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Inhibition of Ricin A-Chain (RTA) Catalytic Activity by a Viral Genome-Linked Protein (VPg)

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Abstract

Ricin is a plant derived protein toxin produced by the castor bean plant (*Ricinus communis*). The Centers for Disease Control (CDC) classifies ricin as a Category B biological agent. Currently, there is neither an effective vaccine that can be used to protect against ricin exposure nor a therapeutic to reverse the effects once exposed. Here we quantitatively characterize interactions between catalytic ricin A-chain (RTA) and a viral genome-linked protein (VPg) from turnip mosaic virus (TuMV). VPg and its *N*-terminal truncated variant, VPg_{1–110}, bind to RTA and abolish ricin's catalytic depurination of 28S rRNA *in vitro* and in a cell-free rabbit reticulocyte translational system. RTA and VPg bind in a 1 to 1 stoichiometric ratio, and their binding affinity increases ten-fold as temperature elevates (5 °C to 37 °C). RTA-VPg binary complex formation is enthalpically driven and favored by entropy, resulting in an overall favorable energy, $G = -136.8$ kJ/mol. Molecular modeling supports our experimental observations and predicts a major contribution of electrostatic interactions, suggesting an allosteric mechanism of downregulation of RTA activity through conformational changes in RTA structure, and/or disruption of binding with the ribosomal stalk. Fluorescence anisotropy studies show that heat affects the rate constant and the activation energy for the RTA-VPg complex, $E_a = -62.1$ kJ/mol. The thermodynamic and kinetic findings presented here are an initial lead study with promising results and provides a rational approach for synthesis of therapeutic peptides that successfully eliminate toxicity of ricin, and other cytotoxic RIPs.

Graphical Abstract

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Keywords

Ricin A-chain (RTA); ribosome inactivating protein (RIP); genome-linked viral protein (VPg); depurination; fluorescence; protein-protein interactions

Introduction

Ricin is a member of type 2 class of ribosome inactivating proteins (RIP). RIPs are produced by many varieties of plants, effectively protecting members of the *Plantae* kingdom against viruses, bacteria, and other parasites (1–3). RIPs are also found in several species of fungi, alga, and bacteria (4–7). Plant RIPs are phytotoxins and deactivate ribosomes (8). Examples of plant-derived RIPs include ricin, abrin (from *Abrus precatorius*), saporin (from *Saponarium officinalis*) and pokeweed antiviral protein (PAP) (from *Phytolacca Americana*) (9–12). Since their discovery, RIPs have been of great scientific interest due to their importance in human health (as both pathogenic agents and therapeutics), and their potential uses in biological warfare and bioterrorism (13–19). The castor bean plant is well-known for its nefarious past. The plant is widely distributed throughout the world. Global cultivation of the plant and the ease of the toxin extraction from its beans made ricin particularly appealing agent with a strong potential to be employed as a biological weapon (20,21). Ricin has been used in sporadic incidents of espionage and bioterrorism (22); therefore, there has been a great deal of interest in developing a safe vaccine or antidote to protect people from ricin poisoning. Although multiple vaccines have been tested and numerous ricin inhibitors have been patented, none possess the ideal properties and standards of an effective vaccine (23).

The cytotoxic effects of ricin have long been attributed to the inhibition of protein synthesis (24–27). The toxin is an RNA *N*-glycosidase that targets and depurinates a specific conserved sarcin/ricin loop (SRL) of the large rRNA (1). The SRL within the rRNA of different ribosomes is universally conserved across all species. For rat liver ribosome, this distinct site is A4324 within the single-stranded SRL (1). Ricin is a type 2 RIP, a heterodimer with a catalytic amino-terminal *N*-glycosidase domain, the A-chain (RTA), joint by a disulfide bond to a divergent carboxyl-terminal domain with lectin properties (carbohydrate-binding domain), the B-chain (RTB), each of approximate M_r of 30,000 (28–

30). The lectin chain binds to the galactosyl moieties of glycoproteins and/or glycolipids on the exterior of eukaryotic cells (31–33) and promotes reverse transport of the RTA to the cytosol, where RTA accesses translational machinery and depurinates ribosomes (34–36). Ricin presents the highest activity toward yeast and mammalian ribosomes but exhibits low activity on plant and *E. coli* ribosomes (37–39). Ricin substrate specificity may be influenced by substitution of residues in ribosomal proteins, which dictate its activity and sensitivity toward different ribosomes (40). The L9 and L10e rat liver ribosomal proteins are targeted by RTA (41). The structure of ricin has been solved, and the presence of various substrate analogs interacting with RTA side chain amino acids has been mapped out (42,43). RTA and PAP reveal a high degree of similarity in their active site residues (23,44,45). Similarly, RTA and Stx1 bind to their target cell via a common receptor, in addition to having analogous amino acid residue distribution within their active sites (3,46,47).

