Pokeweed Antiviral Protein Inhibits Brome Mosaic Virus Replication in Plant Cells*

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Daniel Picard‡, C. Cheng Kao§, and Katalin A. Hudak¶
From the Department of Biology, York University, Toronto, Ontario M3J 1P3, Canada and the Department of Biochemistry and Biophysics, Texas A & M University, College Station, Texas 77843-2128

Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein isolated from the pokeweed plant Phytolacca americana that inhibits the proliferation of several plant and animal viruses. We have shown previously that PAP and nontoxic mutants of PAP can directly depurinate brome mosaic virus (BMV) RNA in vitro, resulting in reduced viral protein translation. Here we expand on these initial studies and, using a barley protoplast system, demonstrate that recombinant PAP and nontoxic mutants isolated from E. coli are able to reduce the accumulation of BMV RNAs in vivo. Pretreatment of only BMV RNA3 with PAP prior to transfection of barley protoplasts reduced the accumulation of all BMV RNAs, with a more severe effect on subgenomic RNA4 levels. Using in vitro RNA synthesis assays, we show that a depurinated template causes the BMV replicase to stall at the template nucleotide adjacent to the missing base. These results provide new insight into the antiviral mechanism of PAP, namely that PAP depurination of BMV RNA impedes both RNA replication and subgenomic RNA transcription. These novel activities are distinct from the PAP-induced reduction of viral RNA translation and represent new targets for the inhibition of viral infection.

Pokeweed antiviral protein (PAP) is a 29-kDa ribosome-inactivating protein of the pokeweed plant Phytolacca americana. Since its initial description as an antiviral agent against tobacco mosaic virus (1), PAP has been demonstrated to reduce the propagation of several plant and animal viruses, including potato virus X, HIV, and influenza (2–4). It therefore holds promise as a broad-spectrum antiviral agent.

Years after its initial discovery, the enzymatic activity of PAP was characterized as an N-glycosylase (5). Like all ribosome-inactivating proteins, PAP efficiently removes a conserved adenine from the sarcin/ricin loop within domain VI of the large ribosomal RNA (6, 7). This depurination slows the elongation step of protein synthesis and is considered to be the reason for cytotoxicity of the protein (reviewed in Refs. 8 and 9).

The accompanying decline in cellular protein translation may cause local cell death and limit virus propagation (10). This model is supported by observations showing a positive correlation between ribosome depurination and inhibition of virus infection (11). The accompanying decline in cellular protein translation, as a result of depurination, is often cited as the cause of antiviral activity. For example, reduction of poliovirus infection of HeLa cells incubated with PAP was attributed to inhibition of translation in virus-infected cells (12). In addition, inhibition of tobacco mosaic virus multiplication in tobacco protoplasts correlated well with PAP-mediated inhibition of translation (13).

More recent results have revealed that many ribosome-inactivating proteins are capable of depurinating RNA substrates apart from the rRNA (14–16). Rajamohan et al. (17) showed that PAP removes both adenines and guanines from HIV-1 when incubated in vitro with the genomic viral RNA. In addition, Hudak et al. (18) have shown that PAP and nontoxic PAP mutants depurate brome mosaic virus (BMV) RNAs in vitro and that this depurination inhibits their translation in a cell-free system. Therefore, the direct depurination of viral RNAs by PAP may contribute to its antiviral activity.

BMV is a model positive-strand RNA virus with a genome composed of three positive sense RNAs designated RNA1, RNA2, and RNA3. Each RNA is 5′-capped and contains a conserved 200-nucleotide tRNA-like structure at the 3′-end (reviewed in Refs. 19 and 20). RNA1 is monocistronic and encodes a 1a protein containing an N-terminal domain with similarity to m7G methyltransferases involved in viral RNA capping and a C-terminal domain with similarity to RNA helicases (21, 22). RNA2 is also monocistronic and encodes a 2a protein that has all of the motifs expected of RNA-dependent RNA polymerases (23). RNA3 is dicistronic and encodes a movement protein and a coat protein that is translated from a subgenomic RNA4 (24–26). Synthesis of the RNAs therefore involves replication of negative and positive strand RNAs and transcription of subgenomic RNA.

