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Inhibition of Pokeweed Antiviral Protein (PAP) by Turnip Mosaic Virus Genome-linked Protein (VPg)*

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Background: PAP is a ribosome-inactivating protein that depurinates RNA and inhibits protein synthesis.

Results: Turnip mosaic VPg inhibits enzymatic activity of PAP in wheat germ extract.

Conclusion: VPg may play a role in overcoming viral resistance by suppressing the plant defense mechanism.

Significance: Depurination inhibition by VPg suggests a novel viral strategy to evade host cell defense and possible anticytotoxic activity against RIPs.

Pokeweed antiviral protein (PAP) from Phytolacca americana is a ribosome-inactivating protein (RIP) and an RNA N-glycosidase that removes specific purine residues from the sarcin/ricin loop of large rRNA, arresting protein synthesis at the translocation step. PAP is also a cap-binding protein and is a potent antiviral agent against many plant, animal, and human viruses. To elucidate the mechanism of RNA depurination, and to understand how PAP recognizes and targets various RNAs, the interactions between PAP and turnip mosaic virus genome-linked protein (VPg) were investigated. VPg can function as a cap analog in cap-independent translation and potentially target PAP to uncapped IRES-containing RNA. In this work, fluorescence spectroscopy and HPLC techniques were used to quantitatively describe PAP depurination activity and PAP-VPg interactions. PAP binds to VPg with high affinity (29.5 nM); the reaction is enthalpically driven and entropically favored. Further, VPg is a potent inhibitor of PAP depurination of RNA in wheat germ lysate and competes with structured RNA derived from tobacco etch virus for PAP binding. VPg may confer an evolutionary advantage by suppressing one of the plant defense mechanisms and also suggests the possible use of this protein against the cytotoxic activity of ribosome-inactivating proteins.

Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein (RIP) that is isolated from the extracts of pokeweed plant leaves (Phytolacca americana) (1). It is known to reduce infectivity of tobacco mosaic virus (2) by inhibiting protein synthesis (3). PAP, ricin, abrin, and other RIPs inactivate ribosomes and inhibit cell-free protein synthesis by means of arresting the function of elongation factor EF-2 (4, 5) in the translocation step (6–8). The N-glycosidase domain of RIPs recognizes a specific and highly conserved region, the sarcin/ricin (S/R) loop (9), within the large 28S rRNA, and cleaves a distinct A4324 residue on the RNA (for rat liver ribosome). This depurination arrests cellular protein synthesis and leads to the activation of apoptotic pathways (10). Ribosomal proteins and structural differences between RIPs themselves account for the diversified activity of RIPs and ribosome substrate specificity (11, 12).

The mode of action for the antiviral activity of RIPs is poorly understood, but there is evidence that this activity does not depend solely on ribosomal inactivation. PAP isoforms cause a concentration-dependent depurination of HIV-1 (13), tobacco mosaic virus (14), poliovirus (15), HSV (16), influenza (17), and brome mosaic virus RNAs (18).

PAP inhibits the in vitro translation of brome mosaic virus and potato virus X RNAs without ribosomal depurination (19) by binding to the cap structure and depurinating the RNA. This may be the primary mechanism for PAP antiviral activity (20); however, it does not clarify the inhibitory effects of PAP on the replication of uncapped viruses, such as influenza (17) and poliovirus (15). Further, PAP does not depurate every capped RNA, and it can inhibit translation of uncapped viral RNAs in vitro without causing detectable depurination at multiple sites (21). Thus, recognition of the cap structure alone is not sufficient for depurination of RNA (21).

In the presence of wheat germ lysate, PAP depurinates uncapped barley yellow dwarf virus transcripts containing a functional WT 3′-translation enhancer sequence but does not depurate messages containing a non-functional mutant

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This paper is dedicated to Dr. Diana E. Friedland, who died in January 2011. Dr. Friedland co-mentor Dr. Domashevskiy and contributed to the design and interpretation of these experiments.

†This article contains supplemental Fig. 1.

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2 The abbreviations used are: PAP, pokeweed antiviral protein; RIP, ribosome-inactivating protein; VPg, viral protein linked to the genome; S/R, sarcin/ricin; TEV, tobacco etch virus; TuMV, turnip mosaic virus; m7G, 7-methylguanosine; ant-m7GTP, anthranoyl 7-methylguanosine triphosphate; eIF, eukaryotic initiation factor; IRES, internal ribosome entry site; NHS, N-hydroxysuccinimide; NHS-fluorescein, N-hydroxysuccinimide ester of fluorescein; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
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3’-translation enhancer sequence (22). This suggests that PAP binding to eIF4G4/iso4G provides a mechanism for PAP to access both uncapped and capped viral RNAs for depurination. PAP binds to the eIFiso4G, and the presence of cap analog increases these protein-protein interactions (23), suggesting that PAP binds to the 5’-m7G cap of mRNA.

To understand how PAP recognizes and selectively targets RNAs, interactions between PAP and TuMV genome-linked protein (VPg) were investigated. VPg is a 22-kDa polyprotein, covalently attached via a Tyr residue (24) to the genomes of one-quarter of the plant positive strand RNA viruses, including the Potyviridae genus. VPg plays a pivotal role in the viral infection cycle, replication, and cell-to-cell movement and also has been implicated in overcoming viral resistance in plants (25). Interactions between VPg of TuMV and the eIFiso4E/iso4G of Arabidopsis thaliana (26, 27) suggest that VPg is important in the initiation of protein synthesis (28). Interactions between VPg and plant eIFiso4E and effects of eIFiso4G on these interactions have been characterized (29-31). VPg stimulates the in vitro translation of uncapped IRES-containing RNA by targeting eIF4F to the IRES and inhibits capped RNA translation in wheat germ extracts (32).