It was shown previously that the *N*-glycosidase activity of RIP PAP is inhibited by a viral peptide (VPg) associated covalently with the potyviral genome and its truncated variant VPg-71 (48,49). VPg of the turnip mosaic virus (TuMV) is linked covalently to the 5'-end of the viral genome through a tyrosine residue (50). The viral protein serves as the 5'-m⁷G cap analog of the mRNAs, interacts with cap-binding proteins (e.g., eIF4E as part of eIF4F or eIFiso4E as part of eIFiso4F), playing an important role in the translation of mRNA (51,52). VPg is important for the viral infectivity (53), cell-to-cell movement (54–57), and has been linked to an array of other functions. VPg has also been shown to have a role in overcoming viral resistance in plants (55,57–62). Based on previous studies, we hypothesized that the *N*-terminal fragment of this viral peptide, VPg_{1–110} (Fig. S1), will serve as an effective ricin activity inhibitor as the wild type protein, and here we employ fluorescence spectroscopy to examine these VPg inhibitory effects on RTA activity. Using a combination of biophysical tools, biochemical assays, and computational techniques, we show that VPg_{1–110} serves as an active inhibitor of RTA activity *in vitro* and in cell-free rabbit reticulocyte translational assays, lowering RTA specificity for the 28S rRNA, when compared to the depurination in control experiments.

Experimental Procedures

Extended experimental materials and methods for protein expression, purification, labeling, steady-state and stopped-flow fluorescence analysis, and molecular modeling and docking are presented in Supplemental Data. All chemicals used (unless otherwise noted) were of molecular biology grade. The pET23a plasmid, containing the *E. coli* codon-optimized RTA gene was provided by Prof. Vern L. Schramm (Albert Einstein College of Medicine, NY), the pETVPg vectors (wt VPg, VPg-71, and VPg-111) were provided by Prof. Hiroshi Miyoshi (St. Mariana University School of Medicine, Kawasaki, Japan), and the pLUC0 plasmid by Prof. Daniel R. Gallie (University of California, Riverside, CA).

Experimental Design and Statistical Rationale -

For quantification of adenine, each experiment included a standard of *N*⁶-ethenoadenine in the appropriate buffer and internal standards, obtained by adding known amounts of *N*⁶-ethenoadenine to the ethanol-soluble fractions from the control and RTA-treated 28S rRNA,

in the presence and absence of VPg. For each datum point, three samples were prepared and analyzed. The concentration of adenine, released from RTA-treated rRNA, was calculated from a standard curve, after the fluorescence reading subtraction, produced by the control RNA samples.

For steady-state fluorescence titrations, for each titration datum point, three samples were prepared. The fluorescence intensity of a protein (e.g., RTA labeled with fluorescein) was measured in the first sample. A second sample, containing a specific amount of titrant protein (e.g., VPg), was also measured, and the corrected intensities of the two samples were summed together (F_s). A third sample, containing equal amounts of RTA and VPg proteins, was mixed together, and the corrected intensity for this complex was obtained (F_c). The difference in fluorescence intensity related to the complex was defined as $F = F_c - F_s$. Similar measurements were also performed for other titrations. The inner filter corrections were applied when appropriate. The normalized fluorescence difference ($F / F_{\max}$) between protein-protein complexes and the sum of the individual fluorescence spectra were used to determine the equilibrium dissociation constant (K_d). A double reciprocal plot was used for determination of $F_{\max}$. The details of the data fitting are described elsewhere. PRISM software (version 5) was used to analyze and plot the data. Non-linear least-squares fitting of the plotted normalized data were used; one-site and two-site binding models were tested. Fraction bound was calculated according to the following equation (63):

$$F_{\text{bound}} = \frac{F_{\text{obs}} - F_0}{((F_{\infty} - F_{\text{obs}}) \times (F_{\infty} / F_0) + (F_{\text{obs}} - F_0))}$$

where, F_{bound} is fraction of RTA bound to VPg in a binary complex, F_{obs} is the observed fluorescence, F_0 is the initial fluorescence, and F_{∞} is the final fluorescence.

Results

VPg Inhibits RTA Depurination of 28S rRNA.