In this report, we expand on our initial in vitro studies to show evidence that PAP and nontoxic PAP mutants inhibit the replication and transcription of BMV RNAs in barley protoplasts. The inhibition caused by PAP is not due to ribosome depurination or decline of cellular translation. Rather, PAP and nontoxic mutants reduced the accumulation of BMV RNAs in protoplasts, by inhibiting both viral RNA replication and transcription. Furthermore, depurinated RNAs were shown to prevent efficient elongative RNA synthesis by the BMV replicase in vitro.

EXPERIMENTAL PROCEDURES

Cloning and Expression of PAPs in E. coli—The mature form of wild-type PAP was amplified from pNT188, a yeast vector expressing the complete unprocessed form of PAP. The 5′ primer (CATGGATCCGTCGATACAAATC) was designed to begin at Val23, and the 3′ primer

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‡ C. Kao acknowledges funding from the National Science Foundation.
¶ K. Hudak is grateful for funding support from the Natural Sciences and Engineering Research Council of Canada. To whom correspondence should be addressed: Dept. of Biology, York University, 4700 Keele St., Toronto, Ontario M3J 1P3, Canada. Tel.: 416-736-2100 (ext. 33470); Fax: 416-736-5698; E-mail: hudak@yorku.ca.

The abbreviations used are: PAP, pokeweed antiviral protein; BMV, brome mosaic virus; PEG, polyethylene glycol; MES, 4-morpholineethanesulfonic acid.

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(CCAAACCTTTAGTTGGTCTGACAGCCTCTCC) was designed to stop at Thr^{36}, thereby amplifying the mature, processed form of the protein present in eukaryotes. The mutants of PAP, namely PAPx, PAPn, and PAPf, were amplified with the same primers, and all PCR products were cloned into the expression vector pET30a (Novagen) at NdeI and HindIII sites. All constructs were confirmed by DNA sequencing and transformed into BL21 cells. The overexpressed wild-type and mutant forms of PAP were purified by affinity chromatography on a Ni^{2+}-nitrilotriacetic acid column. Fractions containing PAP were pooled and concentrated by filtration centrifugation with a 10-KDa cut-off filter (Amicon). Purified proteins were separated by 12% SDS-PAGE and stained with Coomassie Blue.

**RNase Activity Assay**—To determine whether ribonuclease co-purified with preparations of PAP and PAP mutants from *E. coli*, an endoribonuclease assay was adapted from Bhardwaj et al. (27). A chemically synthesized RNA template (DHarmaco, Inc.) of 10 nucleotides was 5'-PA-labeled with T4 polynucleotide kinase and [γ-³²P]ATP. Approximately 100 ng of RNA substrate was incubated with 50 ng of PAP or PAP mutants in 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, and 1 mM dithiothreitol at 30 °C for 30 min. RNA incubated without protein was used as a negative control, and the endoribonuclease of the SARS coronavirus, Nsp15 (50 ng), was used as a positive control (27). The positive control endoribonuclease was induced in vitro by rRNA direction. RNA samples were replaced with 5 mM MnCl₂. Following incubation, samples were separated by a 7.5 % urea, 18% acrylamide gel. The gel was wrapped in plastic and quantification of radiolabeled bands was performed using a PhosphoImager (Amer sham Biosciences).

**Isolation of Ribosomes and Primer Extension of rRNA**—Ribosomes were isolated from barley leaves according to the method described by Turner et al. (30). RNA and protein in each sample were incubated with barley ribosomes (50 µg) in RIP buffer to a final volume of 100 µl for 30 min at 30 °C. RNA was extracted and depurinated by primer extension as previously described (18) using 500 ng of barley RNA. A second primer, which anneals close to the 5'-end of the 28 S rRNA, was included in each sample for primer extension and served as an internal control for RNA loading (28).

**Isolation of Protoplast RNA and Northern Blot Analysis**—Total RNA was isolated from 10^5 protoplasts using TRIzol Reagent. RNA was treated with 0.2 µg of RQ1 DNAase at 37 °C for 10 min. Total RNA was precipitated with 0.2 vol of 100% ethanol, 10 mM Tris, pH 7.0, 1 M NaCl, 0.5 M CuSO₄, 10% mannitol, 2% sucrose, 0.01% gentamycin sulfate. Protoplasts were incubated for 18 h at 27 °C and constant low light (165 µmol/m²/s). As a negative control, 0.5 µg of BMV RNAs were incubated without the addition of PEG, and the positive control was 0.5 µg of BMV RNAs inoculated in the presence of PEG but without prior treatment with PAP.