In this study, fluorescence spectroscopy and HPLC techniques were used to quantitatively describe PAP-VPg interactions. PAP interacts strongly with VPg in a mixed type competition for m7GTP cap analog. PAP binds to and depurinates an S/R oligonucleotide, capped and uncapped tobacco etch virus (TEV), and luciferase mRNA, supporting previous conclusions that the cap structure is the only determinant within the RNA for depurination by PAP. PAP binds to VPg at a different site from the eIFiso4F/eIF4F binding site. The effect of VPg on the depurination of selected RNA molecules, including structured RNA derived from TEV (33-35), showed that VPg decreases depurination of RNAs and competes with IRES containing TEV RNA for PAP binding. These findings correlate with the inhibition of PAP enzymatic activity by VPg in wheat germ lysate. Depurination inhibition by VPg may confer an advantage for viral replication, and may be a novel mechanism to overcome plant defenses.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used (unless otherwise noted) were of molecular biology grade. Tris-HCl, HEPES, KCl, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), aprotinin, soybean trypsin inhibitor, diethylpyrocarbonate, EDTA, and the m7GTP analog were purchased from Sigma. Polyvinylpyrrolidone was purchased from Spectrum. Promega RiboMAX™ large scale RNA production system T7 and SP6 were used for in vitro RNA and S/R oligonucleotide synthesis. Peptone, yeast extract, and NaCl were purchased from Fisher. The Sall and Ncol restriction endonucleases were purchased from New England Biolabs. HiTraps FF chromatography columns (Mono Q and SP) were from GE Healthcare. Plasmid isolation kits and nickel-nitrilotriacetic acid Superflow column were purchased from Qiagen.

Purification of Pokeweed Antiviral Protein—PAP used for these experiments was isolated from the spring leaves of the pokeweed plant (Phytolacca americana) as described previously (23). PAP was purified using the AKTApurifier system from GE Healthcare, equipped with pump P-900, monitor UV-900, monitor UPC-900, valve INV-907, and mixer M-925. The protein fractions were analyzed by 12% SDS-PAGE for purity, and the protein concentration was determined using a Pierce Coomassie assay with BSA as the standard.

Expression and Purification of Wild Type (WT) VPg-His, VPg-71-His, and VPg-220-His—Purification of TuMV VPg was described previously (29). VPg was purified with the AKTApurifier system. Purification of VPg-71-His and VPg-220-His truncated proteins followed an analogous procedure. The purity of all of the proteins was confirmed by 12% SDS-PAGE, and the protein concentration was determined using the Pierce Coomassie assay with BSA as the standard. Prior to spectroscopic measurements, all samples were dialyzed against buffer E (20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl2, 1.0 mM DTT, 1.0 mM EDTA), passed through a 0.22-μm Millipore filter, and concentrated with a Centricon 10 concentrator (Amicon Co.) as necessary.

Expression and Purification of Eukaryotic Translation Initiation Factors (eIFs)—The cap binding and scaffolding initiation factors (eIF4G and eIF4E) were expressed in Escherichia coli containing the constructed pET-3d vector in BL21(DE3)-pLysS cells, as described previously (31). All samples were analyzed by 12% SDS-PAGE and showed homogeneously pure proteins. All protein purification steps were carried out in a cold room at 4°C.

In Vitro Synthesis of TEV RNA, S/R Oligonucleotide RNA, and Luciferase mRNA—TEV DNA constructs were kindly provided by Daniel R. Galie (University of California, Riverside, CA). The full-length TEV construct was cloned as described previously (33). The TEV1–143 leader sequence was positioned next to the SP6 promoter of the PTL7SN.3 GUS vector. DNA was linearized with Ncol. The linearized DNA was treated with proteinase K (100 μg/ml) and 0.5% SDS in 50 mM Tris-HCl, pH 7.5, 5 mM CaCl2 for 30 min at 37°C. DNA was further purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) at pH 8.0 followed by ethanol precipitation. Purity of the resulting DNA was checked on a 1% agarose gel, and the concentration was quantified spectrophotometrically and brought to 0.5 mg/ml. In vitro transcription of the TEV DNA used Promega RiboMAX™ large scale RNA production system SP6 following the manufacturer’s protocol. Cap analog, m7G(5′)ppp(5′)G, was incorporated into the TEV transcript during the RiboMAX™ transcription reaction. The ratio of cap analog/GTP was 5:1 to increase the efficiency of the transcription reaction. Capped and uncapped transcripts were analyzed on 20% denaturing polyacrylamide, 8 M urea gels, and the synthesized products were visualized by ethidium bromide staining. Under the conditions of transcription, more than 75% of RNA transcripts were capped, as determined by the fluorescence intensity of ethidium bromide. Capped RNA transcripts were sliced from the gels, redissolved in a buffer solution, precipitated with ethanol, and repurified. The concentration of TEV RNA was determined by measuring the optical density at 260 nm, and the purity of the synthesized RNA was confirmed by measuring the absorbance ratio A260/A280 in diethylpyrocarbonate-treated water. The S/R oligonucleotide dsDNA template (5′-GGATC-
CTAATACGACTCACTATAGGGTGAACTTATAGTACGAG-
AGGAAACGTTCA-3’; 53 nucleotides) was purchased from Gene Link™ with the sequence corresponding to the universally conserved S/R loop of the large rRNA. The linear DNA template was treated with proteinase K and purified by phenol/ chloroform extraction as for TEV DNA. In vitro synthesis of the S/R oligonucleotide RNA used Promega Ribomax™ large scale RNA production system T7 following the manufacturer’s protocol. Cap analog, m7G(5)ppp(5)G, was incorporated into the RNA transcripts during the Ribomax™ transcription reaction. The plasmid pLUC0 (36) containing the luciferase gene was linearized with DraI and used as template for synthesis of the in vitro transcript. pLUC0 contains a linker sequence (GGCCTAAGCTTGTCGACC) between the T7 promoter and the ATG of luciferase. Following the TAA stop codon of luciferase, synthesis of the gene was linearized with DraI and used as template for synthesis of the transcript. pLUC0 contains a linker sequence (GGCCTAAGCTTGTCGACC) between the T7 promoter and the ATG of luciferase. Following the TAA stop codon of luciferase a poly(A) tail of 50 A nucleotides immediately upstream of the DraI site. Both capped and uncapped luciferase RNAs were synthesized as run-off transcripts of a T7 polymerase reaction (Promega), as described previously (20).