To examine inhibitory effect of VPg on RTA activity, the 28S rRNA was used as a substrate for the depurination. Adenine resulted from the RNA depurination, was converted subsequently to its fluorescent N^6 -ethenoadenine derivative that was separated further on an HPLC, as described elsewhere (64,65). Quantification of the areas under the elution peaks shows VPg inhibiting RTA depurination of 28S rRNA in a dose-dependent manner (Fig. 1). Further, the effects of truncated VPg-71 (70 N-terminal amino acids removed) and VPg-111 (110 N-terminal amino acids removed) proteins on RTA activity were analyzed. The VPg-71 variant inhibits RTA activity as potently as the wild type VPg, whereas VPg-111 exhibits no profound effect on RTA activity, indicative of the 40 amino acid sequence, present in the VPg-71 and absent in the VPg-111 variant, being important in downregulating RTA activity. Further, employing synthetic VPg₁₋₁₁₀ peptide, the inhibitory effect of this peptide on RTA activity can be seen in Figure 1. Depurination assay samples lacking VPg were used as positive controls of the experiment; depurination assay samples lacking RTA, rRNA, or both RTA and VPg, were used as negative controls for the experiments. The RNA *N*-glycosidase activity of RTA and the inhibitory effect of VPg₁₋₁₁₀ peptide were tested further using yeast

(*Saccharomyces cerevisiae*) ribosomes as a substrate for RTA depurination. When the depurinated rRNAs are treated with aniline, cleavage occurs at the depurinated site and a small nucleotide fragment is released from the 26S rRNA (66–69). As shown in Figure S3, RTA depurinates the rRNA, and releases the cleavage product (367-nt), whereas upon incubation of RTA with the stoichiometric amounts of VPg_{1–110}, prior to rRNA addition, resulted in the loss of the *N*-glycosidase activity of RTA, as indicated by the absence of the cleavage products.

VPg Inhibits RTA Activity in Cell-Free Translational Systems.

To monitor ribosomal depurination and the inhibitory effect of VPg peptides on RTA activity, rabbit reticulocyte cell-free translational system was employed (70). Luciferase mRNA (TEV(1–143)-*luc*-A₅₀ RNA) successfully translated in an *in vitro* lysate system (71). Presence of RTA in the translational system causes ribosomal depurination, resulting in the decrease of luciferase production (Figure 2). Prior incubation of RTA with stoichiometric amounts of the wild-type VPg shows inhibition of the RTA activity over time (Figure 2). Similar effects were observed when RTA was incubated with VPg-71 protein, prior to the translational system analysis. Ribosome depurinating activity of RTA-VPg-111 binary complex was evaluated, showing no significant effect on the translation of luciferase mRNA in the cell-free systems (Figure 2). Treatment of the RTA with the synthetic VPg_{1–110} peptide resulted in complete inhibition of RTA activity on ribosomes (Figure 2), suggesting that VPg_{1–110} may serve as a probable inhibitor of RTA toxicity.

Characterization of RTA-VPg Interactions: Thermodynamics and Stoichiometry of Binding.

To quantitatively characterize RTA-VPg interactions and determine the binary complex dissociation constant (K_d), direct fluorescence titrations of fluorescein-labeled RTA with VPg peptides were performed. Figure 3 shows representative binding curves for the interactions of RTA with WT (wild type) VPg, VPg-71, VPg-111, and VPg_{1–110} at 25 °C. Table 1 summarizes these findings. Binding data show that the strongest interactions of RTA occur with VPg_{1–110} synthetic peptide, having K_d of 18.4 ± 2.1 nM at 25 °C, as compared to the full-length WT VPg ($K_d = 138 \pm 12$ nM). To determine the stoichiometry of RTA-VPg_{1–110} binding, the binding data at 25 °C (quenching of the fluorophore's fluorescence) were transformed into the Scatchard plot (Fig. S4). The slope and the intercept of the Scatchard plot $Q/[VPg] \times 10^{-6}$ vs. Q (Fig. S4, *inset*) gave the binding constant ($K_d = 18.4 \pm 2.1$ nM) and the binding capacity ($n = 1.005 \pm 0.005$ at 25 °C) of RTA-VPg_{1–110} binary complex. This suggests that RTA and VPg_{1–110} peptide interact in a 1:1 stoichiometric ratio.

To identify the energy of the RTA-VPg interactions, temperature dependent fluorescence intensity measurements for the binding of RTA with the synthetic VPg_{1–110} peptide were obtained. Figure 4 shows the representative isotherms. The equilibrium dissociation constants, K_d , for the interactions of RTA with VPg decreased with elevation in temperature: from 199.5 ± 19 nM to 4.7 ± 1.2 nM at temperatures ranging from 5 °C to 37 °C (Figure 4). The temperature dependence of the dissociation constant for the binding of RTA with VPg was used to determine the thermodynamic parameters. Thermodynamic parameters, enthalpy (H), entropy (S) and the energy (G) obtained from van't Hoff plots of $-\ln K_{eq}$ versus reciprocal of temperature (T^{-1}) are shown in the *inset* of Figure 4. Thermodynamic

data showed that binding of RTA with VPg was endothermic, yet entropy favored (Table 2). The H and S values for the binding of RTA with VPg₁₋₁₁₀ were $0.0876 \pm 0.012 \text{ kJ}\cdot\text{mol}^{-1}$ and $441.3 \pm 4.3 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, respectively (Table 2). The thermodynamic analysis showed that the binding of RTA to VPg₁₋₁₁₀ has a greater entropic contribution to G at 37 °C. Increase in temperature decreases the enthalpic contribution to about 12% and increases entropic contribution to about 78% at 37 °C. These data suggest that temperature increase induces a conformational change in RTA-VPg complex resulting in reduced hydrogen bonding of the complex. The G values at 37 °C are presented in Table 2.

