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In Vitro Selection of RNA Molecules That Inhibit the Activity of Ricin A-chain*

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The cytotoxin ricin disables translation by depurinating a conserved site in eukaryotic rRNA. *In vitro* selection has been used to generate RNA ligands (aptamers) specific for the catalytic ricin A-chain (RTA). The anti-RTA aptamers bear no resemblance to the normal RTA substrate, the sarcin-ricin loop (SRL), and were not depurinated by RTA. An initial 80-nucleotide RNA ligand was minimized to a 31-nucleotide aptamer that contained all sequences and structures necessary for interacting with RTA. This minimal RNA formed high affinity complexes with RTA (K_d = 7.3 nM) which could compete directly with the SRL for binding to RTA. The aptamer inhibited RTA depurination of the SRL and could partially protect translation from RTA inhibition. The IC_{50} of the aptamer for RTA in an *in vitro* translation assay is 100 nM, roughly 3 orders of magnitude lower than a small molecule inhibitor of ricin, pteroic acid, and 2 orders of magnitude lower than the best known RNA inhibitor. The novel anti-RTA aptamers may find application as diagnostic reagents for a potential biological warfare agent and hold promise as scaffolds for the development of strong ricin inhibitors.

Ribosome-inactivating proteins inhibit protein synthesis by disabling the translation machinery. Class I ribosome-inactivating proteins have a catalytic A-chain that recognizes and depurinates a universally conserved adenosine found in a GAGA tetraloop in the sarcin-ricin loop (SRL)[1] of eukaryotic 23–28 S rRNA (1, 2). Class II ribosome-inactivating proteins have an additional lectin B-chain that is required for cell surface attachment and subsequent endocytosis of the toxin domain (3).

Ricin, from the castor bean plant *Ricinus communis*, is a class II ribosome-inactivating protein that has the potential of being used as a weapon in a biological attack (4, 5). Once inside a cell, ricin is extremely toxic; one resident molecule is sufficient to kill the cell, and the enzyme has been reported to inactivate 1777 ribosomes min^{-1} (2). Aerosolized ricin causes severe respiratory distress and is lethal when injected intravenously at a level of only 3–5 μg/kg (6). Ricin has so far proved to be a relatively inefficient biological weapon but is prepared easily even by Third World nations and terrorist groups. Therefore, facile detection of the toxin and the development of antidotes remain priorities (6, 7).

In vitro selection is a powerful molecular tool that can be used to generate ligands for a wide variety of targets, including both nucleic acid and non-nucleic acid-binding proteins (8, 9). Aptamers can be engineered readily to function as either therapeutics or sensors. For instance, RNA aptamers that bind to human immunodeficiency virus type I Rev also inhibit viral replication (10). Similarly, RNA aptamers that bind to the β_2 integrin leukocyte function-associated antigen 1 specifically inhibit a signal transduction pathway when expressed *in vivo* (11). Consequently, aptamers that recognize and potentially inhibit ricin might be useful prophylactic or therapeutic agents or could be adapted to function as biosensor elements for the detection of aerosolized ricin or areas contaminated by the toxin.

EXPERIMENTAL PROCEDURES

Pools and Oligonucleotides—The random sequence pool (N30) has been described previously (9, 12). Following chemical synthesis, the single-stranded DNA pool was purified on a 6% denaturing polyacrylamide gel and eluted by diffusion. The pool was then amplified via the polymerase chain reaction using primers 41.30 (5′-GATAATACGACTCACTATAGGGAATGGATCCACATCTACG-3′) and 24.30 (5′-AAGCTTCGTCAAGTCTGCAGTGAA-3′). The selected aptamer 80RA was resynthesized as a doped sequence pool (D30). The sequence of the D30 pool was 5′-GGGAATGGATCCACATCTA*CGAATTCAGGGGACGTAGCAATGACTGAGATGCTGGG*TTCACTGCACTTGACGAAGCTT-3′, where italicized residues indicate positions that were doped at 85% wild type and 5% of each non-wild type nucleotide. All other residues were kept constant. The 5′-primer used for amplification was three nucleotides shorter from the 3′-end than 41.30, and the 3′-primer was 24.30.

A small version of the ribosomal SRL binds the catalytic ricin A-chain (RTA) and has been used previously as a substrate in depurination assays (13). The sequence of the 34-nucleotide SRL (34SRL) was 5′-GGAAUCCUGCUCAGUACGAGAGGAACCGCAGGUU-3′; a double-stranded DNA template containing a T7 RNA polymerase promoter was used to transcribe 34SRL.