Fluorescence Assay for Adenine Released by PAP—Experiments were performed by incubating RNA (10 nmol/ml) in Depurination Buffer (20 mM Tris-HCl, pH 7.5, 100 mM NH4Cl, 7 mM magnesium acetate, and 1 mM DTT) for 15 min at 37 °C in the absence and presence of PAP and VPg in 100-μl reaction volumes. At the end of the incubations, 1 volume of cold ethanol was added, and after 10 min at −80 °C, the ethanol-soluble fractions were recovered by centrifugation for 15 min at 14,000 rpm. Free adenine present in the ethanol-soluble fractions was converted into its etheno derivative (37–39); 150 μl portions of the ethanol-soluble fractions were each diluted to 1 ml with diethylpyrocarbonate-treated water, and 0.4 ml of a mixture of 0.14 M chloroacetaldehyde and 0.1 M sodium acetate buffer, pH 5.1, was added to each. The samples were heated in a water bath at 80 °C for 40 min, extracted four times with 1 volume of water-saturated diethyl ether, and passed through 0.45-μm pore size filters. Fractions were analyzed with a Waters high-pressure liquid chromatograph equipped with a Waters 2487 dual λ absorbance detector (set at 254 nm), a Waters 2475 multi λ fluorescence detector (excitation, 315 nm; emission, 415 nm), a Waters 600 controller, and a Waters 717 plus autosampler. The column (4.6 × 150 mm) was a reversed-phase XBridge™ C18 (particle size 5 μm) purchased from Waters Associates. The column was eluted isocratically with 50 mM ammonium acetate buffer, pH 5.0, plus methanol (89:11, v/v) at room temperature. Elution profiles were analyzed with Waters Empower™ chromatography software. Each experiment included a standard of N6-ethenoadenine in the appropriate buffer and internal standards obtained by adding known amounts of N6-ethenoadenine to the ethanol-soluble fractions from the control and PAP-treated RNA. The amount of adenine released from PAP-treated RNA was calculated from the standards after subtraction of the fluorescence reading given by control RNA.

Wheat Germ Lysate Translational Assay—Wheat germ extract was purchased from Promega. Translation of TEV RNA was determined in luciferase assay buffer (25 mM Tricine, pH 8.0, 5 mM MgCl2, 0.1 mM EDTA supplemented with 33 mM DTT, 0.25 mM coenzyme A, and 0.5 mM ATP) for luciferase activity. A 1.0-μg sample of TEV1–143-luc-A50 RNA was translated in a 200-μl reaction mixture containing 50 μl of complete wheat germ lysate extract, 50 units of RNase inhibitor, and 10 μM complete amino acid mixture (Promega). Luciferase activity for brome mosaic virus RNA provided by Promega was used as a control. Equimolar concentrations of PAP, WT VPg, or VPg-220 were added to the translational mixture as described under “Results.” Light emission was measured after the addition of 0.5 mM luciferin as a function of time using the PerkinElmer Gelieco 600 imaging system.

Synthesis of the Fluorescent Anthranoyl-m7GTP—The fluorescent anthranoyl-m7GTP cap analog was synthesized as described previously (40, 41) with the following modifications. The m7GTP cap (10 mg) was dissolved in 1 ml of distilled water at 37 °C. The pH of the resulting solution was adjusted to 9.6 with 2 N NaOH. To this solution with continuous stirring, crystalline isatoic anhydride (5 mg) was added. The pH of the mixture was maintained at 9.6 by titrating 2 N NaOH during the 2-h reaction. After the reaction was complete, the pH of the reaction mixture was adjusted to 7.0 with 1 N HCl solution. The reaction mixture containing the products and the unreacted materials was loaded onto a Sephadex LH-20 (2.4 × 56-cm) column equilibrated with autoclaved distilled water. The column was eluted with the same solvent at a flow rate of about 6 ml/h. Fractions of 1 ml were collected and assayed by TLC on silica gel. The plates were developed in system A (n-propyl alcohol/ammonia/water (6:3:1, v/v/v), containing 0.5 g/liter EDTA). The ant-m7GTP analog had brilliant blue fluorescence (monitored by a UV lamp), whereas anthranilic acid (a byproduct of this reaction) showed dark violet fluorescence. Peak fractions of the fluorescent analog were pooled, combined, and lyophilized in vacuo at liquid nitrogen temperature to prevent degradation. The resulting residue was then dissolved in a minimum amount of water (0.5 ml), and an excess of cold ethanol was added to induce the precipitation of the compound. The fluorescent cap analog was then dried in vacuo over phosphoric anhydride at 4 °C, giving an amorphous powder.