**Incubation of Inoculated Protoplasts with PAP and PAP Mutants**—Aliquots (1 ml) were removed at the indicated time points and analyzed for the presence of BMV RNA1 by Northern blot. Inoculated protoplasts without the addition of PAP were used as a negative control.

**RESULTS**

**Synthesis of Mature PAP and PAP Mutants in *E. coli***—In *E. coli*, BMV replicase was isolated from infected barley leaves as described by Sun et al. (30). Replicase assays were performed essentially according to Adkins et al. (31). Briefly, template RNA (0.5 pmol) and 7 µl of replicase were combined in reaction buffer (20 mM sodium glutamate, pH 8.2, 12 mM dithiothreitol, 4 mM MgCl₂, 2 mM MnCl₂, 500 µM GTP, 200 µM ATP, 200 µM UTP, 242 µM [α³²P]CTP (400 Ci/mmol; Amer sham Biosciences), 0.5% Triton X-100) in a 40-µl final volume. Following incubation at 30 °C for 60 min, the reaction products were extracted with phenol/ chloroform and precipitated in 6 volumes of 100% ethanol, 10 µg of glycogen, and a final concentration of 0.4 M ammonium acetate. Samples were resuspended in formamide loading buffer and separated on 12% denaturing polyacrylamide gels. The amount of label incorporated into newly synthesized RNA was determined with a PhosphoImager and quantified using Amer sham Biosciences software.

To measure the rate of RNA synthesis over time, the same replicase reaction mixture was assembled as described above, and aliquots of 20 µl were removed at the indicated times. Reaction products were precipitated and analyzed as above. The percentages of synthesis were normalized for CMP incorporation relative to the control template analyzed in the same set of reactions.

**In Vivo [³⁵S]Methionine Incorporation**—Protein synthesis in protoplasts was assayed by the incorporation of radiolabeled methionine into protein. Protoplasts (2 x 10⁶ cells/ml) transfected with BMV RNAs were incubated in medium for 4 h. Aliquots of 4 x 10⁵ protoplasts were inoculated with 0.5 µg of BMV RNAs and incubated as described above in 1 ml of incubation medium containing 1.0 µg of PAP or PAP mutants for 1 h at 25 °C. Protoplasts were then pulsed with 10 µCi of [³⁵S]Methionine (1000 Ci/mmol; Amer sham Biosciences), and 100-µl aliquots were removed at the times indicated. Protoplasts were pelleted by centrifugation at 1,000 x g, and 100 µl aliquots were removed from the pellets. Protoplasts were treated with buffer as described above and precipitated in 1 M NaOAc, washed with ice-cold 5% trichloroacetic acid and then ice-cold 95% ethanol. Filters were air-dried, and radioactivity was quantified by scintillation counting.

**BMV Replicase Assays**—RNA templates used in replicase assays were purchased from Dharmaco Inc. (Boulder, CO). BMV replicase was isolated from infected barley leaves as described by Sun et al. (30). Replicase assays were performed essentially according to Adkins et al. (31). Briefly, template RNA (0.5 pmol) and 7 µl of replicase were combined in reaction buffer (20 mM sodium glutamate, pH 8.2, 12 mM dithiothreitol, 4 mM MgCl₂, 2 mM MnCl₂, 500 µM GTP, 200 µM ATP, 200 µM UTP, 242 µM [α³²P]CTP (400 Ci/mmol; Amer sham Biosciences), 0.5% Triton X-100) in a 40-µl final volume. Following incubation at 30 °C for 60 min, the reaction products were extracted with phenol/ chloroform and precipitated in 6 volumes of 100% ethanol, 10 µg of glycogen, and a final concentration of 0.4 M ammonium acetate. Samples were resuspended in formamide loading buffer and separated on 12% denaturing polyacrylamide gels. The amount of label incorporated into newly synthesized RNA was determined with a PhosphoImager and quantified using Amer sham Biosciences software.

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nucleotide (100 ng), followed by separation on a 7.5 M urea, 18% acrylamide gel. PAP mutants (50 ng) were incubated with an end-labeled RNA oligonucleotide (100 ng), followed by separation on a 7.5 M urea, 18% acrylamide gel. RNA substrate incubated without protein was used as a negative control, and the endoribonuclease of the SARS coronavirus, Nsp15, was used as a positive control for replication. In this analysis, it was expected to contain a similar level of contaminating nucleases, since all proteins were isolated in the same manner.