VPg Decreases RTA's Binding Specificity for 28S rRNA.

To examine how VPg peptides affect the rates at which RTA depurinates the 28S rRNA, standard quantification of adenine in the discontinuous assay format was performed. Analysis of the fractions with the HPLC indicated that the 28S rRNA depurination by RTA was virtually 92% complete after 5 min (Fig. 5A). To establish RTA's catalytic constant for the depurination of the 28S rRNA, and then compare the rate of the depurination in the presence of VPg₁₋₁₁₀ peptide, the RNA concentrations were varied. The progress of the depurination of monitored by the absorbance of a UV-absorbing product at the saturating conditions. Experimental depurination rates were then plotted against the corresponding rRNA concentrations, resulting in a Michaelis-Menten type behavior (Fig. 5B). The presence of VPg₁₋₁₁₀ decreases RTA's specificity constant ($k_{\text{cat}} / K_{\text{m}}$) for the 28S rRNA (3.8×10^6 vs. 7.5×10^5) nearly 80-fold (Table 3). As a negative control, poly(C) RNA was employed, displaying no depurination by RTA in the absence (or presence) of VPg₁₋₁₁₀. These data demonstrate that VPg₁₋₁₁₀ decreases depurination of RNA as compared to control experiments, suggesting that VPg₁₋₁₁₀ peptide could be used effectively to downregulate RTA depurination of rRNA by lowering RTA's specificity for the rRNA.

Kinetics of RTA and VPg Binding and the Effect of Temperature: Activation Energy.

The stopped-flow data for the interactions of RTA with VPg peptides were plotted as anisotropy change *versus* time as shown in Figure 6. Stopped-flow kinetic traces were fitted to a single- and double-exponential equation using non-linear regression analysis as described in *Methods*. The kinetic data showed that the binding of RTA to VPg₁₋₁₁₀ synthetic peptide ($k_2 = 32.5 \pm 0.4 \text{ s}^{-1}$) was about 1.8-times faster than the binding of RTA to the wild type VPg ($k_2 = 18.2 \pm 1.8 \text{ s}^{-1}$) (Figure 6 and Table 4). Treatment of the data using a double-exponential function did not improve the fit (data not shown). The residuals representing the deviation between the calculated and experimental data (Figure 6, *lower panel*) indicate that the single-exponential function fits the points over the entire time course of measurements. Under the pseudo-first order conditions, where unlabeled protein (VPg) was in excess, the observed pseudo first-order rate constant is predicted to be a linear function of the concentration of fluorescein-labeled RTA. To test this prediction, RTA-VPg interaction experiments were performed over a concentration range of 0.2–2 μM , 4–40-fold excess. In these experiments, the concentration of fluorescein-labeled RTA was kept constant at 50 nM. Figure 7A shows the rate constants of the RTA-VPg interactions at different concentrations. The RTA-VPg binary complex formation rates were found to be nearly concentration dependent suggesting conformational changes are rate limiting.

A model involving a conformational change, explained in *Methods*, the binding rates have a relationship with the concentration of substrate as described previously, $1/k_{\text{obs}} = 1/k_2 + K_1/k_2[C]$, where k_{obs} is the observed first order rate constant, k_2 is the forward rate constant for the first step, K_1 is the equilibrium constant for the first step, and $[C]$ is the concentration of the unlabeled protein (VPg). The $1/k_{\text{obs}}$ versus $1/[C]$ plot shown in Figure 7A for the RTA-VPg interactions confirm the predicted linear relationship. The k_2 values, obtained from the intercept, were found to be $32.5 \pm 0.4 \text{ s}^{-1}$ for the RTA-VPg interactions at 37°C , respectively.

