Protein, Pepocin*

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RNA Aptamers That Bind to and Inhibit the Ribosome-inactivating Protein, Pepocin*

Ichiro Hirao‡, Kairat Madin§, Yaeta Endo§, Shigeyuki Yokoyama‡, and Andrew D. Ellington**

From the ‡Yokoyama CytoLogic Project, ERATO, JST, c/o RIKEN, Hirosvawa, Wako-shi, Saitama 351-0198, the §Department of Applied Chemistry, Ehime University, Matsuyama, Ehime 790, the ¶Department of Biophysics and Biochemistry, Graduate School of Science, the University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and the ¶University of Texas, Austin, Texas 78712

Pepocin, isolated from Cucurbita pepo, is a ribosome-inactivating protein (RIP). RIPs site-specifically recognize and depurinate an adenosine at position 4324 in rat 28 S rRNA, rendering the ribosome incapable of interacting with essential elongation factors. Aptamers that target pepocin were isolated from a degenerate RNA pool by in vitro selection. A conserved hairpin motif, quite different from the sequence of the toxin-substrate domain in rat 28 S rRNA, was identified in the aptamer sequences. The aptamers selectively bind to pepocin with dissociation constants between 20 and 30 nM and inhibit the N-glycosidase activity of pepocin on rat liver 28 S rRNA. Competitive binding experiments using aptamer variants suggest that the conserved hairpin region in the anti-pepocin aptamer binds near the catalytic site on pepocin and prevents the interaction of pepocin and 28 S rRNA. Anti-RIP aptamers have potential use in diagnostic systems for the detection of pepocin or could be used as therapy to prevent the action of pepocin in mammalian cells.

Ribosome-inactivating proteins (RIPs)† are widely distributed throughout nature (for review, see Ref. 1). They inhibit protein synthesis in mammalian cells by disabling the ribosome. Type II RIPs (e.g. ricin) are heterodimeric proteins; an A-chain catalyzes the depurination of a specific adenosine in rRNA, and a disulfide linked B-chain binds cell surface galactosides (for review, see Ref. 2). Binding of the B-chain to the cell surface results in translocation of the A-chain to the cytosol, where it hydrolyzes the N-glycosidic bond of a universally conserved adenosine in the large subunit of rRNA. Cleavage of the adenine residue disrupts the binding of elongation factors, inactivating the ribosome. Type I RIPs, of which pepocin is a member, are considerably more numerous in nature but much less cytotoxic because they lack the B-chain required for cell entry (3).

The depurination site in the large subunit of rRNA is highly conserved in many eukaryotes and is located in a hairpin loop of 17-mer containing a GAGA tetraloop, in which the second A is removed (4, 5). Recent studies using RNA variants of the toxin-substrate domain have shown that small hairpin fragments containing a GAGA tetraloop are also able to act as substrates for ricin (6). The cleavage reaction is remarkably specific, and the GAGA sequence is essential for toxin recognition. For instance, the A-chain of ricin does not recognize hairpin variants with all of the possible transitions and transversions of each nucleotide in the GAGA tetraloop sequence (6). NMR studies indicate that RNA fragments corresponding to the toxin-substrate domain of rRNAs form the GAGA tetraloop hairpin (7–10). The structure of the GAGA tetraloop is similar to those of stable GAAA and GCAG (GNR) loops, whereas these are not recognized by the toxins (6). These results illustrate the importance of both sequence and structure for recognition in RNA-protein complexes; studies of the contacts between RNA hairpins and RIPs are informative as a model system for scrutinizing RNA-protein interactions.

In vitro selection can be used to generate nucleic acid ligands (aptamers) that bind to target proteins and thus can answer questions regarding which components of an RNA molecule are essential for binding to its target protein (11–13). For instance, crystal structures of RNA aptamers that bind MS2 coat protein shed light on the nature of specific interactions between coat protein and operator (14, 15). Despite the structural differences between the aptamers and the wild type operator, the aptamers bind in the same location on the coat protein as the wild type RNA and maintain many of the same contacts with the protein compared with the wild type RNA. In a similar example, RNA aptamers that bind elongation factor Tu (EF-Tu) were isolated by in vitro selection (16). EF-Tu is known to interact with an RIP recognition domain in rRNA. A consensus sequence identified within the aptamers was found in the toxin-substrate domain of Thermus thermophilus 23 S rRNA.