In vitro depurination of barley rRNA by recombinant wild-type PAP and PAP mutants (50 ng) were incubated with an end-labeled RNA oligonucleotide (100 ng), followed by separation on a 7.5 M urea, 18% acrylamide gel. RNA substrate incubated without protein was used as a negative control, and the endoribonuclease of the SARS coronavirus, Nsp15, was used as a positive control for nuclelease activity. Data are indicated as the percentage of full-length RNA remaining after each incubation and represent the means of four independent experiments ± S.E.

FIG. 1. Characteristics of mature PAP and PAP mutants from E. coli. A, separation of purified proteins. Recombinant wild-type PAP, PAPx, PAPn, and PAPc (2 μg) were separated by 12% SDS-PAGE and visualized with Coomassie stain. PAPp is wild-type PAP isolated from pokeweed (2 μg) as a positive control. M, protein broad range molecular mass marker. B, RNase activity assay. Recombinant wild-type PAP and PAP mutants (50 ng) were incubated with an end-labeled RNA oligonucleotide (100 ng), followed by separation on a 7.5 M urea, 18% acrylamide gel. RNA substrate incubated without protein was used as a negative control, and the endoribonuclease of the SARS coronavirus, Nsp15, was used as a positive control for nuclelease activity. Data are indicated as the percentage of full-length RNA remaining after each incubation and represent the means of four independent experiments ± S.E. Untr, untreated template. C, in vitro depurination of barley rRNA by recombinant wild-type PAP and PAP mutants. Barley ribosomes (50 μg) were incubated with 50 ng of purified PAP or PAP mutants, analyzed by primer extension, and separated by 7 M urea, 6% acrylamide gel. A sample of ribosomes incubated with buffer alone (−PAP) was used as a negative control. Ribosomes incubated with PAP isolated from pokeweed (PAPp) were used as a positive control for depurination. The 28 S rRNA band is an internal control for equal RNA loading.

PAPx is an active site mutant with a point mutation E176V that inactivates the glycosylase activity of this protein (34). PAPn and PAPc contain a point mutation G75D and a termination codon in place of Trp259, respectively, and are nontoxic to yeast growth (34). The purified proteins migrated according to their expected masses, with PAPc moving slightly faster due to the absence of 26 C-terminal residues. Importantly, the purified enzymes lacked detectable contaminating proteins (Fig. 1A) and, at the concentrations of PAP used in protoplast assays, had minimal ribonuclease activities (Fig. 1B). PAPx, which showed the highest level of contamination (% degradation of template), did not affect the accumulation of BMV RNAs in protoplasts (Figs. 2 and 3). Although PAPn was not included in this analysis, it was expected to contain a similar level of contaminating nucleases, since all proteins were isolated in the same manner.

Depurination of Barley Ribosomes by E. coli-expressed PAP and PAP Mutants—The mutants PAPx, PAPn, and PAPc do not depurinate ribosomes when expressed in tobacco or yeast (35, 36). To determine whether mature PAP and PAP mutants expressed in E. coli were able to depurinate ribosomes, the proteins were incubated with ribosomes isolated from barley leaves, and primer extension analysis was performed on the rRNA to detect the missing purine residue. Barley is a host for BMV, the focus of this study. As shown in Fig. 1C, only minimal levels of depurination of the sarcin/ricin loop were evident in barley ribosomes not treated with PAP. This background depurination may be due to the endogenous ribosome-inactivating protein present in barley (37). However, incubation of ribosomes with PAP purified from pokeweed increased rRNA depurination 12-fold over background levels. Efficient levels of depurination were also observed for ribosomes incubated with mature PAP isolated from E. coli. In contrast, the nontoxic mutants PAPx, PAPn, and PAPc did not depurinate barley ribosomes above background levels. These data illustrate that the mature forms of PAP expressed in E. coli exhibit similar depurination properties as the corresponding proteins expressed in transgenic tobacco and yeast.
Inhibition of BMV RNA Accumulation in Barley Protoplasts by Pretreatment with PAP—We have demonstrated previously that wild-type PAP depurinates BMV RNAs in vitro and inhibits the translation of these PAP-treated RNAs in a cell-free system (18). To determine whether PAP treatment could affect BMV RNAs in vivo, the RNAs were incubated with wild-type PAP, followed by phenol/chloroform extraction and ethanol precipitation to remove PAP. Treated RNAs were then inoculated into barley protoplasts, and the amount of replication product that accumulated was monitored by Northern blot analysis (mutant BMV RNAs incapable of replication are not detected by this assay). Fig. 2A illustrates a decline in the level of BMV RNAs that correlated with treatment of increasing concentrations of PAP. These results indicate that prior incubation of BMV RNAs with PAP inhibits the accumulation of these RNAs in barley protoplasts.