In Vitro Selection—The RNA pool (2.2×10^{14} molecules) was transcribed from the amplified DNA template (1.1×10^{14} molecules) using an Ampliscribe T7 *in vitro* transcription kit (Epicenter Technologies, Madison, WI). After purification on an 8% denaturing polyacrylamide gel, the RNA pellet was dissolved in 1 × phosphate-buffered saline and 5 mM $MgCl_2$, then heated to 65 °C for 3 min and allowed to cool to room temperature over 10 min. To exclude filter-binding RNA sequences from the pool, the RNA was passed over a 0.45-μm HAWP filter (Millipore, Bedford, MA) and washed with an equal volume of buffer. RTA was added to the binding reaction (200 μl final volume) in varying amounts throughout the course of the selection (Table I). Binding reaction mixtures were incubated for 1 h at room temperature. After 1 h, the solution was vacuum-filtered over a HAWP filter at 5 p.s.i. and washed twice with 0.5 ml of the selection buffer. RNA retained on the filter was eluted twice with 0.2 ml of 7 M urea, 100 mM sodium citrate (pH 5.0) and 3 mM EDTA for 3 min at 100 °C, and the eluted RNA was precipitated with isopropyl alcohol. For the eighth and ninth rounds, the eluted RNA pool was passed over a HAWP filter after selection and precipitation to remove filter-binding species. Selected RNAs were reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Inc.) and the 3′-primer (24.30). The cDNA products were polymerase chain reaction amplified after the addition of 5′-primer (41.30) and DisplayTaq (PGC Scientific, Gaithersburg, MD). The polymerase chain reaction

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[1] The abbreviations used are: SRL, sarcin-ricin loop; RTA, catalytic ricin A-chain.

TABLE I
Summary of in vitro selection experiments

Selection and round	Input [RNA]	Input [RTA]	[RNA]:[RTA]	% Bound to RTA	% Bound to filter
	nM	*nM*			
N30					
1	2,500	500	5:1		
2	2,500	500	5:1		
3	800	26.7	30:1		
4	800	26.7	30:1	1.06	0.73
5	800	26.7	30:1		
6	800	26.7	30:1		
7	800	26.7	30:1	19.92	7.88
8	800	800	1:1		
9	800	800	1:1	51.09	2.41
D30					
1	1,000	200	5:1	5.62	2.13
2	1,000	200	5:1	15.52	2.86
3	2,000	200	10:1	27.65	2.52
4	2,000	200	10:1	28.72	1.38

products were transcribed and gel purified to begin the next round of selection.

After round 9, the pool was cloned (Topo TA cloning kit, Invitrogen, Carlsbad, CA) and sequenced using standard dideoxy methods. The dominant clone (80RA) and its derivatives (31RA) were characterized using a nitrocellulose filtration assay similar to that used for selection.

The doped sequence selection was carried out using procedures identical to those used in the initial selection. A total of 1.84×10^{13} DNA molecules were used for transcription, and a total of 1.2×10^{14} RNA molecules were introduced at the first round of selection. Based on the rate of mutagenesis and the size of the pool, this should have represented all possible single to hextuple mutations. Table I again summarizes selection conditions.

Competition Assay—31RA and 34SRL were body labeled in a transcription reaction containing [α-^{32}P]UTP (3000 Ci/mmol, NEN Life Science Products). After labeling, products were gel purified and precipitated. In competition assays, the minimal aptamer, 31RA and 34SRL RNAs were incubated at equimolar amounts (0.5 μM) in the presence of limiting RTA (0.1 μM) in the selection buffer. The binding reaction mixture was incubated at 25 °C for 1 h, and 80% of the volume was filtered over nitrocellulose and washed twice with 300 μl of selection buffer. Retained RNA molecules were eluted and precipitated. The remaining 20% of the reaction mixture was also precipitated. Both filtered and unfiltered samples were dissolved in 5 μl of stop dye (7 M urea, 1 × TBE, 0.1% bromphenol blue) and analyzed on a denaturing 12% acrylamide gel. The amount of radioactivity in individual bands was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The relative binding activity was calculated based on the formula: ((counts filtered, 31RA/counts unfiltered, 31RA))/((counts filtered, 34SRL)/(counts unfiltered, 34SRL)). This formula has been used previously to assess the relative binding activities of RNA ligands (14, 15). Assuming that the binding reaction is at equilibrium (a likely assumption, given the length of the binding reaction), the binding ratio represents a ratio of the dissociation constants of individual RNA-protein complexes.