Labeling of PAP with NHS-fluorescein—Pokeweed antiviral protein was labeled with fluorescent N-hydroxysuccinimide (NHS)-fluorescein reagent using the Pierce NHS-fluorescein antibody labeling kit (Thermo Scientific) according to the manufacturer’s protocol. NHS-ester labeling reagents react efficiently with primary amines in the side chains of lysine residues of PAP. PAP storage buffer S was replaced with 50 mM sodium borate, pH 8.5, buffer, containing 1 mM DTT using Microcon® centrifugal filters (Millipore). The fluorescent/protein ratio was estimated spectrophotometrically in phosphate-buffered saline (PBS) by measuring absorbance at 280 and 495 nm (i.e. A280/Amax of NHS-fluorescein). A280/Amax = 0.3. The average amount of labeling was determined to be 65%. Protein concentration was calculated as follows,

Moles of fluorescent/moles of protein

\[
\frac{A_{\text{max}}}{\epsilon_{\text{fluor}} \times [\text{protein}]} \times DF \quad (\text{Eq. 1})
\]

where \(A_{\text{max}}\) is the maximum absorbance of the labeled protein, measured at 495 nm, \(\epsilon_{\text{fluor}} = 70,000\) (NHS-fluorescein molar
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extinction coefficient), [Protein] is the molar protein concentration, and DF is the dilution factor. The degree of PAP labeling was calculated as follows,

\[ [\text{Protein}]_{\text{m}} = \frac{A_{280} - (A_{\text{max}} \times CF)}{\epsilon_{\text{protein}}} \times DF \]  

(Eq. 2)

where \( A_{280} \) is the absorbance of the non-labeled protein, measured at 280 nm, \( A_{\text{max}} \) is the maximum absorbance of the labeled protein, measured at 495 nm, \( \epsilon_{\text{protein}} \) is the molar extinction coefficient of the non-labeled protein, correction factor (CF) = \( A_{280}/A_{\text{max}} \) = 0.3, and DF is the dilution factor.

Fluorescence Data Acquisition and Analysis—Steady state fluorescence was used to monitor protein-protein and protein-nucleic acid interactions (42). Acquisition of steady state fluorescence in the ultraviolet region allows the use of intrinsic protein fluorophores to determine equilibrium constants. A Horiba Jobin Yvon Fluoromax-3.3 fluorometer with a 150-watt xenon lamp with photodiode array detectors was used for all fluorescence measurements. Fluorescence changes (quenching or enhancement, depending on the titrations) were monitored using an excitation wavelength of 280 nm and an emission wavelength of 332 nm (intrinsic protein fluorescence) or using excitation of 332 nm for the anthranoyl group and 493 nm for NHS-fluorescein and an emission wavelength of 420 nm for the anthranoyl group and 516 nm for NHS-fluorescein (for extrinsic fluorophores). All samples were thermoregulated, and the temperatures were monitored by a thermocouple in the sample chamber. All titrations were performed in a titration buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM DTT). For each data point, three samples were prepared. The fluorescence intensity of a protein (e.g. PAP) was measured in the first sample. A second sample containing specific amount of titrant protein (e.g. VPg) was also measured, and the corrected intensities of the two samples were summed together (\( F_c \)). A third sample containing the same amount of PAP and VPg proteins was mixed together, and the corrected fluorescence intensity for this complex was obtained (\( F_s \)). The difference in fluorescence intensity related to the complex was defined as \( \Delta F = F_s - F_c \). Similar measurements were also performed for other titrations. The inner filter corrections for the RNA experiments were applied as described previously (34) using the following equation (42),

\[ F_c = F_s \times \exp\left(\frac{A_{\text{em}} - A_{\text{em}}^0}{2}\right) \]  

(Eq. 3)

where \( F_c \) and \( F_s \) are the corrected and observed fluorescence intensities, respectively. \( A_{\text{em}} \) and \( A_{\text{em}}^0 \) are the absorbance of the excitation and emission wavelengths, respectively. Corrections for the dilutions of the titrated samples were taken into consideration as well. The absorbance of the sample was measured using an Ultrospec 1100 Pro UV-visible absorption spectrophotometer. The normalized fluorescence difference (\( \Delta F/\Delta F_{\text{max}} \)) between the protein-protein and protein-RNA 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 \( \Delta F_{\text{max}} \). The details of the data fitting are described elsewhere (32, 43).

PRISM®, version 5, was used to analyze and plot the data. Non-linear least-squares fitting of plotted normalized data were used; one-site and two-site binding models were tested.

Evaluation of Thermodynamic Parameters—Thermodynamic parameters, \( \Delta H \) (van’t Hoff enthalpy), \( \Delta S \) (entropy), and \( \Delta G \) (free energy), were determined using the following equations,

\[ -\ln K_{eq} = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \]  

(Eq. 4)

\[ \Delta G^0 = -RT\ln K_{eq} = \Delta H - T\Delta S \]  

(Eq. 5)

where \( R \) and \( T \) are the universal gas constant and absolute temperature, respectively. \( K_{eq} \) is the association equilibrium constant, \( \Delta H \) and \( \Delta S \) were determined from the slope and the intercept of a plot of \( \ln K_{eq} \) against \( 1/T \), and \( \Delta G \) was determined from Equation 5.

Determination of the Number of Binding Sites—The quenching of the native fluorescence emission maximum upon the addition of a ligand was monitored for the fluorescence change relative to untitrated PAP or elfiso4F. VPg has very low intrinsic protein fluorescence. The fractional quench, \( Q \), was determined at each PAP/VPg or elfiso4F/VPg molar ratio (\( R \)). For an observed fluorescence intensity, \( F \), the fractional quench, \( Q \), was obtained from Equation 6 (44).

\[ Q = \frac{F_s - F}{F} \]  

(Eq. 6)

Here \( m \) is the maximal quench. Fractional quench is linearly related to ligand binding,

\[ \frac{[\text{Ligand} - \text{Protein}]}{[\text{Protein}]_r} = Q \]  

(Eq. 7)

where \( [\text{Protein}]_r \) represents the total PAP or elfiso4F protein concentration. The average number of binding sites (\( n \)) was determined from the \( x \) intercept of the Scatchard plot \( Q \) versus \( Q/(R - Q)[\text{Protein}]_r \) (44).