This analysis was repeated with 50 ng of nontoxic mutants of PAP, an amount that caused severe inhibition of accumulation with wild-type PAP (Fig. 2A). Fig. 2C shows that both PAPn and PAPc were able to efficiently inhibit accumulation of BMV, despite being inactive for rRNA depurination. The active site mutant PAPx did not inhibit BMV RNA accumulation, since the amount of viral RNA was indistinguishable from samples without PAP treatment. These results indicate that rRNA depurination and inhibition of BMV RNA accumulation both require PAP with a functional active site; however, each has different requirements as revealed by mutants PAPn and PAPc. Samples were also probed for 28S rRNA as an indicator of total RNA loading (Fig. 2, B and D).

Analysis of PAP Activity in Barley Protoplasts—To determine whether PAP could affect BMV RNA accumulation in vivo, without prior treatment of the viral RNAs, PAP or PAP mutants were added to the protoplast incubation medium 30 min after RNA transfection. PAP is known to be able to traverse protoplast membranes; thus, access of PAP to the cytosol was anticipated (13). Northern blot analysis of total protoplast RNA after an 18-h incubation shows a decline in the amount of BMV RNAs for those treated with PAP, PAPn, or PAPc (Fig. 3A). The pattern of BMV RNA accumulation was similar to that seen when the RNAs were incubated with PAP or PAP mutants prior to the transfection of BMV RNAs. Thus, in vitro and in vivo treatments with PAP have comparable effects on BMV accumulation levels in barley protoplasts. Samples were also probed for 28S rRNA as a loading control for total RNA (Fig. 3B).

Next, we examined whether PAP could selectively affect the replication of positive strand BMV RNAs after the initiation of negative strand RNA replication. Three hours after transfection, BMV translation and negative strand RNA replication are known to be well under way, but positive strand RNA synthesis is not detectable (38). Fig. 3C illustrates that PAP addition at either 30 min or 3 h post transfection inhibited the accumulation of positive strand RNAs to approximately the same extent relative to the accumulation in the absence of PAP. However, PAP addition did not obviously affect the accumulation of negative strand RNA. Therefore, synthesis of positive strand RNA is more sensitive to the presence of PAP than negative strand RNA. Consistent RNA loading was shown by probing these samples for 28S rRNA (Fig. 3D).

To test whether PAP affected the half-life of BMV RNAs in protoplasts, in vitro transcript of BMV RNA1 was transfected into protoplasts and then incubated in medium to which PAP was added. Aliquots of protoplasts were removed over a time course and analyzed for the presence of BMV RNA1. This transcript persisted in protoplasts for over 2 h before declining and was barely detectable by Northern blot analysis at 6 h after transfection (Fig. 4A). This temporal pattern was not affected by the presence of PAP, indicating that PAP did not alter the stability of this transcript in protoplasts (Fig. 4C). Samples were probed for 28S rRNA as a loading control for total RNA (Fig. 4, B and D).

To determine whether the decline in BMV RNA accumulation following PAP treatment was due to an inhibition of protein synthesis, barley protoplasts were incubated with PAP or PAP mutants, and the rate of [35S]methionine incorporation, a measure of translational activity, was monitored. Over a 2-h period following the addition of PAPs to the medium, protoplasts incubated with wild-type PAP experienced a significantly lower level of [35S]methionine incorporation compared with control cells incubated in buffer alone (Fig. 5). This result confirms that PAP in the medium is indeed able to access the cytosol of protoplasts to inhibit translation. As expected, the active site mutant PAPx did not cause a reduction in overall protein synthesis. Interestingly, the translation rate of protoplasts incubated with PAPn or PAPc was not obviously inhibited (Fig. 5), consistent with the lack of in vitro depurination of barley ribosomes (Fig. 1C). Therefore, the reduction in accumulation of BMV RNAs seen...
in protoplasts incubated with PAPn or PAPc was not due to general inhibition of total protein synthesis.

