>E49R/D53R RNase A–R9</td>
<td>10.0</td>
<td>48°</td>
<td>1.55 ± 0.03</td>
<td>6.0 ± 0.3</td>
<td>&lt;2.8$^g$</td>
</tr>
<tr>
<td>E49R/D53R/G88R RNase A–R9</td>
<td>10.1</td>
<td>49</td>
<td>3.59 ± 0.05</td>
<td>0.58 ± 0.02</td>
<td>3.0 ± 1.1</td>
</tr>
</tbody>
</table>

ND, not determined.

$^a$Values of $pI$ were estimated from amino-acid composition (Bjellqvist et al., 1993; Bjellqvist et al., 1994).

$^b$Values of $T_m$ (± 2°C) were determined in PBS by ultraviolet spectroscopy.

$^c$Values of $k_{cat}/K_M$ (± SE) are for the catalysis of 6-FAM–dArU(dA)$_2$–6-TAMRA cleavage at 25°C in 10 mM Bis-Tris–HCl buffer, pH 6.0, containing NaCl (0.50 M).

$^d$Values of IC$_{50}$ (± SE) are for the incorporation of [methyl-3H]thymidine into the DNA of K-562 cells (Fig. 2).

$^e$Value from Fuchs and Raines (2005).

$^f$Value from Lee et al. (1989).

$^g$Value was below the lower limit of detection for the assay.

$^h$Raw data from Fuchs and Raines (2005) fitted in the manner of this work.
its high affinity for the cytosolic RI protein. The RI-evasive variant, G88R RNase A, had an IC$_{50}$ value of 6.2 μM. Arginine grafting increased the cytotoxicity of this variant by 3-fold. In contrast, E49R/D53R RNase A was not toxic to K-562 cells.

Finally, the addition of an R$_9$-tag to either G88R RNase A or E49R/D53R/G88R RNase A increased cytotoxicity by an additional 3-fold, with G88R RNase A–R$_9$ and E49R/D53R/G88R RNase A–R$_9$ exhibiting IC$_{50}$ values of 1.9 and 0.58 μM, respectively (Fig. 2B; Table I). Thus, the cumulative effect of arginine grafting and the addition of an R$_9$ tag increased cytotoxic activity by an order of magnitude. E49R/D53R/G88R RNase A–R$_9$ is one of the most toxic of known variants for K-562 cells (Rutkoski et al., 2005).

Surprisingly, E49R/D53R RNase A–R$_9$, which retains high affinity for RI, exhibited cytotoxicity (IC$_{50}$ = 6.0 μM) comparable to that of G88R RNase A. This result was unexpected because RNase A–R$_9$ (Z = +13) does not exhibit cytotoxic activity (Fuchs and Raines, 2005). We suspect that the highly cationic E49R/D53R RNase A–R$_9$ (Z = +17) is internalized so efficiently that its concentration in the cytosol overwhelms endogenous RI. A similar explanation has been put forth to explain the cytotoxic activity of otherwise RI-sensitive ribonucleases (Leich et al., 2006) of which there have been several reports (Notomista et al., 2006; Bosch et al., 2004; Naddeo et al., 2005).

**Summary**

The cytotoxicity of a ribonuclease arises from its degradation of cellular RNA. Herein, we demonstrated that this cytotoxic activity can be enhanced by the installation of an arginine graft. The increased efficiency of internalization presumably afforded by the arginine graft can be used in conjunction with an R$_9$ protein transduction domain to achieve additive enhancements in cytotoxicity. In the absence of an R$_9$ tag, evasion of RI is necessary for a permeant variant to be cytotoxic. Accordingly, the potency of a ribonuclease correlates with its ability to manifest unfettered ribonucleolytic activity in the cytosol. These findings inform the design and engineering of ribonucleases with enhanced cytotoxic activity and clinical utility.

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**References**


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