Here, we further examine the effect of temperature on the kinetic rate of RTA binding to VPg by stopped-flow (rapid-mixing) experiments. The experiments included limiting RTA concentration and excess of VPg, ensuring that the biomolecular RTA-VPg complex formation was pseudo-first order. Stopped-flow data for a typical reaction of RTA binding to VPg were plotted as the anisotropy change vs. time (not shown). The kinetic data were fitted to a single-exponential equation using nonlinear regression analysis as described in *Methods*. Analysis of the data using double exponential components did not improve the fitting results (not shown). The values for the observed rate constants for RTA-VPg complex increased with an increase in temperature (Table 4). The residuals representing the deviation between the calculated and experimental data indicate that the single-exponential function fits the points over the entire time course of measurements. The kinetic data showed that RTA-VPg binding rate ($k_2 = 32.5 \pm 0.4 \text{ s}^{-1}$) at 37°C was 1.2-fold faster than at 5°C ($k_2 = 27.4 \pm 0.4 \text{ s}^{-1}$) (Table 4). In order to determine the activation energy (E_a) for RTA-VPg interactions, the temperature dependent rate constant values (Table 4) were used to construct Arrhenius plot (Figure 7B) according to Eq. S9. The activation energy was obtained from the slope of the linear fit of the plot of $\ln K$ vs. $1/T$. The activation energy for RTA-VPg complex was $-62.1 \pm 1.5 \text{ kJ/mol}$. The overall lower energy for RTA-VPg₁₋₁₁₀ binding ($-67.4 \pm 2.0 \text{ kJ/mol}$) suggests a number of more favorable hydrogen bonds are formed in the transition state of RTA-VPg₁₋₁₁₀ as compared to the RTA-wt VPg complex, favoring the formation of the binary complex.

Discussion and Conclusions

Reports of the use of ricin as a weapon date back to ancient times; ricin has a long history of use in espionage and warfare. Nonetheless, usage of this toxin as customary weapons is quite a modern idea. The ease by which one may acquire large amounts of ricin makes this toxin a prime candidate for bioterrorism. The Centers for Disease Control (CDC) has classified ricin as a Category B biological agent (72). While it is true that ricin, and similar toxins are often cited as major bioterrorism threats, it is also understood that they are not very effective with respect to mass dispersal. It should be stressed that in recent years, bioterrorist mass attacks have given way to a “lone wolf” strategy, where the assault is directed by a single individual or small group, increasing the chances of fatalities. Currently, there is no inhibitor or therapeutic to counteract ricin poisoning, and those that have been investigated induce severe immune responses in animal models. Although multiple types of vaccines have been tested and numerous ricin inhibitors or inactivators have been patented, none possess the ideal properties and standards of an effective vaccine. Many of the inhibitors developed and tested concentrate on structure-based designing of the inhibition of

ricin's *N*-glycosidase activity (23). Recently, an inhibitor of the cellular trafficking of ricin has been reported (73). Schramm *et al.* have reported oligonucleotide-based inhibitors of ricin (74). The RiVax™ (Soligenix) ricin toxin vaccine developed to protect against exposure to ricin toxin is the most advanced vaccine available to date (75,76). New drug candidates have to meet several requirements, including favorable pharmacodynamics (effects of a product related to its clinical activity), pharmacokinetics absorption, distribution, metabolism, and excretion), toxicity and safety issues (77). Peptide attributes originally considered troublesome with respect to drug development may now turn out to be more convenient rather than unfavorable (77). During the past decade, peptides have gained a wide range of applications in medicine and biotechnology, and therapeutic peptide research is also currently experiencing a renaissance for commercial reasons. Peptides are recognized for being highly selective and efficacious and, at the same time, relatively safe and well tolerated. Consequently, there is an increased interest in peptides in pharmaceutical research and development, and over 140 peptides are currently being evaluated in clinical trials as therapeutics (78).

Our findings support the earlier proposed hypothesis that VPg₁₋₁₁₀ peptide shows effective inhibitory effect on RTA activity *in vitro* and in cell-free translational systems. At this time, VPg crystal structure has not been solved nor solution studies performed. Unlike other VPg peptides that are known to be flexible or unstructured, our modeling results suggest that the VPg from TuMV adopts a compact structural fold with prominent helical regions that align well with a consistently predicted secondary structure. The model of the truncated VPg₁₋₁₁₀ peptide reveals distinct acidic surface electrostatic patches that are complementary to the basic surface patches on RTA (Figure 8A and 8B). Docking analyses of VPg₁₋₁₁₀ and RTA also suggest an important electrostatic component to the interaction, corroborating the role of these complimentary surface patches of opposite charge, as they are consistently chosen as the interface for the interaction.