RESULTS

PAP Depurinates both \( m^7 \text{GpppG}-\text{capped and Uncapped S/R, TEV, and Luciferase mRNA—Because PAP can bind to cap analogs, we determined the extent to which the presence of a cap on the RNA affected depurination. To examine the extent to which PAP discriminates between capped and uncapped RNA transcripts, a synthetic S/R oligonucleotide RNA, TEV RNA, and luciferase mRNA were used as substrates for PAP enzymatic activity. PAP recognized a specific and highly conserved adenine residue (A4324) on the RNA (for the rat liver ribosome) (9). Previous reports showed that PAP was able to recognize the cap structure on RNA transcripts (19). It was postulated that PAP binding to the cap structure promotes the depurination of capped mRNAs (20). Other findings indicated that PAP is able to inhibit translation of uncapped RNAs without detectable levels in depurination (21). Both findings are not mutually exclusive but in fact imply that the cap structure itself is not enough to promote the depurination of RNA or inhibi-
tion of RNA translation. To determine whether the cap structure affects depurination of S/R and TEV RNAs and luciferase messenger RNA, the above RNAs were capped during run-off transcription reactions with an m7GpppG cap analog. Separation using HPLC techniques and identification by means of fluorescence allowed for construction of a linear relationship between the amount of 1-N6-ethenoadenosine derived from the depurination of RNAs and the integrated peak area over a wide range of 1-N6-ethenoadenosine concentrations (from 10 to 200 pmol) (supplemental Fig. 1A). The amount of adenine released from WT S/R and WT TEV RNA versus m7GpppG-capped S/R and TEV RNAs with an addition of increasing amounts of PAP was determined. Under the conditions of HPLC, loaded fractions gave a single fluorescent peak with a retention time of 4.5 min (supplemental Fig. 1, B and C). The amount of adenine released upon depurination of capped versus uncapped RNAs was of the same order of magnitude. PAP depurination did not discriminate between either capped or uncapped S/R oligonucleotide and capped or uncapped TEV RNAs (Fig. 1). The amount of adenine released from capped and uncapped S/R RNA was 14.9 ± 0.8 and 13.7 ± 0.7 nM, respectively. The amount of adenine released from capped and uncapped TEV RNA was 6.0 ± 0.4 and 4.1 ± 0.1 nM, respectively. Depurination of uncapped cellular luciferase mRNA yielded 27.5 ± 0.6 nM adenine released compared with 35.6 ± 1.8 nM for m7GpppG-capped RNA. These results indicate that the cap itself had little effect on depurination for naturally uncapped RNA and only a modest effect on luciferase mRNA.

**PAP Has Different Kinetics from Ricin A Chain**—To examine the rates at which PAP depurinates WT TEV RNA, standard quantification of adenine in the discontinuous assay format was performed. Analysis of the fractions on the HPLC indicated that RNA depurination by PAP was virtually 80% complete after 3 min (Fig. 2A). To establish catalytic constants of TEV RNA depurination by PAP, the RNA concentrations were varied. The progress of the reaction was monitored by the appearance of a UV-absorbing product at the saturating conditions. Calculated depurination rates were plotted against the RNA concentrations, resulting in a Michaelis-Menten type behavior (Fig. 2B). The catalytic constant, $k_{cat}$, was calculated to be 2.5 min⁻¹ (0.042 s⁻¹). Fluorescence titrations of NHS-labeled PAP with RNA produced a $K_m$ of 13.6 nM. The specificity constant, $k_{cat}/K_m$, was calculated to be $3.1 \times 10^6$ M⁻¹ s⁻¹. This is compared with the specificity constant of ricin A chain for 80S rabbit ribosomes of $1.4 \times 10^6$ M⁻¹ s⁻¹ (45).
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PAP Binds to VPg with Higher Affinity than to eIFiso4F and m7GTP—The equilibrium binding constants for PAP and VPg interaction over a range of different temperatures were determined from fluorescence titration studies (Fig. 3). The equilibrium constant for PAP-VPg interaction was determined to be 29.5 ± 1.8 nM at 25 °C (Table 1). This compares with VPg-eIFiso4F $K_d$ of 81.3 nM (31) and PAP-m7GTP binding of 43.3 nM (23).

PAP Binding to VPg Is Enthalpically Driven and Entropically Favored—To establish the forces that drive PAP-VPg interactions, the thermodynamics of PAP-VPg binding were determined. Table 1 shows that the affinity of PAP for VPg decreases with the increase in temperature ($K_d = 29.5 ± 1.8$ nM at 25 °C versus 12.5 ± 0.6 nM at 5 °C). The values of $\Delta H^0$ and $\Delta S^0$ were obtained from the intercept and the slope, respectively, of a van’t Hoff plot (Fig. 3, inset) (correlation coefficient of >0.98). The van’t Hoff analyses showed that the van’t Hoff plot is enthalpy-driven ($\Delta H^0 = -29.2 ± 0.9$ kJ/mol) and entropy-favored ($\Delta S^0 = +46.0 ± 3.0$ J/mol), leading to a negative $\Delta G^0$ ($-43.0 ± 1.8$ kJ/mol). The $\Delta G^0$ van’t Hoff component contributes 32% overall to the value of $\Delta G^0$ at 25 °C.

**PAP and VPg Bind in a 1:1 Ratio**—To determine the stoichiometry of PAP-VPg binding, direct fluorescence titration studies of PAP with VPg were performed (Fig. 4). The slope and intercept of the Scatchard plot $Q/([VPg]) \times 10^{-6}$ versus $Q$ (Fig. 4, inset) gave the binding constant ($K_d = 29.5 ± 1.8$ nM) and binding capacity ($n = 0.99 ± 0.01$) of PAP for VPg (44). We conclude that the PAP and VPg interact in a 1:1 stoichiometric ratio.