**Effect of PAP on Viral RNA Replication**—There are several levels at which BMV RNA accumulation could be inhibited, as outlined in Fig. 6A. Depurination of ribosomes could inhibit BMV RNA accumulation. However, results presented here show that PAPn and PAPc do not cause a decline in translation rate of barley protoplasts. BMV RNA accumulation could also be inhibited at the template level, given that we have previously shown that PAP can inhibit the translation of BMV RNAs by direct depurination of the viral RNAs (18). We have also seen PAP-dependent inhibition of positive strand BMV RNA accumulation relative to negative strand; therefore, we hypothesize that PAP activity on BMV RNAs could also affect viral replication or transcription. BMV RNA synthesis can be divided into the processes of RNA replication, the synthesis of genome length negative and positive strand RNAs, and transcription, the synthesis of subgenomic RNA4.

To examine whether PAP could inhibit replication and/or transcription, replication-competent *in vitro* transcripts of each of the three BMV RNAs were synthesized separately, and only BMV RNA3 was treated with wild-type PAP prior to inoculation of all RNAs into protoplasts. We note that BMV RNA1 and -2 can replicate in the absence of RNA3; hence, RNA3 does not code for essential replication factors (38). Northern blot analysis indicates a decrease in the amount of BMV RNA3 following an 18-h incubation of protoplasts (Fig. 6B). Decreases in RNA1 and RNA2 were also evident, possibly due to the movement or capsid protein from RNA3 affecting the accumulation of RNA1 and RNA2 (39). In protoplasts inoculated with RNAs not treated with PAP, subgenomic RNA4, transcribed from a negative strand RNA3, was efficiently produced. Surprisingly, RNA4 was not detected in protoplasts inoculated with PAP-treated BMV RNA3 (Fig. 6B), indicating that pretreatment of BMV RNA3 with PAP preferentially inhibited subgenomic transcription relative to genomic RNA synthesis. Samples were probed for 28 S rRNA as an indicator of total RNA loading (Fig. 6C).

**Effect of RNA Depurination on the BMV Replicase**—The results from pretreatment of RNA3 suggested that PAP also caused a defect at the level of RNA replication and/or transcription. To further demonstrate the feasibility of this mechanism of action, we investigated the effect of template depurination on RNA synthesis *in vitro*. The templates containing depurinated residues at specific locations were synthesized chemically and used for RNA synthesis by the enriched BMV replicase. The prototype, Δ14, is derived from a well-characterized RNA and can direct specific initiation from the penultimate cytidylate in the 3’ CCA sequence (40) (Fig. 7A). Since apurinic nucleotide analogs were available only in the deoxy form at the time, derivatives of Δ14 containing deoxynucleotide (with the base intact) at positions +8, +14, or +19 or at both +8 and +19 (named AbC) were synthesized, and their ability to template RNA synthesis was determined. These molecules generated RNA products at levels comparable with the RNA molecule with no deoxynucleotides (Fig. 7B and data not shown); hence, a modification at the ribose 2’-position was not a significant factor in our analyses. Next, we tested molecules lacking bases at positions +8, +14, and +19 in molecules named Ab8, Ab14, and Ab19. All three templates produced truncated products whose sizes corresponded to the apurinic site within each template. Therefore, apurinic sites within RNA caused the BMV replicase to terminate RNA synthesis at or immediately before these sites. In addition, the synthesis of truncated RNA decreased in relation to the apurinic position along the template. For example, the amount of Ab8 product was 106% of the full-length RNA (normalized for CMP incorporation) compared with Ab19, which was 27% of the full-length RNA. These results suggest that the ability of the BMV replicase to reinitiate RNA synthesis at least partially depended on the position of the abasic residue. Sun *et al.* (30) and Sun and Kao (41) had previously observed that the BMV replicase commits

![Fig. 5](http://www.jbc.org/content/20073/full/f5.large.jpg) **Fig. 5.** Effect of PAP and PAP mutants on protein synthesis in barley protoplasts. Protoplasts were incubated in 1 ml of incubation medium containing 1.0 μg of PAP or PAP mutants and pulsed with [35S]methionine after 1 h of incubation. Aliquots of protoplasts (100) μl were removed at indicated time points, and total protein was trichloroacetate acid-precipitated and scintillation-counted. Protoplasts incubated without PAP were used as a positive control for maximal incorporation of [35S]methionine, and other treatments are presented as values relative to this control. Values are means ± S.E. (n = 3). Line, no PAP; diamonds, PAPwt; squares, PAPn; triangles, PAPc; circles, PAPx.