To identify novel anti-RIP compounds, we used in vitro selection to produce RNA ligands that bind the toxin, pepocin, which is a type I RIP isolated from the sarcocarp of Cucurbita pepo (17). Because the single A-chain of pepocin contains N-glycosidic cleavage activity against the large subunit of rRNA but is not highly toxic, it was considered to be a suitable model system for the study of RIP recognition and inhibition.

EXPERIMENTAL PROCEDURES

Materials—Pepocin and gypsophilin were isolated from the sarcocarp of C. pepo, (17) and from Gypsophila elegans (18), respectively. The purity of proteins was checked by SDS-polyacrylamide gel electrophoresis with silver staining. Oligodeoxyribonucleotides were synthesized using standard phosphoramidite chemistry on a DNA synthesizer (392, Perkin-Elmer). [α-32P]UTP and [γ-32P]ATP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). RNase T1 and RNase V1 were purchased from Amersham Pharmacia Biotech, nuclease S1 was from Takara (Tokyo, Japan), and RNase I was from Promega (Madison, WI).

In Vitro Selection—In vitro selection was carried out using an RNA pool that contained 30 randomized nucleotide positions (19). The RNA pool was transcribed from the DNA template using an Ampliscribe T7
in vitro transcription kit (Epicenter Technologies, Madison, WI). RNA was dissolved in 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 10 mM MgCl₂ and then heated at 75 °C for 3 min and cooled to room temperature. To exclude filter-binding RNA sequences from the pool, the RNA was passed over a 0.45-μm HAWP filter (Millipore, Bedford, MA) two or three times before incubation with target. The RNA was mixed with a pepocin preparation (10 mM sodium phosphate (pH 6.5), 100 mM NaCl, 0.5 mM dithiothreitol, and 40% glycerol). The final solution contained 3 mM sodium phosphate, 3 mM MgCl₂, 45 mM NaCl, 2.5 mM EDTA, 3 mM Tris-HCl (pH 7.5), 0.15 mM dithiothreitol, and 12% glycerol. Table I summarizes the conditions used in each round of the selection. The binding was carried out for 1 h at 37 °C. After 1 h, the solution was vacuum filtered over a HAWP filter at 5 p.s.i. and washed five times with 0.2 ml of the selection buffer. The RNA on the filter was eluted twice with 0.2 ml of 7M urea, 100 mM sodium citrate (pH 5.0), and 3 mM EDTA for 5 min at 100 °C, and the eluted RNAs were precipitated with isopropyl alcohol. In the fifth through eighth rounds, an additional filtration step was carried out on the eluted RNA pool to further exclude filter-binding species (20). Collected RNA was reverse transcribed in 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 6 mM MgCl₂, 0.8 mM dNTPs, 4 μM primer, and 2.5 units of avian myeloblastosis virus reverse transcriptase (Seikagaku, St. Petersburg, FL) for 45 min at 42 °C. The reverse transcription was then added to a polymerase chain reaction amplification mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs, 5% acetic acid, 0.05% Nonidet-P-40, 0.5 μM primers, and 0.5 unit of Taq polymerase (Promega) and thermally cycled. Amplified DNA was used for the next round of selection.

**Cloning and Sequencing**—The polymerase chain reaction products of the eighth round pool were ligated into a TA cloning vector (Invitrogen, Carlsbad, CA) and cloned into Escherichia coli Invata (Invitrogen). Plasmid DNAs were isolated and sequenced using an AutoRead sequencing kit and a DNA sequencer (ALF, Amersham Pharmacia Biotech).

**Filter Binding Assays**—The RNA pools from each round and individual clones from the eighth round were internally labeled with [α-³²P]UTP (3000 Ci/mmol) during T7 transcription. The labeled RNA (0.3 μM final concentration) was mixed with pepocin (0.3 μM final concentration) in 0.1 ml of the selection buffer for 1 h at 37 °C. The solution was then filtered and washed three times with selection buffer. The filter was exposed to a PhosphorImager plate, and the amount of retained radioactivity was determined using a PhosphorImager (Fuji Photo Film, Kanagawa, Japan). The fraction of bound RNA was calculated by comparing the radioactivity on the filter with the total radioactivity of the input RNA.