Filter Binding Assays—After rounds 4, 7, and 9 of the initial selection, and at every round of the doped selection, the RNA pool was body labeled in a transcription reaction using [α-^{32}P]UTP (3000 Ci/mmol, NEN Life Science Products). Products were gel purified and precipitated. The labeled RNA (0.5 μM final concentration) was incubated with RTA (0.5 μM final concentration) in 0.1 ml of selection buffer for 1 h at room temperature. The solution was filtered and washed three times with selection buffer. The filter was exposed to a PhosphorImager plate, and the amount of retained radioactivity was determined. The fraction of the pool or clone that bound RTA was calculated by comparing the counts retained on the filter with the total number of counts in the original RNA.

For the determination of dissociation constants, 1.5 pmol of RNA was end labeled with [γ-^{32}P]ATP (0.03 mCi, 6000 Ci/mmol, ICN Biomedicals, Costa Mesa, CA) after 5′-dephosphorylation. Labeled RNA was purified by two successive precipitations using ammonium acetate. The labeled RNA (2 nM final concentration) was incubated with increasing concentrations of RTA (1.2–450 nM) in 50 μl of the selection buffer for 1 h at room temperature. The mixture was filtered on a vacuum manifold (Schleicher & Schuell) equipped with a piece of pure nitrocellulose membrane (Schleicher & Schuell) over a piece of Hybond (Amersham Pharmacia Biotech) as described previously (16, 17). The radioactivity on the nitrocellulose and nylon filter was quantitated, and the dissociation constant (K_d) for aptamer-RTA complexes was determined using a curve-fitting algorithm on the program Kaleidagraph (Adelbeck Software, Reading, PA).

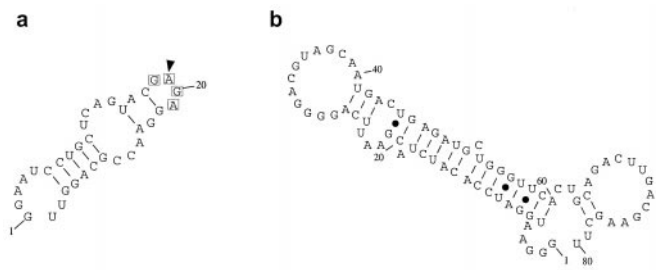

FIG. 1. **Predicted secondary structures of RTA ligands.** *a*, the SRL of 23 S rRNA. The universally conserved GAGA tetraloop of the SRL is *boxed*, and an *arrow* points to the canonical adenosine substrate. *b*, the selected anti-RTA aptamer, RA80.

Assay for RNA Depurination—The assay was performed as described previously (13). RNA (2 μM) was denatured at 65 °C in 1 × phosphate-buffered saline, 5 mM $MgCl_2$ and allowed to cool to room temperature for 10 min. Dithiothreitol and EDTA were added to a 1 mM final concentration (20 μl final volume), and the reaction mixtures were incubated with varying concentrations of RTA for 60 min at 30 °C. After a phenol/$CHCl_3$ extraction and ethanol precipitation, pellets were resuspended in 20 μl of a buffered aniline solution and incubated for 15 min on ice. After a second precipitation, RNA molecules were 5′-end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (0.03 mCi, 6000 Ci/mmol, ICN Biomedicals) in a 5-μl reaction volume. The RNA was precipitated and resuspended in loading dye, and products were analyzed on a 20% denaturing polyacrylamide gel. The gel was fixed, and counts were quantitated using a PhosphorImager.

Protein Synthesis Inhibition Assay—Recombinant RTA was prepared as described previously (18). A standard assay for RTA function is to observe the degradation of translation ability by isolated *Artemia salinas* ribosomes (19). When RTA (2 nM) was added to an *A. salinas in vitro* translation system, the synthesis of ^{14}C-labeled polyphenylalanine was reduced by 80%. Using this basal assay as our standard, different concentrations of 31RA were then assayed for their ability to inhibit RTA degradation of translation ability. Prior to use, solutions containing 31RA were heated at 65 °C for 3 min and allowed to cool at 25 °C for 10 min.