![Fig. 6](http://www.jbc.org/content/20073/full/f6.large.jpg) **Fig. 6.** Effect of PAP on the accumulation of BMV RNA3 in barley protoplasts. *A*, schematic diagram illustrating the possible ways PAP could inhibit the accumulation of BMV RNAs. i, ribosome depurination inhibits translation elongation; ii, viral RNA depurination inhibits its translation; iii, viral RNA depurination inhibits replication of (-) or (+)-strand RNA or transcription of subgenomic RNA. *B*, in *vitro* transcript of BMV RNA3 (1 μg) was incubated with PAP (50 ng), and following incubation, PAP was removed by phenol/chloroform extraction. The treated BMV RNA3 and untreated *in vitro* transcripts of BMV RNA1 and -2 were inoculated into protoplasts and allowed to replicate for 18 h. Total protoplast RNA was analyzed by Northern blot and probed for positive strand BMV RNAs. *C* lane 1–3, 200 ng of untreated *in vitro* transcripts of BMV RNA1, -2, and -3, respectively, loaded directly onto the gel. Lane 4, untreated BMV RNAs inoculated into protoplasts. Lane 5, RNA3 treated with PAP and inoculated with untreated RNA1 and RNA2. C, the same samples probed for 28 S rRNA as a loading control for total RNA.
to elongation after the replicase has synthesized between 10 and 12 nascent RNA residues. Apurinic sites downstream may cause the replicase to stall on the RNA rather than reinitiate.

To test this hypothesis, a time course analysis of incorporation was conducted to compare the amount of product formed from Ab8 compared with Ab19. As illustrated in Fig. 7C, RNA synthesis from Ab8 increased over time relative to Ab19, indicating that multiple reinitiation occurred with template Ab8 but not with Ab19. Therefore, depurination of RNA within 8–10 nucleotides of the initiation start site allows the BMV replicase to reinitiate, whereas depurination downstream causes the replicase to stall on the RNA. These results demonstrate that depurination of BMV RNA by PAP inhibits the synthesis of full-length product, and reinitiation of the viral replicase is dependent on the position of the apurinic site within the template RNA (i.e. should depurination by PAP occur at a stage after initiation, it is likely that the replicase would be inhibited from reinitiation).

**DISCUSSION**

In this study, we have expressed and purified several forms of mature PAP from E. coli and shown that they possess the same properties as their plant- or yeast-expressed counterparts. Specifically, the mutants PAPx, PAPn, and PAPc were unable to depurinate barley 28 S rRNA in vitro and incapable of inhibiting cellular protein synthesis in vivo. However, PAPn and PAPc retained the ability to inhibit virus accumulation in protoplasts without an obvious effect on cellular translation. Further investigation of these proteins and their activities uncovered novel effects of PAP on virus reproduction, namely inhibition of two distinct steps of viral RNA synthesis. Consistent with these observations, templates with apurinic sites inhibited RNA synthesis by the BMV replicase in vitro.

Incubation of BMV RNAs with noncytotoxic PAP mutants inhibited the accumulation of these viral RNAs in barley protoplasts. Several lines of evidence indicate that this inhibition is related to PAP activity. (i) There was a strong negative correlation between PAP concentration and the level of BMV accumulation. (ii) Most strikingly, the catalytic mutant, PAPx, had no effect on BMV accumulation. (iii) The possibility that co-purified contaminants from E. coli or active ribonucleases caused the inhibition is unlikely, since the noninhibitory PAPx was purified under identical conditions as the other inhibitory forms of PAP. These data suggest that the exogenously added PAP was responsible for the inhibition observed, and the results validate protoplasts as an effective system for investigating PAP-mediated effects. Our findings in single cells also confirm and extend previous in planta results, using transgenic tobacco plants and potato virus X, which demonstrated the inhibitory effects of noncytotoxic PAP on viral infection (2). The current data show that PAP and its noncytotoxic forms are also active in a monocotyledonous host and are effective against a multipartite RNA virus. Accordingly, this suggests the productive use of the noncytotoxic forms of PAP against a variety of viruses that infect different host plants.