For the determination of dissociation constants, individual RNA clones were end labeled with [γ-³²P]ATP after 5'-dephosphorylation of

**TABLE I**

| Round | Pool RNA | Pepocin | Total volume | Filtration (pre-post) | PCR cycles | Pepocin binding | Filter binding |
|-------|----------|---------|--------------|----------------------|------------|----------------|---------------|
| 0     |          |         |              |                      |            |                |               |
| 1     | 0.6      | 0.6     | 300          | 3-0                  | 15         | 0.03           | ND            |
| 2     | 0.6      | 0.6     | 200          | 3-0                  | 10         | 0.07           | ND            |
| 3     | 0.6      | 0.6     | 200          | 3-0                  | 7          | 16.6           | 17.4          |
| 4     | 0.6      | 0.6     | 200          | 3-0                  | 7          | 15.4           | 6.4           |
| 5     | 0.6      | 0.6     | 200          | 2-2                  | 12         | 24.3           | 0.2           |
| 6     | 0.6      | 0.3     | 200          | 2-2                  | 13         | 24.2           | 0.09          |

a Times of pre- and postfiltration.  
b ND, not detected.

**FIG. 1.** The 26 sequences located, aligned by their stem-loop sequences.

**FIG. 2.** Conserved hairpin structure between two classes of anti-pepocin aptamers. The stem 1 regions of the class 1 aptamers are depicted as the part of the loop structure. The G-U base pair in stem 2 is underlined. Numbers in parentheses indicate the results of the pepocin filter binding assay described under “Experimental Procedures.” Aptamers are sorted according to their affinities for pepocin from left to right.
the T7 transcript. The labeled RNA (0.6 nm final concentration) was incubated with increasing concentrations of pepocin (1.2–450 nM) in 0.1 ml of the selection buffer for 1 h at 37 °C. The mixture was filtered on a vacuum manifold (Schleicher & Schuell) loaded with a piece of pure nitrocellulose membrane (0.45 μm, Bio-Rad) over a piece of Zeta-Probe blotting membrane (Bio-Rad) (20, 21). The radioactivity on both filters was quantified, and dissociation constants (Kd) were determined by curve fitting with the program Kaleidagraph (Abelbeck Software, Reading, PA).

Binding Competition Assays—The binding activity of each aptamer variant was determined by competition experiments between a 5' labeled aptamer variant and 5'-labeled clone 8-09. Both RNAs (each at a 0.45 μl final concentration) were mixed with pepocin (0.3 μl final concentration) in 100 μl of the selection buffer for 1 h at 37 °C. The mixture was passed over a HAWP filter and washed three times with 0.2 ml of the selection buffer. The RNAs on the filter were eluted with 0.1 ml of 0.025% of bromphenol blue, 7 M urea, 100 mM sodium citrate (pH 5.0), and 3 mM EDTA for 5 min at 100 °C. The solution was applied directly to a gel containing 7 M urea. The radioactivity in each band on the gel was quantified using a PhosphorImager. Binding ratios were calculated by comparing the counts in the retained and input RNA.

Tm Measurements—For Tm measurements, each aptamer (0.7 A260 final concentration) was dissolved in 0.25 ml of a buffer containing 10 mM sodium phosphate (pH 7.0), 1 mM NaCl, and 1 mM EDTA. Before measurement, the aptamer solution was heated at 75 °C for 3 min and then cooled to room temperature. Melting profiles were obtained at 260 nm using a spectrophotometer (DU650, Beckman) at a heating rate of 0.5 °C/min with 1-cm cuvettes. Tm values were calculated from the first derivatives of the melting curves.

Enzymatic Structure Probing—The end-labeled 9-41 and 9-41U20 (3 pmol) were used for digestion experiments. Alkaline digestion was performed in 50 mM sodium carbonate (pH 9.0), 1 mM EDTA, and 5 ng of E. coli tRNAs for 10 min at 90 °C. RNase T1 digestion (0.01 unit) was performed in 20 mM sodium citrate (pH 5.0), 7 M urea, 1 mM EDTA, 0.025% bromphenol blue, and 5 ng of E. coli tRNAs for 5 min at 50 °C.

RNase V1 digestion (0.01 and 0.1 unit) was performed in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 5 ng of E. coli tRNAs for 10 min at 37 °C. S1 nuclease digestion (1 unit) was performed in 30 mM sodium acetate (pH 4.6), 280 mM NaCl, 10 mM ZnSO4, and 5 ng of E. coli tRNAs for 30 min at 37 °C. RNase I digestion (0.3 unit) was performed in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 200 mM sodium acetate, and 5 ng of E. coli tRNAs for 10 min at 37 °C. The digested samples were analyzed by electrophoresis on a 15% polyacrylamide gel containing 7 M urea for 30 min at 90 °C. RNase T1 digestion (0.01 unit) was performed in 20 mM sodium citrate (pH 5.0), 7 M urea, 1 mM EDTA, 0.025% bromphenol blue, and 5 ng of E. coli tRNAs for 5 min at 50 °C.