RESULTS AND DISCUSSION

Selection of Anti-RTA Aptamers—Recombinant RTA was used as a selection target; the recombinant protein has been shown to possess full activity toward rat liver ribosomes as well as toward small variants of the SRL of 28 S rRNA (13) (Fig. 1*a*). *In vitro* selection experiments were initiated with an RNA pool (N30) that contained 30 randomized positions. This pool has been utilized successfully in the past to generate aptamers against both small molecules and proteins (8). In each round of selection, the protein was mixed with the RNA pool, and bound species were separated from unbound by passing the mixture over a modified cellulose filter. After seven rounds of selection, modest protein-dependent binding activity was observed (Table I). To rid the pool of matrix-binding sequences the stringency of the selection was increased by increasing the ratio of input RNA to protein. After an additional two rounds of selection the protein-dependent binding ability of the pool had reached 51% in our standard assay, whereas matrix binding was only 2.5%.

After round 9 of the selection, 24 individual aptamers were cloned and sequenced. Although the initial pool had contained approximately 2.2×10^{14} different sequences, the selected pool had been winnowed to a single consensus sequence. The program MulFold was used to model the secondary structure of the aptamer (RA80). The conformer predicted to be most stable contained a long paired stem topped by a large loop and is shown in Fig. 1*b*.

To determine which sequences in the limit aptamer contrib-

uted to recognition of RTA, a doped sequence population was prepared based on the apparent secondary structure of the aptamer. Each position in the original random sequence region and three positions from the 5′-constant region that contributed to the hypothesized stem structure contained 85% wild type residues and 5% of each non-wild type residue (*e.g.* 85% G, 5% A, 5% T, 5% C at position 21). This level of mutagenesis should have been sufficient to identify sequences and structures required for RTA binding function (21) because the population would have contained all possible single to hextuple sequence substitutions. The constant regions and amplification primers were as before, except that the 5′-primer used for amplification was 3 residues shorter than previously.

After four cycles of selection and amplification the population could bind as well as the parental aptamer to RTA. Early in the selection the population was sequenced to ensure that numerous mutants were present and were competing for RTA (Fig. 2*a*). In contrast, many of the positions that contained mutations in the first round of selection had completely returned to their wild type progenitors by the third round of selection (Fig. 2*b*). Overall, the distribution of mutations within the variants suggests that mutations in the stem portion of the doped region were tolerated, whereas the loop region was much more highly conserved. These results were expected to some extent because the loop region was derived completely from the random sequence region, whereas the stem was established in part by pairing between the random sequence region and the 5′-constant region.

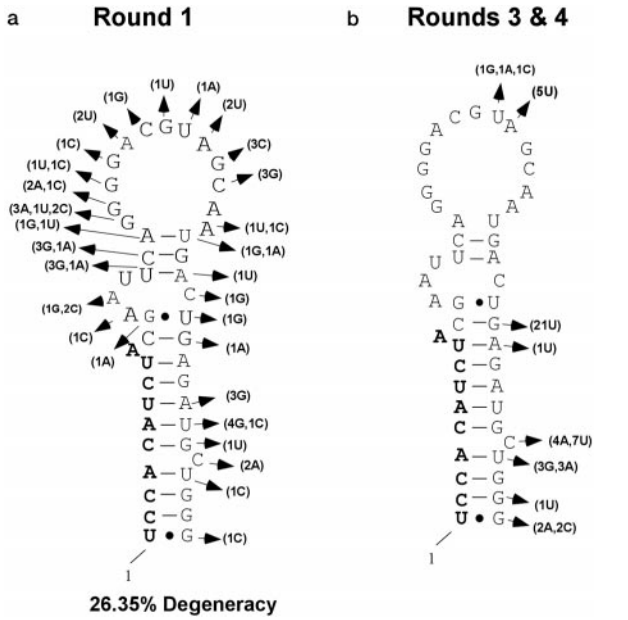

FIG. 2. **The anti-RTA aptamer has high information content.** *a*, mutations present after the first round of selection. The number and type of mutations are superimposed on the predicted secondary structure. Residues that were not doped are shown in *bold*. *b*, mutations present after the third and fourth rounds of selection.

Minimization of an Anti-RTA Aptamer—Surprisingly, neither the limit aptamer nor its predicted secondary structure showed any gross similarity to 28 S rRNA. To determine if more subtle similarities existed, we compared the aptamer with the SRL of 28 S rRNA. The conformation of the SRL has been studied extensively (22–24), and both NMR and crystallography studies have indicated that it is essentially a continuous helix interrupted by a G-bulge and topped by a GAGA tetraloop. The sequence of the tetraloop has been shown to be critical for toxin recognition and binding (25).