**PAP and eIFiso4F Bind VPg at Different Sites**—VPg-71 is a truncated variant of wild type VPg where the N-terminal amino acids 1–70 are removed so that it lacks the eIF4F and eIFiso4F binding sites (32). PAP exhibits 2.8 times stronger binding affinity for VPg (29.5 ± 1.8 nM) than eIFiso4F (81.3 nM) (31). The equilibrium constant for PAP-VPg-71 was found to be 37.4 ± 3.0 nM at 25 °C. Because VPg-71 has the eIF4F/eIFiso4F binding sites removed yet still binds PAP with high affinity, we conclude that the PAP binding site on VPg differs from the eIF4F binding site on VPg.

**VPg and Cap Analog Bind PAP in a Mixed Type Competition**—Competitive binding of VPg and cap to PAP was determined by employing a fluorescent cap analog, ant-m7GTP (40, 41). The competitive substitution reactions were performed at constant ant-m7GTP concentration (100 nM), monitoring the fluorescence change of the analog and increasing amounts of PAP in the absence and presence of VPg. ant-m7GTP was a suitable candidate to study these competition interactions because excitation (332 nm) and emission (420 nm) of this extrinsic fluorophore are far removed from the protein fluorescence, and $K_d$ for the PAP-ant-m7GTP interactions was essentially the same as reported previously for m7GTP interaction with PAP ($K_d = 43.3$ nM) (23). Lineweaver-Burk plots (Fig. 5A) meet at the left of the $y$ axis intercept, indicative of mixed type competitive ligand binding between ant-m7GTP and VPg, sug-

![FIGURE 3. Binding isotherms for the interactions of PAP with VPg.](image)

![FIGURE 4. VPg binds more tightly to PAP than to eIFiso4F (complex of eIFiso4FeIFiso4G). Intrinsic PAP (○) or eIFiso4F (□) fluorescence was monitored upon binding to VPg. VPg has negligible intrinsic fluorescence. Solid lines, fitted theoretical curves. Inset, Scatchard plots showing one binding site for eIFiso4F and PAP with VPg. The slope and intercept of the straight line obtained on the plot Q/[VPg] × 10^{-6} versus Q provided the binding constant ($K_d$) and binding capacity ($n$) of the above proteins with VPg. Q is the fractional quench of fluorescence in titration. n for the PAP-VPg was determined as 0.99 ± 0.01 and, for eIFiso4F-VPg, was 1.05 ± 0.01 (T = 25 °C, $\lambda_{em} = 320$ nm, $\lambda_{ex} = 280$ nm).](image)

**TABLE 1**

<table>
<thead>
<tr>
<th>Complex</th>
<th>5 °C</th>
<th>10 °C</th>
<th>15 °C</th>
<th>20 °C</th>
<th>25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP-VPg</td>
<td>12.5 ± 0.6</td>
<td>17.0 ± 0.7</td>
<td>20.9 ± 1.2</td>
<td>26.7 ± 1.3</td>
<td>29.5 ± 1.8</td>
</tr>
<tr>
<td>PAP-VPg-71</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>37.4 ± 3.0</td>
</tr>
</tbody>
</table>

*ND, not determined.
Inhibition of PAP by VPg

FIGURE 5. A, anti-m7GTP cap analog and VPg show mixed competition binding for PAP. Shown is a Lineweaver-Burk plot for competition of anti-m7GTP and VPg with PAP. The fluorescence change of anti-m7GTP cap analog was measured with increasing concentrations of PAP. VPg concentrations were 0 nM (○), 15 nM (△), and 30 nM (◇). The excitation wavelength was 332 nm, and emission was 420 nm. The spectrum was measured in buffer containing 100 nm ant-m7GTP and VPg as indicated. Data points were fitted using least square analysis. B, elfiso4F and VPg-71 bind competitively to NHS-fluorescein-labeled PAP. Lineweaver-Burk plots of competitive binding are presented. The fluorescence change of NHS-fluorescein-labeled PAP (200 nm) was monitored (λex = 493 nm, λem = 516 nm) with increasing concentrations of VPg-71 in the presence and absence of elfiso4F (0 (○), 50 (△), 100 (△)), and 150 nm (◇)). Data points were fitted using least square analysis. C, VPg and TEV RNA bind competitively to NHS-fluorescein-labeled PAP. Lineweaver-Burk plots show competitive binding. The fluorescence change of NHS-fluorescein-labeled PAP was monitored (λex = 493 nm, λem = 516 nm) with increasing concentrations of TEV RNA in the presence and absence of VPg (0 (○), 50 (△), 100 (△)), and 100 nm (◇)). Data points were fitted using least square analysis.

FIGURE 6. Translation of luciferase reporter TEV RNA constructs in wheat germ extracts. Luciferase relative light units (RLU) were measured for TEV(1–143)-luc-A50 RNA (○), TEV(1–143)-luc-A50 RNA + WT VPg (△), TEV(1–143)-luc-A50 RNA + PAP after 1 min (◇), TEV(1–143)-luc-A50 RNA + WT VPg + PAP after 1 min (▲), and TEV(1–143)-luc-A50 RNA + VPg-220 + PAP after 1 min (●) in wheat germ 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 ml luciferase substrate.
Inhibition of PAP by VPg

...ization of the enzymatic activity of PAP and rapid translation of the luciferase reporter by the wheat germ translational machinery. This indicates that VPg serves as an inhibitor of PAP in wheat germ lysate. VPg-220, a truncated mutant of VPg that lacks the C-terminal portion of the protein, was used as a negative control for the PAP-VPg interactions. The VPg-220 variant did interact with PAP with ∼10-fold lower affinity (Fig. 3); however, it did not affect PAP enzymatic activity, as determined by the fluorescence assay. The presence of VPg-220 in the translational system did not show any increase in translation of the luciferase reporter compared with WT VPg.