One of the classic models for PAP inhibitory activity against viruses has been based on host translational shut-down (12, 13). However, transgenic expression of noncytotoxic PAPc, which does not dephosphorylate ribosomes, was found to inhibit virus infections in plants (2). We also show here that PAP mutants do not cause reduction in cellular translation levels, suggesting that an alternate mechanism is involved in antiviral activity. Indeed, previous studies have shown that treatment of BMV RNAs in vitro with PAP leads to depurination of the templates and inhibition of their translation in a cell-free system (18). Therefore, the observed inhibition of BMV RNA accumulation in protoplasts treated with noncytotoxic PAP could in part be due to inhibition of BMV RNA translation. However, the demonstrated ability of PAP to depurinate BMV RNAs could also manifest itself at other steps in the BMV reproductive process. For example, the inhibition of positive strand RNA accumulation following a 3-h delay for PAP addition suggests that PAP inhibits the replication stage of virus reproduction. Immediately following transfection, the host cellular machinery translates the BMV genome to produce proteins 1a and 2a involved in replication. At 3 h post-transfection, sufficient viral proteins exist for replication of viral RNA to predominate. The observation that PAP primarily affects the accumulation of positive strand RNAs also indicates that translation inhibition is not the dominant reason for lack of RNA accumulation, given that this enzyme replicates both positive- and negative-strand RNAs. Moreover, our finding that pretreatment of BMV RNA3 inhibits accumulation of BMV RNAs in protoplasts is consistent with inhibition at the level of RNA synthesis. Translation of movement or coat protein is not es-
sential for BMV RNA replication, and previous studies have shown that the absence of these proteins does not notably affect BMV RNA accumulation levels in protoplast infections (42). Therefore, inhibition of BMV RNA accumulation is consistent with a PAP-induced defect in viral RNA replication independent of inhibition of protein synthesis. The observed reduction of all three genomic BMV RNAs (Fig. 6B) can be explained by the enhancing activity that RNA3 has on RNA1 and -2 (39). Since PAP is known to depurinate BMV RNAs enhancing activity that RNA3 has on RNA1 and -2 (39). Since PAP treatment of BMV RNA3 transcripts could thus be due to the inhibition of viral RNA replication independent of a stall of the transcript relative to incubation without PAP (Fig. 4). Therefore, we do not have evidence that PAP will decrease the stability of BMV transcript in vivo. Rather, the stalling mechanism is supported by our finding that an apurinic site closer to the initiation nucleotide resulted in higher levels of the truncated product, probably due to reinitiation, than an apurinic site further within the template (Fig. 7C). Sun et al. (30) and Sun and Kao (41, 45) had previously demonstrated that the BMV replicase could undergo abortive synthesis and reinitiation up until positions +10 to +12 of the template. However, after the replicase has synthesized more than 12 nucleotides of the nascent RNA, it is more stably associated with the template RNA (41, 45). It is not known whether viral RNA replicases can escape from a stalled ternary complex, as had been demonstrated for DNA-dependent RNA polymerases (46). These observations could be adapted to study the ternary complexes of RNA-dependent RNA polymerases.

An intriguing finding of this study was that pretreatment of the positive strand RNA3 with PAP resulted in preferential inhibition of subgenomic RNA4 synthesis in comparison with RNA3. Subgenomic RNA4 transcription occurs by internal initiation at a promoter in negative strand RNA3 (47–49). The fact that RNA3 was not able to accumulate in these infections indicates that negative strand RNA3 synthesis may have distinguishing requirements from subgenomic RNA4 transcription. Interestingly, the in vivo PAP treatment or in vitro pre-treatment of all three BMV RNAs did not cause preferential inhibition of subgenomic RNA4 transcription. We do not understand the molecular basis for the preferential inhibition of RNA4 synthesis when only RNA3 was treated with PAP, but the effect was reproducible in three experiments.

Through investigation of the in vitro and in vivo activities of PAP, we have evidence for novel mechanisms by which PAP can inhibit viral reproduction. Specifically, two distinct steps in the virus reproductive cycle were impeded by PAP treatment, RNA replication and subgenomic RNA transcription. These findings not only extend the currently known antiviral activities of PAP, they provide two additional viral targets for inhibiting viral infections. Future studies will focus on gaining a better understanding of the steps involved in these novel mechanisms and defining the extent to which they contribute to viral inhibition in vivo.

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Daniel Picard, C. Cheng Kao and Katalin A. Hudak

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