The original aptamer indeed contained a GAGA motif, but it is ensconced within a stable stem structure (Fig. 1*b*). However, an alternative, less stable conformer was predicted to contain a putative GAGA recognition site in a loop (Fig. 3). To ascertain whether the original (RA80.1) or alternative (RA80.2) conformer was the active ricin-binding species, several deletion variants were prepared. The deletion variants that were predicted to favor the original conformer were active; those that were predicted to favor the alternative conformer were inactive. The selection of a completely non-native, anti-RTA aptamer is not necessarily unusual; many aptamers bear little or no resemblance to corresponding wild type RNA ligands (26, 27).

Multiple mutations occurred in the stem region after mutagenesis and reselection, indicating that many of the residues in the stem might not contribute to RTA binding. To test whether the loop was primarily responsible for interactions with RTA, we prepared a short, 31-mer RNA that contained most of the residues that were conserved on reselection (31RA,

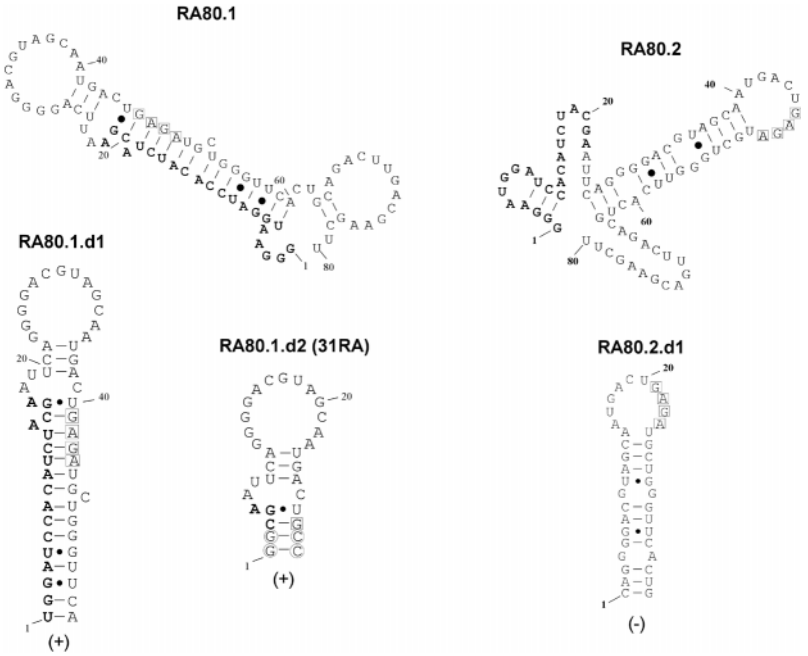

FIG. 3. **Comparison of aptamer conformers.** RA80.1 is the originally predicted conformer; RA80.2 is an alternative conformer that presents a single-stranded GAGA sequence (*boxed*) that might be recognized by ricin. Deletion constructs that were predicted to enforce either the RA80.1 conformer (RA80.1.d1 and RA80.1.d2) or the RA80.2 conformer (RA80.2.d1) were synthesized and assayed for their ability to bind ricin. Residues in the constant regions are in *bold*, and those that differ from the original aptamer are *circled*. Binding is indicated by + (observed binding) and − (no observed binding).

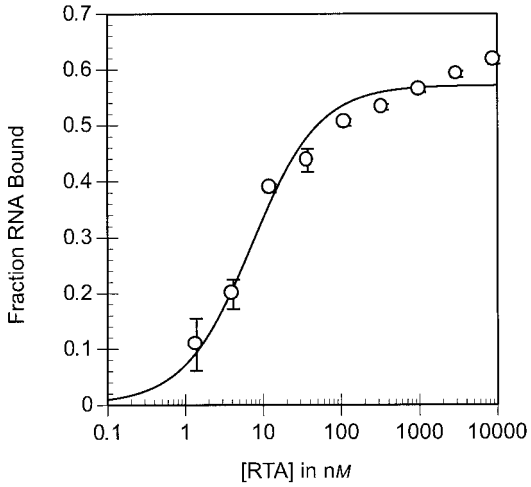

FIG. 4. **Binding isotherm for the minimal anti-RTA aptamer (31RA).** Different concentrations of protein were mixed with a fixed concentration (2 nM) of aptamer. The binding reactions were allowed to come to equilibrium at room temperature over 1 h, and the complexed RNA was separated from free RNA by filtration. 100% binding is likely not observed at saturation because of inefficient capture of complexes. This phenomenon has been noted previously for other aptamer-protein complexes (44, 45). The curve was fit assuming a single RTA binding site for the aptamer, and the calculated dissociation constant (K_d) is 7.4 ± 1.1 nM.