Inhibition Experiments—Pepocin (308 pM final concentration) was incubated with increasing concentrations of anti-pepocin aptamers (0.308–7.7 M) in 25 mM Tris-HCl (pH 7.6), 25 mM KCl, and 5 mM MgCl2 for 15 min at 37 °C. The RIP reaction was stopped with 10% SDS (0.5% final concentration) and 1 mM EDTA. After phenol extraction, RNA was precipitated and dissolved in 5 μl of water. Subsequently, 25 μl of a solution of acetic acid and aniline was added to the RNA, and the cleavage reaction was incubated for 15 min at 37 °C. Cleavage reactions were run on a 4% polyacrylamide gel containing 7 M urea, and RNAs were detected by staining with ethidium bromide.

FIG. 3. Competition experiments using small RNA fragments derived from clone 8-09 or 28 S rRNA. The indicated 32P-labeled RNA fragments were incubated with pepocin and 32P-labeled clone 8-18, and complexes were captured by filtration and subsequently analyzed by electrophoresis on a denaturing polyacrylamide gel. Lane a contains RNAs present at the start of the binding reaction, and lane b contains RNAs recovered after filtration. The binding ratios are indicated in parentheses and were calculated as described under “Experimental Procedures.”

FIG. 4. Possible secondary structures, binding activities, and Tm values of small RNA variants derived from aptamer 9-41. The binding activities shown were derived via competition assays similar to those described previously in Fig. 3.
In Vitro Selection of Anti-pepocin Aptamers—To determine the minimal sequence necessary for anti-pepocin aptamer binding, we prepared various mutant fragments of the class 1 aptamer 8-09, which bound the best in our initial assays. To quantify the binding abilities of each fragment better, competition experiments were carried out. Selected small RNA fragments were competed with 8-18, an aptamer that bound specifically but poorly to pepocin. A 45-mer fragment of aptamer 8-09 (termed 9-45), which contained the AUAAU loop, stems 1–4, and the two bulged cytidines competed roughly twice as well with 8-18 for binding to pepocin (Fig. 3a). The binding activity of aptamer 9-45 was improved slightly by introducing substitutions (UAU to AAU) that closed the apparent internal loop of aptamer 8-09, generating aptamer 9-45b. The fully paired stem was observed in many other class 1 aptamers such as 8-08 and 8-01. A 41-mer fragment, 9-41, had binding activity similar to that of 9-45b, but binding activity decreased after additional truncation (9-37 (37-mer) and 9-33 (33-mer)) (Fig. 3b). These results are consistent with the predicted secondary structure, which shows 10 base pairs between stems 3 and 4. Most of the aptamers isolated from the eighth round pool were also predicted to contain approximately 10 base pairs in stems 3 and 4. It should be noted that all of the aptamers bound much better to pepocin than the isolated toxin-substrate domain of rRNA; for example, aptamer 9-45b binds roughly 100-fold better than the isolated toxin-substrate domain.

There is some sequence degeneracy at specific positions in the loop and stem regions of the aptamers. Four different bases appeared at the first position of the loop, although the UAU motif was highly conserved at the remaining three positions in the loop. We examined the binding abilities of these variants by introducing substitutions into the minimal aptamer 9-41. The binding activities of these variants are shown in Fig. 4. As has been observed in other selection experiments (11), sequence variations between the aptamers provided valuable information about the function. The order of the binding activity of the class 1 loop sequence variants was UUAU > CUAU > AUAU > GUAU. Similarly, 6 of 13 class 1 NUAU loops are UUAU, 3 are CUAU, 4 are AUAU, and no GUAU loops appear. The closest binding aptamer was the class 1 aptamer (9-41U22) containing a UUAU sequence in the loop. The K_d of 9-41U22 was 17.9 ± 2.2 nM, which was similar to that of the full-length aptamers 8-09 (21.0 ± 2.0 nM) and 8-14 (23.4 ± 2.3 nM) (Fig. 5a).

Changes outside the loop region also affected activity. The
second base pair in stem 1L was predicted to be A-A or C-A in class 1 aptamers but U-A in class 2 aptamers. The substitution of the class 1 C-A base pair to a class 2 U-A (9-41U20) decreased binding activity slightly. Interestingly, the class 1 aptamer, 9-41 (T_m = 82.5 °C) was found to be more thermally stable than the class 2 aptamer, 9-41U20 (T_m = 80.0 °C). Finally, both bulged cytidines located in the stem region appear to be important for binding. Substitution of the bulged C to either U or A reduced the binding activity significantly. Overall, it appeared that the sequences in and around stems 1 and 2 contributed to interactions with pepocin.