DISCUSSION

PAP is a highly toxic protein produced by the pokeweed cells and exported outside the cells once synthesized (47, 48). Storage of PAP within extracellular spaces ensures close proximity of PAP to ribosomes. When a pathogen infects the cell, PAP also gains entrance and disrupts cellular protein synthesis, thus killing the pathogen-infected cell and thereby preventing pathogen replication (49).

Khan et al. (32) have characterized interactions between VPg, plant elfiso4E/iso4F, elf4F, and TEV RNA and concluded that VPg increases the binding affinity of elf4F for TEV RNA. The requirement for elf4F in cap-independent translation (34) has been demonstrated, and a mechanism was proposed where VPg substitutes for the cap analog and enhances formation of an elf4F complex with viral IRES (31, 32). We therefore hypothesized that VPg may interact with PAP and possibly target it to uncapped RNA.

The rationale of our investigation was that PAP, being a cap-binding protein, will bind to VPg that functions as a cap analog, and these interactions would affect depurination of uncapped viral RNA or capped cellular RNA. VPg stimulates the in vitro translation of uncapped IRES-containing RNA and inhibits capped RNA translation. Our research indicated that PAP has a high affinity for VPg and that this affinity is almost twice that of the m7GTP analog (23). Greater affinity of PAP for VPg than for the cap structure would produce an advantage for the cell if VPg were to localize PAP to viral RNA for depurination. However, VPg inhibits PAP activity, providing a means to avoid one of the potential host cell defense mechanisms.

The thermodynamic parameters of PAP-VPg binding are similar in magnitude to those of elfiso4E- or elfiso4F-VPg binding. Both interactions are enthalpically driven and entropically favored. The ΔTS van’t Hoff component contributes nearly one-third to the overall value of ΔG° (at 25 °C), suggesting lesser dependence on electrostatic contributions and a greater conformational contribution in the PAP-VPg binding with hydrophobic residues less solvent-exposed in the combined structure. The fact that PAP-VPg interactions are enthalpically driven and entropically favored at biological temperatures supports previous observations by Baldwin et al. (23) that, because PAP is a plant defense protein, it should be able to perform under unpredictable temperature conditions, given its accepted function as a ribosome depurinating agent (23).

Different equilibrium dissociation constant (Kd) values for PAP-VPg compared with elfiso4E- or elfiso4F-VPg binding suggest differences between the active site of PAP and the cap-binding sites of elfs. Léonard et al. (26, 30) have established previously the interactions between VPg and various isoforms of elf4E, and Khan et al. (31) have quantified elf4E/iso4E-VPg interactions as competitive with the m7GTP cap analog (26). Moreover, the binding domain on VPg was mapped to a stretch of 35 amino acids, and substitution of aspartic acid residue found within this region completely abolished interactions of VPg with elf4E/iso4E (26). Plants infected with a TuMV infectious cDNA (p35Tunos) showed viral symptoms with p35Tunos, whereas plants infected with p35TuD77N, a mutant that contained the aspartic acid substitution in the VPg domain, abolished the interaction with elf4E/iso4E (26). VPg-71 lacks the elf4E/iso4F binding sites (32); however, PAP was able to bind VPg-71, indicating that the PAP binding site remains present in VPg-71 and does not include, at least partially, the amino terminus of the protein.

Having determined the binary interactions between PAP, VPg, and elfiso4F, we have examined the ternary interactions. A summary of directly measured binary and ternary complexes is schematically presented in Fig. 7. The equilibrium association constants K1, K2, and K5 were directly measured by fluorescence titration experiments; K6 was from Khan et al. (31). In Fig. 7, K4 and K6 were chosen as the thermodynamically dependent equilibrium constants and were calculated from the relationships shown in Equations 8 and 9.

\[ K_4 = \frac{K_2K_5}{K_1} \quad \text{(Eq. 8)} \]

\[ K_6 = \frac{K_5K_5}{K_3} \quad \text{(Eq. 9)} \]

A comparison of the cross-terms in Fig. 7 shows that the binding of elfiso4F to PAP diminishes the binding of VPg (comparing K1 and K4); similarly, the binding of elfiso4F to VPg diminishes the binding of PAP (comparing K3 and K6).
In order to quantify these interactions, coupling energies were calculated according to the method of Weber (50) and as previously described in detail (51, 52). The coupling energies reflect the overestimation and underestimation of the free energy of binding for the formation of ternary elFiso4F-VPg-PAP complex, $\Delta G^0_{(elFiso4F-VPg-PAP)}$, calculated from the addition of the component binary energies for the interaction of elFiso4F with PAP, $\Delta G^0_{(elFiso4F-PAP)}$, or with VPg, $\Delta G^0_{(elFiso4F-VPg)}$, and PAP with VPg, $\Delta G^0_{(PAP-VPg)}$. These coupling energies therefore represent different binding perspectives and are defined by Equations 10–12.

$$\Delta G^0_{(elFiso4F-VPg-PAP)} = \Delta G^0_{(elFiso4F-VPg)} - \Delta G^0_{(elFiso4F-PAP)} - \Delta G^0_{(elFiso4F-VPg)}$$  (Eq. 10)

$$\Delta G^0_{(PAP, elFiso4F-VPg)} = \Delta G^0_{(elFiso4F-VPg)} + \Delta G^0_{(elFiso4F-PAP)} - \Delta G^0_{(PAP-VPg)}$$  (Eq. 11)

$$\Delta G^0_{(VPg, elFiso4F)} = \Delta G^0_{(elFiso4F-VPg)} + \Delta G^0_{(elFiso4F-PAP)} - \Delta G^0_{(PAP-VPg)}$$  (Eq. 12)