Fig. 3). The secondary structure of the minimal aptamer was stabilized by the inclusion of several designed G:C base pairings. The minimal aptamer was fully competent to bind RTA. The fact that a functional, quadruple mutant (the two designed G:C pairs) could be designed based on the predicted aptamer secondary structure is further evidence in favor of our nascent structural model. Interestingly, the minimal aptamer lacked the GAGA sequence but did contain a GGGG sequence in its loop. It is possible that this altered run of purines encourages tight interactions with RTA while avoiding the presentation of an adenosine substrate.

Anti-RTA Aptamers Bind RTA with High Affinity—The interaction between anti-RTA aptamers and RTA was assessed as a function of protein concentration using a filter binding assay similar to that used for selection. The RTA·RA80.1 complex was found to have a K_d of 240 nM (data not shown), whereas the RTA·31RA complex was found to have a K_d of 7.4 nM (Fig. 4). Because the binding ability of the aptamer actually improved after truncation it can again be argued that sequences in the loop region are primarily responsible for RTA recognition. Indeed, one of the reasons that the binding ability may have improved was that the shorter structure could no longer fold into alternate conformations (such as RA80.2).

Although the affinity of the anti-RTA aptamer for RTA is reminiscent of the affinities of other anti-protein aptamers for their targets (28), it is actually quite extraordinary considering that the SRL itself has a K_m of 13.55 μM in a depurination assay (13). Even a small, SRL-like RNA containing a predesigned transition-state analog has been reported to inhibit RTA activity with a K_i of only 0.73 μM (29). Molecular modeling studies have identified pteroic acid as an inhibitor of RTA activity, but the K_i for pteroic acid is around 0.6 mM (30, 31). The anti-RTA aptamer therefore binds at least 3 orders of magnitude better than the best known small molecule inhibitor of ricin, and 2 orders of magnitude better than a designed RNA analog. The high affinity of the aptamer relative to other ligands exemplifies the dramatic possibilities afforded by *in vitro* selection.

Anti-RTA Aptamers Compete with the SRL but Are Not Depurinated—To prove that anti-RTA aptamers bound to the active site of RTA, a competition assay was employed. Briefly, 31RA and 34SRL were incubated with a limiting amount of RTA. A portion of the reaction was filtered, and bound RNAs were then separated by gel electrophoresis. As Fig. 5 shows, 31RA completely displaced the 34SRL substrate from RTA. This competition assay can also be used to quantitate the relative degree of binding (15). The specific activities of the samples before filtration were compared with the specific activities after filtration. Within the limits of the assay, 31RA binds RTA at least 9-fold better than 34SRL.

Although 31RA does not contain a canonical GAGA recognition sequence, it might still be depurinated (for example, at A32, A36, or even A39). To assess this possibility, we employed a depurination assay in which the accumulation of radiolabeled cleavage fragments is monitored (13). When 31RA was incubated with RTA and the same depurination assay used for the SRL was carried out, no RTA-specific radiolabeled cleavage

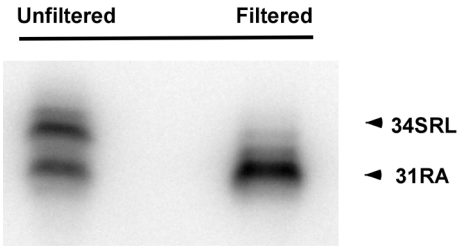

FIG. 5. **The anti-RTA aptamer competes with the SRL for the same site on RTA.** Body-labeled 31RA and 34SRL were incubated in equal amounts with a limiting amount of RTA. A portion of the binding reaction was filtered over nitrocellulose, and filtered and unfiltered RNAs were precipitated and analyzed by electrophoresis. No detectable 34SRL was observed after filtration, and 31RA out-competes 34SRL by at least a factor of 9.