The top electrostatics-favored docking pose suggests VPg clamps the RTA basic surface with its acidic patches (Figure 8C) and this could possibly mediate a conformational change in RTA structure. The predicted RTA-VPg interaction interface does not correspond to or overlap with the active site of RTA but instead binds to the RTA/RTB interface, a region on the RTA surface that is implicated also in binding to P-proteins of the ribosomal stalk (79). Electrostatic mechanism of RTA-VPg interactions is supported further by the fluorescence anisotropy experiments, suggesting VPg binds RTA in a two-step bi-molecular mechanism, leading to an induced conformational change in RTA structure that restricts either the catalytic pocket or prevents binding to ribosomes. The downregulation of RTA activity by VPg suggests that VPg may be not only important in finding a potent inhibitor of ricin toxin, but also represent a general mechanism in viral ability to overcome plant RIPs (PAP and RTA) defense systems, allowing viral access to the cell. VPg interactions with PAP has been described previously (48). Unlike RTA-VPg interactions that become energetically more favorable as the temperature elevates closer to the normal body temperature, favoring the ability of the binary RTA-VPg complex formation at physiological temperature, PAP-VPg binding is favored at lower temperatures. Additionally, PAP-VPg binding affinity at 25 °C ($K_d = 29.5$ nM) is more compatible in strength to the RTA-VPg₁₋₁₁₀ rather than wild type VPg at the same temperature (Table 1). PAP-VPg interactions showed larger enthalpic

contribution but lower entropic involvement (48). The RTA residues consistently predicted to interact with VPg are Gln19, Tyr183, Gly186, Arg189, Thr190, Tyr194, Arg193, Arg234, Phe240, and Ile247. Previous studies have shown that point mutations at some of these residues, i. e., Arg189, Arg193, and Arg234 is associated with reduced the toxicity of RTA (79) and suggests that the predicted binding site of VPg could hinder the recognition between RTA and the ribosomal stalk. Perhaps VPg-eIFs binding, where the viral protein downregulates cellular mRNA translation, while increasing uncapped viral mRNA synthesis, directly relates to these interactions and the ribosome (80).

VP_{g1-110} interacts in a very similar manner with Stx1 and suggests a corresponding region away from the active site of Stx1 (Figure 9A and 9B). Stx (Shiga toxins) are produced by pathogenic STEC (Stx-producing *E. coli*) strains, which are responsible for severe human illnesses: including hemorrhagic colitis, infantile diarrhea and HUS (hemolytic uraemic syndrome) (81). HUS is the main cause of acute renal failure in children and is the consequence of intestinal infection by STEC. A similar set of residues in VPg are predicted to be involved in interacting with both RTA and Stx1. VPg residues consistently predicted in interacting with RTA include Asp110, Glu109, Leu107, Leu106, Phe99, Gln96, Ile92, Asp91, Asp69, and Glu68 and for Stx1 Asp110, Leu106, Ile102, Phe99, Gln96, Val95, Ile92, Asp91, Asp69, Glu68, Tyr25, and Glu23 (Figure 9A and 9B).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RT	ricin toxin
RTA	ricin A-chain
RTB	ricin B-chain
RIP	ribosome inactivating protein
VPg	genome-linked viral protein
TuMV	turnip mosaic virus
SRL	sarcin/ricin loop
K_d	dissociation constant
NHS-fluorescein	N-hydroxysuccinamide fluorescein

Tris-HCl	tris-(hydroxymethyl)aminomethane
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
BSA	bovine serum albumin
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
HPLC	high pressure liquid chromatography
TEV	tobacco etch virus
k_{cat}	catalytic constant
K_m	Michaelis constant
E_a	activation energy
Stx	Shiga toxin
HUS	hemolytic uraemic syndrome
STEC	Shiga toxin-producing <i>E. coli</i>
F	fluorescence
WT	wild type
IPTG	isopropyl β -D-1-thiogalactopyranoside
ATP	adenosine triphosphate
YPD medium	yeast peptone dextrose medium
EGTA	ethylene glycol- <i>bis</i> (β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid

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Significance Statement

Ricin and related Shiga toxins are potentially lethal poisons, which as of yet, have no effective pharmaceutical treatments or preventions. The danger of these poisons is an increasingly significant threat to public health in the current global scenario. Ricin, acquired astoundingly easily, is an ideal toxin for bioterrorism. Shiga toxin is highly relevant in the worldwide health scenario, and the widespread transmission of pathogenic enteric *E. coli* is responsible for severe human illnesses and several fatalities. Results of this study suggest a route to antidotes for these toxins by developing potent variants of VPg peptides that inhibit or effectively counteract the effects brought about by ricin or Shiga toxin poisoning.

Figure 1. Effects of VPg peptides on catalytic activity of RTA towards ribosomes.

RTA concentration in all reactions was 200 nM. Controls: (1) (+) RTA / (-) VPg / (+)

Ribosomes; (2) (-) RTA / (-) VPg / (+) Ribosomes; (3) (+) RTA / (-) VPg / (-) Ribosomes;

wt VPg: (4) 50 nM VPg; (5) 100 nM VPg; (6) 200 nM VPg; VPg-71: (7) 50 nM VPg; (8)

100 nM VPg; (9) 200 nM VPg; VPg-111: (10) 50 nM VPg; (11) 100 nM VPg; (12) 200 nM

VPg; VPg₁₋₁₁₀: (13) 50 nM VPg; (14) 100 nM VPg; (15) 200 nM VPg.