Mapping the Secondary Structure of the Aptamers—Although all sequence and binding data were consistent with the predicted secondary structures, we enzymatically probed the structures of both class 1 (9-41) and class 2 (9-41U20) aptamers. Labeled 9-41 and 9-41U20 were treated with RNase V1, S1 nuclease, and RNase I (Fig. 6). RNase V1 digests double-stranded regions in the RNA specifically. S1 nuclease and small amounts of RNase I preferentially digest single-stranded regions in RNAs.

Both 9-41 and 9-41U20 gave similar digestion patterns, with the exception of RNase V1 digestion in the stem 1L region (positions 18–20 and 26–28 in Fig. 6) and RNase I digestion throughout stem 1 and the loop regions (position 17–27). The digestion pattern of 9-41U20 was consistent with the predicted tetra loop (the NUAA loop) hairpin structure. However, 9-41 may have a structure different from that of 9-41U20 at positions 18–28, including the loop and stems 1 and 2. RNase V1 digestion of 9-41 did not give strong bands at position 18–20 in the stem 1L region compared with 9-41U20. In contrast, RNase I extensively digested 9-41 throughout the loop and the stem 1 regions, although S1 nuclease treatment gave similar digestion patterns for 9-41 and 9-41U20. Under the conditions that we used, the RNase I activity was expected to be slightly stronger than nuclease S1 activity. Therefore, although enzymatic probing confirms that class 1 and class 2 aptamers form secondary structures similar to those predicted, the class 1 aptamers seem to have more flexible stem 1 and loop regions.

Inhibition of Pepocin Activity—To determine whether the anti-pepocin aptamers could inhibit ribosome inactivation by pepocin, we examined the depurination of the specific adenosine in rat liver 28 S rRNA. Rat liver ribosomes and pepocin were incubated together in the presence of increasing concentrations of aptamers. The 28 S rRNA was extracted from the ribosome and treated with aniline to cleave the rRNA at the depurination site. The aniline-treated rRNA was analyzed on a denaturing gel (Fig. 7), and the expected cleavage product of 28 S rRNA was observed (Fig. 7, lane 2).

The addition of a class 1 aptamer, 8-09, in 10,000-fold excess over pepocin inhibited the depurination of 28 S rRNA (Fig. 7, lane 9). The inhibitory effect of a class 2 aptamer, 8-14, was weaker than that of class 1 and was observed at around a 25,000-fold excess (Fig. 7, lane 6). Although the small fragments 9-41U22 and 9-41U22U22 bound tightly to pepocin, they showed no inhibitory effect when present in ten-thousand fold excess (Fig. 7b).

Mechanism of Toxin Inhibition—The simplest explanation of anti-pepocin aptamer inhibition of RNA depurination is that the aptamer competes with RNA for binding to pepocin. However, to account for the large excess of aptamer needed for inhibition, we considered the possibility that the aptamer might also be a substrate for pepocin. To determine if pepocin
depurinated one or more positions in the aptamers, resulting in a loss of activity, the aptamers were incubated with pepocin for 1 day at 37 °C followed by treatment with aniline. No depurination or cleavage products were observed (data not shown).

Because the sequence of the toxin-substrate domain in 28 S rRNA is highly conserved in many eukaryotes, if the anti-pepocin aptamers mimicked the rRNA domain then it seemed possible that the anti-pepocin aptamers might bind and inhibit pepocin. This hypothesis is also consistent with the observed binding or cleavage products were observed (data not shown).

Applications—The identification of anti-RIP aptamers may suggest new approaches to the development of therapeutics or diagnostics for toxic agents. To the extent that the anti-pepocin aptamer has been shown to inhibit RIP activity, it provides proof of principle for the development of prophylactic or therapeutic aptamers to other cytoxins, such as the Vero toxin produced by E. coli O157 (25–27). Such aptamers could be introduced as expression constructs into exposed or at-risk individuals to minimize toxin pathology. Moreover, anti-toxin diagnostics using aptamers might be powerful tools for detecting trace amounts of toxins. For example, anti-pepocin aptamers might be used in biosensors that would detect the presence of pepocin in foods made from pumpkin.

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