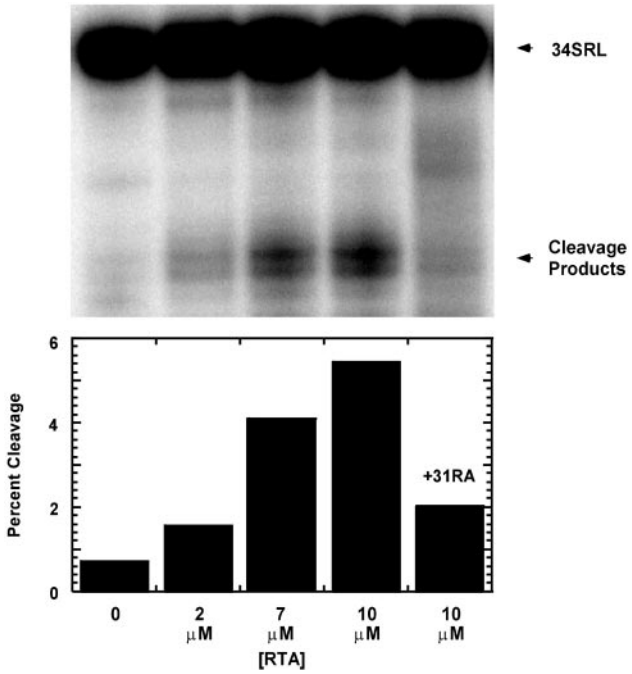

FIG. 6. **The anti-RTA aptamer inhibits depurination of the SRL by RTA.** Different concentrations of RTA were mixed and incubated with a fixed amount of labeled substrate RNA for 1 h. The resulting RNA was treated with aniline to cleave the SRL at depurinated, abasic sites. The products were then analyzed on a 20% gel and quantitated using a PhosphorImager. The expected cleavage product appears in a concentration-dependent manner relative to RTA. The doubling of the band representing the cleavage product is likely due to the presence of untemplated, 3′ additions of nucleotides to the full-length transcription product (20). Competition with a five molar excess of unlabeled 31RA resulted in a 50% decrease in cleavage product.

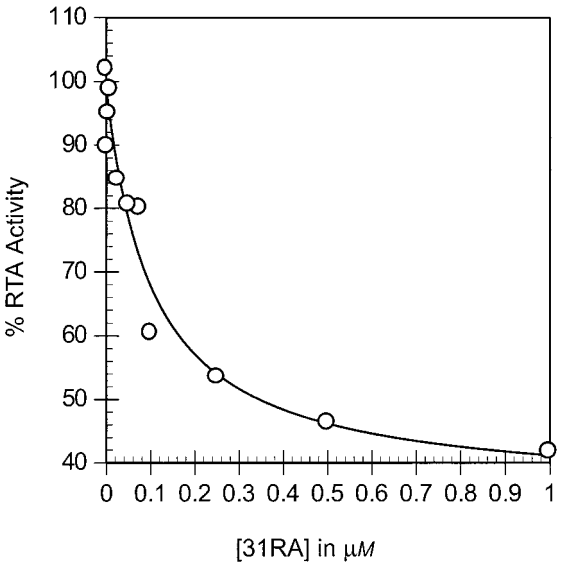

FIG. 7. **The anti-RTA aptamer inhibits RTA activity in an *in vitro* protein synthesis assay.** The degradation of the translation capacity of *A. salinas* ribosomes was judged by determining the amount of radiolabeled protein produced (19). The minimal anti-RTA aptamer 31RA was added at varying concentrations. The percent RTA inhibition was normalized to the amount of protein produced when no aptamer was added. Points are the average of triplicate experiments; a curve-fitting algorithm was used to derive the IC_{50} for 31RA, which was calculated to be 104 ± 25 nM.

products were observed (data not shown). In contrast, when the SRL was incubated with RTA, a characteristic depurination and cleavage product was observed (Fig. 6). When 31RA and the SRL were incubated together (5:1 31RA·SRL), the cleavage of SRL was inhibited, as would be expected if the two RNAs were binding to the same site on the enzyme. No inhibition of depurination was observed with a nonspecific RNA molecule, tRNA. Because of the many steps associated with the depurination assay, it was not feasible to attempt a shorter time course, nor could a K_i value for the aptamer be derived. Overall, though, the data strongly support the nascent hypothesis originally suggested by the sequence and structure of 31RA: the aptamer binds to RTA, competes with the SRL, but is not itself a substrate for RTA.