The values for $\Delta G^0_{(elFiso4F-PAP)}$, $\Delta G^0_{(elFiso4F-VPg)}$, $\Delta G^0_{(PAP-VPg)}$, and $\Delta G^0_{(elFiso4F-VPg)}$ were determined from $K_1$, $K_2$, and $K_3$ in Fig. 7, respectively. $\Delta G^0_{(elFiso4F-VPg)}$ values were determined from the addition of the $\Delta G^0$ values calculated from $K_2$ and $K_3$. These interaction energies indicate how the binding of one component to its site affects the binding of a second component to its site; thus, each component (PAP, VPg, and elFiso4F) is created as if it possesses two binding sites. For instance, $\Delta G^0_{(elFiso4F-PAP, elFiso4F-VPg)}$ shows how the binding of one elFiso4F to one site on PAP affects the affinity of the VPg for its binding site on PAP. The coupling energies may be positive, negative, or zero, depending on whether the interactions are cooperative, cooperative, or noncooperative due to the binding of the second component, respectively (52). The coupling energies calculated in this manner are presented in Fig. 7.

The binding of either PAP or VPg to elFiso4F enhances the subsequent binding of the second factor to elFiso4F, $\Delta G^0_{(elFiso4F-VPg-PAP)}$, is $-1.5$ kJ/mol, which is indicative of cooperative heterotropic interaction between these proteins. On the other hand, the binding of either VPg or elFiso4F to PAP is anticooperative ($\Delta G^0_{(PAP, elFiso4F-VPg)} = +1.1$ kJ/mol) and supports competitive type binding between VPg and elFiso4F, as previously determined. This suggests that the elFiso4F-VPg interaction may prevent VPg from interacting with structural features of the PAP. The binding of PAP or elFiso4F to VPg ($\Delta G^0_{(VPg, elFiso4F-PAP)} = +0.1$ kJ/mol) is relatively indifferent to the subsequent binding of the second component to VPg. From these data, a mechanism can be proposed for the sequence of events leading to the formation of PAP-VPg-elFiso4F complex with subsequent inhibition of depurination of TEV-derived RNA. Two models are possible, where PAP first forms a binary complex with elFiso4F initiation factor, with the subsequent binding to VPg, or PAP binds to a preformed elFiso4F-VPg binary complex. Both models lead to a ternary PAP-VPg-elFiso4F complex formation, which brings PAP to close proximity with viral RNA. However, the above cooperative interactions hinder the enzymatic site of PAP from the depurination of RNA, thus promoting inhibition of the plant’s defense mechanism.

Because cap-binding proteins bind to VPg similarly to cap analogs, and VPg stimulates the in vitro translation of uncapped IRES-containing RNA and inhibits capped RNA translation in wheat germ extracts (32), we have analyzed the extent to which VPg can selectively target PAP to uncapped IRES-containing viral RNA, in contrast to its ability to target elF4F to TEV RNA (32). Instead, VPg inhibits depurination of both capped and uncapped S/R oligonucleotides and IRES-containing TEV transcripts. Inhibition of enzymatic activity of PAP is supported in the wheat germ lysate translational system (Fig. 6), indicating that VPg can inhibit PAP even in the presence of other cellular components. This inhibition of the depurinating activity of PAP is concentration-dependent; equimolar concentrations of VPg completely abolish the enzymatic activity of PAP. VPg has been implicated in overcoming viral resistance in plants (25). Extreme toxicity of PAP to plant cells does not allow expression of the protein in vivo and subsequent studies of PAP interaction with VPg. However, in support of the in vivo relevance of our findings is the fact that pokeweed mosaic virus, a VPg-linked viral species, is the only virus to our knowledge reported to infect P. americana (53).

Our findings further support the notion that VPg may play a role in overcoming viral resistance by suppressing the defense mechanism of the plant. Furthermore, depurination inhibition by VPg also suggests the possible use of this protein against cytotoxic activity of RIPs and inhibition of their biological potency.

REFERENCES


Inhibition of PAP by VPg


SUPPLEMENTAL FIGURE 1. HPLC chromatogram of S/R oligo and TEV RNA depurination by PAP in the absence and presence of increasing amounts of VPg. (A) 1-N⁶-Ethenoadenine standard curve. All points were fitted in triplicate values resulting in \( r^2 = 0.98 \) and \( y = 408x - 5170 \) equation of the line. The peaks were eluted isocratically with 50 mM ammonium acetate buffer (pH 5.0)/methanol (89:11, v/v) at room temperature. (B) m⁷GTP-capped and uncapped S/R oligo RNA; (C) m⁷GTP-capped and uncapped TEV RNA. The amount of 1-N⁶-ethenoadenine was monitored at the excitation wavelength of 315 nm and emission wavelength of 415 nm (1, 100 nM RNA; 2, 100 nM RNA + 50 nM PAP; 3, 100 nM RNA + 100 nM PAP; 4, 100 nM RNA + 100 nM PAP + 25 nM VPg; 5, 100 nM RNA + 100 nM PAP + 50 nM VPg; 6, 100 nM RNA + 100 nM PAP + 100 nM VPg).
SUPPLEMENTAL FIGURE 1

A

Area

[Eteneoadenine], pmol

B

Capped S/R Oligo RNA  Uncapped S/R Oligo RNA

EU

50.0

40.0

30.0

20.0

10.0

0.0

4.2 4.4 4.6 4.8 5.0

Minutes

3 4 2 1 6

C

Capped TEV RNA  Uncapped TEV RNA

EU

24.0

16.0

12.0

8.0

4.0

0.0

4.6 4.8 5.0 5.2 5.4

Minutes

3 4 2 1 6
Inhibition of Pokeweed Antiviral Protein (PAP) by Turnip Mosaic Virus Genome-linked Protein (VPg)
Artem V. Domashevskiy, Hiroshi Miyoshi and Dixie J. Goss

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