Figure 2. Translation of TEV RNA luciferase reporter gene in rabbit reticulocyte extracts. Luciferase relative light units (RLU) were measured for TEV(1-143)-*luc*-A₅₀ RNA (-●-), TEV(1-143)-*luc*-A₅₀ RNA + RTA (-▼-), TEV(1-143)-*luc*-A₅₀ RNA + RTA + wt VPg (-◆-), TEV(1-143)-*luc*-A₅₀ RNA + RTA + VPg-71 (-×-), TEV(1-143)-*luc*-A₅₀ RNA + RTA + VPg-111 (-▲-), and TEV(1-143)-*luc*-A₅₀ RNA + RTA + VPg₁₋₁₁₀ after 30 sec (-●-) in rabbit reticulocyte translational extract as a function of time. The proteins were added in the stoichiometric concentrations (10 nM) in the presence of 1.0 µg of TEV(1-143)-*luc*-A₅₀ RNA, and light emission was measured after the addition of 0.5 mM luciferase substrate.

Figure 3. Binding plots of RTA (200 nM) with VPg peptides.

The values of $\Delta F / \Delta F_{\max}$ for WT VPg (■), VPg-71 (▲), VPg-111 (▼), and synthetic VPg₁₋₁₁₀ (●) versus concentration of VPg peptides at 25 °C. The curves were fit to obtain equilibrium constants as described under *Methods*.

Figure 4. Temperature dependence of fluorescein-labeled RTA-VPg₁₋₁₁₀ interactions.

The normalized fluorescence values ($\lambda_{\text{ex}} = 495$ nm and $\lambda_{\text{em}} = 516$ nm) for the reaction of the bound ligand (VPg₁₋₁₁₀) ($F / F_{\max}$) are plotted versus VPg₁₋₁₁₀ concentration at 5 °C (—■—), 15 °C (—▲—), 25 °C (—●—), and 37 °C (—◆—). Fluorescein-labeled RTA concentration was 200 nM in titration buffer. The curves were fit to obtain dissociation constants (K_d) as described under *Methods*. The *inset* shows van't Hoff plot for the interactions of fluorescein-labeled RTA with VPg₁₋₁₁₀ peptide.

Figure 5. Kinetic studies of RTA depurinating 28S rRNA, and the effects of VPg peptides on the rates of the depurination.

(A) Time course curves of adenine released during depurination of the rRNA in the absence (-■-) and presence of VPg (wt VPg, -▲-; VPg-71, -■-; VPg-111, -◆-; VPg₁₋₁₁₀, -●-) by RTA, as measured by the fluorescence of *N*⁶-ethenoadenine. The controls of the studies included depurination of poly(C) RNA (-x-) by RTA. Aliquots of RTA-rRNA mixtures ($\pm$ VPg) were withdrawn at different times, and loaded onto the HPLC column (excitation and emission wavelengths were 315 nm and 415 nm, respectively). (B) *N*⁶-Ethenoadenine assay kinetic curve for the depurination catalysis of 28S rRNA in the absence (-■-) and presence of VPg (wt VPg, -▲-; VPg-71, -■-; VPg-111, -◆-; VPg₁₋₁₁₀, -●-) by RTA. Controls included samples where the rRNA was substituted for poly(C) RNA (-x-). Each reaction included a sample of RTA (200 nM), treated with the increasing concentrations of RNA, and the amounts of the released adenines were modified, as described under *Experimental Procedures*.

Figure 6. Stopped-flow kinetic binding measurements of fluorescein-labeled RTA and VPg₁₋₁₁₀ peptide.

Representative kinetic data show the time dependent decrease in anisotropy after mixing 50 nM fluorescein-labeled RTA [A] (final concentration) with 0.2 μ M unlabeled VPg peptides ([B], wild type VPg; [C], VPg-71; [D], VPg-111; and [E], VPg₁₋₁₁₀) (final concentration) at 25 °C. Residuals for the fits are shown in the lower panels. The experimental conditions are described under *Experimental Procedures*.

Figure 7.

[A] Kinetic plots of $1/k_{obs}$ versus $1/[C]$ for the interaction of 200 nM fluorescein-labeled RTA with varying concentrations of wt VPg (■), VPg-71 (▲), VPg-111 (▼), and VPg₁₋₁₁₀ (●) peptides. The rate constant k_2 was obtained as the reciprocal of the y-intercept. [B] Arrhenius plots for the interaction of VPg peptides with fluorescein-labeled RTA. The rate constant values for wt VPg (■), VPg-71 (▲), VPg-111 (▼), and VPg₁₋₁₁₀ (●) peptides with labeled-RTA at different temperatures were used to construct an Arrhenius plot according to equation S9. The activation energy was calculated from the slope of the fitted linear plot of $-\ln k_{obs}$ versus reciprocal of absolute temperature T^{-1} (Kelvin).