31RA Inhibits Ribosomal Inactivation by RTA—RTA activity can also be measured by observing the disruption of *in vitro* protein synthesis by *A. salina* ribosomes. This sensitive method has been used previously to assess the activity of other RTA inhibitors (19). The inhibition of RTA activity was assayed as a function of 31RA concentration, and Fig. 7 shows that as the aptamer saturates RTA, the enzyme loses about 60% of its activity. The amount of 31RA that gives 50% of this maximal RTA inhibition (IC_{50}) was approximately 100 nM. It may be that the bound aptamer partially occludes the RTA active site, retarding activity about 60%; partial active site occlusion has been observed previously in aptamers that bind to protein tyrosine phosphatases (12). In the absence of RTA, the aptamer had no toxic effect on ribosomes. A non-cognate, anti-basic fibroblast growth factor aptamer, PS8 (32), was used as a negative control. PS8 was not an inhibitor of RTA function and did not have an effect on *Artemia* ribosomes (data not shown).

The minimal aptamer may have protected translation more effectively than it protected the SRL from depurination because it was in greater excess over RTA in the translation assays. In addition, RTA acted on ribosomes for only 5 min in the *in vitro* translation assay, whereas it acted for 60 min on the isolated SRL. Nonetheless, the ability of the aptamer to inhibit the toxic effects of RTA at relatively low concentrations suggests that it may have promise as a scaffold for the development of gene or more conventional therapies for ricin exposure. For example, aptamers or their derivatives could be conjugated to pteroic acid to produce a highly efficient chimeric inhibitor in which the aptamer binds tightly and the pteroic acid completely inactivates RTA. Similar approaches in which aptamers were conjugated to peptides or transition state analogs yielded inhibitors with very high affinities for human neutrophil elastase (33, 34).

Anti-RTA Aptamers Represent a Novel Class of Nucleic Acid Inhibitors—Aptamers have been shown previously to inhibit a variety of protein targets (35, 36). In general, though, there was little or no possibility that previous aptamers could be acted on by their targets. To our knowledge, RTA is the first selection target that could catalyze the cleavage of individual species in a RNA pool.

It has been shown previously that short stems capped by GAGA tetraloops serve as substrates for RTA (25). Given these minimal requirements for interaction with RTA it is still somewhat surprising that the most bountiful anti-RTA aptamers were not also substrates. Minimal binding motifs frequently overrun *in vitro* selection experiments, a phenomenon that has been termed the "tyranny of small motifs" (37). However, any depurinated anti-RTA aptamers would have been copied inefficiently and would not have contained an adenosine in future generations, likely obviating the tyranny in these experiments. In addition, the selection of a novel sequence and structure is somewhat less startling when it is realized that the natural target of RTA is the ribosome. The SRL is surrounded by other RNA structures in the ribosome (38), and there are multiple amino acid residues in or adjacent to the RTA active site which could interact with these RNA structures (39). Differences between rRNA interactions with RTA and SRL interactions with RTA are highlighted further by the fact that rRNA is depurinated 77,000-fold faster than the SRL (2, 13). Because the relatively short RNA molecules present in the N30 pools lack the structural context of rRNA, very different sequences may be required to bind tightly to RTA.

The anti-RTA aptamer contrived to bind to the active site of the enzyme, but in a way that eluded depurination. RNA molecules that inhibit a RNA glycosidase are reminiscent of proteins that inhibit proteases (40). For example, even though bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor are cleaved by trypsin, the inhibitor remains bound to the active site, allowing resynthesis. In addition, new evidence suggests that these inhibitors may bind in a "nonproductive" mode that is substantially different than the way in which substrates are bound and which preferentially stabilizes the ground state of the reaction (41). Similarly, structural studies of bovine pancreatic trypsin inhibitor complexed with a thrombin mutant reveal that surface loops of the protease are rearranged, leading to inactivation (42). Using this co-crystal structure as a model, mutagenesis studies have shown that a lack of productive electrostatic contacts may also contribute to the ability of plasminogen activator inhibitor-1 to inhibit thrombin (43). Indeed, the "unnatural" interaction of bovine pancreatic trypsin inhibitor with a mutant thrombin may be especially germane to understanding 31RA function, in that it is possible that the unnatural aptamer similarly induces unproductive structural transitions in RTA. Efforts are now under way to determine the precise binding mode of the aptamer-RTA complex by x-ray diffraction analysis.

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