Figure 8. Electrostatic component in RTA-VPg docking interactions.

(A) Surface electrostatic analysis reveals complementary acidic patches in VPg₁₋₁₁₀ (RTA depicted in pale green cartoon format), and (B) Basic patches in RTA (VPg₁₋₁₁₀ depicted in wheat cartoon format). (C) Surface electrostatics of the docked VPg₁₋₁₁₀ (bottom) and RTA (top) showing VPg's acidic surfaces clamp the basic surface of RTA on both sides of the interaction surface.

Figure 9. Predicted mode of interaction of VPg₁₋₁₁₀ with RTA and Stx1.

(A) VPg₁₋₁₁₀ (wheat cartoon) and RTA (pale green cartoon) with the key interaction interface residues shown in red (VPg₁₋₁₁₀) and dark green (RTA). Active site residues are highlighted in orange on the RTA cartoon. (B) VPg₁₋₁₁₀ (wheat cartoon) and Stx1 (pale green cartoon) with the key interaction interface residues shown in red (VPg₁₋₁₁₀) and dark green (Stx1). Active site residues are highlighted in orange on Stx1 cartoon.

Table 1.

Temperature dependence of the equilibrium dissociation constants for the RTA-VPg complexes

Complex	Equilibrium Dissociation Constant, Kd, [nM] *			
	5 °C	15 °C	25 °C	37 °C
RTA · WT VPg	483 ± 18	318 ± 9	138 ± 7	97 ± 6.3
RTA · VPg-71	210 ± 15	166 ± 9	90.4 ± 3.5	37 ± 2.6
RTA · VPg-111	$1 \times 10^5 \pm 10^3$	$0.71 \times 10^4 \pm 256$	$2.35 \times 10^3 \pm 217$	$1.37 \times 10^3 \pm 188$
RTA · VPg ₁₋₁₁₀	200 ± 11	104 ± 6	18.4 ± 2.1	4.7 ± 0.1

* Error is presented as SD.

Table 2.

Thermodynamic parameters for the interactions of RTA with the WT VPg and VPg₁₋₁₁₀ *

Complex	Enthalpy H° (kJ/mol)	Entropy S° (J/K·mol)	Gibbs Free Energy G° (kJ/mol)
RTA-WT VPg	0.0382 ± 0.017	258.1 ± 2.7	-80.0 ± 0.6
RTA-VPg ₁₋₁₁₀	0.0876 ± 0.012	441.3 ± 4.3	-136.8 ± 1.8

* H° and S° values were determined from van't Hoff plot and dissociation constants. G° values were calculated at 37 °C using the equation $G^\circ = H^\circ - T S^\circ$.

Table 3.

Effects of VPg on Kinetic Rates of RTA Depurinating 28S rRNA

Complex	k_{cat} (min ⁻¹)	K_{m} (nM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ ·s ⁻¹)
RTA	1.4 ± 0.1	6.1 ± 0.7	3.8 × 10 ⁶
RTA·WT VPg	11.8 ± 2.6	254 ± 18	7.7 × 10 ⁵
RTA·VPg-71	13.9 ± 4.2	340 ± 24	6.8 × 10 ⁵
RTA·VPg-111	1.9 ± 0.10	11.2 ± 0.90	2.8 × 10 ⁶
RTA·VPg ₁₋₁₁₀	25.3 ± 2.9	560 ± 23	7.5 × 10 ⁵

Table 4.

Kinetic Binding Constants for the Interactions of RTA with VPg peptides

Temperature	Association Rate Constants, k_{obs} (s^{-1})			
	RTA·WT VPg	RTA·VPg-71	RTA·VPg-111	RTA·VPg ₁₋₁₁₀
5 °C	27.4 ± 0.4	35.4 ± 4.0	54.5 ± 2.0	15.3 ± 0.7
15 °C	27.6 ± 0.7	33.7 ± 3.8	60.0 ± 1.1	16.8 ± 1.3
25 °C	31.6 ± 0.5	44.8 ± 2.4	64.0 ± 2.4	20.1 ± 1.5
37 °C	32.5 ± 0.4	43.9 ± 3.1	68.7 ± 1.5	18.2 ± 1.8
E_a (kJ/mol)	− 62.1 ± 1.5	− 90.1 ± 2.4	− 74.0 ± 1.3	− 67.4 ± 2.0